Author's response to reviews

Title: TOX3 is Expressed in Mammary ER+ Epithelial Cells and Regulates ER Target Genes in Luminal Breast Cancer

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Version: 3 Date: 17 November 2014

Author's response to reviews: see over
November 17, 2014

BMC CANCER
BioMed Central

RE: “TOX3 is Expressed in Mammary ER+ Epithelial Cells and Regulates ER Target Genes in Luminal Breast Cancer”

Dear Editors,

We have submitted a revision of our paper. We thank the reviewers for their helpful comments. A point-by-point response to the critiques is below and we hope that this study will now be acceptable for publication.

Sincerely,

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Referee 1

Major Revisions Requested

Figure 2: It is not clear how the authors arrived at the conclusion that the ratio of the TOX3 slice isoforms…
Our conclusion was poorly stated and this has been corrected to indicate that the predominant form detected was the same in normal tissue and tumors.

Minor Revisions Requested

Figure 1: The FOXA1 staining is difficult to see in the image provided.
This has been corrected by a better exposure and addition of arrows to indicate positive and negative cells.

Figure 1: What is the ER/PR status of the areas of the mammary gland that are negative for TOX3 staining?
We have now indicated in the text that some ER+ regions did not have extensive TOX3 staining.

Figure 5…this figure would benefit from reorganization
We thought it best to maintain the original organization, as the data in this Figure concerning CXCR4 flows from the previous microarray data.

Figure 5E. Is TOX3 induced at the protein level.
We have not analyzed this, although the mRNA changes are modest. The point of this experiment was to determine if there was any influence of a pathway that affects FOXA1 expression on regulation of the TOX3 gene. What controls TOX3 expression in vivo in normal tissue or in tumors remains unknown, although it is unlikely to solely be via IGF1.

Referee 2

Major Revisions Requested

Throughout the manuscript both the gene and the protein are written as “TOX3”.
This has been corrected.

In the Background section the authors have a very succinct phrase about risk association with tumour sub-type…
We thank the reviewer for these suggestions. We have added suggested references to Background or Results as we thought appropriate.

The authors mention a report that proposes TOX3 as a TSG…
We have mentioned and referenced the paper noted by the reviewer, although we disagree with the reviewer that this is necessarily supportive of a role for TOX3 as a TSG, as the tumor molecular subtypes were not identified in cells that carried mutated TOX3.

Figure 1B shows “Relative Tox3 expression” in sorted cell populations.
Expression of all qRT-PCR is relative to housekeeping gene expression as indicated in Methods. Also, ‘highly expressed’ was corrected in the text to reflect the relative expression data.

When in line 218 the authors write that the ratio of the two forms…
Corrected as above.
The authors present contradictory results when in Figure 3B…

We had discussed in the text the apparent discordance between microarray molecular subtyping data and IHC subtyping data. We never proposed TOX3 as a biomarker for the LumB subset, but rather as a biomarker for poorer prognosis within the LumB subtype.

The authors attribute a transcriptional activator role to TOX3, nevertheless, they only detect gene expression changes upon transient transfection…

We show both stable and transient changes in gene expression of TFF1 and CXCR4 induced by expression of TOX3. While it is true that we cannot distinguish direct from indirect gene targets, it is clear that TOX-family proteins are DNA-binding transcriptional regulators (including in neurons, as reported for TOX3). We also show here that TOX3 can induce enhancer RNAs, consistent with an activation role in gene expression. Moreover, TOX3 knockdown attenuates E2-induced TFF1 expression, and TOX3 and ER together are sufficient to induce TFF1 in a basal breast cancer cell line. We cannot predict TOX3 binding sites as this protein recognizes structural features of DNA rather than sequence. We consider ChIP-seq analysis important, but outside the scope of this study.

Since the authors have access to microarray data from tumors, they should show that the genes they observe to be up/downregulated upon transfection…

While we have not done a detailed subtype analysis, there is a positive correlation between TOX3 and TFF1 gene expression across the breast cancer samples used in microarray analysis (upper tertile of TOX3 expression shows 4.6-fold increase in TFF1, \( P=2.77E^{-36} \)). Our and other data cited in the paper make a strong case that TOX3 expression is associated with poorer prognosis tumors as a group, and can modulate biological processes associated with tumor progression. However, there is not a clear one-to-one correlation of MCF-7 and molecular subtypes defined for primary tumors (Prat et al., Breast Cancer Res. Treat. 2013, 142:237). In addition, as we show for CXCR4, there is heterogeneity even within TOX3 expressing cells (possibly mimicking intratumor heterogeneity) and some question of whether there is a strict correlation of microarray data and protein expression data. We also purposefully performed our studies in E2-depleted conditions to reveal the intersection of TOX3 and ER gene target regulation. Indeed, we think one of the key findings of the paper is a role for TOX3 in modulating response of an ER target gene, both in presence and absence of E2. For all these reasons we think that it may be difficult to map individual gene expression changes we observed in E2-depleted MCF7 to primary tumors.

Minor Revisions Requested

Line 418…
Corrected.