Author's response to reviews

Title: A stable explant culture of HER2/neu invasive carcinoma supported by alpha-SMA expressing stromal cells to evaluate therapeutic agents

Authors:

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Author's response to reviews: see over
Dear Dr. Browning:

I am very pleased to have been given the opportunity to revise and resubmit my manuscript:

MS: 172071833165815
A stable explant culture of HER2/neu invasive carcinoma supported by alpha-Smooth Muscle Actin expressing stromal cells to evaluate therapeutic agents for reconsideration and publication in BMC Cancer. I hope that a favorable decision will be rendered on behalf of these studies. Every comment and suggestion has been taken into serious consideration and the manuscript has been revised in response to each of the reviewers comments. In the following section is a response to each of the reviewers comments individually in a point-by-point discussion.

REVIEWER 1 (Ewa Gregoraszczuk):

This reviewer was very impressed with the manuscript and had no required revisions.

REVIEWER 3 (Thorarinn Gudjonsson):

Major Comments:
The reviewer has questions concerning the MAM1 co-culture system. It is suggested that a-SMA positive cells should have also been stained with specific myoepithelial cell markers (cytokeratin 14 or 17) to exclude that myoepithelial cells were contaminating the culture.

Response: As stated on p.15, since stromal cells are CD24 negative, it is likely that they are nonepithelial, nevertheless we acknowledge the fact that trace myoepithelial cells may be present in the “stromal cell” subpopulation and don’t think this represents a concern or contamination of the model.

The reviewer is also concerned about the potential presence of endothelial cells in the culture.

Response: We do not anticipate that endothelial cells survive in this co-culture model mainly due to the presence of 10µM dexamethasone in our cell culture medium. Endothelial cells also have a slow rate of growth and limited proliferative potential and would probably be competed out by the tumor and stromal cell populations. We have added these comments on page 7 as suggested.

The reviewer is also concerned about the ability to maintain co-cultures at a 1:1 ratio.
Response: We have elaborated in the methods section on the subculturing techniques we use to address this concern. We now state on page 7 that co-cultures can be expected to maintain a 1:1 ratio during active growth. This ratio may be altered if cultures are kept beyond confluence when stromal cells continue to grow. At time of passage, the majority of stromal cells are collected separately by a brief trypsinization. The remaining culture is further trypsinized and collected separately. Both collections are counted by hemacytometer which readily allows for distinguishing between the tumor(small) and stromal (large) cells. If need be, the cell ratios can be readjusted to accommodate a 1:1 distribution.

The reviewer next is concerned with cell separation and suggests separating the subpopulations in the co-culture and evaluating them separately for drug-responsiveness.

Response: This is an excellent point. We have tried extensively to maintain separate pure “stromal” and tumor populations from this model. Our experience has been in losing the phenotypes of the separate cells. Most notably, we observe a significant loss in the a-SMA and overgrowth of a-SMA negative/low expressing fibroblasts and changes in the morphology and growth pattern of the tumor cells accompanied by a decrease in HER2/neu. Thus, we would not be evaluating the drug effects on the same highly differentiated cells that existed in the original co-culture. The reciprocity between the tumor and stroma is critical to keep each compartment differentiated. We are still pursuing these separate cultures as the reviewer suggested to have a better means of manipulating the MAM-1 model and to understand the factors define the differentiation status of the tumor and stroma.

The reviewer comments on EMT and the possibility of purifying the tumor cells to determine their potential to generate their own stroma by undergoing EMT.

Response: We also feel that EMT may be an important activity in this model. The presence of a-SMA HER2/neu double positive cells may suggest that MAM-1 is capable of EMT, unfortunately this was beyond the scope of the current study and is being developed as a separate project.

Minor comments /suggestion:

The reviewer asks whether cells within MAM1 culture show anchorage independent growth in soft agar assay and how this is compared to purified cancer and stromal cells.

Response: MAM1 grows aggressively in soft agar and forms spheroids even on plastic (as noted on pages 13 and 14 of the text). We have not evaluated the anchorage independent growth of purified subpopulations.

Similarly the reviewer suggests it would be interesting to see if the MAM1 co-culture could regenerate in-vivo like phenotype in three-dimensional collagen assay to potentially create even more physiological conditions than the monolayer.

Response: These are interesting possibilities that we have begun to explore. At present it is clear that MAM1 are invasive when layered on basement membrane type substrates. A whole new class of agents may be evaluated that modulate tumor invasive properties.

The reviewer asks why male Balb-NeuT mice.

Response: Male Balb-NeuT mice are our breeding stock. The strain is heterozygous for the HER2/neu
oncogene and transgenic males are breed with wildtype Balb/c females. Transgenic females are not breed due to their extensive mammary gland disease. Mammary and salivary gland disease is observed in both males and females. However, mammary gland disease is extensive in females and salivary gland disease is most extensive in the males.

The reviewer suggests to spell out SMA and not use the abbreviation in the title.

**Response:** The title has been modified to accommodate this suggestion.

The reviewer asks if other scientists should be included as authors or in the acknowledgement.

**Response:** I was the sole effort on this entire study. I do however, now include Dr. Lonardo and the Pathology department for additional histological analyses and evaluation of the tissue biopsy.

**Response:** As suggested, MAM1 is now defined on page 2 in the abstract, and on page 4, the first time it occurs in the text.

**Response:** As suggested, Bam1a cell line is described briefly on page 17.

The reviewer questions the use of 10% CO2 versus 5% CO2

**Response:** In my experience, 10% CO2 is the most common and appropriate for DMEM-based media that are supplemented with sodium bicarbonate.

The reviewer requests that figure 1 be defined as A, B, C and D and that arrows be added to explain different components.

**Response:** The figure has been modified accordingly

The reviewer inquires about the phrase “finely dispersed chromatin and infrequent macro nucleoli and undergo infrequent mitoses” and states that this is difficult to see on this figure and suggests that this should be explained in more details.

**Response:** This statement comes from the pathology report of the tissue biopsy. These are characteristics of tumor nuclei that are correlated with nuclear grade. The photo was not taken to demonstrate these specific attributes.

The reviewer suggests that the middle panel of figure 2 could be omitted since both phase contrast images are showing the same thing.

**Response:** It is felt that showing two different magnifications of this image provides a better representation of how the co-cultures are organized in vitro.

The reviewer suggests that merged immunofluorescence figures be added to figure 3 in order to localize double positive and double negative cells. This could support the discussion of figure 3A describing the flow cytometry results.

**Response:** Presenting the single color images appears to be sufficient to demonstrate our point. We do not show double positive or double negative cells, rather we show exclusive single positive populations. Thus merging images will not provide additional demonstration that these two
subpopulations are antigenically distinct.

The reviewer inquires whether in figure 3B, that 2N cell population contains two or more cells rather than dividing cells.

**Response:** Flow cytometry records single cell events, therefore, the larger (greater forward scatter) subpopulation records the size of (larger) single cells. Based on cell cycle analysis, we observe that the majority of these large cells are cycling in G2/M phase. Since we have not quantitated the cell cycle distribution of the “small, 1N” and “large, 2N” forward scatter populations we must only refer to these populations as small and large based only on relative size and not nuclear content. This point was also suggested by the final reviewer. It would be necessary for us to record the relative G0/G1, S and G2M distributions of the small and large cells to accurately describe them as 1N or 2N and as cycling or non-cycling. We can only suggest this is the case. Since the histograms we present were not further analyzed by the appropriate tests, we have modified our discussion of these results and now label and refer to these subpopulations as small and large as opposed to 1N and 2N. Nevertheless, we are certain that cell cycle profile of the MAM-1 tumor cells do conform to the typical diploid cell cycle pattern of distribution and do not observe a significant tetraploid population as was a concern of the final reviewer.

**REVIEWER 2 (Mary Helen Barcellos-Hoff):**

**Major compulsory Revisions:**

The reviewer suggests that the identification of the two subpopulations as “stromal” and “tumor” may be premature. This reviewer has great concern that the myofibroblast may also be tumorigenic and feels that it is crucial to genotype the different subpopulations to determine their relationship and test the tumorigenicity of each population.

**Response:** It is highly unlikely that the myofibroblasts in our co-cultures represent the outgrowth of a contaminating normal tissue. This model was derived from a transgenic mouse with a mutated HER2/neu. We expect the genotype to be identical between the stromal cells and tumor cells. Since every cell bears the transforming HER2/neu mutation there is potential for tumor genesis. Indeed, PCR analysis has confirmed, the presence of the HER2/neu transgene in both the stromal and tumor subpopulations. However, expression of the oncogene product is restricted and by definition the hallmark of neoplastic transformation. In our stromal populations, cells are clearly negative for HER2/neu protein. Nevertheless, cancer associated fibroblasts have been shown in other systems to have enhanced tumorigenic potential. This is one reason, that tumor associated fibroblasts have also been suggested as chemotherapeutic targets. It is beyond the scope of this paper to genotype the different subpopulations, but this will be important for future studies. In this simply application we define the stroma and tumor subpopulations on the basis morphology and antigenicity, as is the case in most pathological and immunohistochemical tests and analyses of tumor biopsies. We found this sufficient for our purpose in these studies to characterize the differential drug-response in cell populations that are represented in the tumor microenvironment. In future applications of this model, it will be important to further evaluate the tumorigenic potential of the individual cell populations as the reviewer suggests. Indeed, it is suggested on page 4 that one application of this model would be to treat cultures and test for tumor outgrowth following treatment. It is possible that we would observe a histological distinct tumor, if initiated cells in the stroma emerged to form histological distinct tumors or if drug-resistant subpopulations from the tumor fraction could give rise to a more aggressive or less well-differentiated tumor.
The reviewer feels that the discussion of a putative stem cell component is inconclusive and should not be included in results without functional analysis of sorted cells.

**Response:** The discussion of stem cells is introduced as a suggestion of the biological potential of the tumor cells in this model. There is no harm in acknowledging that the possibility exists and demonstrates a need to consider that stem cells may be an important therapeutic target when developing models.

The reviewer states that the definition of forward scatter as 1N and 2N populations representing cycling and non-cycling cells is not consistent with standard cell cycle descriptive analysis and should be re-evaluated with BrdU incorporation vs. DNA content profiles to determine the cell cycle distribution, the percent of cells in S-phase, and whether the large 2N population represents a cycling tetraploid population.

**Response:** This is a very good point. In our hands, the assumption that the small and large subpopulations represent 1N, non-cycling and 2N, cycling tumor cells, respective is typically the case upon further analysis by staining for DNA content. However, we have not specifically evaluated the G0/G1, S and G2/M distributions of the small and large subpopulation in this figure as needed to confirm this assumption. Therefore we have modified our discussion of these data on page 15 and 16 and only suggest that size difference may reflect a subpopulation enriched in actively cycling cells. We do however know, based on cell cycle analysis of DNA content, that the tumor cell subpopulations do represent the typical diploid pattern in distribution and do not observe a significant tetraploid population in these cells.

In reference to micro array analyses involving MAM-1 and Bam1a, the reviewer argues that the conclusion that “two-fold difference is likely to represent the dilution of tumor cell RNA with stromal cell RNA in the MAM-1 co-culture” and that “Genes uniquely over-expressed by MAM-1 largely reflect the stromal signature of this breast cancer co-culture system” is premature in the absence of the identity of the ‘stromal’ genotype and imprecise given the author’s demonstration that the cell types could be easily separated using cell surface markers.

**Response:** The purpose of using Bam1a, a cloned and characterized BALB-NeuT mammary tumor cell line for comparison with MAM-1 ‘complex’ RNA was to identify common gene sets that: 1) confirm the tumor cell HER2/neu gene signature and 2) identify the divergent gene set as enriched in the stromal cell population. This divergent set clearly represents a set of genes that are consistent with the stromal signatures of breast cancer that have been identified in the published literature and we feel represents the genetic signature of the stromal cells in the MAM-1 co-culture. Additional purification of the cell subpopulations would not change these gene profiles. We did not separate the cells due to the unavailability of flow sorting. Furthermore, our typical staining uses fixed cells and intracellular stains to identify the tumor and stromal subpopulations. RNA could have then been extracted from the fixed cells, but this is not the optimal situation. It is possible to label the tumor cells with a cell surface marker that identifies the rat HER2/neu, so it would be possible to sort fresh cells based on HER2/neu positive and negative fractions. Nevertheless, we felt that the additional manipulations could alter gene expression. Our final decision is that the data presented is valid, interpretable and of considerable value. We do acknowledge that the additional controls provided by analyses of sorted cells would strengthen the data.

The reviewer states the the conclusion that states “data suggest that Iressa preferentially targets signal
transduction from the tumor cell HER2/neu leading to tumor cell death” are premature since cell death has not been demonstrated only reduced cell numbers which may be accounted for by decreased cell proliferation and that PCNA staining is an indirect measure of cycle.

**Response:** We have modified the text on page 20 to state that specific tumor cell apoptosis is observed in Iressa treated MAM-1 cultures based on cellular morphological and nuclear fragmentation evident by DAPI staining (as is shown in Figure 5C) and annexin V binding (not shown) We have also modified the text to reflect that PCNA staining was used as an indirect indication of cycling cell activity.

**Minor Essential Revisions:**

The reviewer is concerned that the figures appear pixilated and may not reproduce well.

**Response:** We will upload our best high resolution figures for the final manuscript and hope that the journal can reproduce them with high quality.

**Discretionary Revisions:**

The reviewer states the itemized list in the introduction is non-standard and distracting.

**Response:** As suggested, we have converted this list on page 4 into a more standard paragraph style and format.

We hope that are responses are adequate to satisfy the reviewers concerns and feel that the manuscript has been improved as a result of the suggestions. We thank you for your continued efforts on behalf of this manuscript and look forward to hearing from you soon.

Sincerely yours,

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