THE
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JOURNAL

Manuscript EMBO-2010-75042

TGF-β switches isoform of FGF receptors and epithelial-mesenchymal transition

Takuya Shirakihara, Kana Horiguchi, Keiji Miyazawa, Shogo Ehata, Tatsuhiro Shibata, Ikuo Morita, Kohei Miyazono and Masao Saitoh

Corresponding author: Kohei Miyazono, University of Tokyo

Review timeline:

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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 14 July 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, the referees all recognise the interest in the topic under investigation, but vary somewhat in their assessment as to the suitability of your study for publication with us: while referee 2 is more positive, both referees 1 and 3 raise significant concerns with the study in its current state. Most notably, all three reviewers highlight the question as to how the CtBP-ZEB1 interaction is regulated, and how this impacts on downstream transcriptional and functional responses. In this direction, it would be important to address the concerns of both referees 1 and 2 as to how CtBP modulates ZEB1 activator vs. repressor functions. Referee 3 also raises two critical points (#s 5 and 8): the effects of CtBP on ZEB1 activity beyond alphaSMA expression, and the question as to how FGF signalling impacts on complex formation. This referee states that the the data regarding CtBP phosphorylation should either be improved or removed; from an editorial standpoint, our assessment is that you would need to provide additional insight into how CtBP activity is regulated by FGF, rather than just removing these data. In addition to these mechanistic points, the functional invasion assays need to be extended - according to the comments of referees 1 and 3.

I do realise that addressing the concerns of all three referees will require a considerable amount of work, but we would regard a successful resolution of the issues highlighted above to be a pre-requisite for eventual publication in the EMBO Journal. Therefore, while I would like to invite you to submit a revised version to us, we would only encourage you to do so should you feel able to address these concerns in full. Given the amount of work involved, and the uncertainty of the outcome, I do understand that you may instead wish to take your work elsewhere without further delay; if so, please let us know.

I also have to raise one other issue with you - as highlighted by referee 1 - regarding the inappropriate processing of figures: both panels 1D and 3E appear to have been generated by splicing together of originally separate lanes. While this is acceptable, it needs to be clearly marked, and we do also need to see the original scans of the gels, showing that all lanes were originally run on the same gel. Alternatively, I would encourage you to repeat the experiment to run all relevant samples next to each other. If and when you submit your revision, please include the original scans of the blots as supplementary files, and make sure that it is clearly stated in the figure legends how these panels have been assembled.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on 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. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

"The manuscript by Shirakihara et al. attempts to address how TGF-beta regulates different isoforms of the FGF receptors and regulates EMT of cancer-associated epithelial cells. The findings are interesting and suggest an active cooperation between TGF-beta and FGF signaling which could lead to an important insight in the invasive and metastatic behavior of epithelial cancer cells. The authors show that the transcriptional activity of the nuclear factor ZEB1 is modulated by TGF-beta and FGF2. Although interesting there are several concerns with the present manuscript that need to be addressed and I offer therefore the following constructive criticisms hoping to help the authors expedite publication of this work in EMBO Journal.

-Fig. 1 D: This figure is clearly a compilation of different figures. As reviewer I cannot judge if the data provided are correct. The original scans of the original gels should be provided or even better the different samples are loaded on one gel so that a firm comparison can be made.

-Fig. 3 D: The Figure annotation is not correct as the blue line should be TGF-β alone and not TGF-β + FGF2.

-It is an interesting observation that the invasive behavior of MCF7 cells is strongly increased upon co-seeding into collagen gels with pretreated NMuMG cells. Some more information how these cells become invasive would be essential to explain the phenomena. Can the authors exclude that this is just normal growth of MCF7 cells into the digested collagen by the NMuMG cells or that this is an active invasive mechanism of the MCF7 cells?

-There is a conceptual problem with the interpretation of the authors on the role of CtBP to repress the transcriptional activity of deltaEF1. The authors give in the result section and in the discussion the impression that deltaEF1 is a transcriptional activator:
• That deltaEF1 is an activator controlled by CtBP this is by no means clearly supported by experimental data in this article. Furthermore the evidence that ZEB transcription factors can act as an activator is so far rather poorly documented in the literature. Therefore direct experimental evidence of an activator role on the alfaSMA promoter should be provided. One simple experiment that could be used to support their statements is the use of deltaEF1 mutant that cannot bind CtBP any more which would support the hypothesis of deltaEF1 being able to act as a transcriptional activator.
• The authors exclude that SRF is not involved in this phenomena but they should at least show the data to illustrate this as this an important finding in the context of previously published data.
• Figure 7 should be modified and the legend should explain in more detail what the reader can learn from the figure. The manner of how deltaEF1 is illustrated outside of the cell is extremely confusing and is not correct. Therefore I would suggest that a new version of this figure is created using for instance color coded nuclei to indicate the deltaEF1 and CtBP status.

Referee #2 (Remarks to the Author):

The authors compared the effect of treatment of epithelial cells with TGFbeta alone to a combination of TGFbeta plus FGF2. They show that TGFbeta alone induces a partial EMT with subsequent differentiation to myofibroblasts. This state in characterized by expression of the marker gene alphaSMA, inhibition of the Erk pathway and most importantly an isotype switch of FGF receptors causing cells to become sensitive to FGF2. Addition of FGF2 induced a stronger EMT with loss of alpha SMA expression and activation of the Erk pathway. They further showed that this is due to switching the EMT activator ZEB1 from a transcriptional activator (of alphaSMA) to a repressor by binding to the co-repressor CtBP. This process could be blocked by FGF2 inhibitors.

The data are new, original and well performed. These findings deepen the understanding of how different extracellular factors cooperate to induce different states of an EMT, and therefore have impact on the how the tumor microenvironment might influence the behaviour of (epithelial) cancer cells.

Some open questions have to be addressed:
1. Fig. 3c: Although, according to the presented data, addition of TGFbeta alone should not allow binding of CtBP to ZEB1/dEF1, epithelial genes such as E-cadherin are already suppressed. Addition of FGF2 inducing a ZEB1/CtBP complex does not increase suppression of E-cadherin. How could this be explained with the proposed model? This should be investigated by further experiments. For instance does knockdown of CtBP affect repression of E-cad in TGFbeta treated cells with or without FGF2? Or are other co-repressors responsible for E-cad repression? Are such cofactors also affected by Erk signaling or addition of FGF2?

Minor points:
1. The indroduction section is very long and could be shortened. For instance it is not necessary to present the results on almost one manuscript page already in this section.
2. Fig. 3b: the blue curve is labeled wrongly.

Referee #3 (Remarks to the Author):

This manuscript describes the role played by FGF signalling in determining Epithelial to Mesemchymal transitions. TGFb signalling leads to a conversion to myo-fibroblast like cells whereas TGFb + FGF2 leads to fibroblasts without significant expression of alphaSMA and a more migratory phenotype. FGF2 signalling is proposed to drive ERK/MAP kinase signalling leading to the interaction of ZEB1/dEF1 and CtBP and alphaSMA repression. The subject is interesting but there are numerous problems. It is hard to recommend publication in its current form. A significant additional amount of work would be required to make this work suitable for EMBO J.

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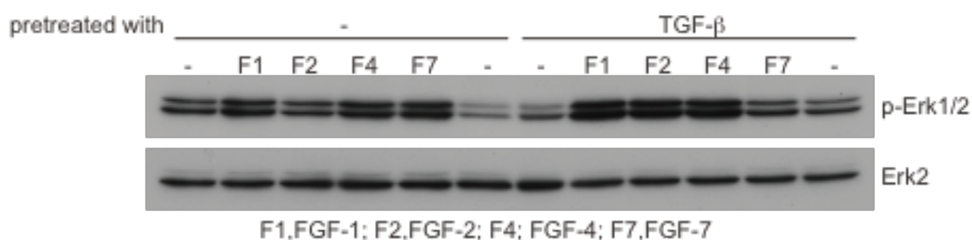
1) It is not clear how different the gene expression patterns of the TGFb vs TGFb + FGF2 cells are. Is it only a couple of genes (aSMA and calponin) that are differentially regulated and FGF2 is promoting motility largely by direct signalling to the cytoskeleton? Or is affecting broad transcriptional programmes.
2) The gel shrinkage assay is a very indirect way of measuring protease production/function. It also reflects complex activities like cell protrusion and actomyosin force generation. It should be not presented as a surrogate assay for matrix degradation.
3) The labelling of Figure 3B must be incorrect.
4) Figure 4 should show multiple siRNA for dEF1/ZEB1. AlphaSMA levels should be shown by western blot.
5) The authors need to show that dEF1/ZEB1 and CtBP1 siRNA also affect cell migration, invasion, gel contraction and calponin. If the ZEB1+CtBP1 complex is only controlling alphaSMA levels but not other genes or functional properties of the cell then the significance of the complex is very unclear. Figure S4D indicates that dEF1/ZEB1 is not important for cell migration triggered by TGFb + FGF2.
6) The labelling of Figure 4D is not clear: is the right hand lane TGFb+FGF2+UO126?
7) It looks like UO126 treatment reduces dEF1/ZEB1 levels. The reduced CtBP1 co-IP is probably because less dEF1/ZEB1 is precipitated and not because the stochiometry of the interaction is altered. These data need improving. A second MEK inhibitor should also be used to mitigate against 'off-target' effects.
8) As the authors acknowledge, the section on whether CtBP1 is directly controlled by phosphorylation is not conclusive. If direct phosphorylation is the mechanism they have not managed to identify the sites. The authors should either map the sites properly or remove this section entirely.
9) Figure 4E should include the input amounts of proteins and not just the IP's.
10) Figures 5 and 6 are rather contrived assays. Their relevance is predicated on the assumption that tumour-associated fibroblasts arise from untransformed epithelial cells that undergo EMT. The evidence for this is rather flimsy and only two reviews are cited. Simple invasion assays should be performed and Figures 5 and 6 could be moved to supplementary figures, which would reflect their rather contrived nature.
11) Figure S2D is not convincing: faint bands are visible in the IgG control lanes and there is not control for the specificity of the DNA fragments pulled down.

1st Revision - authors' response 11 October 2010

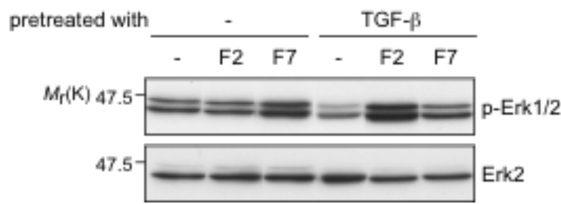
Thank you for your valuable comments. We have modified our manuscript as recommended by the reviewer as follows.

According to the editor’s comments, we show the original blots of Figures 1D and 3E (below). As for Figure 1D, we have repeated the experiment and now present the new data as new Figure 1D. Because the lane assignment of original blot of Figure 3E (see below) had not been coincident with panel assignment of the former Figure 3D, we split and modified them as shown in the former Figure 3E. In our revised version, we have arranged the former Figure 3D as shown in new Figure 3D, and new Figure 3E is shown without splitting lanes (see new Figure 3E).

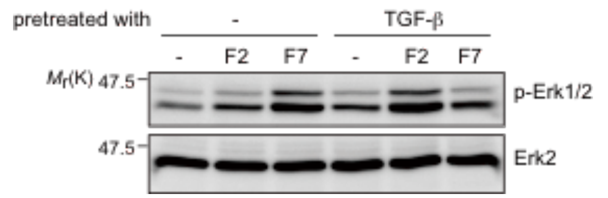
Original blots of Figure 1D



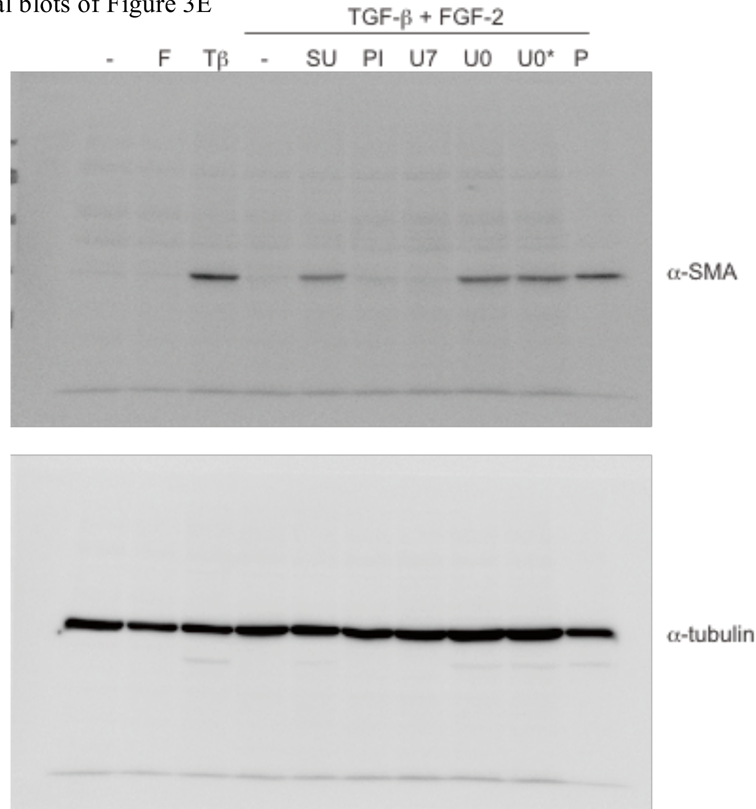
Former Figure 1D



New Figure 1D

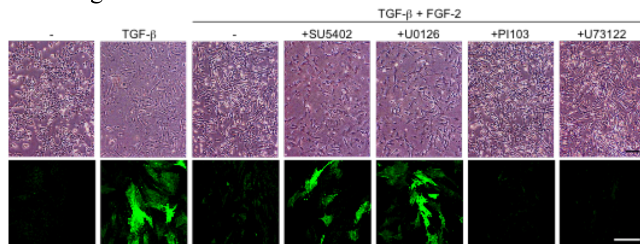


Original blots of Figure 3E

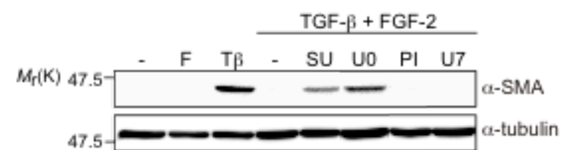


SU, Su5402; PI, PI103; U7, U73122; U0, U0126; U0*,U0126(20uM); P, positive control

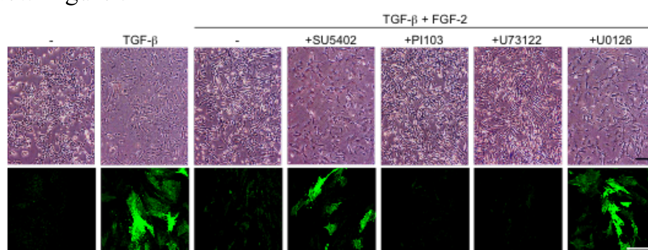
Former Figure 3D



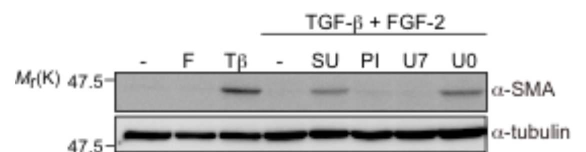
Former Figure 3E



New Figure 3D



New Figure 3E



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Referee #1 (Remarks to the Author):

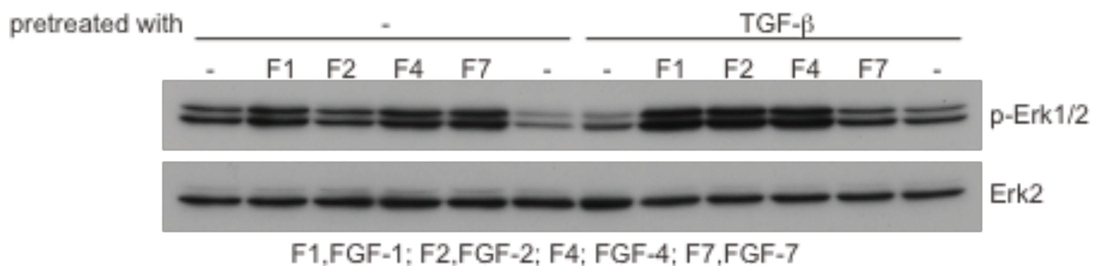
"The manuscript by Shirakihara et al. attempts to address how TGF-beta regulates different isoforms of the FGF receptors and regulates EMT of cancer-associated epithelial cells. The findings are interesting and suggest an active cooperation between TGF-beta and FGF signaling which could lead to an important insight in the invasive and metastatic behavior of epithelial cancer cells. The authors show that the transcriptional activity of the nuclear factor ZEB1 is modulated by TGF-beta and FGF2. Although interesting there are several concerns with the present manuscript that need to be addressed and I offer therefore the following constructive criticisms hoping to help the authors expedite publication of this work in EMBO Journal.

Fig. 1 D: This figure is clearly a compilation of different figures. As reviewer I cannot judge if the data provided are correct. The original scans of the original gels should be provided or even better the different samples are loaded on one gel so that a firm comparison can be made.

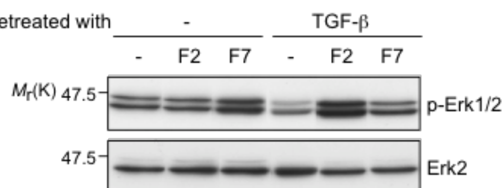
Answer

We show the original blots of Figure 1D (Figure 1 for reviewer 1). To show the representative data, we deleted the lanes of FGF-4 and FGF-1 from original blot and presented it as the former Figure 1D. We repeated the same experiment and present it as new Figure 1D.

Figure 1 for reviewer 1 (original blots of Figure 1D)



Former Figure 1D



New Figure 1D

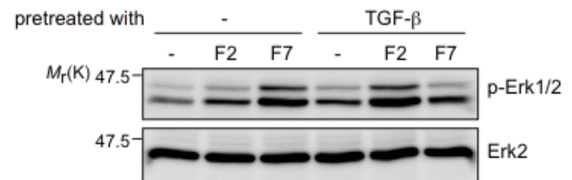


Fig. 3 D: The Figure annotation is not correct as the blue line should be TGF- β alone and not TGF-β + FGF2.

Answer

We think that this comment is on Figure 3B, but not Figure 3D.

We are sorry for this mistake and have corrected the annotation of the Figure 3B. We thoroughly checked for typos in all figures.

It is an interesting observation that the invasive behavior of MCF7 cells is strongly increased upon co-seeding into collagen gels with pretreated NMuMG cells. Some more information how these cells become invasive would be essential to explain the phenomena. Can the authors exclude that this is just normal growth of MCF7 cells into the digested collagen by the NMuMG cells or that this is an active invasive mechanism of the MCF7 cells?

Answer

According to the reviewer's comment, we have performed immunostaining analysis with human specific anti-Ki67 antibody (Figure 2 for reviewer 1), and calculated Ki-67-positive cells (Figure 3 for reviewer 1). Ki67-positive cells were observed only in the absence of TGF-β (left panel of Figure 2 for reviewer 1), suggesting that enhanced invasive behavior does not result from cell

proliferation of MCF-7 cells (see line 11, page 13).

In addition, we have examined the mRNA expressions of MMP2 and MMP9 in crude extracts from coculture of MCF-7 cells with NMuMG cells by RT-PCR using human specific primers. The expression of MMP2 in MCF-7 cells was relatively low and unaffected by TGF-β/FGF-2 or by coculture with NMuMG cells. In contrast, when MCF-7 cells were cocultured with NMuMG and treated with TGF-β and FGF-2, the expression of MMP9 was upregulated, compared with that in MCF-7 cells alone in the presence of TGF-β and FGF-2 (new Figure S5D). However, since it may not be sufficient to explain the phenomena, we are now preparing microarray analyses with human probes using crude extracts from MCF-7 cells cocultured with NMuMG cells. We are much interested in this molecular mechanism, and it will be necessary to elucidate the mechanism for future publications.

Figure 2 for reviewer 1

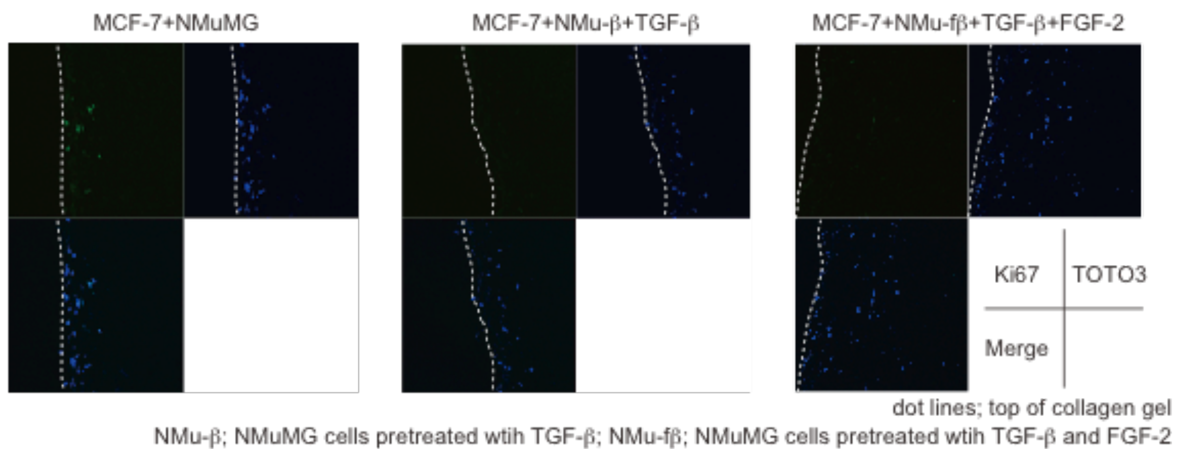
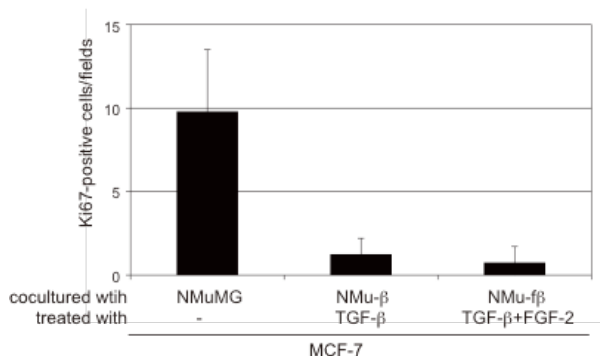


Figure 3 for reviewer 1



There is a conceptual problem with the interpretation of the authors on the role of CtBP to repress the transcriptional activity of deltaEF1. The authors give in the result section and in the discussion the impression that deltaEF1 is a transcriptional activator:
-; That deltaEF1 is an activator controlled by CtBP this is by no means clearly supported by experimental data in this article. Furthermore the evidence that ZEB transcription factors can act as an activator is so far rather poorly documented in the literature. Therefore direct experimental evidence of an activator role on the alfaSMA promoter should be provided. One simple experiment that could be used to support their statements is the use of deltaEF1 mutant that cannot bind CtBP any more which would support the hypothesis of deltaEF1 being able to act as a transcriptional activator.

Answer

We principally agree with the reviewer's comments, and sorry for giving the incorrect impression and for incorrect statement that "δEF1 can bind directly to the E-cadherin promoter and repress its transcriptional activity by interacting with the corepressor CtBP1" (line 20 page 4 in the former

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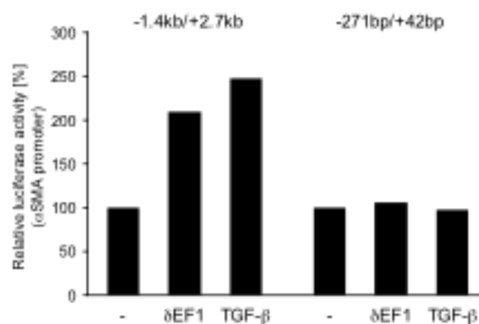
main text). We have properly stated it as "δEF1 can bind directly to the E-cadherin promoter and repress the E-cadherin expression" (line 12 page 4). We have also changed the sentence of "CtBP1 constitutively binds to δEF1 and suppresses its transcriptional activity" (line 13, page 20 in the former main text) to "CtBP1 constitutively binds to δEF1 and suppresses the transcriptional regulatory function of δEF1" (line 15, page 17). In our present and previous studies (Mol. Biol. Cell, 18, 3533-3544, 2007), we could not determine whether δEF1 is an activator or de-repressor of α-SMA transcription, although δEF1 acts as a repressor for the transcription of E-cadherin. Thus, we described "it should be determined in the future whether δEF1 acts as an activator or de-repressor of α-SMA transcription" in the Discussion (line 3, page 18).

According to the reviewer's comments, we have performed α-SMA-promoter reporter assay. The minimal α-SMA-reporter construct, -271bp/+42bp, which was reported to respond to TGF-β, was obtained from Dr. Nishimura's group (Dev. Cell 11, 93-104, 2006). This construct, however, did not work well in NMuMG cells (Figure 4 for reviewer 1, right). We then prepared another α-SMA reporter construct (-1.4kb/+2.7kb) by PCR-based strategy. The α-SMA-reporter construct (-1.4kb/+2.7kb) responded to TGF-β stimulation as well as δEF1 overexpression (Figure 4 for reviewer 1, left, and new Figure S3D). CtBP1 partially inhibited this α-SMA-reporter activity induced by δEF1, while CtBP1 failed to inhibit the activity induced by δEF1 mut that does not bind to CtBP1 (line 20, page 17) (Mol. Cell Biol.19, 8581-8590, 1999). In addition, δEF1 siRNA inhibited TGF-β-induced α-SMA upregulation (Figs. 4A, 4B and new Figure 4C). Thus, these findings suggest that δEF1 acts as an activator or de-repressor, and is indispensable for α-SMA induction by TGF-β in NMuMG cells.

Figure 4 for reviewer 1



- The authors exclude that SRF is not involved in this phenomena but they should at least show the data to illustrate this as this an important finding in the context of previously published data.

Answer

We have already performed co-immunoprecipitation assay with anti-δEF1 antibody. SRF was not apparently coimmunoprecipitated with δEF1, probably due to suppression of the expression of SRF protein by TGF-β (Figure 5 for reviewer 1. We do not show the data because of its negative nature, but describe this result as "data not shown" (line 13, page 17). In addition, we attempted to knock down endogenous SRF by its specific siRNAs. However, these three siRNAs did not completely silence endogenous SRF, and TGF-β faintly upregulated SRF mRNAs in cells transfected with #1 and #2 siRNAs. Under these conditions, α-SMA was not significantly affected at mRNA levels (Figure 6 for reviewer 1). According to the reviewer's suggestion, we comment in Discussion (lines 13 and 19, page 17) and illustrate SRF in new Figure 6.

Figure 5 for reviewer 1

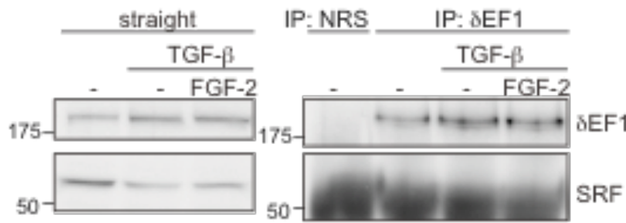
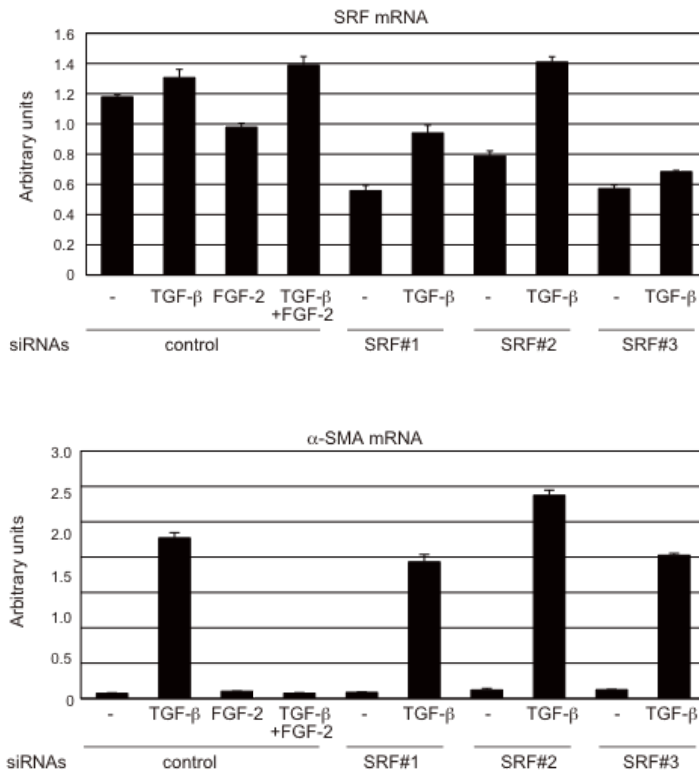


Figure 6 for reviewer 1



- Figure 7 should be modified and the legend should explain in more detail what the reader can learn from the figure. The manner of how deltaEF1 is illustrated outside of the cell is extremely confusing and is not correct. Therefore I would suggest that a new version of this figure is created using for instance color coded nuclei to indicate the deltaEF1 and CtBP status.

Answer

We have modified the schema of the former Figure 7 and present it as new Figure 6.

Referee #2 (Remarks to the Author):

The authors compared the effect of treatment of epithelial cells with TGFbeta alone to a combination of TGFbeta plus FGF2. They show that TGFbeta alone induces a partial EMT with subsequent differentiation to myofibroblasts. This state in characterized by expression of the marker gene alphaSMA, inhibition of the Erk pathway and most importantly an isotype switch of FGF receptors causing cells to become sensitive to FGF2. Addition of FGF2 induced a stronger EMT with loss of alpha SMA expression and activation of the Erk pathway. They further showed that this is due to switching the EMT activator ZEB1 from a transcriptional activator (of alphaSMA) to a repressor by binding to the co-repressor CtBP. This process could be blocked by FGF2 inhibitors.

The data are new, original and well performed. These findings deepen the understanding of how different extracellular factors cooperate to induce different states of an EMT, and therefore have impact on the how the tumor microenvironment might influence the behaviour of (epithelial) cancer cells.

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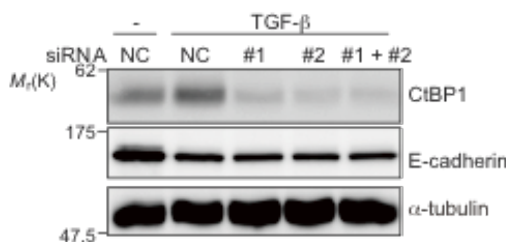
Some open questions have to be addressed:
1. Fig. 3c: Although, according to the presented data, addition of TGFbeta alone should not allow binding of CtBP to ZEB1/deltaEF1, epithelial genes such as E-cadherin are already suppressed. Addition of FGF2 inducing a ZEB1/CtBP complex does not increase suppression of E-cadherin. How could this be explained with the proposed model? This should be investigated by further experiments. For instance does knockdown of CtBP affect repression of E-cad in TGFbeta treated cells with or without FGF2? Or are other co-repressors responsible for E-cad repression? Are such cofactors also affected by Erk signaling or addition of FGF2?
Answer
We are sorry for the incorrect statement that “δEF1 can bind directly to the E-cadherin promoter and repress its transcriptional activity by interacting with the corepressor CtBP1” (line 20 page 4 in the former main text). We have properly stated it as” δEF1 can bind directly to the E-cadherin promoter and repress the E-cadherin expression”(line 12 page 4).δEF1 was identified as one of E-cadherin repressors. Previous study showed that, even though CtBP1 formed complex with δEF1/SIP1, transcriptional suppression of E-cadherin by δEF1/SIP1 was not affected by interaction with CtBP1 (J. Biol. Chem. 278, 26135-26145, 2003). In the present study, FGF-2 increased the interaction between δEF1 and CtBP1, yet FGF-2 did not affect E-cadherin suppression by TGF-β (Figures 3C and S1C). Thus, some molecule(s) other than CtBP1, which is not affected by FGF-2 or Erk signaling, may be responsible for δEF1-mediated E-cadherin repression. One such candidate would be HDAC1 (Cancer Res. 66, 944-950, 2006).

According to the reviewer’s comments, we have performed immunoblot analyses. CtBP1 siRNAs did not affect the levels of E-cadherin repressed by TGF-β(Figure 1 for reviewer 2) orTGF̃βFGF̃2 (new Figure 4D). In contrast, δEF1 acts as an activator or de-repressor for SMA induction, which is inhibited by association with CtBP1. Thus, CtBP1 siRNAs recovered the inhibitory effects of FGF-2 on α-SMA induction by TGF-β (new Figure 4D).

Figure 1 for reviewer 2



Minor points:
1. The indroduction section is very long and could be shortened. For instance it is not necessary to present the results on almost one manuscript page already in this section.
Answer
We have shortened about one page of the introduction section.

2. Fig. 3b: the blue curve is labeled wrongly.
Answer
We are sorry for this mistake and have corrected the annotation of the figure. We thoroughly checked for typos in all figures.

Referee #3 (Remarks to the Author):

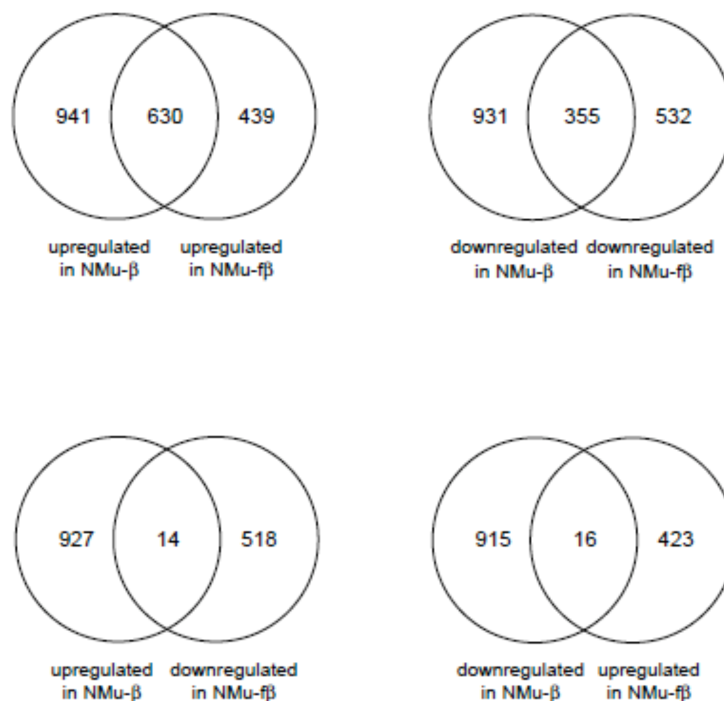
This manuscript describes the role played by FGF signalling in determining Epithelial to Mesemchymal transitions. TGFb signalling leads to a conversion to myo-fibroblast like cells whereas TGFb + FGF2 leads to fibroblasts without significant expression of alphaSMA and a more migratory phenotype. FGF2 signalling is proposed to drive ERK/MAP kinase signalling leading to the interaction of ZEB1/dEF1 and CtBP and alphaSMA repression. The subject is interesting but there are numerous problems. It is hard to recommend publication in its current form. A significant additional amount of work would be required to make this work suitable for EMBO J.

1) It is not clear how different the gene expression patterns of the TGFb vs TGFb + FGF2 cells are. Is it only a couple of genes (aSMA and calponin) that are differentially regulated and FGF2 is promoting motility largely by direct signalling to the cytoskeleton? Or is affecting broad transcriptional programmes.

Answer

We have performed microarray analyses with mouse 45101 probes using NMuMG cells stimulated with TGF-β alone or combination of TGF-β and FGF-2. The summary of the microarray analysis is shown in Figure 1 for reviewer 3. Addition of FGF-2 largely affected transcription of target genes of TGF-β including RGS16 and ELMO-1. These gene products may be involved in enhanced cell motility in the presence of TGF-β and FGF-2, although the contribution of direct signaling to the cytoskeleton cannot be excluded. In addition, EMT regulators, Snail, Slug and Twist, were remarkably upregulated in cells treated with FGF-2 and TGF-β (see line 16, page 18). We will further work on the underlying mechanism, which may be beyond the scope of this paper and thus we plan to publish in the next paper.

Figure 1 for reviewer 3



2) The gel shrinkage assay is a very indirect way of measuring protease production/function. It also reflects complex activities like cell protrusion and actomyosin force generation. It should be not presented as a surrogate assay for matrix degradation.

Answer

Thank you for your critical suggestions. We have performed Zymography analysis and presented it as new Figure 2F.

3) The labelling of Figure 3B must be incorrect.

Answer

We are sorry for this mistake and have corrected the annotation of the figure. We thoroughly checked for typos in all figures.

4) Figure 4 should show multiple siRNA for dEF1/ZEB1. AlphaSMA levels should be shown by western blot.

Answer
According to the reviewer's comment, we have performed immunoblot analysis using 2 different kinds of δEF1 siRNAs (new Figure 4C).

5) The authors need to show that dEF1/ZEB1 and CtBP1 siRNA also affect cell migration, invasion, gel contraction and calponin. If the ZEB1+CtBP1 complex is only controlling alphaSMA levels but not other genes or functional properties of the cell then the significance of the complex is very unclear. Figure S4D indicates that dEF1/ZEB1 is not important for cell migration triggered by TGFb + FGF2.

Answer
As shown in new Figure 6, there are 3 steps during the TGF-β-induced EMT. The first step is a process generating fibroblastic cells from epithelial cells, which is induced by transcriptional upregulation of both δEF1/ZEB1 and SIP1/ZEB2. δEF1 and SIP1 have redundant effects on this process (Mol. Biol. Cell, 18, 3533-3544, 2007). The second step is a process generating SMA-positive myofibroblastic cells from fibroblastic cells (EMyoT), which is caused by increased levels of δEF1. SIP1 is not necessary for this step (Figure 4A). The third step is a process generating activated fibroblastic cells from fibroblastic cells, which is induced by the Erk-dependent interaction of δEF1 with CtBP1. The effects of δEF1 in the first step have been previously published by us (Mol. Biol. Cell, 18, 3533-3544, 2007), and those in the second and third steps are partially elucidated in the present study (Figures 4 and S4). Thus, we focused on the effects of CtBP1 siRNA. Because CtBP1 may have target proteins other than δEF1, phenotypes of CtBP1 knockdown can be different from those of TGF-β stimulation alone. However, we found that calponin induction was restored by CtBP1 siRNAs (new Figure 4D). In addition, knockdown of CtBP1 attenuated FGF-2-induced migratory and invasive properties (Figures 2 and 3 for reviewer 3). We do not show the data, but describe this result as "data not shown" (line 14 page 18). Gelatin zymography (instead of gel contraction assay based on the comment 2 above) triggered by TGF-β or TGF-β+FGF-2 was not remarkably affected by CtBP1 siRNAs (Figure 4 for reviewer 3). Similar to the previous study showing that the inhibitory effect of δEF1 or SIP1 on E-cadherin was not affected by interaction with CtBP1 (J. Biol. Chem. 278, 26135-26145, 2003), E-cadherin repressions by TGF-β alone and TGF-β+FGF-2 were not affected by CtBP1 siRNAs (Figure 5 for reviewer 3, and new Figure 4D). Thus, these finding suggest that CtBP1 regulates the transcription of many genes involved in phenotypic change in the present study.

Figure 2 for reviewer 3 (wounding assay)

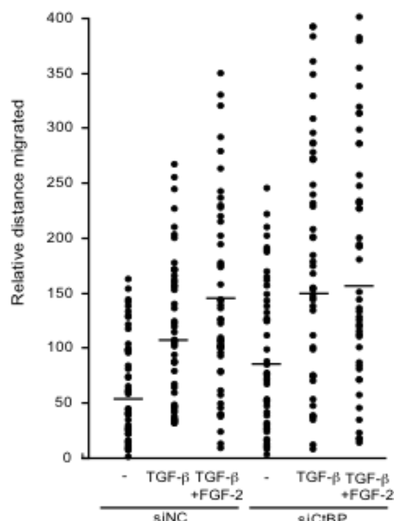


Figure 3 for reviewer 3 (invasion assay)

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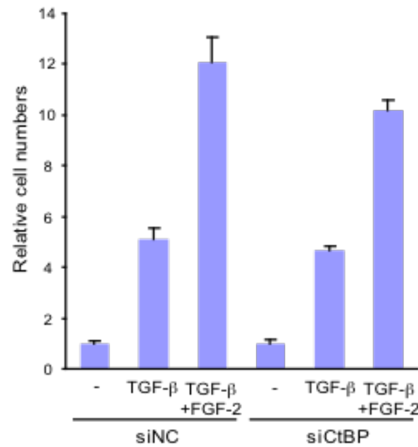


Figure 4 for reviewer 3 (gelatin zymography)

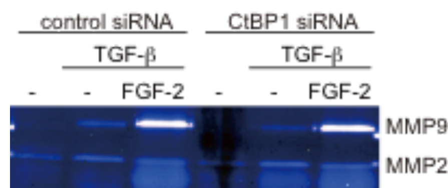
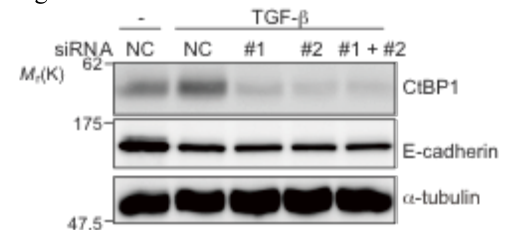


Figure 5 for reviewer 3



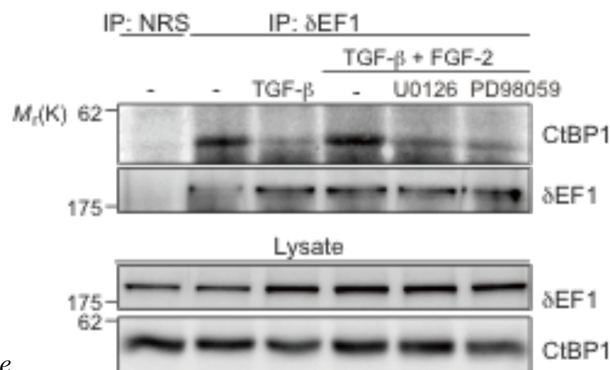
6) The labelling of Figure 4D is not clear: is the right hand lane TGFb+FGF2+UO126?
7) It looks like UO126 treatment reduces dEF1/ZEB1 levels. The reduced CtBP1 co-IP is probably because less dEF1/ZEB1 is precipitated and not because the stochiometry of the interaction is altered. These data need improving. A second MEK inhibitor should also be used to mitigate against 'off-target' effects.

Answer

We are sorry for misleading labeling of the former figure 4D. U0126 was added to the media containing 10% FBS in the absence of TGF-b and FGF-2. U0126 decreased the interaction of dEF1 with CtBP1, compared with that of control (second lane from left in the former Figure 4D), suggesting that constitutive interaction between ofdEF1 with CtBP1 is dependent on Erk kinase activated by serum. However, the data of U0126 appear to be dispensable for the context in this study. To avoid confusion of readers, we would like to delete it (new figure 4E).

According to the reviewer's comment, we have performed immunoprecipitation analyses in the presence of another MEK inhibitor, PD98059. The interaction between dEF1 and CtBP1 was enhanced by treatment with TGF-b and FGF-2, which was decreased by addition of U0126 or PD98059 (Figure 6 for reviewer 3). We do not show the data, but describe this result as "data not shown" (line 12 page 11). These findings support the Erk-dependent interaction between dEF1 and CtBP1.

Figure 6 for reviewer 3



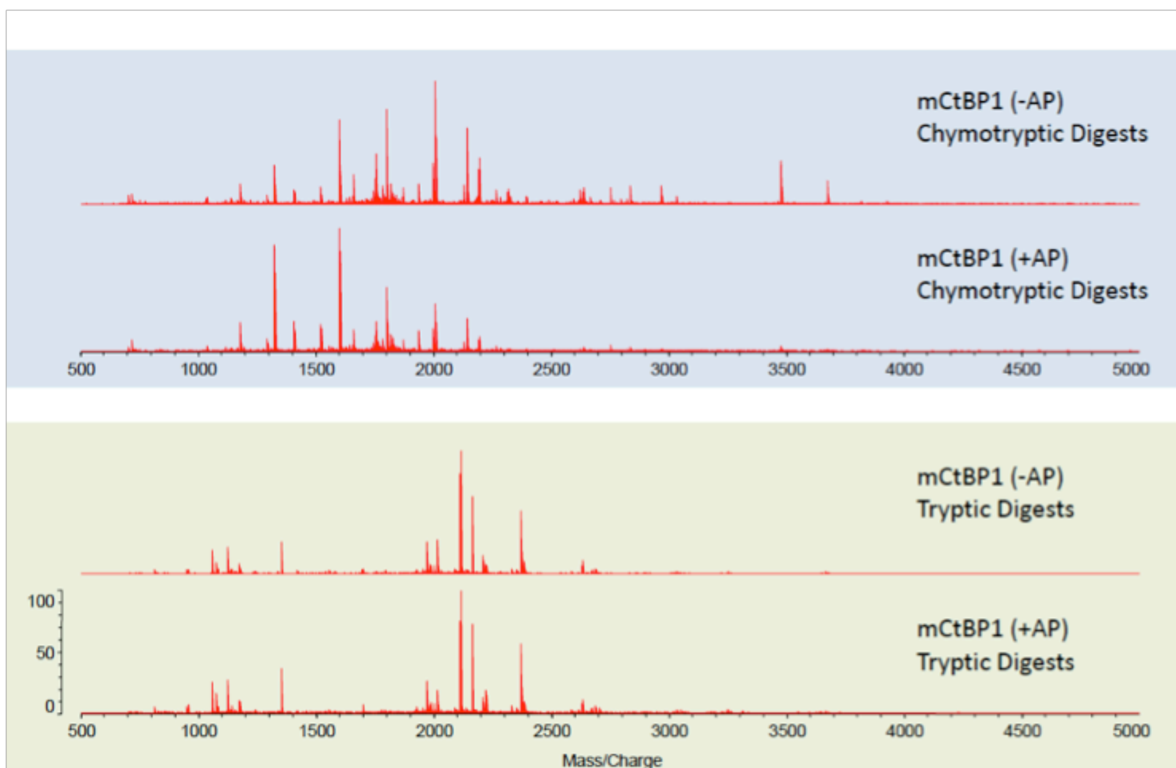
8) As the authors acknowledge, the section on whether CtBP1 is directly controlled by phosphorylation is not conclusive. If direct

phosphorylation is the mechanism they have not managed to identify the sites. The authors should either map the sites properly or remove this section entirely.

Answer

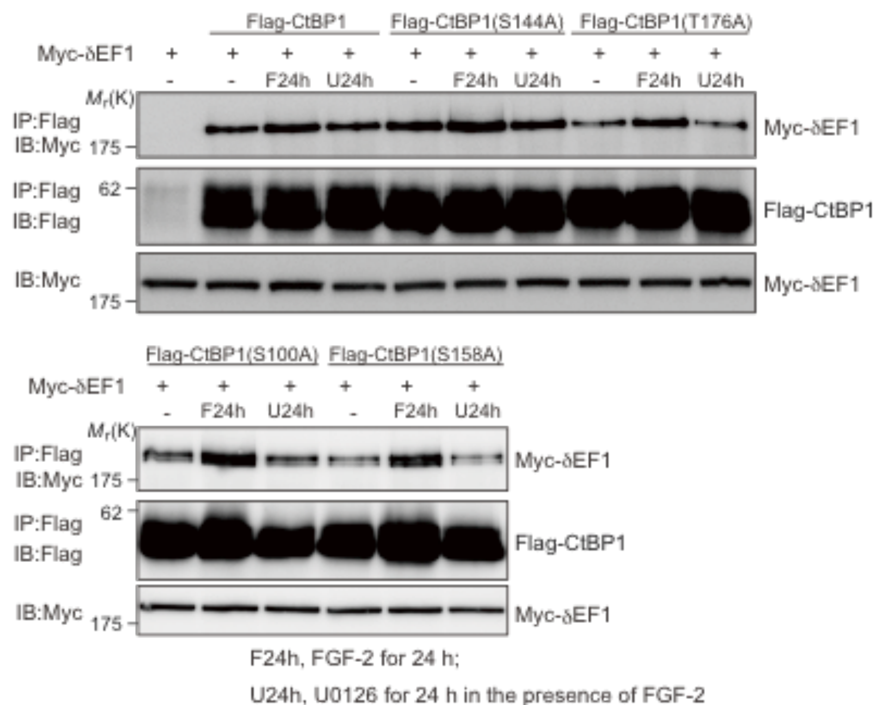
We have performed mass-spectrometry analyses to identify phosphorylation sites of CtBP1 in collaboration with Proteomics Core Laboratory, Ehime Proteo-Medicine Research Center in Ehime University (Japan). However, we failed to detect phosphorylated fragments in this experimental setting (Figure 7 for reviewer 3). Recently, novel phosphorylation sites were identified by mass-spectrometry and published in J. Mol. Biol. (398, 657-671, 2010). Thus, in addition to the phosphorylation sites of 311 and 422 (Figure S3A), we mutated these phosphorylation sites and have performed coimmunoprecipitation assay. Basal interaction between dEF1 and CtBP1 is slightly suppressed by T176 mutation, although FGF-2 still increases the interaction of CtBP1 with dEF1-T176A mutant (Figure 8 for reviewer 3). These findings suggest that basal interaction between dEF1 and CtBP1 is partially dependent on phosphorylation at T176 of CtBP1, and that unidentified phosphorylation site(s) are necessary for FGF-2-dependent complex formation between dEF1 and CtBP1. We do not show the data, but describe this result as “data not shown” (line 13, page 16). We believe that the interaction is partially dependent on phosphorylation of CtBP1.

Figure 7 for reviewer 3



-AP, without treatment with alkaline phosphates
+AP, with treatment with alkaline phosphates

Figure 8 for reviewer 3



9) Figure 4E should include the input amounts of proteins and not just the IP's.

Answer

According to the reviewer's comment, we have performed immunoprecipitation analysis and got almost same results. However, the effect of alkaline phosphatase on the interaction between them *in vitro* was partial, and effect of U0126 was dispensable for the context (similar to the comments 6 and 7 above). Thus, we arranged the former Figure 4E and now present it as supplemental Figure S3B.

10) Figures 5 and 6 are rather contrived assays. Their relevance is predicated on the assumption that tumour-associated fibroblasts arise from untransformed epithelial cells that undergo EMT. The evidence for this is rather flimsy and only two reviews are cited. Simple invasion assays should be performed and Figures 5 and 6 could be moved to supplementary figures, which would reflect their rather contrived nature.

Answer

According to the reviewer's suggestion, the former Figures 6A and 6B are presented as new supplemental Figures S5A and S5B, respectively.

We have performed simple invasion assay using chamber plates (new Figure 5C) and got the results similar to the former Figure 5. The experiments we have performed in the former Figure 5 were originally established in 1981 by Bell (Science, 211, 1052-1054). After modification of strategy by Tsunenaga (Jpn. J. Can. Res. 85, 238-244, 1994), these three dimension assays have been widely-used for investigation on invasion of cancer cells and interaction between fibroblasts and epithelial cells. Recent representative papers are listed as follows, some of which are cited in the main text (bottom line, page12). Therefore, we would like to present Figures 5 A and 5B.

Recent representative references;

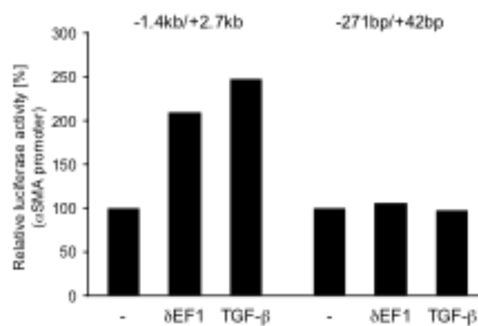
1. Snail-induced down-regulation of DNp63alpha acquires invasive phenotype of human squamous cell carcinoma. Cancer Res. 2007, 67, 9207-9213.
2. Suppression of keratinocyte stratification by a dominant negative JunB mutant without blocking cell proliferation. Genes Cells. 2007, 12, 197-207.
3. Collagen-based co-culture for invasive study on cancer cells-fibroblasts interaction. Biochem Biophys Res Commun. 2006, 346, 268-275.
4. E-cadherin suppression accelerates squamous cell carcinoma progression in three-dimensional, human tissue constructs. Cancer Res. 2005, 65, 1783-1791.

11) Figure S2D is not convincing: faint bands are visible in the IgG control lanes and there is not control for the specificity of the DNA fragments pulled down.

Answer

Based on the report describing the minimal promoter elements (-271bp/+42bp) of SMA responsible for TGF-b induction (Dev, Cell, 11, 93-104, 2006, we generated PCR primers for ChIP analysis and got the results of the former Figure S2D. To confirm the data, we obtained the minimal SMA-reporter construct from Dr. Nishimura's group (Dev. Cell 11, 93-104, 2006). This construct, however, did not work well in NMuMG cells (Figure 7 for the reviewer 3, right). Thus, the primers for ChIP we had designed were not good for ChIP analysis. We prepared another SMA reporter construct (-1.4kb/+2.7kb) by PCR-based strategy. dEF1 induced the promoter activity of this a-SMA-reporter construct to extent similar to that by TGF-b stimulation (Figure 9 for reviewer 3, left). Thus, these findings suggest that dEF1 upregulates the transcription of a-SMA in NMuMG cells. However, we could not design PCR primers for ChIP assay because we have not identified the element responsible for dEF1 and TGF-b in NMuMG cells yet. Thus, we would like to delete the Figure S2D. We hope that the reviewer and editor accept this change. This omitting does not affect our conclusion that "These findings indicate that δEF1 is required for the TGF-β-mediated induction of α-SMA and that FGF-2 reduces α-SMA expression without affecting the levels of δEF1 that are upregulated by TGF-β. Thus, the function of δEF1 is regulated by mechanisms other than those involved in regulating transcription or protein stability."(line 2, page 12 in the former main text and line 1, page 11 in new main text).

Figure 9 for reviewer 3



2nd Editorial Decision 02 November 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-75042R. It has now been seen again by referees 2 and 3; unfortunately referee 1 was unavailable to re-evaluate the study. As you will see, referee 2 is satisfied that you have adequately addressed the concerns raised, but referee 3 - while finding the manuscript to be substantially improved - still highlights a number of remaining issues that would need to be addressed before we could consider publication. While we do usually limit the process to a single round of major revision, we are in this case able to make an exception, since it appears that you do already have most of the data required to address these remaining concerns and/or should be able to conduct the necessary experiments without too much difficulty. Many of the referees' criticisms can be addressed by the inclusion of data presented in your point-by-point response: I would stress that we discourage the referencing of "data not shown" and generally request that authors include all data pertinent to a manuscript as supplementary information. The two points that may require some additional experimental analysis are the related points 5 and 10: further data regarding the function of CtBP and ZEB1 in invasion would be very valuable here. In this context, I would also note that statistical significance should be shown for all relevant data, and the "n" number stated clearly in the figure legends.

I would therefore like to invite you to revise your manuscript according to these remaining concerns of referee. Eventual acceptance of your study is contingent upon your being able to address these criticisms, but I hope that you should be able to do so; please don't hesitate to get in touch if you

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foresee any difficulties here.

I look forward to receiving your revision.

REFEREE REPORTS

Referee #2 (Remarks to the Author):

In the revised version the authors successfully addressed most queries of the reviewers (all of reviewers 1 and 2). Not all proposed experiments suggested by reviewer 3 were done (in particular rev 3, point 1), but I agree to the authors that this would be beyond the scope and focus of this article and should be further addressed in a new manuscript.

Referee #3 (Remarks to the Author):

This manuscript is improved from its original submission. The authors have made a good attempt to address the comments raised. However, there are still some concerns over the relevance of the CtBP + deltaEF1/ZEB1 interaction for cell invasion.

Response to authors' rebuttal

1) The authors need to state clearly that there is a change in the broad transcriptional program, including Snail, Slug and Twist, when TGFb and FGF2 are combined. I was not able to find the comment referred to on line 16, page 18.
2) OK
3) OK
4) siRNA #B shows a rather marginal knock-down. Can the authors provide quantification to demonstrate both the level of knock-down and the reproducibility of the knock-down?
5) The authors have attempted to address the functional significance of the CtBP1 and deltaEF1/ZEB1 in the context of TGFb + FGF2 signalling. However, although they can show altered alphaSMA and calponin levels there is no measurable difference in cell behavior - it is not clear if the data in Figure 3 for Reviewer 3 is statistically significant. This continues to cast doubt on the functional relevance of the regulation of CtBP1 and ZEB1. At the very least the data should be included so that readers can make their own judgement about the functional role of the CtBP1 + ZEB1 complex.
6) OK
7) It is still important to show the effects of UO126 and PD98059 so that the reader can cross-reference this with Supplementary Figure 3a.
8) OK
9) OK
10) The co-culture experiments are still rather contrived. The evidence that cancer-associated fibroblasts really arise from conversion of epithelial cells is rather weak. Furthermore, it is not possible to tell whether the increase invasion results from changes in MCF7, NuMG or both cell types. Figure 5a should be quantified. As stated in point 5, it would greatly improve the manuscript if the effect of CtBP1 and ZEB1 siRNA were thoroughly investigated in the invasion assays.
11) OK

2nd Revision - authors' response 01 December 2010

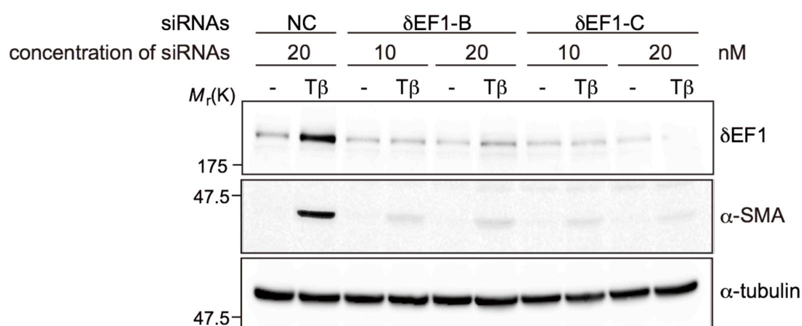
Thank you very much for giving us the opportunity to revise our manuscript again. As recommended by the reviewers and editor, we have modified our manuscript and clearly stated the "n" number in the figure legends. Statistical analyses were performed and indicated in the figures. We now present supplemental Figures S2C, S3E, S3G and S4, which were indicated as "data not shown", and the former Figures 2, 3, 4 and 6 for Reviewer 3 are also presented as supplemental figures S5C, S5D, S5E and S3A, respectively.

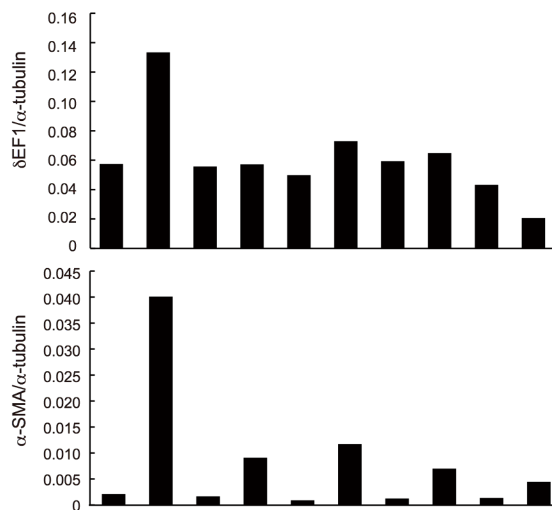
Referee #2 (comments by the reviewer are shown in bold):
In the revised version the authors successfully addressed most queries of the reviewers (all of reviewers 1 and 2). Not all proposed experiments suggested by reviewer 3 were done (in particular rev 3, point 1), but I agree to the authors that this would be beyond the scope and focus of this article and should be further addressed in a new manuscript.
Thank you very much.

Referee #3 (comments by the reviewer are shown in bold):
This manuscript is improved from its original submission. The authors have made a good attempt to address the comments raised. However, there are still some concerns over the relevance of the CtBP + deltaEF1/ZEB1 interaction for cell invasion.
Response to authors' rebuttal
We thank you for acceptance of some of our previous rebuttal. The remaining points have been improved by additional experiments and analyses, which are presented as supplemental figures. We hope that the reviewer will be satisfied with these changes.

1) The authors need to state clearly that there is a change in the broad transcriptional program, including Snail, Slug and Twist, when TGFb and FGF2 are combined. I was not able to find the comment referred to on line 16, page 18.
Reply;
We are sorry not for proper statement of “the broad transcriptional program” in the main text. Based on our results from microarray analyses, we stated that “The expression levels of other EMT regulators, including Snail, Slug and Twist, were also remarkably upregulated during this process (data not shown)” (line 16, page 18 in the former main text). According to the reviewer’s comment, we have decided to confirm the levels of their expressions by quantitative RT-PCR. The expression level of Twist was not significantly increased by TGF-β alone, though it was dramatically enhanced by addition of FGF-2 (Fig. S5A), consistent with the findings from microarray analysis. However, quantitative RT-PCR showed that the induction of Snail and Slug by TGF-β was not markedly enhanced by addition of FGF-2. Therefore, we have changed the statement, as “During this process, FGF-2 altered the broad transcriptional program mediated by TGF-β. Importantly, Twist, a representative of EMT regulators, was included in the program and remarkably upregulated by combination of TGF-β and FGF-2 (Fig. S5A)” (line 10, page 18).

4) siRNA #B shows a rather marginal knock-down. Can the authors provide quantification to demonstrate both the level of knock-down and the reproducibility of the knock-down?
Reply;
According to the comment, quantification of the former Figure 4C has been performed and is now presented as new Figure 4C. In addition, we have confirmed the reproducibility of the experiment and show it with statistical analyses as below.





5) The authors have attempted to address the functional significance of the CtBP1 and deltaEF1/ZEB1 in the context of TGFb + FGF2 signalling. However, although they can show altered alphaSMA and calponin levels there is no measurable difference in cell behavior - it is not clear if the data in Figure 3 for Reviewer 3 is statistically significant. This continues to cast doubt on the functional relevance of the regulation of CtBP1 and ZEB1. At the very least the data should be included so that readers can make their own judgement about the functional role of the CtBP1 + ZEB1 complex.

Reply;

According to the reviewer's comments, we performed statistical analysis of "Figure 3 for reviewer 3" and now present it as supplemental Figure S5D. We state that "silencing CtBP1 in cells treated with FGF-2 and TGF-β affected some properties, including the induction of α-SMA and calponin, cell migration and cell invasion (Figs. 4D, S5C and S5D), while MMP9 activity and E-cadherin expression were not affected by CtBP1 siRNA (Figs. S5E and 4D) (line 15, page 18).

7) It is still important to show the effects of UO126 and PD98059 so that the reader can cross-reference this with Supplementary Figure 3a.

Reply;

We now present it as supplemental Figure S3A.

10) The co-culture experiments are still rather contrived. The evidence that cancer-associated fibroblasts really arise from conversion of epithelial cells is rather weak. Furthermore, it is not possible to tell whether the increase invasion results from changes in MCF7, NuMG or both cell types. Figure 5a should be quantified. As stated in point 5, it would greatly improve the manuscript if the effect of CtBP1 and ZEB1 siRNA were thoroughly investigated in the invasion assays.

Reply;

We have performed the invasion assay of MCF-7 cells in coculture with NMuMG cells transfected with siRNA against either δEF1 or CtBP1 (Fig. S5F). Similar to the finding in Figure S5D, invasive properties were attenuated by CtBP1 siRNA, indicating that CtBP1 in NMuMG cells plays crucial roles in cell migration of NMuMG cells themselves and MCF-7 cells (see line 19, page 18). Thus these findings suggest that both cells cooperatively act for enhancement of collagen gel invasion in the present study. The molecular mechanisms of the enhanced invasion process are still not clear, though we think that they are involved in so-called "collective cancer cell invasion"(see Discussion, page 20).

In addition, as the reviewer commented above, we have not elucidated whether cancer-associated fibroblasts really arise from conversion of epithelial cells. To evaluate this point, specific markers which discriminate fibroblasts (or CAF) in stromal tissue surrounding tumors from fibroblastic cells derived from epithelial cells by EMT, would be absolutely required. Thus, as we described in the former rebuttal (point 3 for reviewer 1 and point 1 for reviewer 3), we are now preparing microarray analyses with combined culture of MCF-7 cells with NMuMG cells in the presence of various combination of ligands. Since we are very much interested in molecular mechanism of enhanced invasion processes and in identification of EMT-derived fibroblastic cells *in vivo*, we will further study them in our future publications.

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Additional Correspondence 02 December 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-75042R1. I have now had the chance to look through it and your point-by-point response to referee 3's remaining concerns. I am pleased to be able to tell you that I am satisfied with your responses, and we will therefore be able to accept your manuscript for publication here. However, there are just a couple of minor text issues that still need to be dealt with.

Firstly, while you do now include data requested by the referee (figure S5), this is only mentioned in the Discussion, and the figure is not referred to at all in the Results section. These are relevant results and should be mentioned in the Results. Secondly, I would ask you to include Author Contributions and Conflict of Interest statements after the Acknowledgements. Finally, we discourage the excessive use of acronyms in titles, and I would therefore suggest you change the title so that it does not use the EMT abbreviation. I would propose the following title: "TGF-beta regulates FGF receptor isoform switching and epithelial-mesenchymal transition".

I suggest that the easiest way forwards at this point would be for you to send me a modified text file, including the changes outlined above, that we can upload in place of the previous version. Once we have this, we should then be able to accept your manuscript for publication without further delay.

Additional Correspondence 09 December 2010

Thank you very much for your positive response to our manuscript. According to your suggestions, we have stated the results of the former Figure S5, which is now presented as Figure S4, in Results section (page 12, and line 14, page 14). Thus, the former Figure S4 is moved to Figure S5.

In addition, we have changed the title to "TGF-b regulates isoform switching of FGF receptors and epithelial-mesenchymal transition", and included the statement of "Author contributions " and "Conflict of interest" after the Acknowledgement (page 26). New Figures S4 and S5, and main text are emailing as attached files.

Thank you very much for your help on our manuscript.

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