Author's response to reviews

Title: Antimetastatic gene expression profiles mediated by retinoic acid receptor beta 2 in MDA-MB-435 breast cancer cells

Authors:

Brett Wallden (bwallden@u.washington.edu)
Mary Emond (emond@u.washington.edu)
Mari E Swift (meswift@u.washington.edu)
Mary L Disis (ndisis@u.washington.edu)
Karen Swisshelm (kswiss@u.washington.edu)

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Dr. Peter Newmark
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We wish to thank the reviewers, including the statistical reviewer, for their suggestions and comments, most of which we have incorporated into this revision. Below we respond, point-by-point to the critiques:

Response to reviewers & point-by-point description of changes made

Reviewer Doris Benbrook:

The reviewer asked that we address the question of whether the retrovirus could have integrated into the Xq28 region. This is an interesting possibility, but a proof of this would entail at a minimum fluorescence in situ hybridization (FISH) studies to map integration site(s), followed by molecular mapping using BACs containing the genes of interest, as there are a total 75 genes that map to the Xq28 region, not taking into account differential promoter usage. If future expression array investigations using tumor RNA reveal additional data for genes on Xq28 being activated by the presence of RARβ2, we will initiate the FISH experiments.

We have added a sentence to the section, “Is Xq28 enriched with beta retinoic acid response elements,” (page 17) to address this issue raised by the reviewer.

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Reviewer Philippe Lefebvre:

Major:
1. Remove paragraph, page 17 (previous page 16), Is Xq28 enriched..., since none of the genes are on Table 3….

We kindly request editor-in-chief judgment in allowing this paragraph to remain. We have added additional discussion, that notes:

- the genes with RAREs are indeed on the array, but were not in the top 100 ranked candidates.
- even though the putative RAREs are nearest to the noted genes, they could be quite distant transcriptional activators/enhancers, as is the case in the HOX gene cluster where activation occurs at distances from 6 to more than 20 kbps. We note that RAREs can occur in regions 3’ to the last coding exon [1, 2].
2. Raised issue as to whether transglutamination of SPP1 could be verified? To our knowledge, there does not exist a reliable biochemical assay to test for transglutamination, which could result in SPP1/osteopontin dimers. We thank the reviewer for an astute question. We would like to modify the discussion on SPP1 to include the possibility that post-translational modifications to the protein could occur at the levels of N- or O-linked glycosylation, phosphorylation, cross-linking with other proteins, deamidation, oxidation and carbamylation. Of note there are at least 30 potential phosphorylation residues that have been identified by mass spectrometry [3, 4]. An overriding hypothesis is that some modifications may impact on the metastatic potential, and this post translational modification may be altered when cells express RARβ2.

3. Request for loading control for western blots. The protein for the western blot was from concentrated cell culture media, since the osteopontin/SPP1 protein is secreted. As clarified in an expanded methods (page 12), we loaded an appropriate volume of medium adjusted to be proportional for the number of cells at the time of harvest. We cannot use actin as a loading control since it is not a secreted protein. That is why we provided duplicate samples. The differences in protein, as the reviewer states, are not dramatic between the vector control cells and RARβ2 containing cells. What is striking is the difference in post translational modification.

Reviewer Danny R Welch:

Major:
1. Candidate genes will need to be evaluated in bioassays for their impact on biological functions (i.e., metastasis). We agree with this request and are in the process of obtaining funds to perform biological assays: this will require the cloning and regulated expression of multiple gene candidates, not just single transfections or transductions into breast tumor cells, with a biological measurement of in vitro invasion or in vivo xenograft studies. These long-term experiments cannot be conducted under the revision time for the manuscript. We feel that we have comprehensively evaluated the expression of ~50% of the unique genes revealed in our array experiments.

2. The manuscript described tumor-as well as metastasis-suppressor functions for RARβ2….are there data that separate these functions for RARβ2…incorporate verbiage. On page 5, we have added statements to address the bi-functional aspects of RARβ2 in the context of breast cancer cells, e.g.: “in cell cultures in which RARβ2 has been introduced via a retroviral vector, we found that all breast cancer cells could be inhibited in their proliferative capacity, even in the absence of the natural ligand, all-trans retinoic acid (AT-RA). Moreover, there is a dichotomy in RARβ2-transduced tumor cells in response to AT-RA: ER-positive cells undergo apoptosis, while ER-negative cells are further reduced in their proliferative potential, in comparison to culture conditions in the absence of AT-RA” [5].
3. **Issue of stromal contamination.**
We agree that there is a degree of murine cell contamination in the primary xenografts, but this does not invalidate our expression microarray results in this manuscript, which were primarily performed with pure tumor cell populations. We, in fact, have information on the relatively low proportion of murine cells within the primary human breast cancer xenografts from two data sources:

1) we performed cytogenetics on the explants at time of resection, and ~13% of the cells were of mouse origin, with the remaining showing a karyotype consistent with the hyperdiploid human karyotype with mixed numerical and structural aberrations; and

2) in order to evaluate vessel (murine contribution) density, we performed CD-31 staining of primary tumor sections. The figure below is typical of the vessels we see in the monotypic primary tumors, both vector control and RARβ2 tumors. There was no difference in microvascular density as published [6].

![Figure shows example of histology of both low [25X] (A) and high [200X] (B) images of primary xenograft tumors stained with an anti-mouse CD-31 primary antibody. Other than endothelial cells in vessels (positive dark blue stain), there is little visible stromal component.]

Minor:

1. **different RNA samples for eight arrays.**
We have added a statement on page 7, under RNA labeling to indicate that we incorporated the use of two independent cell and RNA preparations in our experimental design.

2. **statistical review:**
Performed by the journal’s statistical consultant

3. **Osteopontin irrelevant since arrays are all about RNA**
As we are able to identify reliable antibodies to our gene candidates, we will also validate protein expression. Knowing protein levels, isoforms, or differences in posttranslational modifications is critical to understanding the basic biological consequences of anti-metastatic molecules. We included the protein expression results for osteopontin (SPP1) as it illustrates an important aspect of RNA expression profiling, wherein the protein expression may not mirror the mRNA expression.

4. **Baseline frequency for MDA-MB-435 metastases low compared to the reviewer’s/other’s experience.**
We offer the following possibilities for the differences:

   1. Our protocol [6] employed a model of surgical excision of the primary lesion, in order to mimic a more human situation of ‘lumpectomy.’ It is possible that trophic, circulating factors provided by the initial, and now debulked, primary
tumor curtailed robust metastases. We note that our mammary fat pad xenografts were well encapsulated, and in most cases, we were able to cleanly excise all grossly observable tumor.

2. Differences in animal diets may have profound effects on the metastatic potential. This was demonstrated by Rose and Connolly [7], wherein animals fed a low-fat diet had fewer tumor recurrences, following excisions as well as fewer metastases, similar to our results.

3. Technical details in the surgical procedures, husbandry of the animals, etc.

Moreover, we do not think that our differences should detract from the findings of our expression profiling.

Reviewer Janet Price:

Major:
Address issue of metastatic heterogeneity with transient expression of RARβ2.

In order to address the question of heterogeneity we provide an additional (new) supplemental table 2, which shows that mixed pools of previously isolated clones or newly transduced/selected populations of the RARβ2 gene reflect outcome obtained for the long term cultures, with which the expression arrays were performed. In the newly transduced populations, only one of the seven genes MGC2780, a hypothetical gene of unknown function, resulted in a discordant result (page 14-15) and the new Supplemental Table 2.

In summary, we have added additional data in response to Janet Price, which is now Supplemental Table 2. The previous Supplemental Table 2 is now Supplemental Table 3 in order to follow the order in the text.

We have added additional comments in the text to respond to the reviewers, edited minor details of the text and updated Supplemental Table 1. We have added molecular weight standards guides in Figure 2.

References:

[3] B. Christensen, M.S. Nielsen, K.F. Haselmann, T.E. Petersen, and E.S. Sorensen, Post-translationally modified residues of native human osteopontin are


On behalf of all the authors,

Karen Swisshelm, Ph.D.,
Department of Pathology - Box 357470
University of Washington
Seattle, WA 98195-7470
Phone: 206-616-3182
FAX: 206-543-3644
email: kswiss@u.washington.edu