Author's response to reviews

Title: Gonadotropin-releasing hormone type II (GnRH-II) agonist regulates the invasiveness of endometrial cancer cells through the GnRH-I receptor and mitogen-activated protein kinase (MAPK)-dependent activation of matrix metalloproteinase (MMP)-2

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Version: 8 Date: 8 May 2013

Author's response to reviews: see over
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**Title:** Gonadotropin-releasing hormone type II (GnRH-II) agonist regulates the invasiveness of endometrial cancer cells through the GnRH-I receptor and mitogen-activated protein kinase (MAPK)-dependent activation of matrix metalloproteinase (MMP)-2

**Authors:** Hsien-Ming Wu, Hsin-Shih Wang, Hong-Yuan Huang, Chyong-Huey Lai1, Chyi-Long Lee, Yung-Kuei Soong, Peter C.K. Leung.

Version: 1 Date: 26 April, 2013

We ask a native English speaking colleague in UBC, Canada to help us copyedit the paper as suggestion.

**Author’s response to reviews(1): LAS**

1. Most of the indicated western blot experiments, including gel zymography, do not have their quantification and data are not from different experiments.
   **Ans:** We have revised as Reviewer’s suggestion.

2. In the figure 1A, the quality of the representative figures on cell migration and invasion is not sufficient. It is hard to interpret the dose-dependent effect, which does not seem apparently to match with the chart data on the left. In addition, the measurement units are not accurately written.
   **Ans:** We have replaced with better photos as suggestion and revised labeling for figures.

3. In which cell line siRNA of GnRH-I receptor has been done? Only one blotting is shown, while in the results the authors report that they have knocked down GnRH-I receptor in both cell lines. More importantly, this immunoblot appears to be the same already published by the authors in a previous paper (see Wu et al., Cancer Res 2009, 69, 4204, fig 3A), and still the same is shown in the following figure 3A.
   **Ans:** We have revised the Figure 2A and Figure 3A photos as suggestion.

4. The significance of the figure 2B in the context of work is unclear. It has been already reported that GnRH-I receptor is present in endometrial tumours, and it
has also been hypothesized a correlation between its expression and tumour invasiveness. It would make sense if the authors could be able to associate the GnRH receptors expression with cancer grading.

**Ans:** Previous studies (Longstanding survival without cancer progression in a patient affected by endometrial carcinoma treated primarily with leuprolide. Noci I. et al. Br J Cancer. 2001 Aug 3;85(3):333-6.; Luteinizing hormone increases human endometrial cancer cells invasiveness through activation of protein kinase A. Dabizzi S. et al. Cancer Res. 2003 Jul 15;63(14):4281-6.) have reported the association between endometrial cancer grading, progression and the expression of GnRH receptor in endometrial cancer. Here, we confirm the expression of GnRH-I receptor in type I endometrial cancer tissue and endometrial cancer cell lines for this study.

5. In figure 3A, siRNA suppress almost completely GnRH-I receptor expression, but cell migration and invasion appear to be reduced and not completely blocked. What's about the involvement of the type II GnRH receptor? The authors have to mention about that in the discussion.

**Ans:** Previous studies (Millar R. et al. Proc Natl Acad Sci U S A 2001;98:9636–41.; Neill JD. Endocrinology 2002;143:737–43.; Maudsley S. et al. Cancer Res 2004;64:7533–44.; Pawson AJ. Et al. Endocrinology 2005;146:2639–49.; Kim KY. Endocr Relat Cancer 2006;13:211–20.; Wu HM. Et al. Cancer Res 2009;69:4202–8. ) have demonstrated that GnRH-I receptors may be a common receptor that mediates the effects of both GnRH-I and GnRH-II in gynecologic cancer. We have discussed this issue in the discussion section. We also have revised the Figure 3A photo as suggestion. Based on the data, the GnRH-I receptor was not completely knocked down by GnRH-I receptor si-RNA.

6. MMP-2 protein levels and activity showed in figure 5 have to be completed. Insufficient the description of result quantification, as well as the number of experiments performed (at least three are required). In addition, MMP-9 is also up-regulated in tumours, as well as by treatment with GnRH agonist. The authors claim that cell migration and invasion of endometrial cancer cells depend on MMP-2 activation. But this motility has not been completely abolished by the MMP-2 inhibitor, thus the author should explore also the involvement of MMP-9 in their cell system.

**Ans:** In this study, based on the data, there was no significant effects in MMP-9 by treatment with GnRH-II agonist in endometrial cancer cells.
Minor Essential Revisions

1. Abstract is not well organized, the aim should be moved from methods to the background, and the methods should be re-written.
   **Ans:** We have revised it as suggested.

2. In figures 4 and 5C both chart legends are illegible.
   **Ans:** We have revised them as suggested.

3. Why the author use SKOV-3 cancer cells in figure 2A as positive control? They do not have GnRH-I receptor, if anything "negative" control. No information about this cell line is present in materials and methods.
   **Ans:** We have removed SKOV3 and use GnRH-I receptor siRNA result to show the specificity of GnRH-I receptor antibody.
Author's response to reviews(2): AM

1. By a conceptual point of view, I think that the Authors should investigate whether GnRH-II, under their experimental setting, beside to stimulate cell motility, have an effect on apoptosis and/or cell proliferation.


2. As regards the experimental data, in Fig. 1, the Authors show that GnRH-II stimulates cell migration and invasion. This figure would benefit from data obtained from cytoskeleton analysis using phalloidin and wound scratch assay. Again, GnRH-II concentrations higher than 1 µM could be used and shown, to identify the optimal effective concentration. Furthermore, in Fig. 1A the Authors should explain why “3T3 fibroblast-conditioned medium was added in the lower chamber as a chemo attractant” to assess the cell migration (page 17 – invasion and migration assays). 3T3 fibroblasts could produce both ECM and degrading enzymes as well as growth factors.

**Ans:** In this study, GnRH-II concentrations higher than 1 µM have similar results in stimulating cell migration and invasion to the treatment with 1 µM GnRH-II based on the data. We have corrected and updated the medium in the lower chamber to 10% FBS medium.

3. In Figs. 3B and 3C the Authors should describe the experimental approach used. Do the Authors used a fluorescent probe to identify siRNA transfected cells? If not, data in panels B and C should be, at least corrected for transfection efficiency or even repeated by using a fluorescent marker in co-transfection experiment (i.e. Alexa-Fluor). In addition, the Author should explain why in the Figs. 1A and 1B cells were allowed to migrate for 24 hrs, while in Figs. 3B and 3C were kept for 72 hrs. This time frame seems too long to be used in a siRNA transient transfection.

**Ans:** We have examined the transfection efficiency by using si-GLO which
is the fluorescent-labeled siRNA. As the revised results shown in Figure 3A, after 24 hr si-GLO transfection, almost 90% cells were positive for the florescence. We have revised our migration and invasion section in Material and Methods as well as our Figure legends to clarify the issue regarding our migration and invasion results.

4. In the experiments shown in Fig. 5, the highest MMP-2 expression was observed at a GnRH-II concentration of 1 nM, whereas the maximal cell migration/invasion was observed with concentrations of 1 µM. How the Authors explain this difference? What’s the GnRH concentration used in the experiments shown in Fig. 5C (1 nM or 1 µM)?

**Ans:** There is significant difference for induced MMP-2 expression between Ctrl and GnRH-II treatment. However, there is no statistically significant difference for induced MMP-2 expression between 1nM and 1 µM GnRH-II treatment. The GnRH-II concentration used in the experiments shown in Fig. 5C was 1 µM.

5. Finally, there is another point calling for an explanation: in the experiments shown in Fig. 5C the number of migrated cells is expressed as number of fields. What does it mean? Usually the number of migrating cells is expressed as a percent increase over the control or number of cells passing into lower chamber.

**Ans:** We have revised it as suggestion.

All correspondence and galley proofs should be sent to Dr. Hsien-Ming Wu.

With many thanks for your consideration.

Sincerely yours,

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