Author’s response to reviews

Title: IncompleteDll4/Notch Signaling Inhibition Promotes Functional Angiogenesis Supporting the Growth of Skin Papillomas

Authors:

Dusan Djokovic (dusan.d.djokovic@gmail.com)
Alexandre Trindade (alex.in.trindade@gmail.com)
Joana Gigante (joana.qi@gmail.com)
Mario Pinho (mpinho@fmv.ulisboa.pt)
Adrian L Harris (adrian.haris@oncology.oxford.ac.uk)
Antonio Duarte (aduarte@fmv.utl.pt)

Version: 3
Date: 26 July 2015

Author’s response to reviews: see over
To Dr Daphne Solera  
Executive Editor of BMC Cancer  

Lisbon, 26th July 2015  

Dear Dr Solera,  

we enclose a revised version of our manuscript “Incomplete Dll4/Notch Signaling Inhibition Promotes Functional Angiogenesis Supporting the Growth of Skin Papillomas” for your consideration.  

Following your suggestion we worked to comply with the reviewers requests, changing parts of the text and in some cases carrying out further experiments. Our responses to the reviewer’s are enclosed.  

We would like to thank you for the opportunity to resubmit our manuscript and for the time allowed to carry out further experiments to address the reviewers comments.  

Yours sincerely,  

[Signature]
**Responses to the Reviewers**

**Reviewer 1**

**Major Compulsory Revisions:**

1. The Dll4 +/- tumor angiogenic phenotype was described as “pronounced branching and thin interconnections (Line 249)”. The increased vascular density is clear in Dll4 +/- tumors, but the latter description is not demonstrated in the images presented in Figure 2A. Either replace image to be more representative or adjust the text so that it is data not shown. Similarly, it is stated that the Dll4 tumors vessels have “reduced luminal diameters”. However this data is not presented.

   **Response:** We adjusted the text accordingly.

2. Although there is an increase in VEGFR2/VEGFR1 ratio in circulation in Dll4 +/- tumor baring mice, it is unclear why levels of both circulating VEGFR1 and VEGFR2 levels are down. If Dll4/Notch signaling in endothelial cells induces VEGFR1 and suppresses VEGFR2 expression, then circulating VEGFR2 levels should be increased in Dll4 +/- and not decreased. Moreover, it is unclear how this data relates to specifically to the tumor vasculature as the mouse is globally heterozygous and all vessels are missing a copy of Dll4. Finally, it is unclear whether reduced circulating VEGFR1 levels are due to a decrease in endothelial expression of full-length VEGFR1 or sVEGFR-1 (soluble-Flt1). This is particularly important as Jagged1/Notch signaling has been shown to down-regulate sVEGFR-1 (Kangsamaksin, Cancer Discovery 2015). Staining of the tumor tissues for endothelial VEGFR2 or VEGFR1 expression would help to clarify these concerns.

   **Response:** We performed VEGFR2 staining of WT and Dll4 +/- tumors and found that it is significantly increased in Dll4 +/- papillomas in comparison with the WT lesions. The information was included in modified Figure 3 (panel B) and modified section “Dll4 down-regulation increases VEGF function in the skin papillomas “ (the 2nd paragraph).

3. The observation that PDGFR-beta levels go up in Dll4 +/- tumors is counterintuitive. PDGFR-beta is expressed in the aSMA+ VSMCs that are reduced in Dll4 +/- tumors. Is this tumor expression of PDGFR-beta that is affected? Staining of tumor tissues would address this concern. It would be more appropriate to look at PDGF-B transcript levels as it is expressed in endothelial cells. A reduction of PDGF-B levels would be consistent with the VSMC phenotype observed.

   **Response:** We performed tumor PDGFR-beta immunostaining (Figure 3. D) and modified the section “Dll4 deletion affects the expression of factors regulating perivascular cell recruitment in chemically-induced skin papillomas”.

4. Missing comment / There is not comment no. 4.

5. The authors infer a lot from reduced tumor volume in the sorafenib studies. As the focus is on the effects of Dll4 +/- on tumor vessels, tumor sections should have been characterized for the vascular phenotype.

   **Response:** Vascular response was examined by double PECAM/α-SMA immunostaining indicating reduced sprouting and recruitment of perivascular cells in sorafenib-treated vs. vehicle-treated WT mice, as well as enhanced endothelial proliferation, however, with increasingly impaired vessel wall assembly in sorafenib-treated Dll4 +/- vs. WT mice (Figure 4. C; modified section “Partially inhibited Dll4/Notch signaling decreases the tumor suppressive effect of sorafenib on chemically-induced skin tumors”).
Minor Compulsory Revisions:

1. Alpha smooth muscle actin recognizes vascular smooth muscle cells and not pericytes that are aSMA negative. Please correct in the text: line 131.
   
   Response: We corrected accordingly.

2. Line 274: Typo: “VGFR2/VGFR1”
   
   Response: Corrected.

3. Figure 1A, upper left panel x-axis labels are cut off. This may be due to PDF rendering, but should be verified.
   
   Response: Yes, this was due to PDF rendering and is correct in the original image.

4. Figure 1B is a bit dark and should be lightened.
   
   Response: Corrected.

5. Figure 1C is described thoroughly in the text of the results (Lines 229-232), but it is unclear from the image and the figure legend how this translates. The image should be labeled and figure legend expanded to help the reader.
   
   Response: We corrected and expanded the legend as suggested.

6. Figure 3A: No statistics are provided for this averaged data from sera analysis. Thus it is unclear what changes in expression are significant. Changes VEGF-A levels were not significant, whereas the decrease in circulating VEGFR1 and VEGFR2 is significant?
   
   Response: The error bars have been provided (please, amplify this vectorial image). ”**” has been also provided where the statistical significance exists (Figure 3. A, down right). VRGF-A, VRGFR1 (R1) and VRGFR2 (R2) level differences are not significant while R2/R1 differs significantly between the two experimental groups. We emphasized this in the legend.

7. Figure 3B: What do the asterisks represent? This is not addressed in the figure legend. Labels in the figure should match the text of the figure legend, “WT vs Dll4+/+”.
   
   Response: The asterisks represent the level of statistical significance between 2 tumor sets. We mentioned that in the Material and Methods – Statistical Analysis: “All results are presented as mean ± SEM. P-values < 0.05 and <0.01 were considered significant (indicated in the figures with *) and highly significant (indicated with **), respectively.”

8. Figure 4B bottom panel: The bars marking the significance are too crowded and an asterisk is missing.
   
   Response: We corrected accordingly.

Reviewer 2

1. The question on how those chemically induced papillomas are related to human tumours, especially H Ras mutated lesions, like in Costello syndrome and epidermal nevi.
2. It is not stated that DMBA alone can cause angiomatic lesions, and it is not stated how many vascular lesions, if any, in parallel to papilloma, were induced in mutant mice.

Response: We included this information (section “Dll4 allelic deletion promotes the growth of induced skin papillomas”).

3. Considering the effect of sorafenib, it could be also speculated that it is not only VEGF signalling specific.

Response: We mentioned this in the section “Partially inhibited Dll4/Notch signaling decreases the tumor suppressive effect of sorafenib on chemically-induced skin tumors”.