December 30, 2014
Xiao-Fan Wang
Associate Editor
The Journal of Biological Chemistry

RE: 2014/610915

Dear Dr. Wang:

Thank you very much for reviewing our manuscript. We also greatly appreciate the reviewers for their complimentary comments and suggestions. We have carried out the experiments that the reviewers suggested and revised the manuscript accordingly

Please find attached a point-by-point response to reviewer's concerns. We hope that you find our responses satisfactory and that the manuscript is now acceptable for publication.

Sincerely,

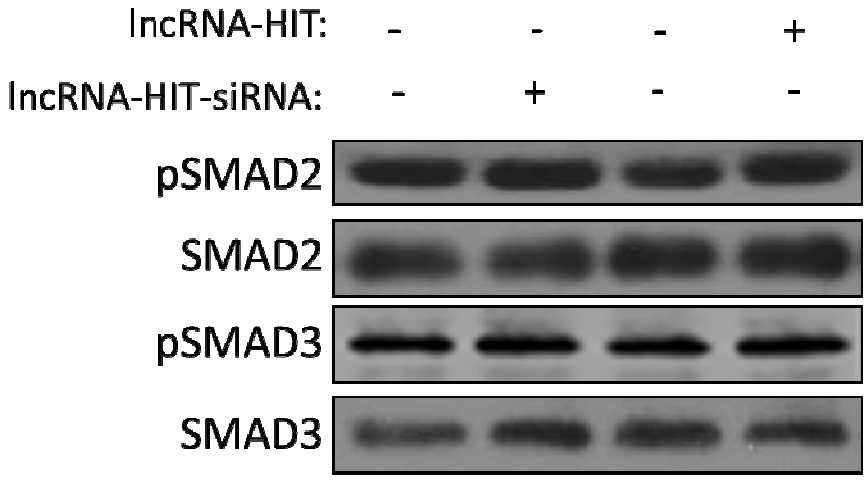
Jin Q. Cheng, Ph.D., M.D.
Professor
Department of Molecular Oncology
H. Lee Moffitt Cancer Center & Research Institute
Tampa, FL 33612
Email: jin.cheng@moffitt.org

Reviewer 1

We appreciate that the reviewer's comments. The followings are our point-by-point responses:

1) Does lncRNA-HIT affect TGFβ-induced Smad phosphorylation or cytostasis?

Response: As suggested by the reviewer, we have performed Western blot and found that knockdown or enforced expression in NMuMG of lncRNA-HIT didn't change pSMAD2/3 levels.



2) Does lncRNA-HIT affect expression of EMT-inducing transcription factors. Snail, ZEB1, SIP1 and Twist in 4T1 cells and TGF-beta-treated NMuMG cells?

Response: We have carried out Western blot and Affymetrix gene expression array analysis with lncRNA-HIT expressing and vector treated NMuMG cells. E-cadherin, but not Snail, ZEB1, SIP1 and Twist, was significantly increased upon knockdown of lncRNA-HIT. In addition expression of lncRNA-HIT reduces E-cadherin expression but not EMT-inducing transcription factors (see Figure 6).

3) Is overexpression of lncRNA-HIT sufficient for inducing EMT phenotypes including enhanced cell motility and invasion?

Response: As suggested by the reviewer, we transfected lncRNA-HIT into NMuMG cells and observed that ectopic expression of lncRNA-HIT induces cell migration and invasion (see Fig. 6).

Other specific points:
4) A recent paper by Yuan et al. (Cancer Cell 25, 666-681, 2014) reported lncRNA with a similar function in hepatocellular carcinoma. This paper should be cited and discussed.

Response: We agree with the reviewer and have discussed this publication in the manuscript.

5) Figure 4C: How could the authors exclude the possibility of genomic contamination? An RNA that is localized in the cytoplasm should be used as
control.

Response: As suggested by the reviewer, we have included PAI-1 and U6, which were amplified with primers crossing 2 exons, as controls (see Fig. 4C).

6) Page 2, Methods, invasion and migration assay: 4T1 is described only in the
first sentence.

Response: We have added information of cell lines used in this manuscript including 4T1 cells in **EXPERIMENTAL PROCEDURES**

Reviewer 2

We appreciate that the reviewer's comments. The followings are our point-by-point responses:

1) They must show how lncRNA-HIT promotes EMT and cell motility at molecular level. Does this lncRNA affect transcription of certain target gene because it is localized in nucleus based on their results? If so, they must find a target molecule(s).

Response: We agreed with the reviewer and have carried out Affymetrix gene expression array analysis with lncRNA-HIT expressing and vector transfected NMuMG cells. Using 3 fold as cutoff, 483 and 160 genes are downregulated and upregulated upon expression of lncRNA-HIT, respectively. E-cadherin is one of the most dowregualated genes. Previous studies have shown that knockdown of E-cadherin in mammary epithelial cells induced EMT, indicating that downregulation of E-cadherin is not only a marker but also a driver of EMT. We have confirmed our microarray finding on E-cadherin and have also shown that expression of E-cadherin largely abrogated lncRNA-HIT induced cell migration, invasion and tight junction disruption. These findings indicate that E-cadherin is a key target gene of lncRNA-HIT (see Fig. 6).

2) The highest expression of lncRNA-HIT can be detected in 4T1 cells among cells examined. Does knocked-down of lncRNA-HIT in 4T1 cells decrease metastasis in the xenograft model and anchor independent growth in in vitro model?

Response: As suggested by the reviewer, we have performed the orthotopic model which shows that knockdown of lncRNA-HIT significantly reduces lung metastasis and tumor growth in 4T1 cells as well as colony growth (see Fig. 7 E-G).

3) How about overexpression of lncRNA-HIT?

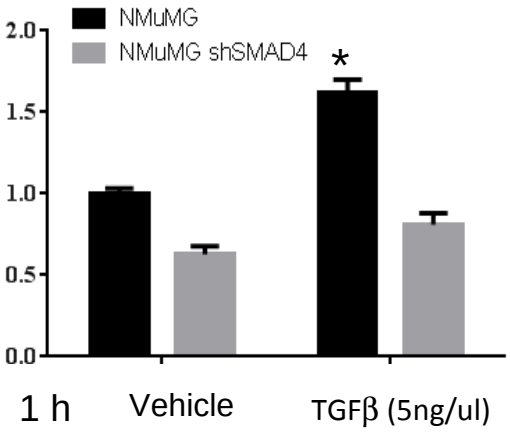
Response: As suggested by the reviewer, we have transfected lncRNA-HIT in NMuMG cells and examined its effect on EMT, cell migration and invasion (see Fig. 6).

4) Is lncRNA-HIT a direct target gene for TGF-beta? How about BMP

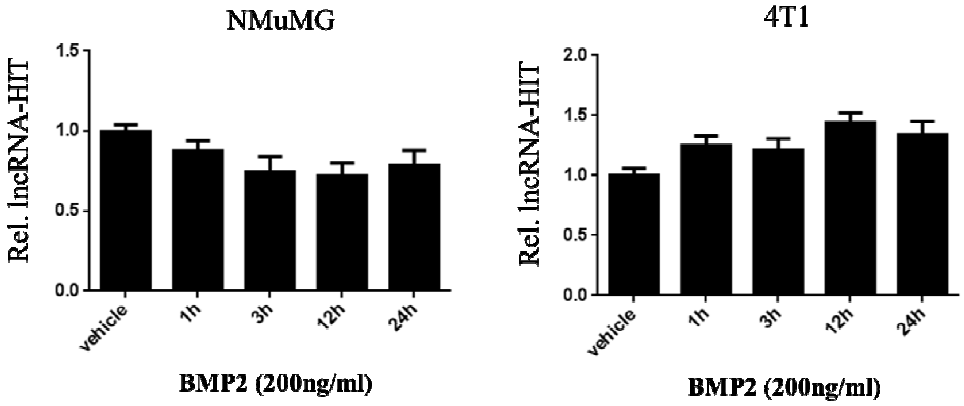
signaling?

Response:

We have treated parental and stable knockdown SMAD4-NMuMG cell line with TGF-beta for 1 hour. Induction of lncRNA-HIT is observed within 1 hour of TGF-beta treatment in the parental line, however this induction is abrogated in the SMAD4 deficient line. This result suggests lncRNA is a direct target of TGF-beta pathway.



We have evaluated the effect of BMP on lncRNA-HIT expression and found that BMP had not significant effects on lncRNA-HIT expression in both NMuMG and 4T1 cells.



5) They have to show time course experiments in Fig. 4B.

Response: As suggested by the reviewer, we have repeated the experiment and have shown the time course of TGFb induction of lncRNA-HIT (see Fig. 4B)

6) In Fig. 4C, they only show the expression of lncRNA-HIT in nucleus.
This reviewer is wondering if RNA separation was succeeded. Can they show localization of Smad7, PAI-1 or JunB mRNA together with lncRNA-HIT as a control?

Response: We agreed with the reviewer and have provided PA1 and U6 RNAs as controls (see Fig. 4C).

7) In each siRNA, the absence of TGF-beta is required in Fig. 5A-E.

Response: As suggested by the reviewer, we have provided absence TGF-beta for each siRNA

8) In 4T1 cells, is lncRNA-HIT induced by TGF-beta? In Fig. 6 C & D, the presence of TGF-beta must be required.

Response: We did not observe induction of lncRNA-HIT by TGF-beta in 4T1 cells while TGF-beta enhances 4T1 cell migration and invasion (*Int J Cancer.* 91:76-82, 2001). Knockdown of lncRNA-HIT did not affect TGFb-induced 4T1 cell migration and invasion.