Author's response to reviews

**Title:** Downregulation of Cyclophilin A by siRNA diminishes non-small cell lung cancer cell growth and metastasis via the regulation of matrix metallopeptidase 9

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**Version:** 5  **Date:** 10 September 2012

**Author's response to reviews:** see over
Dear editor Marinette Lacson:

Thanks you very much for your comments and suggestions on our submission “Downregulation of Cyclophilin A by siRNA diminishes non-small cell lung cancer cell growth and metastasis via the regulation of matrix metallopeptidase 9” (MS: 1504236017237805). The manuscript has been revised according to the editor’s and reviewers’ comments. In the revised version of the manuscript we have addressed all issues raised by the reviewer and have incorporated additional experiments as requested. The number of figures was changed and the panels in some figures were also modified. The reference was also changed in the manuscript. Please find a detailed response below. All the changes are made in “Track Changes” in the revised manuscript. We hope that the manuscript is now suitable for publication in your journal. Thank you very much for your consideration.

Sincerely yours,

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1 Reviewer: Giovanni Luca Beretta

Major Compulsory Revisions

1) To dissect the role of the mass protein and enzyme activity it would be interesting to evaluate the effect of small molecule/s targeting the CypA activity. In this regard some inhibitors have been already published by Ni and coworkers (Ni S et al. Discovering potent small molecule inhibitors of cyclophilin A using de novo drug design approach. J Med Chem. 2009 Sep 10;52(17):5295-8) and one of these compound (239836 Cyclophilin A Inhibitor) is commercially available. It should be interesting to evaluate the cell growth, motility, invasion and, most importantly, MMP9 activity of NSCLC cell line/s exposed to this inhibitor.

Thank you very much for the comments on our submission. The effect of 239836 Cyclophilin A Inhibitor on cell proliferation and MMP9 activity was detected. Cell growth and MMP9 activity was inhibited by 239836 in a dose-dependent manner. And corresponding results were added in the revised manuscript (Fig. 3B and Fig.6B).

2) The authors refer to different growth-related signaling molecules that are stimulated by CypA in cancer (e.g., ERK1/2, Jak2, p38, Stat5) but they did not evaluate possible changes (expression levels and phosphorylation) of these actors after CypA silencing.

In the revised manuscript, western-blot analysis was taken to detect the levels of ERK1/2, JAK2, p38, STAT5 and their phosphorylation forms. The Date shown CypA silencing can deregulates the phosphorylation of ERK1/2 and p38 in A549cells.

3) In the Discussion section the authors reported that CypA accelerates cell growth by stimulating cell proliferation, tumorigenesis, and metabolism, and by inhibiting apoptosis. However, they do not evaluate apoptosis in CypA silenced cells. What is the effect of CypA silencing on apoptosis in NSCLC cell lines.
In our research, we determined whether cell apoptosis was regulated by CypA. But cell apoptosis was not affected when CypA expression was inhibited (data not shown).

4) The authors report that CypA enhances the activity of secreted MMP9 but they did not elucidate the mechanism/s. In my opinion this is the most important conclusion of the paper that requires explanation. Again, is there a direct interaction between CypA and MMP9? A detailed analysis of the protein sequence of MMP9 could help in defining this point.

In order to explore whether there is a direct interaction between CypA and MMP9, PIPs human protein-protein interaction prediction was used [1, 2]. But no direct interaction between CypA and MMP9 were found.


2 Scott, MS and Barton, GJ Probabilistic prediction and ranking of human protein-protein interactions BMC Bioinformatics 2007 8:239-260 Abstract

Minor Essential Revisions

1) Abstract, conclusions: change the sentence “CypA was correlated with decreased NSCLC cell tumorigenesis and metastasis” to “The suppression of CypA expression was correlated with decreased NSCLC cell tumorigenesis and metastasis”.

Thank you very much for your comments. The sentence was changed in the revised manuscript.

2) Figure legends of figure 3 and 4: change + SEM to ± SEM.

I am sorry that I made a written mistake and I have changed the + SEM to ± SEM in the manuscript.
2 Reviewer: Valentina Zuco

Major Compulsory Revisions

1) The data reported in the manuscript does not add much to the well-known knowledge in this field. The manuscript doesn't explain any mechanism concerning the tumor growth inhibition and the decrease of tumor invasion caused by the knockdown of CypA. Your research is pretty interesting, but unfortunately there are any explanation about the final results that you are claiming. I suggest you to go deeper in your research in order to complete your work.

Thank you very much your suggestion. To determine the effect of CypA, 239836 CypA inhibitor was used. In our results, cell proliferation and MMP9 activity was weakened by the treatment of 239836 CypA inhibitor in NSCLC cell lines (Fig. 3B). We have also done some more research on the mechanism of CypA promoting cell proliferation (Fig. 4).

2) Clarification of the quantitative Real Time PCR method. The normalization approach employed for quantitative Real Time PCR should be explained in a clearer way. In materials and methods paragraph and in figure 1 legend the authors say: “relative mRNA levels are presented as $2^{[Ct(b\text{-actin})-Ct(gene\ of\ interest)]}$. What does that mean? Have they used the ##Ct approach? What is “1” in the y axes of graphs in figure 1A?

I am sorry that the description of normalization approach employed for quantitative Real Time PCR was not clear. The data of real-time was analyzed using $2^{-\Delta CT}$ ($\Delta CT= Ct$ for target gene - Ct for β-actin) method, we have also revised the description in the manuscript. In figure 1A, “1” in the y axes means the expression level of CypA mRNA is the same as β-actin mRNA.

3) It’s extremely important to know protein levels of CypA in cell lines of the figure 1A. Western-blot analysis of the CypA protein levels, it is recommended.

The expression level of CypA protein was detected by western blot analysis and
immunofluorescence staining. The results of immunofluorescence staining experiment were quantified by Cellomics ArrayScan HCS Reader using the ArrayScanTM software. The data was shown in Fig. 1B in the revised manuscript.

4) Staining of cells in the invasion assay should be performed after 24h and not 48h. Thus, it is necessary to repeat this assay after 24h of incubation.

   In the invasion assay, cells attached to the lower surface after 24h of incubation was also detected. Unfortunately, little cells were observed. Then we count the number of cells on the lower surface of the membrane at 48h. We had revised the description in the manuscript.

5) In invasion and migration assay, usually conditioned medium was added to the lower chamber. Are there any reason why are you using a medium containing 10% FBS?

   I am sorry that the description in the methods was not clear enough. We have modified the manuscript. The invasion and migration assay was done as previous report (reference: Jiang L, Huang Q, Zhang S, Zhang Q, Chang J, Qiu X, Wang E: **Hsa-miR-125a-3p and hsa-miR-125a-5p are downregulated in non-small cell lung cancer and have inverse effects on invasion and migration of lung cancer cells. BMC Cancer 2010, 10:318-330**). Cells were seeded in upper compartments of the chambers with serum-free RPMI 1640. The lower compartments of the chambers contained 10% FBS for use as a chemoattractant.

6) To indicate the doubling-time of cells in figure 1B, 2C and D.

   In figure1B, 2C and D (figure 1 C, figure 3A in revised manuscript), cell proliferation activity was detected by MTS assay. Cell doubling-time was calculated using doubling time online calculator (Roth V. 2006, [http://www.doubling-time.com/compute.php](http://www.doubling-time.com/compute.php)) and it was indicated in the revised manuscript.
7) I’m not agree with your claim in page 8, line 17-18 (our data demonstrate that the suppression of CypA resulted in marked inhibition of tumor formation) and in page 9, lines 20-21 (our data indicate that CypA plays a CRUCIAL role in the progression of NSCLC). The results reported in your manuscript are not supporting your conclusion. There are not any real and objective results that can provide the involvement of CypA in the inhibition of tumor formation and the crucial role of CypA in the progression of NSCLC. In fact, your results clearly show an inhibition of the tumor growth and not of the tumor formation.

Thank you very much for your comments. These sentences may not be very precise. We have modified the sentences in the revised manuscript.

8) The reference 22 in page 10, lines 20, is not corrected: the reference 20 is related to breast cancer. The reference 22 is related to metastatic melanoma cell lines. Please, to check references.

I am sorry that I made a mistake and I checked all the references. I have corrected it in the revised manuscript.

9) Style and language may be checked carefully.

Thanks for your comments. We had corrected all grammatical errors that need corrected. To improve the language level, the entire manuscript has been proofread by an English native speaker to avoid possible spelling and grammatical errors.