Author’s response to reviews

Title: FKBPL and its peptide derivatives inhibit cancer stem cells and breast cancer metastasis by downregulating DLL4 and Notch4

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Responses to reviewers’ comments:

We would like to thank both reviewers for their constructive and thorough comments. We believe the modifications to our manuscript as explained below have improved its quality. In particular, we have toned down language to suggest that DLL4/Notch 4 are FKBPL/AD-01/ALM201 targets, but rather are downregulated after treatment which results in anti-stemness
activity. We highlight that future mechanistic signalling work will help to unravel the associated mechanisms, which are beyond the scope of this manuscript.

Reviewer 1:

Concern 1: The peptide AD-01 and its effect on endocrine therapy resistance (Figure 5-6) were covered in McKeen CancerRes2010, migratory properties (Figure 3) were covered in Yakkundi PLoSOne2013 and mammosphere formation/cancer stem cell properties were covered in McClements ClincancRes2013 previously by the same group in breast cancer cells. In fact, some very similar figure panels were published elsewhere by the same group [Fig. 1A, B; this study vs Fig. 4B; (McClements ClinCancerRes2013)]; [Fig. 3A; this study vs Fig. 3B (Yakkundi PLoSOne2013)] removing some luster on the originality of this manuscript. The true novelty here gets down to the attenuation of breast cancer metastatic progression through DLL4 and NOTCH4 by FKBPL.

Response: We thank the reviewer for taking the time to assess our previously published papers. Whilst there is some overlap, we respectfully disagree that Fig. 5-6 were covered in McKeen et al (Cancer Res 2010) study. In McKeen et al there was no mention of the effect of FKBPL on the cancer stem cell subpopulation but rather on cancer cell survival where we showed that high levels of FKBPL (not treatment with AD-01 or ALM201 peptide) sensitise MCF7 cells to endocrine therapies. In this study, we demonstrated that our novel therapeutic peptide, ALM201, can specifically target tamoxifen or endocrine therapy resistant cancer stem cells, rather than the general cancer cell population.

In relation to Yakkundi et al (PLoS One 2013) we agree that we showed before the effect of AD-01 on HMEC-1 endothelial cell migration via CD44. However, we have never previously demonstrated the effect of AD-01 on breast cancer, MDA-MB-231 cell invasion. This is supported by the additional novel data presented within our current manuscript, in the same figure, showing in vivo inhibition of metastasis by our novel therapeutic peptide, AD-01.

In McClements et al (Clin Cancer Res 2013) we published the effect of the pre-clinical candidate peptide, AD-01, on breast cancer stem cells. However, in this paper we demonstrated the effect of the clinical candidate peptide, ALM201, on cancer stem cells, both using in vitro and in vivo models. In addition, we also treated MCF-7 cells or xenografts with tamoxifen since cancer stem cells have been implicated in tamoxifen resistance. Furthermore, in relation to Fig. 1A&B, whilst we looked at the effect of AD-01 on holoclones/meroclones/paraclones, we never investigated the effect of endogenous FKBPL on these colonies using our breast cancer cell lines with stable FKBPL overexpression therefore confirming FKBPL’s endogenous inhibitory role on cancer stem cells. In Yakkundi et al (PLoS One 2013), Fig. 3B demonstrates a CD44-dependent effect of AD-01 on MDA-MB-231 cell migration whereas here we demonstrate in Fig. 3A AD-01’s anti-migratory effect on MDA-MB-231 cells without CD44 knockdown. Nevertheless, we do agree with the reviewer that this figure is not the most novel data within our current study, but does add to and support our previously published work.
In summary, we have demonstrated a number of novel effects of FKBPL, AD-01 and ALM201 which include the (1) inhibitory effects of tamoxifen-resistant cancer stem cells by the novel clinical candidate anti-cancer agent, ALM201, which is currently in clinical trials, (2) inhibition of both cell migration and invasion of breast cancer cells by AD-01 which was translated in vivo, demonstrating potent anti-metastatic activity, and (3) implication of a novel mechanism of action, suggesting that FKBPL and its therapeutic peptides also downregulate the DLL4/Notch4 pathway, in addition to CD44. Therefore, we have made a substantial addition to the published literature in this area.

Concern 2: The mechanism of action postulate by the author (through modulation of DLL4 and NOTCH4) is highly speculative and solely rely on correlative experiment. Thorough rescue experiment should be carried out to conclude that FKBPL inhibit CSC and metastasis by downregulating DLL4 and Notch4. From the data presented, DLL4 is at best a potentially good marker that correlate with AD-01 treatment / FKBPL level.

Response: We agree with the reviewer that we have demonstrated an involvement of DLL4 and Notch4 in the mechanism of action of FKBPL-based therapeutic peptides, AD-01 and ALM201. Previously, in McClements et al (Clinical Can Res 2013) we showed that when we co-treat with DAPT (Notch inhibitor) and AD-01 we see additive inhibitory effects on the cancer stem cells suggesting an independent mechanism from CD44. The involvement of the highly important DLL4 and Notch4 proteins is very exciting given the wealth of literature demonstrating their importance in the treatment of breast cancer and their role in CSC signalling. However, to elucidate the precise mechanisms is beyond the scope of this paper, given the wealth of other novel data we presented, that was translated into the in vivo setting in terms of sensitising cancer stem cells to endocrine therapy and reducing tumour metastasis; both highly novel biological endpoints. This study is of a particular importance as ALM201 is a novel anti-cancer agent currently in clinical development and which has recently been granted Orphan Drug Designation by the FDA. Nevertheless, we have now added a statement in the discussion to suggest that further experiments will be necessary to further interrogate the role of the DLL4/Notch4 pathway in ALM201-mediated impact on CSC signalling (page 17, line 17). Furthermore, throughout the manuscript we have removed any reference to FKBPL/ALM201/AD-01 in terms of targeting the Notch/DLL4 pathway and rather state that FKBPL/ALM201/AD-01 all independently downregulate the levels of these proteins across two cell lines, which may then lead to their anti-stemness activity. Please also see further comments in relation to this topic in response to reviewer 2 below. In particular, to upregulate these proteins would undoubtedly enhance stemness, as previously reported (Harrison et al. Cancer Res 2010; Simoes et al. Cell Rep 2015; Lombardo et al Breast Cancer Res 2014), and their levels in all cells lines tested are already quite high; so one would question whether this would really help address the questions the reviewers refer to. Instead, we have toned down comments to state that FKBPL/AD-01/ALM201 appear or is likely to regulate the Notch4/DLL4 pathway, which might explain the FKBPL-mediated effects on stemness.

Concern 3: The experiment in Figure 3 C-D is interesting but required further attention to conclude. In fact, the reason why two different set of experiments (panel C with pre-treatment
and panel D without pre-treatment) is presented. Does the FKBPL actually modify the metastatic microenvironment before cancer cells reach it? Does it interfere with cell grafting? Also, the in vivo experiment relies on bioluminescence as a single readout which is a surrogate for the number of cancer cells. The author should complement this figure by measuring tumor growth with other readout such as tumor weight, tumor volume estimate.

Response: We agree with the reviewer that Figs. 3 C/D are very interesting and show that FKBPL pre-clinical peptide, AD-01, has multiple anti-tumour effects, unlike other clinically available anti-cancer agents, including anti-angiogenic, anti-cancer stem cell and anti-metastatic effects. In relation to the design of our in vivo metastasis experiments, we did carefully explain the rationale for employing the pre-treatment in the results as follows: “Since it has been previously demonstrated by Ebos and colleagues(38) that anti-angiogenic agents can promote metastases, we investigated whether AD-01 pre-treatment can prevent metastatic invasion using two separate in vivo MDA-MB-231 experimental lung metastasis models.”

We are not able to comment on whether FKBPL modifies the metastatic microenvironment or cell grafting as we did not investigate this effect in this study; however, we would hypothesise that its ability to prevent migration and invasion is likely to prevent engraftment of the tumour cells – we have added a statement to that effect in the discussion (page 16, line 39). Since our experiment involved intravenous injection of MDA-MB-231-lucD3H1, an established in vivo metastasis model, and not a xenograft model we were unable to measure tumour growth but rather lung colonization. Nevertheless, all of our previously published studies indicate that AD-01 does inhibit xenograft growth through targeting angiogenesis and CSCs (Valentine et al., 2011 and McClements et al., 2013). It would be unethical to perform this experiment again, to measure metastatic growth, when we know that our therapeutic peptides significantly inhibit tumour growth.

Concern 4: Overall, the figure flow, their legends and data presentation are difficult to follow

We apologize for this. To make it clearer we have reordered Fig. 3&4 and corrected the text (page 11&25) so we feel that now the order is more logical starting with the effect of endogenous FKBPL on cancer stem cells, the implication of DLL4 and Notch4 mechanism, the effects on metastasis and the inhibitory effect on endocrine therapy resistant cancer stem cells.

- The knockdown validation is presented in the middle of the figure (Fig.1, 2, 4).

Response: We are not sure what the reviewer means by this comment but we will try to clarify that we only knocked down CD44 in Fig 2D to demonstrate the involvement of another pathway, likely DLL4 and Notch4, independently of CD44.

- Labels on x axis should be something like: control, FKBPL overexpression. Not the name of the cell line and a code like A3, D2, etc... Also please indicate what is the negative control used in all experiments (PBS?, empty vector?)
Response: We created MCF-7 and MDA-231 cell lines with stable FKBPL overexpression which we named D2 and A3. The control was parental cell line used i.e. MCF-7 as a control for D2 and MDA-231 as a control for A3. We have now added in the methods section that MCF-7 and MDA-231 were parental cell lines for D2 and A3, respectively (page 5, line 51).

- Figure 3C and D should be separated in individual panel and presented/discussed in the main manuscript

Response: We have already done this and discussed the results in the result section of the manuscript.

Concern 5: There is a couple of citations that are duplicated in the reference section. Please double check all references as each should have a unique number.

Response: Many thanks for spotting this. We have rectified this now.

Other comments

-In Figure 2C and 4D. Please indicate which band is the correct one in the NOTCH4 ICD blot

Response: Actually both bands belong to the Notch ICD. This has been previously shown by Harrison et al (Cancer Res 2010). We have now indicated this in the figure legends (Page 24, line 58; page 25, line 2).

-In Figure 2D. Could the authors comments on the CD44 KD not having an impact by itself (comparison between both control in panel D). Statistic should be provided between the two treated condition (the authors provide stats between control and treated but not between treated and treated)

Response: We have performed statistical analyses between all the groups in Fig. 2D however there was no statistically significant difference between ALM201 treated group without CD44 knockdown and ALM201 treated parental (MDA-MB-231) cell group (p=0.8). We have explained this now in the text for clarification (page 11, line 24).

-According to the data presented, FKBPL/AD-01 influence the % of stem cell-like cells population. Did the authors test the impact of AD-01 on "pure" stem cells and differentiated cells as sorted by FACS?

Response: We have investigated the effects of AD-01 and ALM201 on cancer stem cells using a functional mammosphere assay, however in our supplementary data we showed that the
%ESA+/CD44+/CD24- is substantially increased within mammospheres (MS) compared to the cell monolayer (ML) of both MDA-231 (2.9% vs 34.9%) and MCF-7 (4.1% vs 8.5%). In McClements et al. (Clinical Can Res 2013) we demonstrated that AD-01 is able to reduce ESA+/CD44+/CD24- stem-like cell subpopulation in both MCF-7 and MDA-231.

What are the best evidences indicating that AD-01 is not a prodrug? (that a smaller peptide is not the active substance?)

Response: When designing both AD-01 and ALM201 we carried out full analysis of the 24 and 23 mer peptide using ala and D amino acid scans and truncated peptides. We needed at least 23 amino acids to see activity. We lost activity with shorter peptides. So at this time we do not believe that AD-01 or ALM201 is a prodrug. This is beyond the scope of this manuscript and we have not commented further on this within the manuscript.

In introduction, Page 4, line 35. Please precise which cohort are you discussing and add the appropriate reference

Response: This is a cohort of five independent TMAs that we stained for FKBPL and published the data in Nelson et al (Oncotarget 2015).

Reviewer 2

The overall writing of this article is good and logical, but there are still some problems as follows:

1. There is no p value showing in the picture C and D of figure 6, and in the picture D-2 of figure 2.

2. The manuscript reveals that FKBPL and its peptide derivatives inhibit cancer stem cells and breast cancer metastasis by downregulating DLL4 and Notch4. So my suggestion is that the author should upregulate DLL4 and Notch4, and then observe that if FKBPL and its peptide derivatives can still inhibit cancer in vitro or vivo.

Response: We thank the reviewer on commending us on the article standard.

1. These are not statistically significant which is why we did not include any p-value representation on the Figures. In relation to Fig. 2D we only have n=1 to show the proof of downregulation and therefore we could not perform statistical analysis. Nevertheless, this cell line has a stable knockdown of CD44 which has been shown in previous publications (McFarlane et al, Oncotarget 2015).
We agree with the reviewer that we have not shown direct involvement of DLL4 and Notch4 in the mechanisms of AD-01 or ALM201. We have responded to a similar comment by reviewer 1 and have modified the text within the manuscript accordingly to suggest that further studies would be needed to robustly demonstrate a role for FKBPL/ALM201/AD-01 in the Notch4/DLL4 pathway (page 17, line 17). We also feel that a more thorough/in depth analysis of the role of Notch4/DLL4 would have been more likely to have targeted a higher impact journal. Nevertheless, the fact that both proteins were modulated by the full length FKBPL transgene as well as both therapeutic peptides, AD-01/ALM201 across two different cell lines, should be sufficient to convince the reviewers that the data are real. Furthermore, because both DLL4/Notch4 have known biological roles in controlling stemness, it would be unlikely that the modulation of these proteins by FKBPL/ALM201/AD-01 were irrelevant or not biologically significant. Furthermore, to upregulate these proteins would undoubtedly enhance stemness, as previously reported (Harrison et al. Cancer Res 2010; Simoes et al. Cell Rep 2015; Lombardo et al Breast Cancer Res 2014), and their levels in all cells lines tested are already quite high; so one would question whether this would really help address the questions the reviewers refer to. Instead, we have toned down comments to state that FKBPL/AD-01/ALM201 appear or is likely to regulate the Notch4/DLL4 pathway, which might explain the FKBPL-mediated effects on stemness. As mentioned previously, in McClements et al (Clinical Can Res 2013), we showed that when we co-treat with DAPT (a Notch inhibitor) and AD-01 we see additive inhibitory effects on cancer stem cells, further supporting our hypothesis that this pathway would be important for the FKBPL-mediated effect on in addition to the signalling through CD44. In figure 2D we also showed that when we knock down CD44, ALM201 is still able to inhibit cancer stem cells suggesting that another pathway is involved, likely DLL4 and/or Notch4. In summary, whilst there is further work that could be done to support a role for DLL4 and Notch, we feel that this is beyond the scope of this paper and journal and would be something we could pursue in our future studies on mechanism.