Author's response to reviews

Title: Mammary tumors that become independent of the type I insulin-like growth factor receptor express elevated levels of platelet-derived growth factor receptors

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Author's response to reviews: see over
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Dear Editor

I would like to thank the reviewers for their constructive criticism of our manuscript. I have outlined the concerns of Reviewer 1 below and the steps taken to address these concerns. Reviewer 2 did not have any concerns.

I hope the manuscript in its current form is now acceptable for publication.

Sincerely,

Roger Moorehead
Response to Reviewer 1

1. **HER family and BCRP in IGF-IR Resistance**
   We have measured the levels of EGFR1, ErbB2, and ErbB3 in our mammary tumors following doxycycline withdrawal and in primary mammary tumors and recurrent spindle tumors. Neither study showed any consistent increase in total EGFR, ErbB2 or ErbB3 or the activated forms of these receptors in mammary tumors with lower levels of IGF-IR (see westerns below). Therefore, we did not pursue the ErbB family any further. We have however shown in another publication that elevated expression of ErbB2 in our RM11A cells does enhance tumor formation and elevated ErbB2 expression can suppress tumor regression following IGF-IR downregulation and increase metastasis (Mol Cancer 9: 235, 2010). A comment about the EGFR/ErbB status in our tumors has been added to the discussion in the first paragraph on page 19.

   ![Western Blot Images](image)

2. **PDGFR upregulation as a resistance mechanism**
   The reviewer is correct in that the term “resistance mechanism” is not appropriate to describe the function of PDGFRs in mammary tumor cells that have become independent of IGF-IR signalling. Reference to a resistance mechanism have been removed from the manuscript and I have altered the manuscript to indicate that PDGFRs are upregulated following loss of IGF-IR and the PDGFRs mediate migration but do not compensate for IGF-IR induced signalling.

3. **mechanism underlying PDGFR upregulation**
   The mechanism underlying PDGFR upregulation is currently being investigated but remains unknown at this time.

4. **Insulin receptor in IGF-IR independent tumors**
The levels of the insulin receptor are similar in IGF-IR-dependent and IGF-IR-independent mammary tumors. This data has been published in Oncogene (in press).

5. **Scratch assays influenced by proliferation and apoptosis**
The reviewer is correct in that increases in proliferation or decreases in apoptosis can artificially increase the closure of scratch wounds. That is why the scratch wound assays were complemented with the Boyden chamber Assays. In the Boyden chamber assays cells must first degrade the artificial basement membrane and then migrate through the membrane. In this assay, alterations in proliferation or apoptosis should have less impact than on the scratch wound assays. Since both assays show similar results we are confident that PDGFR knockdown impacts cell migration. If the reviewer wants us to remove the scratch assay figure and simply show the Boyden Chamber figure then the manuscript can be altered.

6. **MTT assays**
My lab does not have expertise in flow cytometry and that is why we chose to compliment the MTT results with experiments directly assessing proliferation (Ki67 and phosphorylated histone H3) and apoptosis (JC1 and cleaved caspase 3). It is unclear what additional information regarding proliferation or apoptosis that flow cytometric analysis would provide other than information regarding cell cycle time or distribution of cells in different cell cycle phases.

7. **Differences in apoptosis measured by JC-1 and cleaved caspase 3**
JC-1 and cleaved caspase 3 measure two different steps in the apoptotic cascade. JC-1 measures the loss of mitochondrial membrane potential using a fluorescent cationic die while cleaved caspase 3 is measured by an antibody that specifically recognizes the cleaved form of caspase 3. Since these two assays measure different aspects of the apoptotic cascade and likely have different sensitivities, it is not surprising that the magnitude measured by these two assays is different. PCNA and Ki67 are both used as measures of proliferation but since the half-life of the proteins are different; they may provide different magnitudes in changes in proliferation. I would also not be surprised if immunofluorescence and western analysis of cleaved caspase 3 provided different magnitudes of apoptosis since these two assays have different levels of sensitivity and one assay is measuring each cell (immunofluorescence) while the other assay provides an average of the cell population (western). The important point is that two independent measures of a cellular process found consistent trends.

8. **Show IGF-IR in Figures 1 and 2**
The levels of IGF-IR protein in the IGF-IR independent mammary tumors have been previously published (Oncogene 2009 28:2152-2162). All tumors had very low levels of IGF-IR that were either undetectable or barely detectable by western blotting.

9. **Transfection efficiency**
Transfection efficiency was not measured in this study. We routinely performed western blotting following PDGFR RNAi treatment throughout the study to ensure we could achieve consistent knockdown of the PDGFRs.

10. **Define HPRT**
Done