**Author’s response to reviews**

**Title:** A novel long non-coding RNA from the HOXA6-HOXA5 locus facilitates colon cancer cell growth

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Point-by-point responses to the reviewer’s comments

Reviewer reports:

Anurag Singh, Ph.D. (Reviewer 1): This paper by Saijo et al. describes the identification and characterization of a novel long non-coding RNA (lncRNA), which is transcribed as a short RNA isoform for the HOXA5/HOXA6 loci. While HOXA5 has been implicated as a potential tumor suppressor gene, the results presented here support the conclusion that the HOXA5-short lncRNA can promote cell proliferation and tumor growth. Overall, the studies are very well conducted and the major conclusions of the paper are well supported by the data. However, some concerns should be addressed before the paper is acceptable for publication:

1. In Fig. 1A, HOXA5 mRNA levels are shown in RNAi-transfected cells. HOXA5 protein levels should also be shown.

   **Reply:** As suggested by the reviewer 1, HOXA5 protein levels in the siRNAs-treated cells have been shown in Figure 1B. We also mentioned this result in this revised manuscript. (page 13, line 239)

2. The identification of HOXA5 transcripts was performed in HCT116 cells. It is unclear how HOXA5-short RNA abundance in HCT116 compares to the other colon cancer cell lines (DLD-1 and HT29), as well to normal colonic epithelial cells. The authors should show relative HOXA5-short RNA expression levels in the various cell lines.
Reply: Thank you for your comment. According to the reviewer’s suggestion, we have analyzed expression of HOXA5-short RNA in colorectal cancer cell lines (HCT116, DLD1 and HT-29) as well as normal colonic epithelial cells (HCEC-1CT). As shown in new Figure 5A, HOXA5 short RNA is abundantly expressed in cancer cell lines, especially HCT116 cells, compared to normal colonic epithelial cells. This new result is more informative than the previous data (comparison between lung normal and cancer cells). We have added this new obtained data in Figure 5A. According to this change, we have mentioned this finding in this revised manuscript. (page 20, line 374-376)

3. In Fig. 4C, the authors state that increased phospho-EGFR is associated with increased total EGFR protein levels. This is not the case as shown in the figure. The text should be changed accordingly.

Reply: As suggested by the reviewer, we have corrected the statement about this observation. (page 20, line 366-367)

4. The Discussion section is too long. Results are presented again, which is unnecessary. Description of results should be removed from the Discussion.

Reply: Thank you for your suggestion, and we accepted it. We have removed several descriptions of results from the Discussion. We have tried to shorten the Discussion section; however, we had to add several important discussions for responses to the following comments 5 and 6. We are sorry.

5. HOXA5-short causes increased EGFR phosphorylation but the mechanistic consequences of this are not addressed. How are downstream signaling pathways impacted? Are there changes in ERK and AKT activation? Do these pathways show up in the IPA analysis?

Reply: Thank you for the comment, and we agreed with the reviewer’s criticism. As suggested by the reviewer, we have assessed ERK and AKT activation by western blotting (Figure S7). Under the current experimental condition, HOXA5 short RNA dramatically altered neither ERK nor AKT activation in the HCT116, DLD1 and HT-29 cells. We also reanalyzed altered expression genes among HOXA5 short overexpressed and knockdown cells using the IPA software. However, IPA showed no activation of ERK or AKT signaling at the mRNA level. Further studies are needed to reveal the mechanistic consequence of EGFR phosphorylation promoted by HOXA5 short RNA. We have discussed this unrevealed issue in the Discussion. (page 24, line 446-451)

6. Fig. 4B shows that the estrogen receptor (ESR1) and beta-estradiol pathways are upregulated in HOXA5-short expressing cells. The ESR1 pathway is not mentioned or discussed. Could these pathways contribute to the proliferative and tumorigenic effects of HOXA5-short?
Reply: As pointed out by the reviewer, we should consider and discuss the possibility of involving ESR1 pathway in the proliferative and tumorigenic effects of HOXA5-short RNA. As shown in Fig. 4B, IPA predicted upregulation of both ESR1 (estradiol receptor alpha) and estradiol, which acts as a ligand of ESR1. In general, ESR1 increases cellular proliferation in tumor cell lines, such as lung, prostate and breast cancer cells. However, ESR1 function in colon cancer has not been elucidated, because ESR1 expression was limited in normal and malignant colonic epithelium. We have added this issue in the Discussion. (page 24, line 451-455)

Sriram Seshadri, Ph. D. (Reviewer 2): The authors have structured the manuscript appropriately with all relevant controls and justifying their hypothesis and objectives.

Reply: Thank you for the review of our manuscript, and for your encouragement.