Author's response to reviews

**Title:** Odontogenic Ameloblast-Associated Protein (ODAM) inhibits growth and migration of human melanoma cells and elicits PTEN elevation and inactivation of PI3K/AKT signaling

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**Author's response to reviews:** see over
Response to queries by reviewer # 2

Major Points

1. The reviewer states that due to the *in vivo* correlation of nuclear ODAM expression and disease aggressiveness it would be “interesting to assess whether ODAM protein is located in the nucleus or the cytoplasm in ODAM positive cells”.

We have looked at ODAM localization both in the melanoma cell lines utilized in this study and in earlier published studies with MDA-MB-231 breast cancer cells. The first two exons in ODAM encode a leucine/isoleucine-rich secretory leader and studies in our lab and others [1] [2] indicate the protein expressed upon transfection is largely secreted, with as little as 0.5% remaining cell-associated after 48 hr. culture. Earlier studies in our lab also demonstrated that recombinant ODAM binds to the exterior of cultured cells and to extracellular matrix proteins.

Immunostaining of cultured, transfected cells shows ODAM protein localized predominantly in the golgi apparatus with faint vesicular staining in the cytoplasm, and at the cell periphery [1] [2]. At this time, we have not observed nuclear localization of ODAM in cultured cells of any type including the melanoma cell lines described in this report. This has been assessed both by photomicroscopy and through western blotting of lysates from isolated nuclei. That said, IHC analysis of a range of normal and malignant tissues readily demonstrates specific instances of nuclear ODAM staining, and nuclear ODAM has been observed in cultured primary ameloblasts [3]. These varied findings do, however, leave the mechanisms of nuclear import for ODAM, and the functional significance of ODAM localization unclear, as the reviewer has noted.

We do not feel that addition of photomicroscopy for ODAM localization will augment this report since essentially identical images of transfected (MDA-MB-231) cells were included in an earlier publication [1]. A description of the localization pattern for ODAM has, however, been added to the text.

2. The reviewer indicates that the observed increase in PTEN expression for C8161-ODAM cells is unconvincing (Figure 3B). Admittedly, the increase in PTEN for C8161-ODAM cells, as submitted in the original figure, was not as robust as that observed in A375-ODAM cells.

In the course of further work we have utilized a different primary antibody (optimized rabbit monoclonal) for PTEN detection on western blots and IHC, and upon reprobing of lysates analyzed in figure 3 we now show a more clear-cut increase in PTEN protein in C8161-ODAM cultures that is more comparable to that in A375-ODAM cells. Figure 3B has been updated with images derived via the new antibody reflecting this improvement. The reviewer also requested the inclusion of quantitative PTEN mRNA expression data for C8161-ODAM and control cultures as support for the protein data. This data has been added in figure 3C.
3. The reviewer states “Figure 4A. Surprisingly, in this western blot, PTEN basal expression shows an important increase in C8161 parental cells versus C8161-ODAM cells (contrary to results shown in Figure 3B….) Please discuss.”

In our view the data as given in figure 4A (the PTEN panel is shown below) again show higher PTEN in untreated ODAM-expressing cells and do not contradict those in figure 3B in this respect. The intent of the figure centers on the effects of PTEN siRNA on PTEN expression and p-AKT levels.

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The figure as submitted showed the panel probed for phosphor-AKT above the PTEN panel and perhaps led to some confusion. We have rearranged the figure for clarity and revised the legend to match.

4. The reviewer states that the text concerning Figure 4B states “that p-AKT is not increased after PTEN silencing in A375-ODAM cells; contrary to that statement figure 4B shows an increase in p-AKT in these cells.”

The data as given does indeed show an increase in p-AKT as the reviewer states, and it is our intent to indicate this in the text. The text as submitted was:

“While PTEN siRNA treatment reduced PTEN protein levels to a lesser degree in A375-ODAM cells, AKT phosphorylation was nonetheless increased (figure 4B).”

We have modified the text to clarify this point. It now reads:

“While PTEN siRNA treatment reduced PTEN protein levels to a lesser degree in A375-ODAM cells, AKT phosphorylation was increased (figure 4B).”

5. The reviewer asks if it was possible to achieve better PTEN silencing in A375 cells. Regrettably, after multiple attempts the answer is no. Whether due to inefficient transfection, the reportedly long half-life of PTEN protein, or other factors, the data as given seem to reflect the limit of PTEN silencing through transfected siRNA in these cells.

Nonetheless silencing as described was sufficiently effective to demonstrate significant recovery of AKT activation in ODAM-expressing cells, which was the experimental intent. This is in accordance with the gene-dose (and protein-dose) dependency of PTEN action [4] [5].
6. The reviewer indicates that confirmatory experiments downregulating ODAM in cell lines expressing endogenous would be desirable.

We would, of course, be very interested in the results of such experiments on a variety of fronts. Unfortunately we are as yet unaware of any continuous cell line that expresses endogenous ODAM (we have tested numerous lines as have others). Only primary ameloblast cultures and cultured primary ameloblastoid tumors have been definitively demonstrated to maintain expression of the protein in culture. For that matter we have found that most tumor cell lines exhibit growth suppression in response to ODAM transfection, and will not tolerate long term ectopic expression of the protein.

Our preliminary data suggests that the protein is expressed in primary Human mammary epithelial cells (HMEC) but experiments with respect to that effort are just beginning and are beyond the scope of this report.

7. The reviewer requests that discussion of the role of ODAM in malignancy be enlarged and clarified with respect to the apparent dichotomy in ODAM action as relates to nuclear/non-nuclear expression. Our understanding on this point is preliminary at best, and real answers lie well beyond the scope of this report. We have thus added some context within the discussion to meet this end, but do not wish to speculate further.

Minor Points
1. The reviewer requests a reference for c-Raf (S259) as a target of AKT action. This reference has been provided in the text (reference # 24, page 13).
2. The reviewer states that it is “surprising that ODAM transfected melanoma cells do not modify their extracellular adhesion properties. Please discuss.” Our data show that ODAM-expressing melanoma cells exhibit increased adhesion to extracellular matrix in vitro (Figure 1) yet, as the reviewer states other aspects (cell-cell adhesion and catenin-adherens junction rearrangement) are not consistent across all the cell types where we have enforced ODAM expression. To our mind this is not unexpected given the complexity of cellular adhesion and the broad array of adhesion anomalies associated with tumor cell lines (alterations in cadherins, integrins, catenins, and associated signaling molecules….). The accumulated knowledge indicates a role for ODAM in promoting cellular adhesions at the dental junctional epithelium, in cultured cells, and likely in a range of other tissues. This underlies suggestions that ODAM be classified as a matricellular protein [6]. There remains much to define as to the specifics of ODAM interactions at cell-cell, cell-matrix, and cell-enamel interfaces. Therefore a clear interpretation of the observed variation is not possible at this time.

Summary of changes to figures
-reprobed western blots for PTEN substituted in figure 3B.
-analysis of PTEN RNA expression for C8161 cells added in figure 3C.
-order of p-AKT and PTEN western blot panels reversed in figure 4 for clarity.
-Figures 1, 2, and 5 are unchanged.
-a minor labeling error was fixed in figure 5
References


