Author's response to reviews

Title: Changes in Claudin tight junction protein distribution patterns in the progression of mouse skin tumorigenesis

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Author's response to reviews: see over
Dear Dr. Le Good:

RE: Changes in the distribution pattern of Claudin tight junction proteins during the progression of mouse skin tumorigenesis by Azadeh Arabzadeh, Tammy-Claire Troy and Kursad Turksen.

Thank you for handling this manuscript for us (2763970041454058). We appreciate the invaluable and positive comments of the four Reviewers. We have incorporated nearly all of their requested revisions, and are very pleased to forward it to you today. We hope that you will now find our manuscript acceptable for publication in *BMC Cancer*.

We have addressed the Reviewers’ comments using colored text. Please find below specific comments to each Reviewer’s suggestions:

**Reviewer (Michael H Koval)**

Major Compulsory Revisions:
1) As suggested by Dr Koval, we have tried to make our figures more easily interpretable to readers outside of the skin field. Rather than using double-labeling for this, which in this case would not be feasible for every marker, we have marked the epidermal basal layer and the suprabasal compartment on representative panels of each figure in this revision.
2) Dr Koval is correct in pointing out that our immunohistochemical analysis does not address whether Cldn localization is really cytoplasmic or rather localized to an intracellular membranous compartment (like the Golgi or endosomes). We have therefore re-worded our revised manuscript to describe Cldns as “not associated with cell membranes” rather than being “cytoplasmic”.
3) Although we agree with Dr. Koval that an immunoblot analysis of Cldn expression would improve this study, unfortunately we face technical roadblocks. With the exception of Cldn1, our custom-made Cldn antibodies (Cldn6, Cldn11, Cldn12 and Cldn18) do not work for immunoblot analysis. This is something we are working on, but will not be able to address in a timely manner to include in this revision.

Minor Essential Revisions:
1) As suggested, we have re-labeled the figures to make the typeset bigger and we have also marked the epidermal basal and suprabasal compartments as described above. We agree that this will make our revised manuscript more reader-friendly.

Discretionary Revisions:
1) In our revised manuscript, we have included Dr Koval’s suggested possibility that an inhibition of a post-translational insertion into the ER may be responsible for the lack of Cldn localization to the cell membrane.

**Reviewer (Zsuzsa Schaff)**

Major Compulsory Revisions:
1) In our laboratory we routinely use Bouin’s solution for tissue fixation prior to paraffin processing. However in our experience, our Cldn1 antibody is not best-suited for Bouin’s fixed tissue. Alternatively, we have found that our custom generated antibodies against Cldn6, Cldn11,
Cldn12 and Cldn18 work extremely well on Bouin’s fixed tissues, and in fact look the same as they do on frozen sections. Therefore in response to Dr. Schaff’s suggestion we have described this in the Methods section of our revised manuscript.

2) We do not believe that the “shift” of Cldn localization away from the cell membrane observed is in result of tangential sectioning in more advanced samples, nor thickening or folding of the epidermis. The observations reported in this manuscript are highly reproducible using many different sections obtained from different animals undergoing the same treatments at different times. Therefore in response to Dr. Schaff’s comment, we have included an explanation of our sampling size in the Methods section of our revised manuscript.

Minor Essential Revisions:
1) In response to Dr. Schaff’s suggestion, in this revised manuscript we have re-worded our description of the “downregulated expression” of different Cldns. We indeed used “downregulated” to refer to either a less-intense staining or a reduction in the number of epithelial cell layers where localization is observed.
2) As suggested, we have added reference to city and country for materials listed in the Materials section of our revised manuscript.
3) Each of our figure panels has a scale bar on it (marking 20 µm, or 1 cm). In response to Dr. Schaff’s comment, we have replaced Figure 4a in this revised manuscript with a larger image in order to provide a better demonstration of the basal to suprabasal localization of Cldn1.

Discretionary Revisions:
Dr. Scaff had no comments of this type to be addressed.

Reviewer (Karen Swisshelm)
Major Compulsory Revisions:
1) In response to Dr. Swisshelm’s comments, we have included an explanation of our sampling size in the Methods section of our revised manuscript. We have analysed many different biopsies of each animal, three animals for each time point in three different experimental repeats. Therefore for each time point, we have assayed 9 different mice using many different biopsy sites from each animal.
2) As requested by Dr. Swisshelm, we have added information on our commercially available and custom generated antibodies in this revised manuscript.

Minor Essential Revisions:
1) As suggested by Dr. Swisshelm, we have better described Figure 1F as a photograph of the dorsal view of the DMBA/TPA-treated mouse; in addition we have added a 1 cm scale bar.
2) Each of our figure panels has a scale bar on it marking 20µm. In response to Dr. Swisshelm’s comment, we have included arrows and stars on Figure 3 to point out non-membranous Cldn localization and Cldn-null epidermal cells in our revised manuscript.

Discretionary Revisions:
Dr. Swisshelm did not have any comments of this type indicated.

Reviewer (Hideki Chiba)
Major Compulsory Revisions:
1) We have re-structured the abstract in our revised manuscript as requested by Dr. Chiba.
2) To address the concerns of Dr. Chiba, we have omitted panel a of Figure 1 and replaced it with a new panel showing a vehicle-treated control (12 weeks). Also, we have clarified the description of our histological samples in our revised manuscript.
3) In our revised manuscript, we have clarified the confusion describing Figure 2. Indeed, the Figure describes 2, 6 and 12 weeks of TPA treatment, and the text was confusing this description. In addition, in response to Dr. Chiba’s comments, we have included panels showing the vehicle-
treated epidermis at each time point. We have also clarified our description of this figure within the revised manuscript.

Minor Essential Revisions:
1) In response to Dr. Chiba’s suggestion, we have revised the title of our manuscript such that there is no longer so many nouns arranged in a row.
2) We have clarified lines 9-11 on page 3 of our revised manuscript as per Dr. Chiba’s comments.
3) We have included a space before the units on page 4.
4) As pointed out by Dr. Chiba, we have mentioned the observation of pseudohorn cysts in the epidermis after 6, 8 and 12 weeks of treatment in the revised manuscript.
5) As described above, we have replaced Figure 4a in our revised manuscript in order to show a better representation of ClDN1 associated to the basal and suprabasal epidermal compartments.
6) Although we agree with Dr. Chiba that a description of ClDN4 after exposure to chemical carcinogens would be of great interest, our ClDN4 antibody (Panomics; cat. # E3984) does not work on either Bouin’s fixed or frozen sections (although it was raised against human ClDN4, and it apparently cross-reacts with mouse). As indeed ClDN4 has been documented to be expressed in the adult epidermis, we did not include our data in this report. Although it would be quite feasible for us to purchase another antibody, we will not be able to complete the necessary experiments in a timely manner for the data to be included in this revision.

Discretionary Revisions:
1) In response to Dr. Chiba’s comment, we have had our manuscript edited for stylistic problems. We hope that Dr. Chiba will now agree that our revised manuscript reads much better than our previous submission.

I look forward to hearing from you.
Sincerely,
Kursad

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