Author’s response to reviews

Title: Leptin-induced ER–Positive Breast Cancer Cell Viability and Migration is mediated by Suppressing CCN5-Signaling via Activating JAK/AKT/STAT-pathway

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To

Editorial Board Members

BMC Cancer

Subject: Revised Manuscript- Leptin-induced ER--Positive Breast Cancer Cell Viability and Migration is mediated by Suppressing CCN5-Signaling via Activating JAK/Stat-Akt pathway by Inamul Haque, Arnab Ghosh, Seth Acup, Snigdha Banerjee, Kakali Dhar, Amitabha Ray and Sushanta K. Banerjee
Dear Members:

We appreciate your timely review of our manuscript and the opportunity to revise, correct and improve the manuscript. The reviewers’ vital comments and suggestions have been considered carefully, and we addressed each one in the attached pages as well as in the revised manuscript (yellow highlighted).

We believe that the quality of the manuscript has been significantly enhanced by these modifications.

Once again, we appreciate the efforts and time you and the reviewers have spent evaluating this manuscript. We look forward to your comments on this revised manuscript.

Sincerely

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And

Snigdha Banerjee, Ph.D.
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REVIEWER 1:

1. Factors like Leptin, hrCCN5 should be used as molarity and not as ng/ml.

RESPONSE: We modified from ng/ml to molarity. Most of the publications used ng/ml and thus, we used both in this manuscript so that it would cover all the readers.

2. I need to know what is the difference for you between viability and proliferation. You have to choose between MTT or tritiated thymidine uptake.

RESPONSE: We measured cell viability or survival upon leptin or CCN5 treatment using Crystal Violet Assay, a standard and established protocol for the detection of viable cells.

3. You extend your viability studies to 24h and 48h. However, the doubling time for MCF-7 is 29h and the doubling time for ZR-75-1 is 80h. Therefore, your viability studies should go to 96h (4 days) to give a chance to both cell lines to grow.

RESPONSE: Cell viability can be measured any time point of the growing cells as this assay only identify survival cells under tissue culture conditions. However, as per reviewer’s suggestion, we performed viability assay in a dose and time dependent fashion. Results are included in the revised manuscript (See the Figure 1).
4. Results analysis of Fig1 ABC: I don't see any dramatic effect there, just a simple variation because your conditions are not optimal.

RESPONSE: Although the reviewer found the effect was not dramatic and the variation is due to lack of optimal condition, we found that the effect was highly significant and we are very confident about the results as the effect was validated by three different experimental approaches. These include Northern blot, qPCR and finally Western blotting. The experimental conditions were optimal and identical.

5. As for Fig 2A, B: The effect is not dramatic at all. Fig2C, I don't see a suppression of apoptosis, your results show some 10-30% decrease.

RESPONSE: Reviewer’s comments are well taken and thus we mentioned in the revised manuscript that since the leptin effect on inhibition of apoptosis in MCF-7 and ZR-75-1 cells were not so dramatic as compared to the leptin effect on cell viability, we anticipate that other cell physiological factors could be linked with leptin-induced cell viability. Thus, further studies are warranted, which is our next goal.

6. Fig3, the effects are not impressive at all. There is no need for you to explore the effect on EMT or MET, Those results are not credible.

RESPONSE: Epithelial-mesenchymal transition (EMT) is the hallmark of invasion and metastasis and this vital pathobiological events can be reversed by CCN5. Consistent with previous work (THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 11, pp. 8598–8612, March 9, 2012), we found that leptin promotes EMT in breast cancer cells. In addition, we first time showed that leptin promotes EMT by suppressing CCN5 activity. Thus, we believe that this study is an innovative finding and results are credible.
REVIEWER 2:

1. The authors have observed the effect of leptin on epithelial-mesenchymal transition (EMT) and suggested that the transcriptional suppression of CCN5 by leptin might be a underpinning mechanism in ER positive breast cancer. But, the data interpretation need to be clearer because the experimental findings presented in this paper did not show the direct mechanistic relationship between CCN5 and leptin. In fact, the data just indicated competitive antagonistic effect of GCN5 on leptin action and vice versa.

RESPONSE: We extended our experiments and provided new mechanistic data.

2. Major Revisions

1. Figure 1: The authors said that the CCN5 expression can be repressed in MCF-7 and ZR-75-1 cells by leptin treatment. To support this idea, the authors presented data from Northern blot, qPCR, Western blot and CAT assay. However, the author did not show any evidence showing if the leptin treatment was properly working in both cell lines or not.

RESPONSE: Except CAT assay, we used both MCF-7 and ZR-75 cell lines to support the idea (see Figure 2). We believe that performing CAT assay in one cell line is sufficient for this study.

3. The authors said that leptin can stimulate JAK/STAT3, ERK1/2 and PI3K pathways. The authors at least presented which kind of signal pathway was able to be activated in both cell lines by leptin treatment. After they find major signal pathway activated by leptin, the authors may want to perform inhibitor studies to verify leptin-induced CCN5 transcriptional repression.

RESPONSE: New signaling data has been included in the revised manuscript indicating leptin blocks CCN5 expression via JAK/Stat3-Akt pathway.

4. Figure 2 and 3: Again, the authors need to present the signal pathways involved in leptin-induced cell proliferation, invasion and migration combined corresponding inhibitor studies.

RESPONSE: These studies have already been done and published by several laboratories. Thus, we felt it would be the duplication of the existing published works.
5. Unfortunately, the data in these figures did not show leptin-induced CCN5 transcriptional repression. They have shown the repression of leptin effect on cell biology by CCN5. This issue is quite important because the title of this paper is "Leptin-induced ER--Positive Breast Cancer Cell Growth and Migration is mediated by Suppressing CCN5-Signaling". The authors need to show the results from new experiments showing the effect of leptin treatment on CCN5-null systems.

RESPONSE: Figure 2A-D strongly support that leptin suppresses the CCN5 expression at the transcriptional level. We tested in CCN5 negative cell line (Figure 1). The effect of leptin on cell viability has been found to increase in CCN5-negative MCF-7 cells. However, this data is not included in this manuscript as more experiments are required, which have not yet been completed.

6. Minor Revisions 1. Figure 2: The data on this figure did not show the dose dependent effect of leptin, but presented time-dependent effect. Please check the description for this figure (Page 13, Line 3). 2. Figure 4B: The authors may want to present p-values. 3. Figure 5: The summary figure is not based on the data presented in this paper. It should be revised properly after revision experiments.

RESPONSE: We included new data in support of the statement. We modified the summary figure in the revised manuscript. We included P-value in the revised manuscript

REVIEWER 3:

1. Overall, these experiments are straightforward and seem reasonable. However, there is weakness in the attention to detail regarding why CCN5 was pursued in ER positive breast cancer. The overall data is interesting, however the final figure is a bit misleading and is too narrowly focused. CCN5 may be a key player, but it needs to be placed in context with other key protein players in the field. As I mentioned in my comments below, other labs have looked at the role of leptin in ER positive breast cancer. I strongly suggest the authors review the literature and place their protein of interest in context with the work in the field. Otherwise, this work stands alone and it is hard for readers to appreciate the significance of this work in light of what has already been done and how this work may help to move the field forward. Once this work is
placed into context with other work in the field, the authors may find that other experiments may be needed to provide a more comprehensive perspective.

RESPONSE: We modified the final figure. We included the role of some key signaling pathway that involved in CCN5 regulation by leptin. We included other works in the revised manuscript.

2. Other comments are provided below: 1. Could use more clarification in the background of why CCN5 was investigated: The author's state that "the primary aim of the present study was to evaluate whether leptin has any influence on CCN5 to promote BC progression". However, the rationale provided in the Background section for pursuing the direction to investigate the role of CCN5 is quite abrupt and could use a description that provides a better transition on why CCN5 was chosen to pursue out of all the other candidates (e.g. is this work completely exploratory?)

RESPONSE: We modified the background section with substantial information and references.

3. Has any other papers reported on CCN5 or is this the first paper to do so?) Actually, some of the information in the discussion section would be more appropriately placed in the intro section. For example on page 16 it states- "Multiple studies from our laboratory and others have shown that CCN5-signaling plays a vital role in orchestrating the growth and behavior of cancer cells. CCN5 acts as an anti-invasive 3 element in cancer cells of the breast, pancreas and GI tract. [29-31, 50, 61, 62]. CCN5 is a 29-35 kDa secreted protein and is overexpressed in preneoplastic disorders in the human breast, including atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS) compared with adja- cent invasive cancer cells where expression levels were undetected, minimally detected, or only sporadically detected [29]." I would've preferred to see this at the beginning

RESPONSE: This is the first report indicating a negative role of CCN5 in leptin-induced breast cancer viability and invasive phenotypes. As per the reviewer’s instruction, some of the information are included in the INTRODUCTION section.

4. 2. Experiments are straightforward and seem reasonable. However, it is not clear how/why 50ng/ml was the only dose chosen for Leptin. Is there a dose RESPONSE? Is this the standard concentration used for prior studies?
RESPONSE: Dose of leptin was initially selected from different publications (Gut 2005;54:1136–1145. doi: 10.1136/gut.2004.060533; THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 18, pp. 13316–13325, May 4, 2007; Int J Oncol. 2007 Jun;30(6):1499-509.), and then we also performed dose-dependent effects of leptin in MCF-7 and ZR-75-1 cell lines. Based on the data, we considered 50ng/ml (3.125nM) for these studies.

5. Notes on References: 1. I thought it was rather odd and very unclear to me why the authors would select the very "specific" research article (ref. 1) to support the "general "statement in the background section. General statement: Breast cancer (BC) is the most commonly occurring cancer and second most common cause of cancer deaths among American women [1]. Specific Reference cited: Winer A, Janosky M, Harrison B, Zhong J, Moussai D, Siyah P, Schatz-Siemers N, Zeng 4 J, Adams S, Mignatti P: Inhibition of Breast Cancer Metastasis by Presurgical 5 Treatment with an Oral Matrix Metalloproteinase Inhibitor: A Preclinical Proof-of- 6 Principle Study. Mol Cancer Ther 2016, 15(10):2370-2377. 2. The manuscript lacks inclusion of the more recent articles highlighting research on leptin and estrogen positive breast cancer, for example: Strong AL, Ohlstein JF, Biagas BA, Rhodes LV, Pei DT, Tucker HA, Llamas C, Bowles AC, Dutreil MF, Zhang S, Gimble JM, Burow ME, Bunnell BA. Leptin produced by obese adipose stromal/stem cells enhances proliferation and metastasis of estrogen receptor positive breast cancers Breast Cancer Res. 2015 Aug 19;17:112. The majority of the articles cited are prior to 2010. The authors should make sure that their literature search in the background is current. Leptin-regulated gene expression in MCF-7 breast cancer cells: mechanistic insights into leptin-regulated mammary tumor growth and progression.

RESPONSE: Suggestions of the reviewer are well-taken. We modified the revised manuscript.