Author's response to reviews

Title: Gain in cellular organization of inflammatory breast cancer: A 3D in vitro model that mimics the in vivo metastasis

Authors:

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

The authors have addressed all criticisms and concerns of the three reviewers and made the necessary revisions to the manuscript. The major criticisms and concerns included clarification of the model and methods of sample preparation. Also, significant was assurance of the robustness and accuracy of the data sampling. In response, the authors of the manuscript have clarified the nature of the xenograft model (MARY-X), incorporated additional text for clarification to detail sample (spheroid) preparation, introduced additional data (Figures and figure panels) and included in the Materials and Methods section the sampling procedure. The authors of the manuscript thank the reviewers for their time and effort and wholeheartedly believe their critique has strengthened the manuscript.

Below is a point-by-point response to the reviewers’ criticisms and concerns. Note, the reviewers’ requested revisions are written in regular text and author’s response is written in italics.

Respectfully,

Mary L. Alpaugh
Reviewer 1 (Dr. Wendy A. Woodward)

Major Revisions:
There are interesting microscopy findings here with the conclusion that in vitro Mary-X spheroids recapitulate the structural findings of tumor emboli, and that this is unique to IBC.

The paper would be stronger if the claims were less broad, more specific to the explicit findings. As it is, there is only one image in figure 5 of a tumor emboli in vivo. Since this is a major conclusion, comparative in vivo emboli images would be appropriate for Figure 1-3 as well, and in figure 5 align in vivo and in vitro images to demonstrate clearly the similarity.

Additional TEM, SEM and H&E images (panels) were added to existing Figures and additional Figures were incorporated to specifically detail the gain in cellular organization on an ultrastructural level of both the MARY-X in vitro spheroid and in vivo emboli (either of a primary tumor or pulmonary metastasis). This gain in cellular organization is depicted as canals with apical surface microvilli. Neighboring cells which make up the lumen, display the lumenally-positioned tight junctions, followed by adherens junctions and desmosomes at the apex of the apical/lateral surface. Specific attention was paid to Figure 8 (formerly Figure 5) to further clarify the findings of this manuscript.

There is a statement on page 14 that these findings imply this structural difference is maintained through metastasis – if the method of culture (described in the methods as mincing, growing what then sounds like an organoid into spheres of various sizes then straining) is not disrupting the existing architecture this does not show a persistence through the process of metastasis but rather a persistence of emboli architecture through the mincing process. Still important, but a technical means of recapitulating the emboli rather than a biological implication.

This is a valid point, however, a more explicit explanation of the preparation of the in vitro spheroids and description of the model will assist in showing that these architectural findings are unique in the assembly of the in vitro spheroid and the in vivo embolus and persistent through metastasis.

With respect to the production of the MARY-X spheroids the following points must be clarified: The IBC spheroids are a cellular derivative (i.e. primary cell line) of MARY-X tumor explants. Upon mincing the tumor, cells are released into the media as sheets of cells and single cells (i.e. do not resemble an intact spheroid). The sheets of tumor cells and single cells form tight, compact clumps or aggregates of cells termed “MARY-X spheroids”. These spheroids can be further purified or partitioned from the cellular debris by employing cell strainers of varying pore sizes (e.g. 40, 70 and 100 µm; BD Biosciences) [Alpaugh ML, et al. Relationship of sialyl-Lewis(x/a) underexpression and E-cadherin overexpression in the lymphovascular embolus of inflammatory breast carcinoma. Am J Pathol 2002, 161(2):619-628.]. The resultant preparation is 100%
human IBC cells (termed MARY-X spheroids) which can be maintained in culture for periods up to three months.

These purified MARY-X spheroids (purified human IBC cells) when injected into severe-combined immune deficient (SCID) mice will form a complex primary tumor (and distant lung metastases) where the tumor cell emboli are found nestled within the murine lymphatics and blood vessels (i.e. lymphovascular invasion) [Alpaugh ML, et al. A novel human xenograft model of inflammatory breast cancer. Cancer Res 1999, 59(20):5079-5084]. The primary tumor is composed of a 30% murine component (surrounding stroma, lymphatic vessels and blood vessels) and 70% human inflammatory breast cancer cell component (tumor cell emboli) [Alpaugh ML, et al. A novel human xenograft model of inflammatory breast cancer. Cancer Res 1999, 59(20):5079-5084]. The emboli of the tumor (and distant lung metastasis) proliferate substantially to form numerous new emboli – all of which retain the cellular organization detailed within the manuscript. Therefore, it is not merely an interim “disruption” of architecture but rather a programmed mechanism (i.e. innate ability) of the IBC tumor cells. Also, the MARY-X spheroids can be disadhered into a single cell suspension upon Ca++ depletion. Replenishing the Ca++ within a 3-hour time period will render reformed spheroids. These reformed spheroids when injected into severe-combined immune deficient (SCID) mice will again form a complex primary tumor (and distant lung metastases) [Alpaugh ML, Barsky SH: Reversible model of spheroid formation allows for high efficiency of gene delivery ex vivo and accurate gene assessment in vivo. Hum Gene Ther 2002, 13(10):1245-1258]. Therefore, the fact that the spheroids form from sheets of cells and single cells, and that these purified spheroids when injected into SCID/nude mice proliferate and produce numerous new emboli, both of which display a gain in cellular organization indicates that this structured architecture is a programmed mechanism of the IBC tumor cells.

Most significantly and further indication that this “gain in cellular organization” is innate to the IBC cells (and persistent through metastasis) is confirmed by the existence of lung metastases. The mechanism by which the emboli metastasize is unknown. However, what could be accepted as a physiological impossibility is for a large tight embolus of the primary tumor to travel unimpeded through a vessel, subsequently making its way to the lungs. What is more plausible is a cell or two breaking away (mechanism unknown and presently being explored) from an embolus of a primary tumor, traveling through the vasculature until becoming trapped within the capillary network of the lungs. From these few cells, form an embolus (and subsequent complex metastasis i.e. metastasis consists of emboli nestled within stroma) within the lung where they proliferate and reconstruct the structural architecture of canals with polar elements. In this scenario, the emboli of lung metastases do begin as single cells.

If these spheres are from single cells state that explicitly. If not, images from cells grown from Mary-X single cells would be of interest as this would better substantiate the biology that inherently these cells are programmed for a more complex architecture that can be reproduced de novo in vitro.
This has now been stated explicitly within the text of the revised manuscript (see above).

I wouldn’t expect mcf-7 spheres grown from single cells in serum to be very primitive or capable of much other than replicating into a homogenous sphere. As such the lack of architecture is not surprising. If the MCF-7 spheres were grown in a stem cell promoting culture (Dontu et al) they indeed cavitate and this might change the results. A more interesting comparison would be one to support the claim made herein - these findings are never seen in cancer. A better control would be a non-IBC xenograft minced in the same way and grown as an explant into spheres organoids if possible. This is one approach for digesting primary human biopsies so may be feasible from a technical standpoint.

The reviewer is correct in stating that when MCF-7 (like MCF10A) are grown in reconstituted basement membrane under appropriate conditions these cells will form polarized, acini-like spheroids very comparable to normal breast epithelial [Debnath J. et al. 2003]. These multicellular models are often used to explore the changes i.e. oncogenic influences which convert normal epithelial (acini-like spheroid) to an aberrant phenotype. However, this was not the intent of this manuscript. Using the MARY-X model, the intent of this manuscript was to investigate the ultrastructural architecture of the aggressive, highly metastatic inflammatory breast carcinoma (IBC), which unlike other metastatic cancers retains expression of E-cadherin. E-cadherin is the key molecule that maintains architectural integrity in normal epithelium and in IBC/MARY-X mediates the formation of the tight, compact in vitro spheroids and in vivo emboli. To determine if E-cadherin expression only or possibly an innate mechanism of spheroid/embolus IBC assembly contributed to the gain in cellular organization, a comparison was made between MARY-X and the non-IBC cell line, MCF-7.

MCF-7 cells express E-cadherin and are known to form spheroids when grown under conditions which prohibit attachment (coating of plates with 1% agarose) and are commonly used in this fashion to test therapeutic efficacy [Oktem G. et al. Oncology Reports 2006, Guirado D. et al. The British Journal of Radiology 2003, Monazzam A. et al BMC Cancer Cell International 2006]. These artificial multicellular spheroids more closely resemble the in vivo metastasis (i.e. compact clump of cells). It is for the above reasons (E-cadherin expression and spheroid formation that closely resembles the in vivo metastasis i.e. clump of cells rather than acini-like structures which resemble normal epithelial) that this cell line and spheroid formation protocol was chosen as a comparable in vitro model. Furthermore, if the MCF-7 agarose-induced spheroids did show a gain in cellular organization similar to what was found in IBC/MARY-X then it would be fair to conclude that this architecture was not unique to IBC and does not confer the IBC/MARY-X malignant phenotype since it is found in a more indolent cell type (MCF-7) with an invasive phenotype. However, this was not found to be the case in this study.

Minor revisions:
Epithelial is misspelled in line 2 of the abstract
This was corrected within the text of the manuscript.
A schematic will help the unfamiliar reader with the introduction of junctions

*The focus of the manuscript was to report a gain in cellular organization not typically found in a carcinoma with a malignant phenotype. A schematic of polarity common within normal epithelium would be appropriate within a review article but detracts from the focus in the context of this research manuscript.*

The second sentence of the concluding paragraph of the introduction is a run-on. *This was corrected within the text of the manuscript.*

More detail in the method of spheroid formation will clarify. *This was corrected within the text of the manuscript (also, see above).*

On page 10 the statement that findings are “never seen” in cancer needs a reference and qualification as to who has looked and how similar the conditions were.

*A thorough literature search was performed to see if architectural findings of other aggressive, highly metastatic carcinoma models (of in vitro spheroids and primary tumors) were reported. No such papers were found. However, the language within the manuscript has been changed to more accurately report the findings (i.e. change from “never seen” to “uncommon”).*

In the comparison of types of junctions between MCF-7 and Mary-X, this should be quantitiated rather than the qualitative comparison “few”. *This has been more accurately reported within the revised manuscript.*

The last paragraph of the results seems like discussion. *This is true and has now been incorporated within the conclusion.*
Reviewer 2 (Dr. Massimo Cristofanilli)

Minor essential revisions:
There is no determination of E-cadherin, vimentin in the spheroid analyzed to actually support that claim.

The author can only assume that reviewer two was referring to a sentence in the above section (last sentence) stating “In this experiment, Dr Morales suggest that the spheroids associated with MARY-X have a tightly maintained and regulated polarity and this is assumed related to the persistent over-expression of E-cadherin.” If the author is correct in this assumption reviewer two is questioning the claim that E-cadherin is responsible for the compaction and maintenance of polarity in the MARY-X spheroid. E-cadherin’s role in compaction of the MARY-X spheroid and potential role in survival of the MARY-X in vitro spheroid and in vivo emboli has already been determined in past publications [Alpaugh M.L. et al. American Journal of Pathology 2002 and Tomlinson J.S. et al. Cancer Research 2001]. The presence and significance of various adhesion molecules has been pursued in MARY-X as reported in past publications [Alpaugh M.L. et al. Oncogene 2002]. This manuscript is making the claim that the persistent over-expression of E-cadherin along with compaction of the MARY-X in vitro spheroid and in vivo embolus as previously reported, also contributes to the gain in cellular organization.

It is possible that the spheroids are actually associated with a heterogeneous population of cells in which the centrally located forming lumens have higher expression and are more polarized than the remaining of the spheroids.

The reviewer is correct in that it is possible that the MARY-X spheroid/emboli may be composed of a heterogeneous population of cells. With respect to the inverse relationship of E-cadherin and sialyl Lewis X expression of MARY-X, indeed there is a heterogeneous expression (Pinho, S. et al. PlosOne 4: 2009). However, the dominant population of cells express E-cadherin. The author has introduced additional data (New Figure 1; image analysis, IHC) of the MARY-X spheroid/emboli and metastases that show that the dominant population of cells express membrane positioned E-cadherin evenly distributed through out the spheroid and embolus.

Furthermore, the characteristics associated with spheroids formation should be fundamentally similar in MCF-7 models and not associated with levels of expression of E-cadherin. The subsequent dissolution of spheroids is described as associated with cytoplasmic translocation of E-cadherin and induction of apoptosis but, this is a well known and already reported finding.

The manuscript reports that upon dissolution of MARY-X spheroids there is a nuclear translocation of the cytoplasmic domain of E-cadherin – not “cytoplasmic translocation of E-cadherin. To the author’s knowledge, the only other reported finding of nuclear translocation of E-cadherin was in Ferber E.C. et al. JBC 2008 and this was cited in the manuscript. This finding was in the MCF-7 cell line.
Reviewer 3 (Dr. Barbara Vanderhyden)

Major revisions:

1) The authors should revise the descriptions in the Abstract, Introduction and Methods to reflect the accurate identity of Mary-X. This does not take away from the value of this model, but its nature needs to be clearly described.

Below changes were made within the manuscript:

ABSTRACT (BACKGROUND): The human xenograft model of IBC (MARY-X), like IBC, displays the signature phenotype of an exaggerated degree of lymphovascular invasion (LVI) in situ by tumor emboli.

INTRODUCTION: In this study, using the human xenograft model of IBC (MARY-X) we show that malignant IBC displays architectural features or a gain in cellular organization that is not typically found in aggressive carcinomas.


METHODS: MARY-X was established from a patient with inflammatory breast cancer (IBC) [Alpaugh ML, et al. A novel human xenograft model of inflammatory breast cancer. Cancer Res 1999, 59(20):5079-5084]. In vivo, MARY-X recapitulates the human IBC phenotype of extensive lymphovascular invasion of the tumor cell emboli. The IBC spheroids are a cellular derivative (i.e. primary cell line) of MARY-X primary tumor explants. Upon mincing the tumor cells are released into the media as sheets of cells and single cells. The tumor cells form tight, compact clumps or aggregates of cells termed “MARY-X spheroids”. These spheroids can be further purified or partitioned from the cellular debris by employing cell strainers of varying pore sizes (e.g. 40, 70 and 100 µm; BD Biosciences) [Alpaugh ML, et al. Relationship of sialyl-Lewis(x/a) underexpression and E-cadherin overexpression in the lymphovascular embolus of inflammatory breast carcinoma. Am J Pathol 2002, 161(2):619-628]. The resultant preparation is 100% human IBC cells (termed MARY-X spheroids) which can be maintained in culture for periods up to three months.

The MARY-X spheroids (human IBC cells) when injected into severe-combined immune deficient (SCID) mice, form complex primary tumors (and distant lung metastases) where the tumor cell emboli are found nestled within the murine lymphatics and blood vessels (i.e. lymphovascular invasion) [Alpaugh ML, et al. A novel human xenograft model of inflammatory breast cancer. Cancer Res 1999, 59(20):5079-5084]. The tumor is composed of a 30% murine component (surrounding stroma, lymphatic vessels and blood vessels) and 70% human inflammatory breast cancer cell component (tumor cell emboli).
2) The Methods section also needs to clarify the description of the methods used to generate the spheroids and the tumor emboli. The description of the methods provided in the authors’ response to reviewers describes the spheroids as being obtained from minced tumors, but does not clarify the origin of the tumor (i.e. primary or lung metastases) or the method for isolation of the tumor emboli.

Please see above.

3) Since the interpretation/conclusion of the results is largely based on the similarity of the Mary-X spheroids to tumor emboli, additional images that show these similarities in the tumor emboli would be appropriate.

Additional TEM, SEM and H&E images (panels) were added to existing Figures and additional Figures were incorporated to specifically detail the gain in cellular organization on an ultrastructural level of both the MARY-X in vitro spheroid and in vivo emboli (either of a primary tumor or pulmonary metastasis).

4) As noted by Reviewer 1, the influence of the culture system used to generate the MCF-7 on the type of spheroids obtained needs to be discussed. In their response, the authors have provided the rationale for the use of a specific method for MCF-7 spheroid formation, but they did not provide this rationale in the manuscript. The rationale for not using a culture system that supports more complex organization in MCF-7 spheroids is important. Essentially, it appears that the comparison being made is an IBC/high E-cadherin expressing tumor explant (Mary-X) with a non-IBC/lower E-cadherin expressing cell line (MCF-7), and so it's not surprising that the behavior of these cell lines is different (whether because of the different nature of the cell line or its level of E-cadherin expression). Some indication of the relative level of E-cadherin expression in the Mary-X and MCF-7 spheroids used in this study would also be appropriate.

The following rationale was incorporated in the manuscript: MCF-7 is a non-IBC cell line and one that has significant E-cadherin expression. This cell line is commonly used to form agarose-induced multicellular spheroids. Cultured in this manner the MCF-7 spheroids more closely resemble the in vivo metastasis and are often used to test therapeutics. IBC/MARY-X forms tight, compact aggregates of cells both in vitro and in vivo as spheroids and tumor emboli, respectively. The point of this experiment was to determine if the architectural findings were solely due to E-cadherin expression or innate to IBC spheroid/embolus assembly. Therefore, the non-IBC, E-cadherin expressing MCF-7 agarose-induced spheroid was used as a comparative model.

The relative level of E-cadherin expression of MARY-X spheroids and other breast cancer cell lines, including MCF-7, has been previously reported in a past publication on
MARY-X [Tomlinson, J.S. et al. Cancer Research 61, 5231- 5241]. This will be referenced within the manuscript.

5) Quantitative assessment of the observations made for the types of junctions present in Mary-X and MCF-7 spheroids should be included. There is no indication of how many spheroids were examined. Some assurance needs to be made that sufficient numbers (ideally with quantitation) have been examined to ensure accuracy of the observations.

With respect to quantification of junction complexes, there were image distinguishable differences in the ultrastructural analysis of both the MARY-X spheroids and MCF-7 spheroids, namely lack of tripartite complexes in MCF-7 and lack of internal canals, as proven by both TEM, SEM and SEM/FIB. Most significantly, another independent laboratory performed ultrastructural analysis on MCF-7 agarose-induced spheroids (referenced within the manuscript) and reported an increase in the number of cell-cell junctions in the form of tight junctions and desmosomes but did not report the tripartite complexes (this has been explicitly stated within the revised manuscript). The findings of our manuscript concur with this earlier reported data. The authors of this manuscript have also incorporated additional data (TEM, SEM and SEM/FIB) to further validate the findings i.e. existence polar elements in IBC and lack thereof in a non-IBC cell line, MCF-7. Also, the number of spheroids analyzed has been added to the Material and Methods section of the manuscript.

In order to address the concerns of the reviewers’ we incorporated within the text of the revised manuscript is the following: 1) Analysis of spheroid preparations was performed on spheroid pellets (5,000 – 10,000 spheroids/pellet). TEM viewing was performed on 20 - 30 randomly chosen fields of multiple spheroid pellet preparations. For SEM analysis, spheroid pellet preparations (5,000 – 10,000 spheroids/pellet) are mounted (scattered) onto pins and sputter-coated as described. Observations were performed on 10 – 20 individual spheroids of multiple preparations. 2) Additional figures for TEM, SEM and SEM/FIB of both MARY-X and MCF-7 spheroids further validating the findings of this manuscript.

The number of spheroids/pellet, multiple preparations and number of randomly chosen fields correspond with previously reported ultrastructural data of an independent laboratory. Our laboratory even did additional analyses in the form of SEM and SEM/FIB to confirm lack of structural architecture (i.e. canalis) of the MCF-7 spheroids. All of the above adds to the robustness of this manuscript’s findings.