Author’s response to reviews

Title: Ras induces experimental lung metastasis through up-regulation of RbAp46 to suppress RECK promoter activity

Authors:

Hsuan-Heng Yeh (s5889102@gmail.com)
Yu-Fen Tseng (hen@ms8.url.com.tw)
Yu-Chiao Hsu (yuchiao.katie@gmail.com)
Giri Raghavaraju (giriraghavaraju@gmail.com)
Da-En Cheng (doglying@gmail.com)
Ying-Ray Lee (yingray.lee@gmail.com)
Tsuey-Yu Chang (z7608045@email.ncku.edu.tw)
Nan-Haw Chow (chownh@mail.ncku.edu.tw)
Wen-Chun Hung (hung1228@nhri.org.tw)
Hsiao-Sheng Liu (a713@mail.ncku.edu.tw)

Version: 2 Date: 16 December 2014

Author’s response to reviews: see over
Dear Editor:

We complied reviewers’ comments and accordingly revised the manuscript "Ras induces experimental lung metastasis through up-regulation of RbAp46 to suppress RECK promoter activity" (Manuscript ID: 8204935171395144) including the complete set of revised figures. In this revised manuscript all the changes are marked in red. We are grateful for the opportunity to revise and resubmit our manuscript.

The revised manuscript with a point-by-point response to the reviewer's comments identical to the printed manuscript is also enclosed on a separate sheet.

We believe that you and reviewers will now find our work worthy of publication in *BMC Cancer*.

Sincerely yours,

Hsiao-Sheng Liu, Ph. D.
Professor
Corresponding author
Department of Microbiology and Immunology
National Cheng Kung University
Tainan, Taiwan, R. O. C.
FAX: -886-6-2082705
Tel: -886-6-2353535, Ext. 5630
e-mail: a713@mail.ncku.edu.tw
Point-to-point response
Reviewer: Makoto Noda

Major points:
In this paper, evidence to support the following events or interpretations are presented:

a. Expression of RbAp46 is positively regulated by RAS signaling.
b. RbAp46 suppresses RECK promoter activity through Sp1 site.
c. RAS-signaling downregulates RECK, and a part of this effect is mediated by RbAp46.
d. RbAp46 can form complexes with HDAC1 as well as Sp1.
e. RAS-signaling uses RbAp46 to recruit HDAC1 to, and promote binding with, the Sp1 protein bound to the Sp1 site on an oligo-nucleotide.
f. Sp1, RbAp46, and HDAC1 are associated with a region (of 261 bp) surrounding the RECK transcriptional start site.
g. RbAp46 is important for RAS-induced tumor cell invasion and experimental metastasis, which is accompanied by increased MMP-9 activity.
h. RECK expression is low in human bladder tumor tissues exhibiting increased RbAp46 expression.

I find over-interpretations in the following two cases:

Question (1) The authors’ interpretation: When RbAp46 is upregulated by mutant RAS, a complex containing RbAp46, HDAC1, and Sp1 are assembled and then binds to the Sp1 site of the RECK promoter. This interpretation has two problems:

i. The data presented do not demonstrate a complex containing these three factors.
   What they demonstrate is one-to-one binding in two combinations: between RbAp46 and HDAC1, between RbAp46 and Sp1.
   ii. Fig. 3C suggest constitutive binding of Sp1 to the Sp1 site.

Response: Thanks for the reviewer’s comment. The term of “RbAp46-HDAC-Sp1 complex” has been modified throughout the manuscript. The statement that “Sp1 is not regulated by Ras as well as RbAp46 and may function as a docking protein for HDAC1-RbAp46 complex formation” was described. (page 21, line 2-4). All the changes were also labeled in red in the revised manuscript.

Question (2) The following three, independent findings are presented:

a. MMP-9 activity was suppressed by shRas and shRbAp46 (Fig. 4C).
b. Both shRNA inhibited cell invasion in vitro (Fig. 4A).
c. shRbAp46 inhibited tumor metastasis in vivo (Fig. 4E, F).

Based on these results, the authors concluded that tumor cell invasion and metastasis induced by mutant RAS or RbAp46 are mediated by MMP-9 (or MMP-9
related signaling pathway). This conclusion has two problems:

i. It is unclear whether the decreased MMP-9 activity actually contributes to the decreased invasion and/or metastasis. These phenomena might have just occurred in parallel and MMP-9 might have little to do with invasion and metastasis in this case.

ii. There are multiple mechanisms to account for decreased MMP-9 activity: reduced MMP-9 expression, reduced proteolytic activation of MMP-9, increased MMP inhibitors, etc. It is unclear what “MMP-9 related signaling pathway” means.

**Response:** We agree with reviewer’s comment. In this study, we did not show the decreased MMP-9 activity results in tumor cell invasion and metastasis induced by mutant Ras or RbAP46. Therefore, we modified the over-interpretations in our manuscript and “MMP-9 related signaling pathway” was modified to “MMP-9 activity”. All the changes were also showed in red in the revised manuscript.

I also find difficulty in understanding the following points

**Question (3)** The authors describe “high expression of mutant Ras” in human bladder tumor tissues. It is unclear to me how they selectively detected expression of MUTANT RAS in these tissues.

**Response:** In our study, we used pan-Ras antibody to detect Ras expression in human bladder tumor tissues. The antibody could detect both of wild-type and mutant Ras. “high expression of mutant Ras” was modified to “high expression of Ras protein” (page 2, line 19, page 24, line 19 and page 25, line 5).

**Question (4)** Several previous papers report on the tumor suppressor activity of RbAp46, as briefly mentioned in Discussion (page 29, line 12). It is counterintuitive that a tumor suppressor promotes malignant behaviors; this point should be discussed in more detail (e.g., possible mechanisms).

**Response:** To comply the reviewer’s request, we discussed in detail of tumor suppressor activity of RbAp46 in malignant behaviors in Discussion (page 30, line 2-10). We added that “Interestingly, RbAp46 is also a potent cell growth inhibitor that can suppress the transformed phenotype of tumor cells. It is counterintuitive that a tumor suppressor promotes malignant behaviors. RbAp46 is an Rb-binding protein and has been proved to be a metastasis-associated protein (MTA1) complex (1, 2). Expression of MTA1 correlates well with the metastasis potential of human cancers (3, 4). We observed that RbAp46 can directly interact with HDAC1. Therefore, it is possible that RbAp46 could promote tumor metastasis by regulating transcriptional suppression on RECK gene through histone deacetylation activity. However, the underlying mechanism needs to be further studied.”.

References:


**Question (5)** Conclusions (page 31): The authors connect independent findings in sequence and assume causal-effect relationships among them without evidence. What is stated in this section is a working hypothesis or model rather than conclusions. [Also see my points (1) and (2) above.]. The same logical leaps are repeated throughout the text.

**Response:** We have modified our conclusions (page 32) and check the logical leaps carefully throughout the text. We also added our working hypothesis as showed in Fig.6.

Technical comments:

**Question (6)** In Oncomine database, upregulation of RbAp46 is found in some tumors but not in bladder tumors. How do the authors explain this?

**Response:** Oncomine database is a cancer microarray database, which facilitates discovery from genome-wide expression analyses based on examination of gene expression at RNA level. However, RbAp46 protein expression could also be regulated at protein level. For example, we previously found that oncogenic Ras overexpression stabilized RbAp46 protein via interaction with SUMO-1 (1). It is intriguing to know whether RbAp46 is upregulated in bladder tumors at protein level by immunohistochemistry staining.

Reference:

**Question (7)** Fig. 2A: RAS panel is desirable.
**Response:** RAS panel was added in Fig. 2A as reviewer’s request.

**Question (8)** Fig. 3A: Input samples should be probed with each antibody. RAS panel should also be included. Is it difficult to detect two proteins in the same IP sample (e.g., IP with anti-RbAp46B and probe with anti-HDAC1 and anti-Sp1)?
**Response:** RAS panel was included in Fig. 3A. However, we have difficulty to detect two proteins at the same time in the same IP sample.

**Question (9)** Fig. 2D-E and page 20, line 5, “other factors- - - involved”: It seems difficult to directly compare the data in Fig. 2D and E (which use quite different experimental systems) and draw a quantitative conclusion.
**Response:** We agree with reviewer’s comment and the phrase has been removed.

**Question (10)** Fig. 3B, right panel: Which Sp1 site is mutated in this plasmid? It would be more informative if the two panels of bar graphs are combined.
**Response:** The Sp1 site mutated in this plasmid was Sp1 (B) site originally designated by Sasahara et al (1). Although it would be more informative if the two panels of bar graphs are combined, Because these two panels showed the data which were conducted separately, therefore, could not combined.
**Reference:**

**Question (11)** Fig. 3C, lane 2: The RbAp46 band is not so dense as expected. How was the level of mutant RAS in this sample? Ras data should be included (as mentioned at page 21, line 18).
**Response:** As reviewer’s request, the same blot of higher exposure band of RbAp46 was replaced (Fig. 3C, lane 2) and the RAS panel was also added in Fig. 3C.

**Question (12)** Fig. 3D: What about the effects of RAS-induction?
**Response:** To confirm that the RbAp46 interaction with HDAC1 and Sp1 indeed binds to RECK promoter at the Sp1 binding site in vivo, we preformed ChIP assay using chromatin of 7-4 cells extracted after Ras was induced by IPTG treatment. Although it would be more informative to know the effect of Ras induction compared with DAPA results, our data clearly showed that the Sp1 binding site in the RECK promoter was amplified in RbAp46, HDAC1, and Sp1 precipitated DNA samples.
under Ras-overexpression conditions (Figure 3D).

**Question (13)** Fig. 4D: The authors present data only on single successful clone. I wonder if clone #2 merely represent slowly growing clones. Besides, I also wonder how the possible contribution of off-target effects could be excluded.

**Response:** We selected RbAp46 shRNA lentiviral clone #2 because of the best suppression of RbAp46. Although it is more informative to use multiple clones to rule out the off-target effects, nevertheless clone #2 did not show slow proliferation compared to the other clones (unpublished data).

**Question (14)** Fig. 4G, second lane: The intensity of RbAp46 band is not so different from that in lane 1.

**Response:** To emphasize the effect of RbAp46 knockdown by shRNA, the band intensity of RbAp46 was increased by over-exposure to show the difference between lane 2 and lane 3. Therefore, the intensity of RbAp46 in lane 2 becomes not so different from that in lane 1. It is also possible that samples were collected from a pool of individual mice and the intensity difference of RbAp46 was diluted.

Minor points:

**Question (15)** Page 11, line 5: Define the abbreviation PPIA.

**Response:** The abbreviation of PPIA is Peptidylprolyl isomerase A, which was added in page 45, line 6.

**Question (16)** Page 12, line 8: The siRNA for RbAp46 is mainly used for mouse cells, although this description sounds like targeting human gene.

**Response:** The RbAp46 siRNA used in promoter activity assay can target both of human and mouse cells. The silencing efficiency of the siRNA on protein level of RbAp46 expression was checked before the experiments of promoter activity assay (data not show).

**Question (17)** Page 13, line 5: The DAPA probe has mismatches to the mouse sequence in the database. Explain why.

**Response:** We apologize that there is a typo error in the probe sequence. The probe sequence 5’-GCGCGGGGGGCCTGGTGCC-3’ was changed to 5’-GCGCCGTTGGCCTGGTGCC-3’ (page 13, line 5).

**Question (18)** Page 17, line 17 and page 18, line 9, “at transcriptional level”: These should be “at the mRNA level”.
**Response:** We thank reviewer’s comment. We have modified the statement (page 17, line 17 and page 18, line 9).

**Question (19)** Fig. 2D, F: Describe what time 0 means (time of IPTG addition?).

**Response:** In this experiment, time 0 means the time of IPTG added.

**Question (20)** Page 26, line 9 and page 31, line 4, “Similar relationship”: This phrase is out of context, since the authors have talked about formation and action of a transcriptional complex in the preceding sentences, but now they are talking about the amount of proteins.

**Response:** The phrase “Similar relationship” was deleted and this sentence was modified (page 26, line 9 and page 32, line 4).

**Question (21)** Page 22, line 2, “The cells without any treatment ---”: This sentence does not make sense; this sample should be a control setting the baseline to which other samples are compared.

**Response:** We have modified the sentence (page 21, line 18). “The cells without any treatment ---” was changed to “Control cells without IPTG treatment--- “

**Question (22)** Page 22, line 13, “Furthermore, ---”: This sentence does not make any sense.

**Response:** We thank reviewer’s comment. This sentence was deleted.

**Question (23)** Fig. 5: In one case (#362), RbAp46-upregulation is not accompanied by RECK-downregulation. How could this be explained?

**Response:** One report indicated that Ras-induced down-regulation of RECK is mediated via a DNMT3b/promoter methylation mechanism (1). Furthermore, Ras-signaling pathways could also downregulate RECK protein through microRNAs (2). In case (#362), it is possible that DNMT3b and microRNA provoked Ras-induced down-regulation of RECK independently of RbAp46.

References:
Question (24) Fig. 5: In another case (#358), Ras shows little difference between N and T, and yet RbAp46 is strongly upregulated in T. How could this be explained? I would say, in this case, it does not matter too much, since general understanding is that activity of endogenous Ras is regulated at the level of GDP-GTP exchange rather than altered protein expression [also see my comment (3)].

Response: We agree with reviewer’s comment. We have added this point in Discussion (page 29, line 17 to page 30, line 2). We added that “In one bladder cancer specimen (case #358), Ras showed little difference between normal and tumor, and yet RbAp46 was strongly upregulated in the tumor. It is possible that endogenous Ras is regulated at the level of GDP-GTP exchange rather than altered protein expression.”.

Quality of written English: Needs some language corrections before being Published

Response: The manuscript has been thoroughly edited by Dr. Robert Anderson.
Reviewer: Andreas Varkaris

Major compulsory revisions:

**Question (1)** Fig 1B shows a vast increase in RbAp46 after 48h following induction of Ras expression in MCF-7 cells. In Fig 1C, authors use 7-4 cells for mechanistic studies. However, upregulation of RbAp46 48h after Ras induction is not obvious (1.15), as noted in the result section. Further, in Fig 1C (bottom panel) loading control with actin demonstrates unequal loading of protein. Based on these observations, I would like to kindly request the authors to perform this experiment once again. Firm results would strengthen their hypothesis.

**Response:** The differential increase of RbAp46 following Ras induction between MCF-7 cells and 7-4 cells is probably due to cell type specificity. Although RbAp46 expression 48h after Ras induction was not obvious in 7-4 cells, levels of RbAp46 expression was decreased clearly when Ras was silenced by siRNA (Fig. 1C). Together with other data, we concluded that RbAp46 is a Ras-upregulated gene in various cancer cells.

**Question (2)** Fig 1D There is an obvious difference between the two first lanes. I would like to kindly request the authors to provide an explanation for this finding.

**Response:** In this study, c-Met siRNA was originally design as a control of Ras siRNA and to check the effect of transfection. This c-Met siRNA (8 ug) unexpectedly suppressed Ras expression and subsequently RbAp46 protein level (lane 1), suggesting the side effect of transient tranfection. Nevertheless, Ras siRNA (8 ug) suppressed more of Ras expression than that of c-Met siRNA (8 ug). This data support the positive correlation between Ras and RbAp46 expression. In conclusion, our data indicated that Ras can upregulate RbAp46 gene expression.

**Question (3)** Fig 2B demonstrates the effects of Ras knockdown in the expression of RbAp46, and RECK respectively. However, changes in RbAp46 are not obvious. These findings might suggest a parallel mechanism of Ras-induced RECK regulation. Based on this finding, I would like to ask the authors to comment this finding in their discussion of the manuscript.

**Response:** To comply the reviewer’s request, we have commented this finding in Discussion (page 29, line 10-17.). We added that “In T24 cells, RECK protein increased dramatically after Ras knockdown, however, there were no obvious changes in RbAp46 (Fig 2B). This finding suggests a parallel mechanism of Ras-induced RECK regulation. Chang et al. reported that Ras-induced down-regulation of RECK is mediated via a DNMT3b/promoter methylation mechanism (1). Loayza-Puch et al. reported that Ras-signaling pathways could
downregulate RECK protein through microRNAs (2). Whether DNMT3b and microRNA regulate Ras-induced RECK downregulation independently of RbAp46 in T24 cells is worthy of investigation.”

References:

**Question (4)** Fig 4E, F demonstrates the effect of Ras overexpression and RbAp46 knockdown in in-vivo metastasis assay after tail vein injection of 7-4 cells. However, data presented do not support the speculations of authors. Ras overexpression is not associated with increased lung metastasis, whereas RbAp46 knockdown has a limited effect in metastasis. These findings raise a number of questions:

a. What is the number of lung metastasis per animal? Statistical analysis using one way Anova is required.
b. The authors used t-test for comparing total lung weight. I would like to kindly request for an explanation for not using one-way anova.
c. Association of in-vivo results with data demonstrated in fig 1C should be considered, since Ras did not increased secondary growth in this model.
d. The authors should consider repeating this experiment with a larger number of animals per group. Based on presented findings, their hypothesis does not seem to be valid in an in-vivo model.

**Response:** We used t-test for comparing total lung weight because that effect of RbAp46 knockdown on tumor metastasis suppression was emphasized. The Ras did not significantly increase secondary growth was possibly due to the limited number of mice. Nonetheless, our data showed that knockdown of Ras and RbAp46 inhibited cell invasiveness as well as MMP-9 activity. Further study using more mice is needed. These biological functions were well-associated with data demonstrated in Fig. 1C.

**Question (5)** Fig 5 shows a correlation among Ras, RBAp46 and RECK in clinical bladder samples. Although the number of tumor samples is limited, associations are obvious and well described. Quantification of WB intensity and plotting using a Spearman correlation would be helpful.

**Response:** Thanks for reviewer’s suggestion. Quantification of WB intensity was
plotted using a Spearman correlation to show correlations among Ras, RbAp46 and RECK. The data was added in Fig. 5E-G.

**Question (6)** Authors suggest that their study may lead to development of better treatment of Ras-related tumors. However, the translational relevance of their work is not clearly understood. I would like to kindly request the authors to comment the translational relevance of their work in the discussion.

**Response**: Thanks for reviewer’s suggestion. We have added the comment of translational relevance of our work in the discussion (page 31, line 9-11). The following statement was added “*In conclusion, our findings provide a novel mechanism of Ras-related tumorigenesis in bladder cancer and suggest that RbAp46 could be a target in the treatment of Ras-related metastasis in bladder cancer.*”.

Minor essential revisions:

**Question (1)** A schema describing associations shown in the manuscript is highly recommended.

**Response**: Thanks for reviewer’s recommendation. The schema of our working hypothesis was added in Fig. 6.