Author's response to reviews

Title: Ephrin-A1 Inhibits NSCLC Tumor Growth via Induction of Cdx-2 a Tumor Suppressor Gene

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Author's response to reviews: see over
We thank the editors for giving an opportunity to revise our manuscript. We have fully addressed all the reviewer’s comments in the revised manuscript. We have included new additional data from four additional NSCLC cell lines to validate our key finding in this study. The new data is incorporated into the text of the revised manuscript. As result the old Figure -1 now appears as Figure-2 in the revised manuscript and the old Figure 2 had been incorporated as Panel E into the Figure-2 in the revised manuscript. The detailed response to reviewer’s comments is as follows:

**Reviewer:** Dr. Tommaso Dragani

We thank Dr. Dragani for his valuable comments and suggestions on our manuscript. We have addressed the comments and all the changes are incorporated in the text of revised manuscript.

1. **Comment:** The manuscript of Sukka-Ganesh et al., describes results of an in vitro study that has been carried out in a single cell line (A549) on Ephrin-A1 effects. The authors over-expressed Ephrin-A1 by transfection of a recombinant vector and over-expressed and silenced EphA2, which is the primary Ephrin-A1 receptor. They found that EphA2 activation by ephrin-A1 induces tumor suppressor gene cdx-2, which attenuates tumor cell proliferation. Overall, the study is well conducted and the results support the conclusion. A limitation of the study is represented by the single cancer cell line that has been used.

**Response:** We concur with the reviewer that the studies have been carried out in one cell line A549. We have now added four NSCLC cell lines to demonstrate the key data in our manuscript. The new data appears as Figure 1 panel A-C. The results are discussed on page 10, and paragraph 1 in the revised manuscript.

2. **Comment:** Minor points: p.6, last line: 100 nM, not 100nm.

**Response:** We regret the error. Now we have corrected the 100 nm to 100 nM and it appears on page 6, paragraph 1 and line 6 in the revised manuscript.

**Reviewer:** Dr. Tyson Sharp

We thank Dr. Sharp for finding our manuscript “well written, concise clearly defined hypothesis.” and for the valuable suggestions on our manuscript. We have addressed the comments and all the changes are incorporated in the text of revised manuscript.

**Minor Essential Revisions:**

1. **Comments:** This is a well written and concise study with a clearly defined hypothesis that is addressed well and with some nice (if not a little standard) techniques. There are a few minor errors: Page 7 line 6 ‘80ml’ is an error.

**Response:** We regret the error. We have now corrected the error and it appears as 80 microliters (µl) on page 7, and line 6 in the revised manuscript.

2. **Comment:** Page 7 line 14 reads ‘and the at acquired’ this is clearly wrong and needs a re-write.

**Response:** We fully concur with the reviewer. This error has been corrected and it appears on page 7, line 14 in the revised manuscript.
3. **Comment:** Page 12 the last two lines of first paragraphs on this page I think is not a true statement. The authors have not depleted cdx-2 in this study and thus to state that depletion results in an increase of EphA2 is incorrect.

**Response:** We concur with the reviewer and now we have modified this statement and it appears on page 12, paragraph 1, and lines 12-14 in the revised manuscript.

4. **Comment:** Also throughout the manuscript the authors refer to NSCLC cells or NSCLC cell lines. This again is misleading as they have only used A549 in this study. Thus they should re-write and state such. For example ‘cdx-2 expression was lost/unregulated in the A549 NSCLC cell line.’

**Response:** We agree with reviewer. We have now included data from four additional NSCLC cell lines supporting key findings of this study and rephrased the statements in the revised manuscript.

**Reviewer:** Dr. Christine Sers

We thank Dr. Sers for her insightful comments and valuable suggestions on our manuscript. We have addressed all the comments and all changes are incorporated in the text of revised manuscript.

1. **Comment:** The manuscript of Bhagyalaxmi A. et al describes EphA2 receptor (EphA2) activation through the ligand EphrinA1 (EFNA1), a subsequent induction of cdx-2, a tumor suppressor gene, which finally leads to decreased cell proliferation and tumor growth via suppression of the tight junction protein Claudin-2. In general, the manuscript is weak with regard to the content. The authors describe a potential role for EphA2 signaling in lung malignancies, yet the complete manuscript deals with just one single cell line and nothing has been tested in other NSCLC cells.

**Response:** We fully concur with the reviewer. We have now added four additional NSCLC cell lines to validate the key findings of our study. The expression of receptor EphA2 and Claudin-2 is now shown in five NSCLC cell lines and it appears as Figure 1, Panel-A in the revised manuscript. The new data has been discussed under results section and it appears on page 10, paragraph 2 and line 1 to 4 in the revised manuscript.

2. **Comment:** EphA2 signaling is described to play a major role, however this is not shown, because no phosphorylation of the receptor or signaling molecules has been tested as an indicator of pathway activation.

**Response:** We agree and thank the reviewer for this valuable suggestion. Now we have demonstrated that the activation of receptor EphA2 with its ligand EphrinA1 leads to phosphorylation of EphA2 and it appears as Figure 1C in the revised manuscript. In addition, we have also measured the downstream signaling MAP kinase erk1/erk2 phosphorylation in A549 cells. Furthermore, we have tested the activation of receptor EphA2 by transfection of NSCLC cells with vector containing EphrinA1 construct (pcDNA-EFNA1) in all the five cell lines which inhibited the expression of receptor EphA2 compared to empty vector. This new data appears
3. **Comment:** What is the functional relationship between cdx and Claudin-2? EphA2 signaling is likely to affect a whole lot of genes, why should cdx be the one-and-only Claudin-2 master regulator?

**Response:** It is true that EphA2 signaling affects several genes. However, in the present study experiments were designed to study if EphA2 signaling affects claudin-2 expression and promotes NSCLC growth. Claudins form an important component of Tight Junctions (TJ) proteins and play a key role in variety of pathological conditions including tumorogenesis. In addition, claudin-2 is modulated by transcription factors including caudal-related homeobox gene-2 (cdx-2). Cdx-2 is positively involved in the regulation of the human claudin-2 promoter activity [Sakaguchi et. al.; The Journal of biological chemistry 2002, 277(24):21361-21370]. Therefore EphA2 signaling may have caused reduced expression of transcription factor cdx-2 that hinder its binding to claudin-2 promoter and thus cause dysregulated expression of claudin-2 which is reported to be increased in NSCLC cells in the present study. We hope this response clarifies the comment.

4. **Comment:** In general the manuscript lacks several controls, e.g. there is no Western blot or ELISA for EFNA1 overexpression is shown. In addition, there is no control for all of the siRNA effects. Several figures are documented quite poorly and don’t show scientific standards. For example, Figure 1D shows a cut-out lane for EphA2. The blot is very hard to judge. I don’t see an overexpression of EphA2.

**Response:** We regret for not making it clear in our manuscript. Now we have revised the Figures to show the controls for the siRNA. The old Figure 1, panel D has been revised to show the bands clearly and now it appears as Figure 2, panel D in the revised manuscript.

5. **Comment:** On the protein level the Western Blot for Claudin (which is nice) does not provide any difference between the control and the EphA2 transfected cells; obviously this only works at the mRNA level, however the authors don’t comment on this.

**Response:** Now we have discussed this in the revised manuscript and it appears on page 12; paragraph 2, and lines 3 to 7.

6. **Comment:** Paragraph 1 and 2 should be combined; they describe the Figure 1 in a lengthy manner. In Figure 3C the Y-axis does not contain a legend. What do we see there? If it is optical density, what is it normalized against? Most of the (control) blots are heavily overloaded and individual lanes cannot be judged reasonably.

**Response:** We regret the error. We have now reduced the description for old Figure 1, which now appears as Figure 2 in the revised manuscript. The y-axis legend has been added to the Figure 3 panel C in the revised manuscript. We have used 20 µg of protein per lane and normalized the data with β-actin that was detected to demonstrate equal sample loading.
7. **Comment:** In Figure 2B the downregulation cannot be seen due to the scale of the X-axis. Could it be that Figure 1 and Figure 2 are very similar? Just the one is a Western blot and the other is immunofluorescence. What kind of additional information do we get from Figure 2?

**Response:** We regret for not discussing the data clearly. We have confirmed the expression of claudin-2 by immunofluorescence analysis in addition to Western blot analysis. Since claudin-2 is a TJ protein, immunofluorescence microscopy will allow analyzing the cellular distribution/localization upon various treatments. Furthermore, it showed the localization of protein and cytoskeletal changes in activated cells. The old Figure 2 now appears as Figure 2 panel E in the revised manuscript. This is addressed under results section on page 12, paragraph 2, and lines 3 to 7 in the revised manuscript.

8. **Comment:** In Figure 5C the authors shown no significant increase in proliferation upon siRNA of cdx2 following treatment with EFNA1 ligand or transfection with EFNA1 vector. In the result chapter, the author describe that there is a significant increase. This discordance should be clarified.

**Response:** We regret for the confusion. We have revised Figure 5 panel C. This has been discussed under results section and it appears on page 14, paragraph 2, and lines 7 to 14 in the revised manuscript.

9. **Comment:** Further, proliferation of cells with cdx over expression and cxd-2 siRNA should be shown to analyze, whether proliferation of cells depends directly on the cdx tumor suppressor gene activated via EFNA1.

**Response:** We have now added the new data to show the effect of cdx2-siRNA on NSCLC proliferation. It appears in Figure 5, panel C in the revised manuscript.

10. **Comment:** In Figure 6 letters are missing which was indicated in the text. The methods are not described in detail, but the authors describe the same for EphA2 and EFNA1 vector design and transfection in two paragraphs. This should be combined.

**Response:** No we have corrected these errors. We regret for not describing the methods in detail. We have now revised the methods. The method for vector design has been combined in the revised manuscript as suggested. This appears on pages 5 and 6, in the revised manuscript.

11. **Comment:** In general, there are a numerous spelling and grammar mistakes and also the format of the manuscript is not acceptable. For example, it is spelled claudin not caludin or in Figure 2 it is EFNA1 not EFA1. It would be helpful to use a word processing program.

**Response:** We regret the errors. We have now corrected these errors in the revised manuscript and the entire document is now corrected using word processing program.

12. **Comment:** In the discussion there is even a reference lacking (insert reference), maybe the authors submitted a preliminary version?

**Response:** We apologize for this error due to our oversight. We have now inserted the reference and it appears as reference 28 on page 17, paragraph 1, and line 3 in the revised manuscript.