Title: Overexpression of CD44 accompanies acquired tamoxifen resistance in MCF7 cells and augments their sensitivity to the stromal factors, heregulin and hyaluronan.

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Author's response to reviews: see over
Dear Sir/Madam,

Thank you very much for kindly allowing us an extended period in which we could conduct a revision of our manuscript.

One of the key issues raised by reviewer #1 (Dr Plaza-Menacho), and mentioned in the editors letter, was that our study contained no data to demonstrate that CD44 was a mediator of tamoxifen resistance. However, our study did not set out to investigate whether CD44 promotes a tamoxifen-insensitive phenotype, rather, our aims were to explore the functional consequence of CD44 overexpression in a state of tamoxifen resistance as our in vitro models of acquired resistance suggested that CD44 was upregulated in this context. We subsequently present data that show that upregulation of CD44 in tamoxifen resistance enhances cellular sensitivity to ligands (hyaluronan and heregulin) commonly present in the stromal microenvironment. Thus these data have potentially important clinical consequences. In our revised manuscript, we have taken greater care to try and clearly state that our aims are to explore CD44 in the tamoxifen-resistant context and have changed the title to better reflect these aims. We agree, however, that the issue regarding CD44 and resistance is an important one and are currently investigating this link. Previous studies (Ahmed et al, Breast Cancer Re Treat 2011) reveal that CD44s is associated with a favourable prognosis clinically whilst in contrast to in vitro data, suggesting the true picture of CD44 clinically is complex, in that it may rely of the stromal composition (e.g. presence of HA/growth factors in microenvironment) and differential contribution from individual CD44 family members. We have now performed further studies in this area and now present what we feel is novel and important preliminary clinical data in our revised manuscript that suggest a link between increased CD44v3 expression and a poorer response to tamoxifen. Since these data are not the main focus of our studies however, we have presented them as supplementary data.

A number of additional points were raised by all three reviewers which have led to the extensive revision of our manuscript which we hope will be re-considered for publication. Please see below for specific details on how we have addressed the reviewers comments.
Kind regards,

Stephen Hiscox
Reviewer: Dr Plaza-Menacho

A number of important issues concerning our paper were raised by the first reviewer for which we are grateful, as in addressing these (as detailed below), we have been able to improve our manuscript.

Major issues

- One of the major concerns of this reviewer appears to be that there are no data in the study which demonstrate CD44 as a mediator of tamoxifen resistance. However, this study does not suggest that CD44 promotes a tamoxifen-insensitive phenotype, rather, that in a state of tamoxifen resistance, CD44 expression is elevated, where it acts to modulate heregulin and hyaluronan sensitivity. Although we initially felt that this was clearly reflected in the title of the manuscript, we have now revised the manuscript title and abstract to better reflect our study aims.

- We do agree with the reviewers’ comments regarding the relevance of CD44 in clinical breast cancer as an important issue. However, obtaining clinical material from tamoxifen-resistant tumours is problematic due to it frequently presenting as local and/or distant metastases of which sampling cannot routinely be done. We certainly agree that exploring the link between CD44 and endocrine response is of relevance in the light of our findings presented here. Previous studies (Ahmed et al, Breast Can Res Treat 2011) suggest CD44 is associated with favourable outcome in breast cancer. In our revised manuscript, we have extended such analysis and are able to show clinical data that reveals an association between elevated CD44v3 and a poor outcome (shorter response) on tamoxifen in ER+ breast cancer. This data is presented as supplementary figure 1.

- Figure 2: Staining of CD44 and erbB members in tamoxifen-sensitive MCF7 cells is problematic in that MCF7 cells have very low levels of erbB receptors (previously reported by us: Knowlden et al, 2003) and we felt that showing immunofluorescence staining of just CD44 in MCF7 cells was repetition of data demonstrated in figure 1. We have discussed lack of erbB expression in MCF7 cells in the relevant results section.

- Figure 3: The data in this figure clearly show CD44 siRNA to have a greater effect on EGFR phosphorylation versus erbB2 phosphorylation in the cells, suggesting that CD44:EGFR interplay may be more dominant versus CD44:erbB2. Although we originally hypothesised that CD44 - erbB2 interactions might be dominant in tamoxifen resistant cells (at least in the endogenous context) based on previous data supporting the interaction of these receptors in other cell types (e.g. see Bourguignon et al, 2007), our data (that CD44:EGFR are dominant in TamR cells) is further supported by our additional experiments that show a greater suppressive effect on EGFR activity versus erbB2 activity when CD44 is knocked down prior to stimulation of the cells with the erbB ligand, heregulin (figure 5) or
hyaluronan (figure 7). We have revised our description of these data in the text to more accurately reflect these observations.

- Figure 6: Western blots showing dose-dependent stimulation of MAPK by HA in TamR versus MCF7 cells have been replaced to more clearly show the effects of HA in TamR cells versus MCF7 cells on MAPK activation.

- Ongoing studies in our laboratory have investigated whether knockdown of CD44 in TamR cells affects tamoxifen response (as we think is suggested by the reviewers’ comments); these experiments have failed to show any significant affect on cell growth. However, the focus of this paper is an investigation of the consequences of CD44 upregulation in tamoxifen-resistance, rather than its potential link with the development of resistance. The latter, however, is an important issue and form part of our current research although we have discussed these aspects in our revised version of the manuscript and have now included clinical data which in part addresses this question (see our comments above)

- Figure 7: The Western blots for figure 7A showing that CD44 is required for HA-induced phosphorylation of erbB2 and EGFR have been replaced since our original images were not representative of our data as a whole, which clearly show this link.

- Figure 8: We have replaced the IP blots which we felt were the poorest quality in our original submission.

Additional comments

- Cell ‘wounding’ assays are very widely used to determine the migratory capacity of cells in culture as 2D monolayers. Whilst there are a number of alternatives (e.g. transwell migration systems based on Boyden chambers), wounding assays provide a reliable and straightforward method providing they include appropriate controls since, as quite correctly pointed out by the reviewer, wound areas can differ between samples. However, in our experiments we have taken numerous measurements for each sample prior to treatment and after the treatment period. This allowed calculation of wound closure at the 24hr time point as a percentage of its own control. These data were then collated and a mean % closure calculated. Overall, we consider wound healing analysis as an appropriate means to determine cellular migration in a simple, 2D cell culture system.

- Western blotting alignment has been checked to ensure that all align correctly

- Figure 6A and C have been replaced with figures of better resolution
Reviewer #2 (Dr Dunbier)

The comments from the second Reviewer were very much appreciated as they pointed out a number of grammatical errors within our original manuscript. These have now been addressed in our revised version as detailed below.

*Minor essential revisions*

- The word ‘students’ should have been ‘studies’ in the abstract – this has now been rectified
- Page 5, line 17: text has been changes to state ‘these data’
- Page 6, line 5: the word ‘by’ has been removed from the text
- Page 6, line 19: text now correctly refers to figure 8 for immunoprecipitation data
- Page 11, line 23: ‘An’ has been changed to ‘a’
- Figure 1 legend: a key for figure 1A has been added to the legend
- Figure 2 legend: the legend has been corrected to refer only to the data shown (immunofluorescence staining of CD44 and erbB receptors in TamR cells)
Reviewer #2 (Dr Klingbell)

The comments by the third reviewer have been very helpful in assisting us to revise our manuscript with the aim of improving its clarity and presentation and addressing some key issues within our data to better explore the function of CD44 in the context of acquired tamoxifen resistance. We have addressed this reviewers’ comments as follows:

Discretionary revisions

- In the experiments where MCF7 cells were compared to their tamoxifen-resistant counterparts, both cell lines were maintained in phenol red-free RPMI containing charcoal-stripped serum in order to reduce exogenous steroid hormone levels. This is primarily performed to enable us to study antagonist-occupation of the estrogen receptor (in this case, tamoxifen) versus either no occupation (use of media containing stripped serum) or estradiol-bound ER (using stripped media containing estrogen \(10^{-12}\)M). In our experience, we do not see any differences in CD44 expression or its ability to induce erbB signalling in MCF7 cells. In addition, levels of growth factors appear also to be at a minimum in our experimental media as evidenced by the fact that the EGFR (barely detectable in these cells) is not activated; in contrast to this, short-term treatment of MCF7 cells with tamoxifen in experimental medium results in induction of EGFR expression which can then be phosphorylated by exogenous ligands. We have clarified the methodology section to state the conditions that MCF7 cells were cultured in for these comparisons.

- Immunocytochemical analysis of CD44 expression has been used in figures 3 and 4 to demonstrate loss of CD44 on the cells following siRNA treatment. Although we wanted to include all these data as it demonstrated the reproducibility of this siRNA approach, we do agree that this is in effect presenting the same data twice. As such, the staining has been removed from figure 3 and the text amended to just refer to the ICC data retained in figure 4.

- We would like to retain parts A-D in figure 6 as they demonstrate clearly the difference between MAPK activation in response to HA in MCF7 versus tamoxifen-resistant cells.

- We agree that the supplementary table summarising the IP data detracts from the manuscript and does not improve its clarity. We have removed this table.

- Sample loading in Western blots is controlled for by re-probing the blots with either beta-actin or GAPDH. These have now been included for figure 5A.

- All the labelling of the Western blots have now been checked for consistency regarding the nomenclature used to detail the antibodies used for probing. Details of the specific phosphorylation sites that these antibodies detect as given in the methodology section.
Minor revisions

- Spelling mistakes have now been rectified throughout the manuscript.

- Figure 2: We fully accept that dual immunofluorescence staining alone does not directly suggest co-localisation of CD44 and erbB members but, in the absence of a confocal microscope and together with our immunoprecipitation and siRNA data, we believe that our data implies such a relationship in tamoxifen-resistant cells and would therefore wish to retain these images. That the location of erbB3 appears nuclear has been reported previously and suggests that this is not an artefact in staining. We have now incorporated these issues into the text of our revised manuscript.

- The immunoprecipitation data refereed to in figure 2 was an error and has been removed.

- Figure 5: All ‘controls’ in figures B, C and D were ‘non-targeting’ siRNA. The figure legend has been amended to reflect this.

- Figure 8: The legend for this figure has now been amended to correctly state that HA induces a loss of EGFR:erbB3 dimerisation and a gain in erbB2:erbB3, erbB2:EGFR and CD44:EGFR heterodimerisation.

- Figure 7: Figure 7B originally showed migration data which had been calculated as a % of non HA-treated (i.e. untreated) cells. We have now revised this figure to show effects of inhibitors on the basal migration capacity of these cells. The figure legend incorrectly referred to the use of the MAPK inhibitor, PD098059. We removed this data from the original manuscript as we felt that data for MAPK inhibition was incomplete and did not add anything of significance to our report. The legend has been amended to remove reference to these data.

- Materials and methods:
  o Both Westerns and immunoprecipitation utilised the same Triton X100-based buffer, details of which have been provided in the paper.
  o Concentrations of the inhibitors used were 1µM (gefitinib) and 100nM (trastuzumab). These have been stated in the methodology section of the paper.
  o Statistical analysis has now been performed for figure 6E and 7B with p-values included in figure legends.

Major revisions

- We used a pool of siRNA (Dhamrmacol Smartpool) to suppress CD44 expression in our tamoxifen-resistant cell line as a means to circumvent using individual siRNAs which can prove problematic with respect to lack of consistency in the level of knockdown achieved. We routinely saw >80% suppression in CD44 using this approach. In our revised manuscript, we have stated that this was a pooled CD44 siRNA rather than an individual one.
• Figure 3C. We generally saw a small reduction in total erbB2 protein when cells were treated with CD44 siRNA, and this may therefore have a bearing on our interpretation of the effects of CD44 siRNA on erbB2 activation. We have amended our text to more appropriately describe these data.

• Figure 5A: the text has been amended to more accurately describe the data in terms of a weak suppression of erbB2 and MAPK in contrast to the effects seen on EGFR and Akt when HA was used in the absence of CD44.

• Figure 6F was present in our original submission and showed that CD44siRNA prevented HA-induced cell migration. Figure 6 was presented over three pages in order to maintain a large enough size of the wounding assays to provide the reviewers with good enough resolution.

• Figure 7A: We have provided a replacement figure that is more representative of the effects on EGFR by HA.

• Figure 8: We have replaced the IP blots which we felt were of poor quality. Whilst we ran IgG controls for these IP experiments, these samples were not run on lanes adjacent to the treatment samples which made it difficult to present all samples in a clear figure. Rather than add an IgG control as a separate element to each figure, we have described in the text that no signal could be seen in these samples. We have also removed the supplementary table that originally described the IP data as we agree that this probably confused the issue.