Author’s response to reviews

Title: A Plausible Role for Actin Gamma Smooth Muscle 2 (ACTG2) in Small Intestinal Neuroendocrine Tumorigenesis

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Replay to Reviewer:

1. Many details are missing from Materials and Methods section. Either more details or reference to previous publication should be provided for Immunohistochemistry and immunofluorescence methodologies. The time, conditions and concentration of antibodies should be indicated. It should be stated which secondary antibodies have been used and manufacturers for all reagents and antibodies provided.

    - Detailed information about immunohistochemistry procedure are now referred to references 25 and 26, line 104. More detailed information has been added about immunofluorescence procedure, headline “Immunofluorescence” line 115 and forward.

2. The authors state that 'successful DNase I treatment of all RNA preparations was recognized by RNA template in the qRT-PCR'. What do they mean? The only way to ensure successful DNase I treatment is to show lack of amplification after additional RNase treatment.

    - This sentence has been clarified. Line 139: “Successful DNase I treatment of all RNA preparations was established by PCR analysis of the MYC promoter.”
3. In the drug treatment experiments, the manufacturers of compounds should be indicated as well as solvents used. How did the authors choose the concentration of drugs? Were the EC50s established for CNDT2.5 cells? Since 5-aza-dC and EPZ-6438 did not have any effect on ACTG2 expression the authors should use a control to be able to show that the drugs worked as expected. Ideally, they would be able to show a decrease in global DNA methylation after 5-aza-dC treatment (if the cells are slowly dividing than 72h might not be enough to achieve enough demethylation) and decrease in global H3K27 methylation after EPZ-6438 treatment. These data shouldn't be difficult to collect but if the authors are not able to provide them then the conclusions should be modified accordingly taking into account that the desired effect of the drugs was not confirmed.

- The manufacturers of compounds and solvents have been added, line 150 and 151.

The concentrations of the drugs were chosen after titration. This has been added to the manuscript, line 148: “CNDT2.5 cells were seeded onto 6 well plates and treated with increasing concentrations of 5-aza-dC (5-aza-2’-deoxycytidine) (0.025, 0.5, 1.0, 1.25, 1.5 µM) and DZNep (3-deazaneplanocin A) (2.5, 5.0, 10.0, 12.5, 15 µM). The non-toxic concentrations 1 µM for 5-aza-dC and 10 µM DZNep were chosen for further experiments.”

To achieve the effect of 5-aza-dC only freshly prepared drug was used. This has been clarified in line 151; “Freshly prepared 5-aza-dC was used in the experiments.”

Following has been added to the results of the manuscript, Line 226: “It should be noted that positive controls for treatments with 5-aza-dC and EPZ-6438 were not included here.

4. The colony formation and viability assays were performed with ACTG2-expression vector. It should be stated which vector. Since the authors use anti-DDK (anti-FLAG) antibody it must have been introduced to the insert or tagged vector had been used. Any modifications to the insert or the vector should be described. One can only assume that ACTG2-expression vector is also a pcDNA3.1 but since the authors do not state it, it is possible another type of vector was used to express ACTG2 which could affect the results and conclusion from proliferation and viability assays.

- Vector information has been added in Line 176; “The ACTG2 expression vector consisted of an expression-validated cDNA clone (TrueORF Gold, catalog no. RC203151. Origene Technologies, Inc., Rockville, MD, USA).”
5. In the first results section the authors say that 'eight primary tumours and two lymph node metastases displayed positive staining for ACTG2 in chromogranin-positive cells' while in the second section the analysis 'did not reveal staining of both ACTG2 and chromogranin A in the same cell'. Could the authors explain the discrepancy? Perhaps the first conclusion should be modified. Also, in the second case it is not clear what tissue was used. As the authors look for the expression in enterochromaffin cells from which the tumours originate, shouldn't normal intestinal mucosa be examined here?

- The headline has been clarified, line 208: “ACTG2 protein is not detected in enterochromaffin cells of the normal small intestine” and also clarified in the text, line 209; “In order to determine whether ACTG2 is expressed in chromogranin-positive cells of the normal intestinal mucosa (enterochromaffin cells)…”

6. It appears either stroma or tumour cells can express ACTG2 in a fraction of tumours, therefore the origins of the cells expressing ACTG2 are different. Could the authors comment on it?

- This has been commented; line 262 “Expression of ACTG2 was detected in stromal cells and whether ACTG2 can display growth effects here remains to be investigated.”

7. The western blotting results shown in Fig 1E should show a bigger section of the blot and include protein ladder. The lanes should be labelled on the figure.

- We have now included a protein ladder and clarified the fact that only one band was detected (ACTG2) in the Western blot analysis.

8. Fig. 3B shows ACTG expression after EPZ-6438 treatment. Which EPZ-6438 concentration was used in this experiment?

- The concentration of EPZ-6438 was 1.0 μM. This has been added to the legend of Figure 3.
9. In both results and discussion section the authors comment on miR-145 being significantly less expressed in the metastasis compared to primary tumours however Fig5A indicates this to be true only for liver metastasis but not for lymph node metastasis. Further on, it is stated 'comparing primary tumours with their respective lymph node metastasis showed lower levels in lymph node metastases in five out of six pairs'. These data are not shown at the moment but should be included in the figure together with paired statistical analysis.

- In Figure 5A there is a higher p-value for the comparison between primary tumors and liver metastases but the p-value is 0.01 when comparing primary tumors to lymph node metastases.

The comparison between matched pair was unfortunately wrong, and this has been removed from the manuscript.

10. Figure 5B based on qPCR data carried out on the current cohort or the one included in the previous publication (Edfeldt et al, 2011)? If it's based on the current cohort, the data should be included in the Supplementary Table 1. Was there a correlation between ACTG qPCR data and immunohistochemistry data?

- The qPCR data is based on previously published data, this has been clarified in the text, line 237 “In line with these results, previously published experiments have shown significantly reduced expression of ACTG2 mRNA in lymph node metastases compared to primary tumors (Figure 5B) [10]”.

Six of the stained tumours were included in the quantitative RNA analysis, but no clear relation between ACTG2 protein and mRNA expression was evident. This could be due to the fact that immunohistochemistry is not quantitative.
11. The last sentence in the conclusion section needs revising.

- This sentence has been revised: Line 292; “Further investigations are needed to determine whether introducing miR-145 to SI-NET cells could have therapeutic advantages.”

Ethics:

If your study involves humans, human data or animals, then your article should contain an ethics statement which includes the name of the committee that approved your study.

If ethics was not required for your study, then this should be clearly stated and a rationale provided.

Consent:

If your article is a prospective study involving human participants then your article should include a statement detailing consent for participation.

If individual clinical data is presented in your article, then you must clarify whether consent for publication of these data was obtained.

- This is stated in line 89: “This study was approved by the regional ethical review board in Uppsala (11-375/1.1.2011, Regionala etikprövningsnämnden i Uppsala). Written informed consent for participation and publication of individual clinical details was obtained from all patients.”
Availability of supporting data:

BioMed Central strongly encourages all data sets on which the conclusions of the paper rely be either deposited in publicly available repositories (where available and appropriate) or presented in the main papers or additional supporting files, in machine-readable format whenever possible. Authors must include an Availability of Data and Materials section in their article detailing where the data supporting their findings can be found. The Accession Numbers of any nucleic acid sequences, protein sequences or atomic coordinates cited in the manuscript must be provided and include the corresponding database name.

Authors Contributions:

Your 'Authors Contributions' section must detail the individual contribution for each individual author listed on your manuscript.

- Authors contributions are described; line 302.