**Author’s response to reviews**

**Title:** The oncogenic effects of HES1 on salivary adenoid cystic carcinoma cell growth and metastasis

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**Version:** 1  **Date:** 19 Dec 2017

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Response to reviewers

Zhengyu Zha, ph.D (Reviewer 1):

In this manuscript, Xiao-Yu Huang and his/her colleagues reported that HES1, which contributes to SACC proliferation, apoptosis and metastasis, is a specific downstream gene of NOTCH1. The authors showed the systematic mechanism and function assay to prove their conclusions. Overall, the study is interesting and the data is solid and impressive. Here are some minor questions:

1. Fig1, A, B, C&D could move to supplemental data.
Response: Thank you for your suggestion. We have moved Fig1, A, B, C&D to the supplemental data (Fig.S1).

2. Fig 1E &1F, please show the clear and enlarged figures.

Response: Thank you for your suggestion. We have enlarged the Fig 1E&1F to make Fig.1 clearer.

3. Fig1I, the WB figures are too narrow.

Response: Thank you for your suggestion. We have reselected a clearer WB image to present in Fig1.

4. Fig5A, please quantify the differences.

Response: Thank you for your suggestion. In Fig5, we have quantified the width of injury line and compared the differences among the groups.

Vladislav Korobeynikov, M.D. (Reviewer 2):

In this article the authors describe a role of HES1 in regulating proliferation, migration and apoptosis in salivary adenoid cystic carcinoma (SACC). In general, the manuscript is well written but it raises several concerns that should be addressed prior to publication.
Major concerns:

1. The authors do not make it clear what cell lines for which experiments they used. The Materials and Methods section includes two SACC cell lines, however, the figures apparently show the sets of experiments from only one cell line. Could you please specify which cell lines was used for which sets of experiments? In addition, in order to make any statements about the mechanism a full set of experiments in at least two independent cell lines is required.

Response: Thank you for your suggestion. The SACC LM cell line was used in vivo and vitro and we have specified the name of cell line used in every set of experiments in the paper. We know normally all the experiments should be performed in at least two independent cell lines in cancer research. But SACC is an uncommon cancer, and 6 SACC cell lines (ACC2, ACC3, ACCM, ACCNS, ACCS, CAC2) were found to be cross-contaminated and misidentified (Phuchareon J, et al. Plos One. 2009; 4: e6040) and now there are not good cell lines available for us.

2. Elevated levels of full length caspases (figure 3F) does not necessarily mean an increase in apoptosis. The authors should provide a Western blot image with bands for cleaved species of caspases 3 and 9 and report a ratio of cleaved to full-length forms.

Response: Thank you for your suggestion. Actually, we showed the cleaved bands in our previous version. According to the reviewer’s comments, we showed the Western blot images with full-length and cleaved species of caspases 3 and 9 in Fig.3. But the bands of full-length of CASP3 and CASP9 are too weak to get reliable quantitative data, so we still used the quantification of the cleaved bands to show the data in Fig 3.

3. If the authors speculate that HES1 affects cell growth they should provide a data of cell-cycle assay or similar method showing different distribution of cell cycle phases in cells upon HES1 knockdown.
Response: Thank you for your suggestion. We have applied the PI staining flow cytometry cycle tests to explore whether HES1 knockdown affects the distribution of cell cycle phases. The results didn’t show consistent trend and there were not significant difference upon the siRNAs transfection. So we put the results in Supplementary Fig.S2.

4. The authors used siRNA knockdown for xenografts study with an endpoint at 18 days after inoculation. siRNA knockdown is not stable and lasts on average for around 5-7 days depending on the cell type. Taking this in consideration, the authors should provide IHC images of tumors stained for HES1 and/or Western blot from tumor lysate to compare the levels of HES1 at the endpoint.

Response: Thank you for your suggestion. We have added the IHC images of tumors stained for HES1 and also provided the percentages of positive cells in different groups (Fig.4D), which proves that the siRNA transfection is stable and reliable in our xenografts study.

5. In the sentence in page 16 line 2 the authors state "...reductions in HES1 expression in SACC cells could induce cellular apoptosis to inhibit cell growth." This is misleading. Induction of apoptosis will result in inhibition of tumor growth, not necessarily cell growth (proliferation). If the authors state that HES1 knockdown also affects cell proliferation in vivo they should provide a confirmation to this hypothesis such as IHC images of tumors stained for Ki67 and calculate proliferative index.

Response: Thank you for your suggestion. We have added the IHC images of tumors stained for Ki67 and also calculated its proliferative index (Fig.4D), which proves that HES1 knockdown also affects cell proliferation in vivo.

6. The authors' data suggests that HES1 knockdown affects cell migration. Could that be observed in vivo? Do the mice injected with HES1 knockdown cells develop less metastatic lesions than the mice injected with the control cells?
Response: Thank you for your suggestion. We already checked the lymph nodes and other tissues for metastasis in the end of experiments but we can’t find any metastatic lesions. We think it may due to the fast growing of the xenograft tumors and the short period of the experiments.

Minor concerns:

1. The article contains orthographic and grammatical errors that, however, do not significantly affect the clarity of the text. Several examples are provided below.

a. Page 6 line 1 "…pathway that could active HES1."

Response: Thank you for your suggestion. We have used “activate” to replace active.

b. Page 13 lines 10-13 contain a confusing sentence.

Response: Thank you for your suggestion. We have changed this sentence to “CASP9 can process and activate CASP3”.

c. The sentence on page 18 line 13 is confusing.

Response: Thank you for your suggestion. We have changed this sentence to “Furthermore, we tested the expression of HES1 by western blot analysis (Fig.1E), and the SACC cells transfected with NOTCH1 overexpressed plasmid displayed a higher expression of HES1 compared with control group”.
2. Table 1 is a bit misleading - it contains two sets of sequences per single siRNA. Could you please clarify what exactly what used in the study?

Response: Thank you for your suggestion. In Table 1, we showed the sequence of siRNAs both sense and antisense. We added the label in Table 1 for easier understanding.

3. Some links to the figures in the text seem to be incorrect. For example, page 13 line 10 has a link to figure 2I although it apparently should be 1I, page 14 line 10 should have a link to figure 2B instead of 2A.

Response: Thank you for your suggestion. We have corrected the mistakes about the links you mentioned above.

4. All Western blot images lack indication of molecular weights of the bands and quantification.

Response: Thank you for your suggestion. We have added the indication of molecular weights of the markers and quantified the bands for all the Western blot images.