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The ZEB1 / miR-200 feedback loop controls Notch signaling in cancer cells

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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 01 September 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, all three express interest in the study and are broadly in favour of publication. However, all three raise a number of concerns that would need to be addressed first. Most critically, both referees 1 and 2 comment on the largely correlative nature of the study, and argue that epistasis analyses would be required to show that the regulation of Jagged1 accounts for the effects of ZEB1 and miR-200 on Notch signalling and on the cellular phenotype (referee 1 paragraph 3, referee 2 point 3). In addition, as highlighted by both referees 2 and 3, clarification and statistical analysis of the data presented in figure 6 would be important.

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.

Best wishes,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Brabletz and colleagues provide molecular evidence for a cancer relevant connection between EMT, stemness and Notch signaling through the miR-200/ZEB1 signaling conduit. Jag1 is identified as a novel miR-200 target and EMT and Notch signaling components positively correlate in two human cancers.

Overall the data are convincing and of high technical quality. However, while suggestive the study suffers from too much correlative data.

Figures 4C and D and 5A, B and C all only show correlations. What needs to be done in each case is to inhibit miR-200 and then knock down Jag1 in order to determine the contribution of Jag1 and Notch signaling to the miR-200 mediated effects.

Proper statistics need to be added to Fig. 4C and Fig. S1E.

Figure 3B: Protein levels of ZEB1 do not change much when compared to those of Jag1. Does this mean that Jag1 is a better target than ZEB1 with its multiple miR-200 seed matches? In addition it is not clear what the bands mean that migrate just below ZEB1. I am not a big fan of developing blots with antibody mixtures if this is what was done here. The nature and origin of all bands needs to be clear.

I am sure since the submission of the manuscript the authors have succeeded in mutating sites 1 and 2 in the Jag1 3'UTR. These data should be added.

The use of different clones in Figs. 2A and 3A should be explained in the figure legends.

Referee #2 (Remarks to the Author):

Comments

Brabletz et al show in this paper a connection between the regulation of EMT by the ZEB1/miR-200 feedback loop and the activity of the Notch pathway. ZEB1, which is known to promote EMT, is shown to be required in breast and pancreatic cancer cell lines for activity of the Notch pathway. The extracellular Notch ligand, Jagged1, is shown to be a target of miR-200 family members, providing one point of intersection between the pathways, although the influence of ZEB1 is fully evident in cells in which the Notch pathway is activated intracellularly, indicating that ZEB1 also connects with the Notch pathway downstream of any Jagged1 effect. The Notch pathway transcription activators Maml2 and Maml3 are suggested as potential intermediates in this further connection between the pathways, but this is not tested experimentally. The authors confirm that Jagged1 is important for Notch pathway effects on cells in vitro and show that Jagged1 level correlates with ZEB1 and EMT in a sampling of basal breast cancers and pancreatic adenocarcinomas.

Effects of the Notch pathway on cancer, on EMT and on stemness, survival and proliferation have been reported previously although the demonstration of a connection between the ZEB1-miR-200

feedback loop and the Notch pathway is novel and important, as is the demonstration that miR-200 targets Jagged1. However, to warrant publication in EMBO Journal some aspects should be strengthened, in particular the evidence that targeting of Jagged1 by miR-200 is responsible for the link between ZEB1 levels and Notch pathway in cancer.

Major points

1. Although there is impressive correlated expression of ZEB1 and Jag1 in the single breast and pancreatic tumors shown in Fig. 6B and 6C, the total number of tumors assessed in Fig 6A is low. A statistical test should be applied to confirm the correlation is statistically significant.

2. The proposal in the paper's title that miR-200 mediates the activation of Notch by ZEB1 should be better supported with miR-200 data in Fig 6. There is no description at all of how miR-200 was measured for the table (Fig 6A), and no description of the criteria for the classification of "reduced miR-200". Inclusion of in situ hybridization for miR-200 in Fig 6B and 6C would add further weight.

3. To demonstrate that the effects of ZEB1 on Notch pathway activity are via effects on miR-200, it should be shown that Notch reporter activity in shZEB1 cells is restored by transfection with miR-200 antagomirs.

3. To determine whether Jagged1 is the major target mediating the effect of ZEB1 knock down on the Notch pathway (via miR-200), it should be demonstrated that pathway activity is rescued by introducing a Jagged1 expression construct devoid of the 3'UTR into shZEB1 cells and is also rescued in cells transfected with miR-200.

4. The last paragraph of the introduction overstates the conclusions, because it appears to claim that ZEB1 can trigger Notch signaling by increasing expression of Maml2 and Maml3 through inhibition of miR-200, but this has not been shown here. Although the targeting of Jagged1 by miR-200 is convincingly demonstrated, most of the effect of ZEB1 on the Notch pathway appears to be downstream of this, because the signaling in cells transfected with Notch ICD is also strongly inhibited by ZEB1 siRNA. Verifying the Notch coactivators Maml2 and/or Maml3 are also targets of miR-200c would provide a mechanistic explanation for this downstream effect and would substantially enhance the paper. This could be done in conjunction with demonstration that knock down of Maml2 and Maml3 also reduces Notch reporter activity and/or Hey1 expression in these cells.
This would allow a more definitive statement in the abstract than the current "miR-200 family members also target Notch pathway components such as Jagged1". The "such as" is without substance and should be removed.

Minor points

1. Comparing luciferase reporter activities measured in different cell lines requires careful normalization to ensure there is no bias from transfection efficiency differences, but there is no mention of normalization. The data in Fig 2 should be accompanied by a description of how this was done.

2. The legend to Fig 5D should state how many tumors were measured to obtain these data.

3. The size bar mentioned in the legend to Fig 6B is missing in the inserts.

Referee #3 (Remarks to the Author):

Brabletz et al

The manuscript identifies Jagged1 as a target of mir200c/141 miRNAs in human cancer cell lines. The manuscript also provides evidence that this regulatory connection may be relevant to cancer metastasis in vivo, since mir200c/141 miRNAs appear to have reduced expression in metastatic breast cancer, while Jagged1 expression is increased (Fig 6a). This is a key result and the authors

must make clear in the both the text and figure legend (1) in which cell lines this experiment was done, (2) how the experiment was done (QPCR?). Ideally, this result would be extended by performing in situ hybridisation (or QPCR) for mir200c/141 on tissue samples from normal and metastatic cancers.

Instead, the authors rely on their previous work, showing that ZEB1 represses expression of mir200c/141, and show that ZEB1 expression correlates with Jagged1 expression by immunohistochemistry in cancer tissue sections. Together with knockdown experiments showing that ZEB1 is required for normal expression of Jagged1 (in Panc1 cancer cell lines in culture and in assays for tumourigenicity in vivo), these results make a reasonable substitute for direct examination of mir200c/141 expression in cancer tissue sections.

Finally, the authors provide some evidence that Jagged1 and Notch signalling might contribute to tumour formation, using colony forming assays. While not as convincing as a mouse knockout experiment deleting Jagged1 in a metastatic tumour model, these data do support the authors' speculation that regulation of Jagged1 by ZEB1 (via mir200c/141) might be an important regulatory network in certain types of metastatic tumour formation in vivo. Thus, the manuscript is suitable for publication in EMBO Journal.

Notes
Throughout the manuscript, the authors deliberately conflate and blurr the lines between human cancer cell lines in culture and human cancers in vivo, equating EMT in vitro with metastasis in vivo, and assuming in vitro results are generally relevant for cancers of different tissue origin vivo. This is a bad habit, and the authors should try to clarify and qualify their descriptions more rigorously.

For example, as far as I am aware, there is zero *in vivo* evidence that ectopic activation of Notch signalling induces, or is required for, EMT or cancer metastasis (rather than tumour growth) in any direct way. The authors seem to imply that Notch signalling drives EMT in vivo, but then cite work that only features experiments in cell culture.

Furthermore, the authors should not leave it to the last line of the discussion to admit that their work is mainly restricted to a few cancer cell lines and does not demonstrate the general physiological importance of this regulatory network in vivo:

"Future work will show if the proposed link between ZEB1, miR-200 and Notch activation is also active in other cancer types and is controlling physiological processes in embryonic development and adult tissue homeostasis as well."

1st Revision - authors' response 23 November 2010

Revision of manuscript EMBOJ-2010-75565

Reply to Reviewer #1:

Thank you for the positive review of our manuscript. Based on the very helpful comments we could improve the manuscript and included additional data, informations and new figures. Please see our point by point reply below your specific comments.

Brabletz and colleagues provide molecular evidence for a cancer relevant connection between EMT, stemness and Notch signaling through the miR-200/ZEB1 signaling conduit. Jag1 is identified as a novel miR-200 target and EMT and Notch signaling components positively correlate in two human cancers.

Overall the data are convincing and of high technical quality. However, while suggestive the study suffers from too much correlative data.

Figures 4C and D and 5A, B and C all only show correlations. What needs to be done in each case

is to inhibit miR-200 and then knock down Jag1 in order to determine the contribution of Jag1 and Notch signaling to the miR-200 mediated effects.

This was a helpful suggestion. We have now done the proposed experiments, however this was not possible in the undifferentiated Panc1 and MDA-MB231 cell lines, since they do not express endogenous miR-200. Instead we used the differentiated HPAF2 pancreatic and MCF7 breast cancer cell lines, which express miR-200. We could show that antagomirs against miR-200 increased the Notch reporter activity and sphere forming capacity, but knockdown of Jag1 could only partially reduce antagomir-induced Notch reporter activation (new Fig. 4F and G, suppl. Fig. 2B. There was no significant effect on proliferation in these undiffentiated cell lines (new Fig. 4H and suppl. Fig. 2C). The partial rescue of Notch activity was also confirmed by a reverse experiment, where overexpression of Jag1 lacking its 3íUTR could not fully rescue Notch reporter activitity suppressed by miR-200 or ZEB1 knockdown in the undifferentiated cancer cell line Panc1 (new Fig. 4E).
Together this data indicated that Jag1 is not contributing alone to the effect of ZEB1 and miR-200 on Notch signaling. We further investigated other potential miR-200 targets which could in particular explain the inhibitory effect of a ZEB1 knockdown also on reporter assays after overexpression of NICD (new Fig. 4 I). This lead to the identification of the Notch co-activators Maml2 and Maml3 as additional targets of miR-200 (new Fig. 5A-I), and further shows that miR-200 affects Notch signaling at different levels and not only by inhibiting Jag1 expression.

Proper statistics need to be added to Fig. 4C and Fig. S1E.
Standard deviations and p-values were now calculated and included (now new Fig. 3C, 4H, suppl. Fig. 1E and 2C).

Figure 3B: Protein levels of ZEB1 do not change much when compared to those of Jag1. Does this mean that Jag1 is a better target than ZEB1 with its multiple miR-200 seed matches? In addition it is not clear what the bands mean that migrate just below ZEB1. I am not a big fan of developing blots with antibody mixtures if this is what was done here. The nature and origin of all bands needs to be clear.
Indeed ZEB1 was not always reduced after stable overexpression of miR-200 members in Panc1 cells. An explanation for miR-141 could be, that ZEB1 is not a good target for this miRNA. A reduction by miR-200c was indeed only seen for one of two clones. We have no explanation in the moment, why Jag1 is reduced in both miR-200c, clones but ZEB1 only in one, and will try to clarify it in further work. The band below ZEB1 is a degradation product, which is seen relatively often for this large 180kd protein. ZEB1 and Jag1 were not developed in one antibody mixture, since both have similar size (180kd vs. 170 kd). We agree that the initial picture panel gave this implication and now separated both blots again.

I am sure since the submission of the manuscript the authors have succeeded in mutating sites 1 and 2 in the Jag1 3'UTR. These data should be added.
We now succeeded in constructing compound mutants including mutants for all predicted binding sites. Subsequent mutation of all bindings sites steadily increased the reporter activity in differentiated HPAF2 cell, expressing endogenous miR-200 family members, indicating that all putative binding sites are functional (new Fig. 2F).

The use of different clones in Figs. 2A and 3A should be explained in the figure legends.
This is now explained in the figure legends (now new Fig. 1F and 2A).

Revision of manuscript EMBOJ-2010-75565

Reply to Reviewer #2:

Thank you for the positive review of our manuscript. Based on the very helpful comments we could improve the manuscript and included additional data, informations and new figures. Please see our point by point reply below your specific comments.

Brabletz et al show in this paper a connection between the regulation of EMT by the ZEB1/miR-200

feedback loop and the activity of the Notch pathway. ZEB1, which is known to promote EMT, is shown to be required in breast and pancreatic cancer cell lines for activity of the Notch pathway. The extracellular Notch ligand, Jagged1, is shown to be a target of miR-200 family members, providing one point of intersection between the pathways, although the influence of ZEB1 is fully evident in cells in which the Notch pathway is activated intracellularly, indicating that ZEB1 also connects with the Notch pathway downstream of any Jagged1 effect. The Notch pathway transcription activators Maml2 and Maml3 are suggested as potential intermediates in this further connection between the pathways, but this is not tested experimentally. The authors confirm that Jagged1 is important for Notch pathway effects on cells in vitro and show that Jagged1 level correlates with ZEB1 and EMT in a sampl ing of basal breast cancers and pancreatic adenocarcinomas.

Effects of the Notch pathway on cancer, on EMT and on stemness, survival and proliferation have been reported previously although the demonstration of a connection between the ZEB1-miR-200 feedback loop and the Notch pathway is novel and important, as is the demonstration that miR-200 targets Jagged1. However, to warrant publication in EMBO Journal some aspects should be strengthened, in particular the evidence that targeting of Jagged1 by miR-200 is responsible for the link between ZEB1 levels and Notch pathway in cancer.

Major points

1. Although there is impressive correlated expression of ZEB1 and Jag1 in the single breast and pancreatic tumors shown in Fig. 6B and 6C, the total number of tumors assessed in Fig 6A is low. A statistical test should be applied to confirm the correlation is statistically significant.
We agree that the number of cases in this initial study is low, and now performed a two-sided Fishers exact test, which confirmed significant association of ZEB1 and Jag1 expression in basal type of breast cancers and undifferentiated pancreatic cancers (new suppl. Fig. 2D). This will be confirmed on lager tumor collection in future work.

2. The proposal in the paper's title that miR-200 mediates the activation of Notch by ZEB1 should be better supported with miR-200 data in Fig 6. There is no description at all of how miR-200 was measured for the table (Fig 6A), and no description of the criteria for the classification of "reduced miR-200". Inclusion of in situ hybridization for miR-200 in Fig 6B and 6C would add further weight.
Since we were not able to establish an in situ hybridisation for miR-141 and miR-200c, we performed a quantitative qPCR after microdissection of tumor areas and isolation of small RNAs from paraffin-embedded tumor sections. We were able to determine miRNA expression in all eight available basal type of breast cancers and in five of the undifferentiated pancreatic cancers. Comparison of undifferentiated types of human cancers (basal type of breast cancers and undifferentiated pancreatic adenocarcinomas) to differentiated (ductual invasive) breast cancer and pancreatic cancers, as well as to corresponding normal breast and pancreatic epithelia, revealed a highly significant reduction of miR-141/200c and increase of ZEB1 expression in the undifferentiated cancers (new Fig. 6A).

3a. To demonstrate that the effects of ZEB1 on Notch pathway activity are via effects on miR-200, it should be shown that Notch reporter activity in shZEB1 cells is restored by transfection with miR-200 antagomirs.
We have done the suggested experiments, which demonstrated that miR-200 antagomirs largely (but not fully) restored decreased Notch pathway avtivity in shZEB1 cells (new Fig. 1G).

3b. To determine whether Jagged1 is the major target mediating the effect of ZEB1 knock down on the Notch pathway (via miR-200), it should be demonstrated that pathway activity is rescued by introducing a Jagged1 expression construct devoid of the 3'UTR into shZEB1 cells and is also rescued in cells transfected with miR-200.

We fully agree that (together with the next point , Maml2 as miR-200 target) this was a valid and helpful suggestion. We have now done the suggested experiments, and demonstrated that overexpression of Jag1 lacking its 3iUTR could not fully rescue Notch reporter activitity, which was suppressed by miR-200 or ZEB1 knockdown in the undifferentiated cancer cell line Panc1 (new Fig. 4E). We confirmed this result by a reverse experiment in differentiated HPAF2 pancreatic and MCF7 breast cancer cells, expressing endogenous miR-200. We could show that antagomirs against

miR-200 increased the Notch reporter activity and sphere forming capacity, but knockdown of Jag1 could only partially reduce antagomir-induced Notch reporter activation (new Fig. 4F and G, suppl. Fig. 2B. Together this data indicated that Jag1 is not solely responsible for the effects of ZEB1 and miR-200 on Notch signaling.
Also in this light your suggestion to further investigate Maml2 and Maml3 as potential miR-200 targets was very straight forward (see your next point).

4. The last paragraph of the introduction overstates the conclusions, because it appears to claim that ZEB1 can trigger Notch signaling by increasing expression of Maml2 and Maml3 through inhibition of miR-200, but this has not been shown here. Although the targeting of Jagged1 by miR-200 is convincingly demonstrated, most of the effect of ZEB1 on the Notch pathway appears to be downstream of this, because the signaling in cells transfected with Notch ICD is also strongly inhibited by ZEB1 siRNA. Verifying the Notch coactivators Maml2 and/or Maml3 are also targets of miR-200c would provide a mechanistic explanation for this downstream effect and would substantially enhance the paper. This could be done in conjunction with demonstration that knock down of Maml2 and Maml3 also reduces Notch reporter activity and/or Hey1 expression in these cells.
This would allow a more definitive statement in the abstract than the current "miR-200 family members also target Notch pathway components such as Jagged1". The "such as" is without substance and should be removed.
Thank you for encouraging us to determine the role of Jag1 in mediating the miR-200 effect on Notch activation (your previous point) and to validate Maml2 and Maml3 as Notch target. This was done by showing that miR-200 overexpression reduces expression of Maml2 and to a lesser extend Maml3 (new Fig. 5 B-D), as well as the activity of Maml2- and Maml3-3íUTR driven reporter constructs (new Fig. 5 E). Moreover, as suggested we showed that knockdown of Maml2 and Maml2 affected Notch reporter activity (new Fig. 5F) and expression of the Notch target Hey1 (new Fig. 5 G and H), as well as the capacity of the undifferentiated cancer cell line Panc1 to form spheres in a ëcancer stem cellí-sphere assay (new Fig. 5I).
Together with the new results answering your previous point, these data show that miR-200 affects Notch signaling at different levels and not only by inhibiting Jag1 expression. We are convinced that, based on your suggestions, these new data improved the relevance of our manuscript.

Minor points

1. Comparing luciferase reporter activities measured in different cell lines requires careful normalization to ensure there is no bias from transfection efficiency differences, but there is no mention of normalization. The data in Fig 2 should be accompanied by a description of how this was done.
The descriptions of how we normalized luciferase activities to cotransfected values of a pCMV-Renilla construct is now included in the Material and Methods section and valid for all shown reporter assays (see “Cell culture and various assays”).

2. The legend to Fig 5D should state how many tumors were measured to obtain these data.
Numbers are now included in the figure legend (new Fig. 4D).

3. The size bar mentioned in the legend to Fig 6B is missing in the inserts.
Size bar is now included.

Revision of manuscript EMBOJ-2010-75565

Reply to Reviewer #3:
Thank you for the positive review of our manuscript. Based on the very helpful comments we rewrote the manuscript and included additional data, informations and new figures. Please see our point by point reply below your specific comments.

The manuscript identifies Jagged1 as a target of mir200c/141 miRNAs in human cancer cell lines. The manuscript also provides evidence that this regulatory connection may be relevant to cancer metastasis in vivo, since mir200c/141 miRNAs appear to have reduced expression in metastatic

breast cancer, while Jagged1 expression is increased (Fig 6a). This is a key result and the authors must make clear in the both the text and figure legend (1) in which cell lines this experiment was done, (2) how the experiment was done (QPCR?). Ideally, this result would be extended by performing in situ hybridisation (or QPCR) for mir200c/141 on tissue samples from normal and metastatic cancers.
Instead, the authors rely on their previous work, showing that ZEB1 represses expression of mir200c/141, and show that ZEB1 expression correlates with Jagged1 expression by immunohistochemistry in cancer tissue sections. Together with knockdown experiments showing that ZEB1 is required for normal expression of Jagged1 (in Panc1 cancer cell lines in culture and in assays for tumourigenicity in vivo), these results make a reasonable substitute for direct examination of mir200c/141 expression in cancer tissue sections.

We are sorry for the misleading explanation of Fig. 6A, and thank the reviewer to alert us to the pontential of misunderstanding. The correlation of ZEB1 and Jag1 expression was not analysed in metastatic breast cancer or cell lines, but in undifferentiated, aggressive types of human cancers (basal type of breast cancers and undifferentiated pancreatic adenocarcinomas), as detected by immunohistochemistry. This was now better explained in the figure legend (new suppl. Fig. 2D).
As suggested by the reviewer we now additionally analysed miR-141 and miR-200c expression in human cancers, comparing undifferentiated, aggressive vs. differentiated types of cancers. We performed a quantitative PCR after microdissection of tumor areas and isolation of small RNAs from paraffin-embedded tumor sections. We were able to determine miRNA expression in all eight available basal type of breast cancers and in five of the undifferentiated pancreatic cancers. Comparison of undifferentiated types of human cancers (basal type of breast cancers and undifferentiated pancreatic adenocarcinomas) to differentiated (ductual invasive) breast cancer and pancreatic cancers, as well as to corresponding normal breast and pancreatic epithelia, revealed a highly significant reduction of miR-141/200c and increase of ZEB1 expression in the undifferentiated cancers (new Fig. 6A).

Finally, the authors provide some evidence that Jagged1 and Notch signalling might contribute to tumour formation, using colony forming assays. While not as convincing as a mouse knockout experiment deleting Jagged1 in a metastatic tumour model, these data do support the authors' speculation that regulation of Jagged1 by ZEB1 (via mir200c/141) might be an important regulatory network in certain types of metastatic tumour formation in vivo. Thus, the manuscript is suitable for publication in EMBO Journal.

Notes
Throughout the manuscript, the authors deliberately conflate and blurr the lines between human cancer cell lines in culture and human cancers in vivo, equating EMT in vitro with metastasis in vivo, and assuming in vitro results are generally relevant for cancers of different tissue origin vivo. This is a bad habit, and the authors should try to clarify and qualify their descriptions more rigorously.
Thank you for alerting us to potential incorrect and misleading descriptions (see also above our statement to your critics on Fig. 6A of the first manuscript). We carefully corrected such passages in the new manuscript.

*For example, as far as I am aware, there is zero *in vivo* evidence that ectopic activation of Notch signalling induces, or is required for, EMT or cancer metastasis (rather than tumour growth) in any direct way. The authors seem to imply that Notch signalling drives EMT in vivo, but then cite work that only features experiments in cell culture.*
It was never our intention to directly link Notch signaling to cancer metastasis. Also we agree that till now there is no evidence that Notch drives EMT in vivo. Wherever our manuscript was misleading to such a direct association, we carefully rewrote it to avoid any misunderstanding.

Furthermore, the authors should not leave it to the last line of the discussion to admit that their work is mainly restricted to a few cancer cell lines and does not demonstrate the general physiological importance of this regulatory network in vivo:

"Future work will show if the proposed link between ZEB1, miR-200 and Notch activation is also active in other cancer types and is controlling physiological processes in embryonic development and adult tissue homeostasis as well."

As suggested we moved this issue to the paragraph describing the results for human breast and pancreatic cancers in the discussion section.

Additional Correspondence 03 December 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-75565R. It has now been seen again by referees 1 and 3, who are happy with the revision and do not request any further changes before publication (their brief comments are enclosed below). I am therefore pleased to tell you that we will be able to accept your manuscript for publication here at EMBOJ.

I have just a couple of things from the editorial side. Firstly, please can I ask you for an author contributions statement? If you could just send us an updated version of the text including this, we can replace the previous version in our system.

Secondly, we ask that the Supplementary Information is all contained in a single file, rather than as multiple separate files. We combined all your supplementary information into one PDF (attached), but I would like to check with you that you are happy with this; if not, please can you send us a Supplementary Information file that you would like to be the published version.

Many thanks and best wishes,

REFEREE REPORTS

Referee 1:

The authors have addressed all concerns by the three reviewers and have improved the manuscript to my satisfaction.

Referee 3:

The manuscript is much improved and I recommend publication.

