Author's response to reviews

Title: Identification of genes regulating migration and invasion using a new model of metastatic prostate cancer

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Author's response to reviews: see over
Dear Editors and Reviewers,

We thank you for your review and consideration of our manuscript entitled “Identification of genes regulating migration and invasion using a new model of metastatic prostate cancer” by Banyard, et al. We have included new data in Figures 5, 6 and S1. We have addressed both reviewers comments below.

Response to Reviewer #1.

_The establishment of the DU145-derived lines here described has already been reported in the paper from this group in Sci Reports 2013._

The Reviewer is correct, and we would like to clarify this issue. Our laboratories had two separate projects dealing with this new DU145 model and both papers were originally submitted in the fall of 2013. One manuscript focusing on microRNA-424 and -200 is now published in Scientific Reports 3:3151, Nov 2013. We now refer to this paper in our revised manuscript (now ref #21).

_Figure 1 and 2 of the here submitted manuscript are very similar (partly identical) to Fig 1, A, B and C from the published paper._

We apologize for the confusion. The study submitted herein focuses on a new metastatic model in greater detail and presents a microarray study of genes differentially expressed between the cell lines, and the role of selected genes in cell migration. Only one panel of Figure 1 from Sci Reports, a photograph of original tumors, is used in our revised manuscript. We have properly cited this in the legend and have written permission from Sci Reports to reproduce this image.

_“Also in that paper, Western blots are shown for EpCam, vimentin, Ecadherin and Cytokeratin, for which in the submitted manuscript immunohistochemistry now demonstrates the same changes.”_

Again, we apologize for any confusion. The Western blots presented in the Sci Reports manuscript are from total cell lysate from cultured cell lines _in vitro_. In this report, we expanded beyond cell culture and focused on Ecadherin and EpCAM proteins expressed _in vivo_ in the tumor tissue. We do not show vimentin or cytokeratin.

_First paragraph: PSA screening is no longer accepted by FDA. Please enter this important information here._

We thank the reviewer for this important point and have omitted this sentence based upon his comment.

2. There is a conceptual compromise made during the generation of this model for which the rationale is unclear: DU145 cells were injected in the prostate of nude mice. After 4 weeks or longer, the sentinel para-aortic lymph node was minced, and human cells growing out of homogenized lymph nodes were reinjected into prostate again. Why are cells taken from the lymph nodes reinjected in the prostate? This is different from the situation in patients, where there is obviously communication between the prostate (tumor) and the draining lymph nodes, but the route back (cfr to reinjecting cells back into the prostate) is less obvious. This should be well explained in the discussion.
We apologize for the lack of rationale regarding our new model. We have revised the text to clarify this point. As the reviewer suggests, we would indeed have no expectation that the lymph node metastases would return to the prostate.

Our intention was to select for a highly reproducible metastatic line. Previous studies (J Natl Cancer Inst 1992, 84:951; Clin Cancer Res 1996, 2:1627) have demonstrated that repeated rounds of in vivo cycling from prostate to lymph node can create lines with increased metastatic potential.

3. Page 6: ‘DU145 cells ... still have AR.’ This is a controversial statement. Except for this 2006 paper the authors refer to, most people that tried to detect AR in DU145 were unsuccessful. It is crucial therefor to prove with appropriate qRT-PCR that there is AR mRNA present in these cells. Otherwise it would be better to leave out this statement and rather emphasize the fact that DU145 cells might represent a dedifferentiated state and thus be a good model for AR- mCRPC which arises after enzalutamide and/or abiraterone treatment.

We thank the reviewer for this important point and have omitted this sentence based upon his suggestion. The AR status of the cell lines should have no bearing on the data or conclusions we present in this manuscript.

Minor essential points
1. In the introduction, some details on how the original DU145 cell line was established should be discussed.

Thank you for this suggestion. We have now edited the introduction to include this detail.

2. On p.14 ‘metastatic incidence varied’ Do the authors mean ‘local metastasis’, or did they check for metastasis in bone and soft tissues?

We apologize for the lack of specificity regarding the location of metastasis. We meant local metastasis and have edited the text to reflect this.

3. On p.17, the ref 25 is used. Apparently, the new metastatic model presented in the her submitted manuscript is also presented in that paper. It is unclear how much overlap there is between ref 25 and this study.

We agree and have now attempted to clarify this point and distinguish the studies. We now reference this publication as #21 where appropriate.
Reviewer 2

Major compulsory Revisions:

1) A major reservation this reviewer has with the present form of the manuscript is the lack of any mechanistic data to implicate signaling pathways downstream of the candidate genes in imparting metastatic potential to DU145 cells.

We appreciate the comment of the reviewer and although a thorough study of signaling pathways in this model is beyond the time frame for revision requested by the editor and beyond the scope of this manuscript, we have added two new figures regarding signaling. We plan to present a more detailed signaling study in the next year.

In response to this reviewer’s comment, we have used Ingenuity analysis software to examine signaling genes differentially expressed in the various cell lines. We now present this data in Supplemental Figure 1. Some interesting molecules which increase in the metastatic lines include Dusp4, which is a MAPK phosphatase and MAPK13. Others include CD24, a cell adhesion molecule and Flt1, a VEGF receptor.

In addition, we have investigated the downstream pathways of uPA signaling in the DU145LN4 line. We examined signaling pathways following serum stimulation (to model the response to serum in the migration and invasion assays). We present new data in Figure 6 that the phosphorylation of AKT and p70-S6K was affected by inhibiting uPA expression.

2) In addition the essential rigor of establishing causative effect of gene expression by re-expressing or overexpressing ITGB4, uPA, and EpCAM in parental DU145 is a necessary set of experiment that would further validate the authors’ conclusion that these candidate genes do play a role in providing metastatic phenotype to DU145 cells. Without mechanistic data to implicate the phenotypic change in the cycled cell lines in this model, though well done and well-presented, this work remains largely descriptive.

We apologize for any confusion regarding causation. We agree that overexpression of each of these genes either individually or in combination may be informative. A careful and robust study of these genes will require a greater time frame and a different scope for a manuscript. We plan these experiments in the future.

We believe our data goes beyond description since we used microarray analysis to uncover genes upregulated during metastasis. Then we chose 3 genes upregulated in metastatic DU145 cells and used siRNA to demonstrate their involvement and necessity in cell migration and/or cell invasion. Each of these genes may not be individually competent to induce metastasis in parental cells. We have edited our text to reflect this comment.

3) It would be interesting to find the nature of signaling pathways downstream of ITGB4, uPAR and EpCAM in the panel of DU145 derived cell lines. …understanding mechanisms behind biology of metastasis would bolster this work and afford better visibility.

We agree that evaluation of signaling pathways in the DU145 cell series would be interesting. We now include new data evaluating AKT and S6K following depletion of uPa in Figure 6.
Minor Essential Revisions

1) The Results and Discussion are well written. However the text contains redundant information in some sections that can be deleted. For example the p values are repeated in the text while they are also presented in the figure and explained in the figure legend.

Thank you for this comment. We have edited the text to remove redundant information.

2) Remove Supplementary Figure 1 since it is not very informative as it is similar to the dendogram shown in Figure 5A.

We have removed this Supplemental Figure based on the reviewer’s suggestion.

3) This reviewer is unaware of the section that the authors mention in page 25 citing reference 45. As it reads it is a confusing statement that appears to contradict the author’s current results.

Former Reference 45 (now reference #41) refers to Yoshioka et al, J. Clin Invest 2013. This publication shows transgenic mice with an ITGB4 signaling domain mutation that resulted in reduced prostate tumor formation and progression, thus supporting our data that ITGB4 is involved in tumor cell migration and metastasis. We have now edited this section for clarification.

4) Figure 3A. Need higher magnification images to highlight phenotypic difference.

In Figure 3A, we now include close-up images as insets in the lower magnification images as suggested.

5) Figure 3B. Quantitation on fold difference of staining would be useful.

We respectfully suggest that quantification of IHC should be done only on larger cohorts of images and preferably on a single slide such as those found in a tissue microarray analysis in order to establish a cut-off for “significant” staining (Banyard et al, Clin Can Res 2007, 13:2634; Spivey et al, Cancer Epidemiol Biomarkers Prev 2010, 19:1362).

6) Figure 4C. To be consistent with rest of the images remove DU145-PAR and list just DU145.

We have modified Figure 4C as requested.

7) Figure 5A. This is a very dramatic figure that essentially encapsulated the paper’s key point, i.e. change in expression levels of select set of genes. However, the reasoning to choosing only a few from the whole list is not apparent in the text. Is the choice based on p-value, Q-value (after corrected for False Discovery Rates)? Would it be better to list the gene names according to their statistical values rather than alphabetically if the choice was based on FDR?

Selection was not based on significance value. All of these genes in Figure 5A had qualified for inclusion in this dataset by being >1.5 fold changed. We selected genes of interest to our groups, and three individual genes seemed a reasonable number to study. We have edited the text to describe our rationale.
8) Figure 5B and C should be supported by graphs that show normalized expression of EpCAM, ITGB4, uPA from “n” number of experiments. As such a single experimental result of Western blots are not very convincing when juxtaposed next to the dramatic change in RNA levels in (A).

We thank the reviewer for this comment. RNA levels do appear dramatic in the heat map. Absolute RNA differences are shown in Supplemental Table 1. Based on the reviewer’s comment, we have created new graphs quantifying several western blots for each protein. The new data is now shown in our revised Figure 5C.

9) Figure 5 C. Needs labels. Although it is the same cell lines as in (B), the conditioned media analysis in (C) looks “orphaned” without the labels! An internal control should be included to show equal loading, say with some other secreted protein.

We were unable to find another secreted protein to use for equal loading and have therefore omitted the western of conditioned media from Figure 5C (version 1 of paper).

10) Merge 6 H & I therefore there is no need to duplicate the same GAPDH blot. Remove the notation to this effect in the figure legend.

We have now merged former Figure 6H & I as requested into new Figure 6H. Panel I is now a new western.

11) Figure 6D & E, even though not significant provide the p value and indicate (n.s)

We have provided the p value and noted that these treatments were not significant in the legend for Figure 6.

We hope that all of the reviewers’ comments have been addressed to your satisfaction. We appreciate the opportunity to publish with BMC Cancer.

Yours Sincerely,

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