Author's response to reviews

Title: Vascular measurements correlate with estrogen receptor status

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Author's response to reviews: see over
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Editor
BioMed Central

Dear Sir/Madam,

My colleagues and I would like to resubmit an article titled “Vascular measurements correlate with estrogen receptor status” for consideration by the BioMed Central.

Revisions to this manuscript have directly responded to reviewer’s comments following our initial submission. We believe that the comments provided by the reviewers have given us the opportunity to significantly strengthen this manuscript. For that, we thank the reviewers and look forward to publishing these works.

Specific responses to the reviews are available here:

Reviewer’s report
Title: Environmental conditions correlate with estrogen receptor status
Version: 0 Date: 15 August 2013
Reviewer: Rachael Natrajan

Reviewer’s report:
The manuscript by Lloyd et al correlates ER status with tumor vasculature given the working hypothesis that estrogen is carried to the tumors via blood and diffuses through the tumor. This is an interesting study attempting to address the issue of heterogeneity with ER staining vasculature, however a number of issues need to be addressed:

Major Compulsory Revisions

1. Are the authors sufficiently powered to detect significant differences with only 6 ER negative patients? Why did they only look at cases >90% ER+ cells if one of their aims was to assess the regional distribution of ER+ and ER- cells.
   The authors agree that the number of patients used in this study is low and that an increase in the variance of ER+ patients could strengthen the results. The authors also note that the differences were significant enough that even with the number of patients used statistical relevance was possible. In the manuscript this was addressed on page 13 with the comment “While the number of patients evaluated in this study is limited, and a larger patient population would be desirable, the number of individual vessels evaluated is on the order of $10^3$ to $10^4$ per patient. For this reason, our results indicate statistical significance to detect differences between ER positive and negative patients.”

Although the numbers are small, did the authors look at the tumors with heterogeneous ER staining and look at the vessel distribution? Did the ER+ cells congregate nearer to the vessels within the tumor?
   A spatial analysis of ER+ cells around vessels is a strong future direction for this research. This was addressed on page 16 with the following statement: “Furthermore, it may be possible that ER+ cells cluster around vasculature and effectively act as a barrier. While this is a future direction of this research and has not yet been tested, it may explain how both populations coexist spatially in a single tumor.”

2. Based on the findings the title would better be suited to being a little more specific. “Vessel size correlates with Estrogen receptor status” or something similar.
The authors absolutely agree and have changed the title to “Vascular measurements correlate with estrogen receptor status”.

3. The authors hypothesize that ER will be expressed only if there is estrogen in the microenvironment. Is there a way to test this?
   It is plausible that ER expression only occurs in the microenvironment when estrogen is present may be a testable hypothesis. This was addressed on page 14 with the statement “Regardless, this unavailability of estrogen is one reasonable explanation for estrogen-independent tissue selection. This may be a testable hypothesis in vitro or using techniques including laser capture microdissection to isolate specific regions of high vascularity within patient tumors and evaluating the estrogen concentrations. This is a key future direction for this research.”

4. Is ER status correlated with the cellularity of the tumor? i.e. more densely packed the cells, perhaps the estrogen is more difficult to diffuse through the stroma to adjacent cells?
This was an excellent suggestion and was tested by the authors. The following statement can be found on page 12 “In order to test cell density as a plausible barrier of diffusion we evaluated the mean cell number per mm2 and did not find any significant difference in the 24 samples evaluated (p=0.586). This suggests cell density alone does not correlate with ER status.”

5. A number of times the authors quote p values >0.05 (page 9). P values >0.05 are NOT significant. Thank you for identifying this error. All p values on page 9 have now been explicitly stated to reduce confusion.

6. On page 9, the authors state that ‘Vessel size was surprisingly not found to be correlated with disease progression (p=0.295)’. Why was this surprising? The word surprising was removed from the text to simplify and clarify this statement.

7. The authors conclude that ‘This suggests that as ductal carcinoma in situ progresses towards invasion, if necrosis does not increase with the cancer progression, then ER+ cells are more likely to dominate the population.’ Isn’t it more plausible here that the blood vessel size means the larger they are the less necrosis you get? and is actually determined quite early on? Is there a difference between vasculature in the adjacent normal breast and whether the tumor/DCIS lesion is ER+ or ER-? These are very good questions and the authors address these questions on page 13 with the statement “Our second hypothesis, that ER status would be inversely correlated with necrosis, was even more strongly supported. This suggests that as ductal carcinoma in situ progresses towards invasion, 1) the larger the vasculature is early in disease progression, the lower the volume of necrosis and 2) if necrosis does not increase with the cancer progression, then ER+ cells are more likely to dominate the population.” The vasculature in normal breast samples was not investigated.

8. On page 15 the authors state “Furthermore, it may be possible that ER+ cells cluster around vasculature and effectively act as a barrier’. Did the authors see this? The authors have not observed ER cells clustering around vasculature with the current samples. This was stated on page 16 with the statement ‘Furthermore, it may be possible that ER+ cells cluster around vasculature and effectively act as a barrier. While this is a future direction of this research and has not yet been tested, it may explain how both populations coexist spatially in a single tumor.”

Minor Essential Revisions

9. Throughout the manuscript, the authors quote R2 values as percentages. It would be better to quote these as fractions. This revision was not addressed due to the preferences of one of the authors.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests

Reviewer’s report
Title: Environmental conditions correlate with estrogen receptor status
Version: 0 Date: 2 August 2013
Reviewer: Andriy Marusyk

Reviewer’s report:

The authors used whole slide image acquisition and analysis algorithms to study the relationship between ER expression status, vasculization and extent of necrosis on a panel of primary breast cancer samples. The study revealed that vasculization positively correlates with expression of ER, whereas necrotic areas are more extensive in ER- tumors. Authors used these results to support a priori hypothesis that the correlation between expression of ER and vasculization arises due to selective pressures, where ER expression provides a selective advantage under conditions of adequate blood supply, but is disadvantageous under the conditions of poor vasculization and therefore undergoes negative Darwinian selection.

Contributions of selective pressures in tumor evolution appear to be underappreciated in the mainstream of cancer biology and studies on how tumor biology can be shaped by selective pressures can potentially alleviate this deficiency. However, current study makes an unjustifiable leap of faith between the data and the conclusions ignoring alternative explanations that appear to be much more likely based on current knowledge on the biology of ER.

Authors assume that ER expression is a fixed phenotypic trait that can serve as a substrate for Darwinian selection under positive selection pressure upon estrogen availability and negative selective pressure in estrogen absence. Whereas this assumption is formally
possible, large body of evidence from many labs strongly indicates that ER expression is a dynamic trait under complex regulation (for review see PMID 15143157).

Of relevance to the current study
1) Genetically homogeneous breast cancer derived cell lines display variability in ER expression even in presumably homogeneous in vitro culture conditions.

2) Similarly to cells from normal mammary epithelia, in breast cancer ER expression is absent in progenitor like cells and abundant in cells with luminal differentiation phenotype (PMID 17349583), i.e. heterogeneity in ER expression within tumor might reflect remnants of differentiation hierarchy.

3) Hypoxia has been reported to induce proteosomal degradation of ER (PMID 19952428, 12351689), therefore findings of the correlation between ER expression and vascularization can be explained on the basis of published knowledge.

These are very important points. The authors embrace this critique and have included the following statement and references:
“Furthermore, we acknowledge that a large body of work exists which addresses the complex dynamics of ER expression in vitro (Pinzone et al 2004 and Stoner et al 2002) and in vivo (Shipitson et al 2007). We embrace these works and do not suggest that phenotypic adaptation alone is sufficient explain variation in ER expression.”

Additional caveat, majorly overlooked by the study is that drawing the conclusions on selective pressures based on comparisons between different tumors is hardly justifiable, especially in the case of comparisons between ER+ and ER- cancers which represent clinically and biologically distinct group of disease, with major differences in evolutionary trajectories (including commonly presumed difference in target cells for cancer initiation, different patterns of gene expression, different patterns of chromosomal instability and different putative drivers), different biological and clinical properties and are thought to originate from different target cells. The particular panel of samples with used in this study (with nearly uniform expression of ER) appears to be ill suited for addressing the roles of selective pressures; analysis of intra-tumor differences would be more revealing (while dealing with heritable differences, which is unlikely to include differences in ER expression).

This is a poignant point. The authors agree that intratumoral differences would be more revealing and aim to make that the key future direction of this work. However, the authors also believe a great deal can be learned from the evaluation of significant intertumoral differences observed between the vasculature in ER+ versus ER- patient cohorts.

I strongly disagree with the assumption “As with any common phenotypic trait, we assume that ER expression will be observed only if it provides an adaptive advantage. When estrogen is absent, ER expression represents a needless expenditure of resources and will be selected against”. Whereas these assumptions would be applicable to populations of unicellular organisms under competitive growth limiting conditions, it makes little sense in context of population of tumor cells. Overexpression or deletion of many genes has little to no effect on competitive fitness in tissue culture or xenograft studies, suggesting that energy considerations required for expression of a single gene are unlikely to play significant role selection pressure wise.

The reviewer raises an important point here. It is unknown how important ER expression is for selection. We have amended the statement on page 4 to read “We propose that ER expression will be observed if it provides an adaptive advantage. Specifically, we propose that ER will be expressed only when estrogen is present in the microenvironment.”

It should be at least noted in the discussion of the data that due to tumor-specific alterations in vasculature bed not all of the endothelial cells would necessarily display CD34 staining, therefore there is a possibility that some differences might be over or underestimated with the use of the antibody employed in the study.

The authors absolutely agree with this critique and have added the following text on pages 12 and 13: “Our results do show that ER+ tumors are associated with larger blood vessels and a lower percentage of tissue necrosis. It should however be noted that differences in CD34 staining may over or underestimate the vascularity due to tumor-specific alterations in the vascular bed such that not all of the endothelial cells may be appreciated.”

I would like to advise more careful referencing to figures. For example, authors state “The average diameter of ER+ DCIS without central necrosis was 702 µM (Figure 4)”, whereas figure 4 contains no quantitative data instead showing some representative H&E pictures.

Similar inaccuracies can be also found in references to other figures.

The authors thank this reviewer for the careful review and have made changes to the text referring to Figure 4 on page 13.

Throughout the manuscript some data is presented as mean numbers only without showing deviation, which hinders adequate evaluation of the results.

Likewise, I suggest greater care with references. Reference Alfarouk et al 2013 mentioned in the Introduction is missing in the list of references.

The authors thank this reviewer for the careful review and have made applicable changes to the references.
To support statement “Similarly to molecular heterogeneity found in other cancers” authors reference 2 reviews, 2 primary papers and 1 mathematical modeling paper, which is a questionable choice, as the 2 primary papers do not exhaust massive body of experimental literature on the subject, referencing 1-2 comprehensive reviews would be sufficient, also see comment below regarding use of term “molecular heterogeneity”.

Authors appear to be using genetic heterogeneity, cellular heterogeneity and molecular heterogeneity interchangeably, which not only lacks precision, but can also lead to unnecessary confusion, as terms cellular heterogeneity and molecular heterogeneity are commonly used to describe an entirely different phenomena of meaningful phenotypic differences within cell populations that arise due to stochastic fluctuations in gene expression networks (for example see high profile review PMID 20478246).

The authors agree that the previously used terminology was causing unnecessary confusion and the terms cellular heterogeneity and molecular heterogeneity have both been changed.

Statement “.. the source of estrogen in the breast is typically serum” is incorrect, serum is blood plasma depleted of coagulation factors, whereas cells in normal tissues and tumors are bathed in interstitial fluid, not serum.

This is a very important critique and the authors changed this term on page 4: “Since the source of estrogen in the breast is typically (although not always) interstitial fluid and moves from the vessels into the cell by a simple reaction diffusion model identical to oxygen, nutrients, etc. (Jiang et al 2002), we propose the hypothesis that ER+ cells will be found in regions of high blood flow while ER- cells will be present in regions of poor blood flow.”

In summary, the data presented in the paper might be useful for the cancer research community. However, sound support of author’s conclusions would require refuting large body of evidence on dynamic regulation of ER expression and its dependence on oxygenation levels. I suggest complete re-evaluation of conclusions and discussion of the

The authors aim not to refute existing work, but rather offer an additional perspective. It was the goal of the authors to make this clear throughout the manuscript, but in particular, in the conclusions.

My colleagues and I thank you for your consideration.

Best wishes.

Mark Lloyd