Author's response to reviews

Title: Increased Diacylglycerol Kinase zeta Expression in Human Metastatic Colon Cancer Cells Augments Rho GTPase Activity and Contributes to Enhanced Invasion

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Response to Reviewers

We thank the reviewers for their careful reading of the manuscript and for pointing out critical omissions and errors. We have corrected these in the revised version of the manuscript. We appreciate Dr. Topham’s positive comments: We agree that the work is “solid” and “adds relevance” to our previous studies. The main criticism of this paper, agreed upon by all three reviewers, was the limited number of cell lines tested. We concur that this was a limitation of the original manuscript. To address these concerns, we have included new data from two additional cell lines, the highly metastatic MDA-MB-231 breast cancer line and the PC-3 prostate cancer line. We depleted DGKζ expression in these cells in two different ways. For PC-3 cells, we created stable cell lines using a shRNA in the pLKO plasmid, as we did for SW620 cells. For MDA-MB-231 cells, we used three different shRNA-expressing lentivirus particles. This method allows relatively long term knockdown of DGKζ expression in a stable population of cells and allowed us to obtain efficient knockdown (see Fig. x).

We are happy to report that we have essentially replicated the findings we obtained from SW620 cells; namely, that silencing DGKζ expression decreases the invasiveness of PC-3 and MDA-MB-231 cells. Since DGKζ is ubiquitously expressed, these new findings suggest silencing DGKζ expression is a potential strategy to attenuate invasion in a variety of cell types and not only in cells where DGKζ expression is elevated.

We agree with Dr. Taketomi that testing the silenced cell lines for their metastatic potential in scid or other immune-compromised mice would provide in vivo data that would nicely complement our in vitro results however, we feel that those studies are beyond the scope of the present work. Moreover, we feel strongly that our new findings greatly strengthen the manuscript and would be of significant interest to researchers in the DGK and Rho GTPase fields, as well as to those studying metastatic cancers.

We agree with Drs. Topham and Xiao that evaluating DGKζ (protein) expression in patient samples would strengthen the clinical relevance of our work. However, as noted by Dr. Topham, clinical specimens are difficult to come by and we are presently not in a position to purchase commercial tissue microarrays. As suggested, we have added a statement in the discussion that acknowledges this limitation:

Thus, DGKζ and its downstream signaling pathways may be important factors influencing colon cancer progression. However, a limitation of our studies is the lack of correlative clinical data showing the DGKζ protein level is elevated in metastatic cancer. Thus, it will be important to validate our findings by comparing DGKζ protein levels in primary and metastatic tumor specimens. Moreover, the effect of silencing DGKζ expression on the in vivo metastatic potential of tumor cells with elevated DGKζ levels or high Rho GTPase activity remains to be investigated.

Below, I have addressed Dr. Xiao’s comments point by point.
Major Revisions

1. We respectfully disagree with the reviewer regarding the physiological meaning of the present work – it is, in fact, quite clear. We previously showed that DGKζ regulates the activity of Rac1 (Abramovici et al. 2009) and RhoA (Ard et al. 2012) and that DGKζ-deficient cells have reduced activity of both GTPases. Moreover, we showed that DGKζ-deficient mouse embryonic fibroblasts (MEFs) migrated and invaded less than wild type MEFs. Showing that DGKζ expression is increased in cancer cells with increased Rho GTPase activity and invasiveness suggests targeting DGKζ is a possible route to decreasing invasiveness and metastases.

2. It is presumptuous and disingenuous to expect us to cite the reviewer’s own publications when discussing the role of DGKζ in cell motility. The references in question deal with the role of atypical protein kinase C (PKCζ) in cell motility, not DGKζ or any other DGK isoform. I performed a text search of each of the three articles cited by the reviewer and DGKζ is not mentioned anywhere in them. It is not clear if the reviewer expects us to discuss the role of PKCζ in cell motility. If that is what is meant, then we feel that is well beyond the scope of this manuscript.

3. See the response to all three reviewers.

4. We have added more detail in the Materials and Methods to clarify how we quantified invasion. We consulted numerous references to determine the optimal conditions for invasion for each cell line and outlined the methods in a style similar to those references.

5 and 8. This is a good suggestion but there are no commercial antibodies to phosphorylated DGKζ available, nor any that we have made ourselves. We plan to make antibodies to phospho-DGKζ in the future.

6. We realize that the relative RhoA activity is normalized to tubulin. The data were presented this way to reflect the fact that total RhoA levels also increased. However, we recognize that this may have caused some confusion, so we have modified the discussion as follows to clarify this point:

*RhoA activity was increased approximately 3-fold in SW620 cells compared to SW480 cells. Furthermore, there was a comparable increase in both RhoA and DGKζ expression. Since DGKζ is required for efficient RhoA activation {Ard, 2012}, the combination of increased DGKζ and RhoA expression likely accounts for the increased RhoA activity in SW620 cells. However, since the level of RhoA activity but not protein was decreased by DGKζ silencing, it appears unlikely that DGKζ directly regulates RhoA expression. Thus, our findings in SW620 cells are consistent with our previous studies in mouse embryonic fibroblasts, which indicated that DGKζ regulates RhoA activity {Ard, 2012}.*
We thank the reviewer for noticing that the legends for Fig. 2D and E were missing. We have included them in the revised manuscript.

7. We have included a control for tubulin in Fig. 3A as requested.

8. See response to point 5.

9 and 10. See the response to all three reviewers.

We did not perform a tubulin control for the Rac1 activity assays shown in Figure 5 because the activity is normalized to total Rac1 and the levels were approximately equal. A tubulin blot was included as a control for the RhoA activity assay because the immunoblot of the SW480 and SW620 extracts revealed a substantial difference in RhoA levels.

**Minor Revisions**

1. We thank the reviewer for catching the error with regard to cell migration instead of cell invasion. The running title has been changed accordingly.

2. SiRNA-mediated silencing was changed to shRNA-mediated silencing.

3. We have included sources and catalogue number for antibodies used in this study and the concentrations used in the experiments.