Author's response to reviews

Title: STAMP alters the growth of transformed and ovarian cancer cells

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Author's response to reviews: see over
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Re: MS #1682725227301436

Dear Ms. Neilan,

We were pleased to learn that our paper on STAMP and cancer may be acceptable for publication pending suitable revision. We thank both referees, especially Referee #1, for their extensive efforts with, and constructive criticism for, our paper. We have seriously considered and responded to each concern in the attached revised manuscript. We believe the incorporation of their suggestions has led to a much clearer and better paper. The reviewers’ comments are given below in italics: our response is in regular type. We look forward to your decision.

Sincerely yours,

S. Stoney Simons, Jr., Ph.D.
Chief, Steroid Hormones Section
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Referee #1

1. Is the question posed by the authors well defined? Not entirely. It seems to be to further investigate the physiological role of STAMP. In general the paper doesn’t seem to connect the various sections into a cohesive whole. The last section in particular seems disconnected from the ovarian cancer data. (However, I am not very familiar with the type of experiments in Figure 4, so perhaps I am missing the connection.) We have revised sections of the Abstract, Background, Results, and Discussion to try to better present the data as a cohesive and logically related study. Briefly, the last section shows that the seemingly opposite effects of STAMP on ovarian cancer cell growth (i.e., to both increase and decrease growth) are not unusual because we also see opposite effects with the modulator activity of STAMP on GR transactivation (i.e., to both decrease and increase the EC\textsubscript{50} and partial agonist activity).

2. Are the methods appropriate and well described? Yes Thank you. No response necessary.

3. Are the data sound? Major:
My main worry is with the QPCR data. It is not clear how the QPCR data has been analysed. The y axis in 2A is given as “relative amount” – relative to what? Also there are no error bars on these graphs – were the QPCRs replicated? This should be stated. In general, I would always do QPCR in triplicate, however I am not familiar with the Tissue Scan cDNA and validated Taqman assay used – perhaps these are sufficiently QC controlled to provide accurate results after 1 assay. The tissue scan panels from Origene are pre-normalized cDNA arrays prepared from pathologist-verified tumor tissues. Each well of the panel has the same total amount of cDNA. Thus, any target gene (e.g. STAMP) variation in different wells reflects real changes between tumor samples. The qPCR results are presented as relative to the STAMP mRNA level of the first sample in the Breast panel so that the reader can also appreciate the absolute levels of STAMP mRNA across the different tissues. A brief description of these facts is now included on new page 8 and in the legend for Fig. 2A. Replicates of the samples are not possible (without the considerable expense of buying another plate) because they are presented as individual wells on a plate by the supplier, OriGene. A statement describing this has been inserted on new page 8. Of note is that control experiments on other samples show that our qPCR results on a given sample are very reproducible.

For the first set of Tissue Scan panels they do, I am not convinced it is appropriate to draw a conclusion from just 12 samples per tumour type. Given how variable the results are, I would much prefer to see the results for the wider tissue panels performed. The purpose of these panels was to quickly scan a variety of tumor samples, which were again commercially available. We agree that we might be missing something but decided at the outset that we would concentrate only on tumors that displayed a dramatic difference in such a small panel of tumors. This is now explicitly stated in the Abstract.

For the larger panels I would also suggest using a plot that shows individual sample data or a box-whisker plot to indicate the spread of values, rather than the average shown in Fig 2B. The use of standard error is not appropriate here, standard deviation should be shown as the samples are not replicates but drawn from a population. Done.

There is a major problem, often underappreciated for ovarian cancer, which is that ovarian cancer subtypes are considered to be different diseases (see work by the group at BCC in Canada, eg Kobel et al., 2008., Plos Medicine and Gilks et al. 2008, Hum Pathol) and conclusions about expression and clinical outcome must take subtype into account. Grade should also be considered, as grade 1 serous are not the same as grade 2 & 3 serous. Similarly grade 3 endometrioid are likely to be different from grade 1 & 2. When considering stage, subtype is key, as erroneous conclusions can be drawn on stage, which are actually based on subtype – for example, most serous cases are diagnosed late, while most mucinous cases are diagnosed early. Immunohistochemical markers for example are usually consistent within a subtype over multiple stages, more so than between subtypes. We agree with the referee completely. We thought that we had pointed this out on pages 3, 5, 16, and 17. Additional emphasis has been added on new page 16. In addition, our
ovarian cancer expert (ECK) has reviewed and improved the language.

For the data shown in Figure 2B, I have a problem with the comparison with the normal samples provided in the panel. This tissue appears to be whole ovary, which is not an appropriate control for ovarian cancer, as it is mostly stromal cell types, whereas the tumours are epithelial in origin. Thus the difference observed may be a difference between stroma and epithelium rather than tumour and normal. A better control would be microdissected ovarian surface epithelial cells, though this is very difficult to obtain. An alternative would be fallopian tube epithelium, which may be a control suitable for the serous subtype. Even a comparison with benign or borderline epithelial tumours of the same subtype would be preferable. Alternatively, the data would be strengthened by some sort of in situ data showing protein or RNA expression differences between normal ovarian epithelium and tumour epithelium. At the very least the data should not be interpreted as it is at present. Again, we agree with the referee. We thought that we had addressed this on pages 3, 5, 11, 16, and 17. Due to the importance of this matter, though, we have re-emphasized the need for caution and further qualified our conclusions on new pages 6, 11, 16, and 18.

Also, ovarian borderline tumours are not considered to be malignant and should be considered separately. We failed to clearly state that the IGB samples are “low malignant potential, borderline tumors.” We have added this definition on new page 11 and the legend for Fig. 2B, in which case we have considered low malignant potential tissue as a separate category, as recommended by the referee. We also felt that this is a better comparator for the epithelial tumor absent normal epithelial surface epithelium.

Minor:

In describing the very first experiment (Page 9) the authors give the cell counts for the two clones, but could they also give the count for the empty vector control for comparison? The data presented are for the number of STAMP-transfected cells as the percent of that seen for the empty vector-transfected cells (i.e., [no. of STAMP-transfected cells]/[no. of empty vector-transfected cells]), where equal numbers of each cell type were plated at time 0, as indicated on page 7. In an effort to clarify this, we have added a phrase on new page 10.

When discussing the ovarian panel, the authors use the phrase “endometrio[ ]id cancers” but presumably they mean ovarian cancers, as the subtypes of the ovarian cases in the panel is a mixture of subtypes. We appreciate the referee’s concern regarding the correct designation of the cancer samples. However, according to both the OriGene pathologist and independent analysis by one of us (ECK), the tumor samples in Fig. 2B are indeed endometrioid ovarian cancer, a well recognized histologic subtype of epithelial ovarian cancer.

What is “IGB”? I can’t find a definition for this term. IGB stands for “low malignant potential, borderline tumors” or borderline neoplasm. We have added this definition on new page 11 and the legend for Fig. 2B.

Discretionary:

I think the data in Table 1 would be better expressed as a bar graph, which would enable error bars to be shown to indicate the reproducibility of the values obtained by QPCR. Also I can’t see why for Table 1A all the cell lines were not done, when they are then used in the subsequent analysis. Why was Hey A8 chosen as the reference cell line? We prefer to retain the table because quantitative values are easier to determine from a table. To indicate the reproducibility, we have added the SEMs for each value. The absolute values of Table 1A changed slightly because we have now normalized before averaging. Not all of the cells are listed in Table 1A because, at the time that the levels of STAMP mRNA were determined for Table 1A, we did not have the other cell lines. When we obtained the additional cell lines, we simply proceeded and quantitated the levels of STAMP mRNA with control or STAMP siRNA with both the old and new cells. Hey A8 was arbitrarily chosen as the reference cell line simply because it was one of the first cell lines examined and was used throughout the experiments. This is now stated on new page 12 and the legend for
Table 1.

I don’t really understand the normalisation of the QPCR done for Table 1B. Is the normalisation as written just (cell A Stamp siRNA)/(Hey Lamin siRNA) because the “cell A Lamin siRNA” cancels out? It is more usual to see a comparison of each cell line with a percentage knock-down relative to a control siRNA, although this is mentioned in the text. Using the SEM is not appropriate here (page 12, line 7) as the cell lines are not replicates but part of a population. Perhaps the range of percentage knockdown could be given, to show that all the cell lines were knocked down efficiently. We realize that the normalization of Table 1B can be confusing. Unfortunately the normalization is not quite as simple as described by the referee. In both cases, the denominator concerns “STAMP mRNA with Lamin siRNA” but in one case it is for “cell A” and in the other case it is for “Hey A8 cells”. We have reworded the normalization on new page 12 in an effort to more clearly describe conceptually what was done. We agree that it is customary to give the percent decrease relative to a control siRNA. We give that average value (now ± S.D. as requested) in the text. We performed the more elaborate calculation in Table 1B so that we could then use these data (as described at the top of new page 13) to make the plot of Fig. 3B, in which we use the relative amount of STAMP mRNA after siRNA treatment.

4. Does the manuscript adhere to the relevant standards for reporting and data deposition? Yes. The authors should be complimented on defining their error bars. Thank you.

5. Are the discussion and conclusions well balanced and adequately supported by the data? No. I don’t believe the interpretation of the correlation of STAMP mRNA and ovarian cancer is justified due to the use of whole ovary as a control. This contention could be supported by data mining of microarray data sets to see if other studies have detected this gene as overexpressed. For the reasons state above, we feel that it is appropriate to speculate that increased levels of STAMP in samples of ovarian tissue are of interest and warrant further investigation. Any statement regarding STAMP as a putative biomarker has been markedly softened and qualified as requiring validation in a larger study. We have tried to clarify this point on new pages 11 and 16. We recognize that this finding has to be validated in a larger, independent, and better controlled sample set.

6. Are limitations of the work clearly stated? No, I don’t think the authors are sufficiently familiar with some of the inherent difficulties associated with analysing ovarian cancer data, and these should be acknowledged in the discussion. The appropriate difficulties of working with ovarian cancers, along with the accepted limitations, have now been explicitly added to this revised paper by one of us (ECK), who is a recognized ovarian cancer specialist.

7. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? Yes No action needed.

Discretionary:

Sometimes the choice of references could be improved. E.g. ref 22 seems a bit odd, wouldn’t similar information be held in one of the 3 “Cancer statistics” reports cited? Or the FIGO annual report can be a useful resource for ovarian cancer stats(Heintz et al., 2006). E.g. On page 6 the authors cite 2 papers to describe multiple pathways in ovarian cancer, but neither paper is about ovarian cancer. Better references have been used, as suggested by the referee.

8. Do the title and abstract accurately convey what has been found? Yes. No action needed.

9. Is the writing acceptable? Yes. No action needed.

Minor:

Page 10 line 6. Change “14-fold after being withdrawal” to “14-fold after withdrawal” Done.

Page 24 legend to fig 2 “was determine as in A” to “was determined as in A” Done.

Discretionary:

Page 5 line 4. The authors use the term “homologues”. This is a bit confusing here as the term is often used to compare genes between different species (i.e. orthologs). Perhaps this sentence
could be rephrased or the term “paralogs” used. Done.

Page 11 line 18. “expressed” used here is a bit confusing given it has a more biological connotation as well. Perhaps change to “shown” or something similar. Done.

Page 11 line 21. change “that in the above control” to “that in the control.” Done.

Additional points (discretionary): I wonder given the suggestion of interaction of STAMP with steroid hormones, whether the authors had looked at the ER/PR status of breast tumours in relation to STAMP expression. I don’t know if that information is available with the Tissue Scan data, but perhaps could be obtained from publicly available expression data sets. This is a reasonable suggestion. However, we not yet pursued this possibility because we found that STAMP does not interact with ERs (see top of new page 5).

Referee #2

Major comment:

New data explaining the mechanism(s) underlining differential effects (positive and negative) of STAMP on cell growth are necessary for further processing this manuscript in the Journal. We agree that such data would improve this paper. However, we also respectfully believe that our findings of growth affects (and associated reduction in death) due to STAMP are a new observation relative to a mechanism of action of STAMP in the 293 cell experiments (Fig 1). In addition, we provide negative data related to key signaling growth and survival kinases and proteins, commonly found associated with growth advantages. While negative data, they add to our body of understanding of STAMP action. The contributions of STAMP to cell growth are thus many and diverse. We have added a statement on new page 17 indicating that these results advance our knowledge but do not point to a defined mechanism of how STAMP is affecting cell growth.

Minor comments:

(1) Results shown in Figure 1 are well organized and demonstrated. However, difference in cell growth rates between VA and S13 cells might have come from non-specific alteration in cell characteristics during multiple rounds of cell duplication for establishment of these cell lines. Thus, the authors should include results using transient knockdown/overexpression of STAMP in the same cell line by using, for example, STAMP siRNA or tetracycline-induced STAMP expression in stable cell lines. We appreciate, and initially shared, the referee’s concern. However, four sets of observations convinced us that we are not looking at simple non-specific alterations in cell growth. First, as originally stated (see top of new page 10), the same effects of STAMP on the proliferation of the stably transfected 293 cells was observed in two independently isolated, stable STAMP-overexpressing clones. Second, different frozen samples of both clone S13 and the VA control gave similar differences in growth rates. Third, the differences in growth rates appear to decrease with time of withdrawal of G418 and decrease in STAMP mRNA levels in the cells, which is consistent with a dosage effect where decreased STAMP DNA results in less inhibition of cell growth (as described on page 10 with regard to Fig. 1D). Fourth, we have used STAMP siRNA to also alter the STAMP levels and growth rates of several ovarian cancer cell lines, as shown in Fig. 3A.

(2) Figure 2: Please add brief explanation to staging of each cancer. Does different staging indicate virulence of cancers or difference in growth rates? I believe most of the cancer stagings are based on the degree of cancer spread inside/over organs. Thus, staging does not always correlate with malignant features of cancers or their growth rates. Staging of ovarian cancer is standardized throughout the world and depends upon the pathologic subtype and the surgical findings (FIGO criteria). The staging was provided by the supplier of the tissue scan panels (OriGene) and is based upon the pathologic information provided with the cases they received (and confirmed by one of us [ECK]). A statement to this effect has been added on new page 8.
(3) Figure 4 A: How did you estimate that right-shift of the titration curves was statistically meaningful? Statistical significance was established by analyzing the EC$_{50}$ values from four independent experiments, as described in the figure legend and the Statistical Analysis section of the Methods. The wording in the figure legend has been modified in an effort to clarify how statistical significance was determined.