Author's response to reviews

Title: STAT6 expression in glioblastoma promotes invasive growth

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Author's response to reviews: see over
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Melissa Norton, MD
Editor in Chief, BMC Cancer

SUBMISSION OF REVISED MANUSCRIPT

Dear Dr. Norton,

Enclosed is a revised version of our manuscript entitled “STAT6 expression in glioblastoma promotes invasive growth” for your consideration as a research article in BMC Cancer. We appreciate the opportunity to re-submit this article, and would like to thank the reviewers for their helpful feedback and suggestions, which we have incorporated into the manuscript. Below, please find our point-by-point responses to each reviewer’s comments; we have also highlighted sections of the manuscript that have been modified from the original, as requested.

Thank you for your consideration of this manuscript.

Sincerely yours,

Barbara Merk
Reviewer: Ian Lorimer

1) We thank the reviewer for pointing out the inaccuracy of our statement regarding temozolomide. We have corrected our statement, and have added the suggested reference to the manuscript.

2) We apologize for not being clearer when describing how the clones were generated. We clarify that each clone was in fact knocked down using a distinct hairpin vector, and that at least 2 clones containing vectors of different sequence were used in each experiment. The first number in individual clones’ names as they appear in the figures (i.e. U-1242 Clone 11-22 or 13-13) refers to the sequence used to knock down STAT6 in that clone. As an additional control for off-target effects, cells containing a non-target shRNA were included in each experiment as well. Please see the expanded Methods section for a more detailed description and sequence information.

3) It is always difficult to ensure that decreased proliferation does not affect invasion; however we took several steps to minimize such effects. First, the invasion assays were performed in serum-free medium, and neither cell line (U-1242MG or U-87MG) actively proliferates in the absence of serum. In addition, we chose the 8-hour time point since with a 24-hour cell cycle, only 1/3 fewer cells would be expected even if the shRNA had caused complete growth cessation, which was not the case (see Figure 5). Lastly, there is no obvious correlation between individual clones’ growth rate and invasiveness: the clones with the greatest decrease in proliferation were not less invasive than the others (please see Figures 5 and 6).

4) We have expanded Figure 7 to include separate survival curves of only GBM patients (excluding the lower grade gliomas) and only patients with grade II/III astrocytoma.

5) We thank the reviewer for pointing out this apparent inconsistency, and have expanded the manuscript to include possible explanations. The most likely scenario is that STAT6, by means of its transcriptional targets, promotes invasion only in the presence of additional pro-invasive factors, which are not expressed in early stage tumors.

6) We have modified the abstract to clearly state the type of microarray data that was used in the survival analysis.

7) We agree that mRNA stabilization is a possible alternative explanation, and have incorporated it into the manuscript.

8) We and another reviewer feel that this has not been demonstrated previously in GBM cells and is worth showing.

9) We thank the reviewer for pointing this out and have corrected the discrepancy between the text and the figure legend.
Reviewer: Julie Carrier

Response to Major Comments:

1) We agree with the reviewer that investigation of genes that are co-induced with STAT6 is worthwhile. We have performed additional experiments to provide this information; specifically, we used a microarray to compare genome-wide expression levels in wild-type GBM cells and the STAT6 knockdown clones in both U-87MG and U-1242MG. Two tables listing the most significantly affected genes has been added to the manuscript as tables 2 and 3; the complete array data is included as supplementary data.

2) We have seen nothing in the literature to suggest a direct cross talk between STAT6 and STAT3. The microarray data also do not suggest that one is a downstream transcriptional target of the other. In our opinion, the reduction in STAT3 seen in some cells after treatment with STAT6 shRNA is due to a high degree of homology between the two STATs, leading to non-specific knock down by the shRNA.

STAT3 and STAT5b were chosen as controls for off-target effects based on their expression levels in the two GBM cell lines, as well as their demonstrated importance in GBM. STAT3, which has received the most attention in GBM, is expressed at very low levels in the U-87MG cell line (see Figure 1) and thus is not an ideal control.

We did test each shRNA sequence for specificity against STAT3, STAT5a and STAT5b early in the process of generating clones. For each cell line, the two sequences resulting in the best STAT6 knockdown in combination with the fewest off-target effects were chosen to generate the stable knockdown clones. We apologize for not making this clearer. The Methods section has been modified to explain the rationale for the different controls.

Response to Minor Comments:

1) We did not observe STAT4 expression in any of our GBM cell lines. We did perform a positive control experiment, and are thus confident that the antibody was functioning properly. STAT4 expression is limited to myeloid cells, thymus and testis (Zhong et al., 1994).

2) We apologize for lack of clarity when explaining the normalization of mRNA expression. We have corrected the discrepancy between the legend and text, and expanded the Methods section to include details on how the real-time (quantitative) PCR was performed.

3) We did attempt to use a phospho-specific STAT-6 antibody as well; however the staining was too weak to be interpretable. Patient survival data unfortunately was not available for this set of samples.

4) We have added Figures 7b) and 7c) which show the correlation between STAT-6 expression and survival in patients with GBM, excluding the lower grade gliomas (7b) or grade II/III astrocytomas only (7c). We also included a comment regarding the possibility of false positives in a microarray.
5) We used non-replicative lentiviruses to make pools of stable knockdown cells. During the 2 weeks of selection and expansion, the interferon response should return to normal. In addition, control cells were infected with similar viral preparations expressing irrelevant shRNAs.

Reviewer: Janusz Rak

1) Thanks to the reviewer for pointing out that the TMA expression data can be simplified to include only negative and positive descriptors. We have incorporated that change. Regarding the discordance between the protein expression seen in the TMA and the mRNA expression noted in the Rembrandt data, there are several possible explanations. First, protein and mRNA expression is discordant in GBM cell lines (see for example figure 1). This suggests that post-transcriptional control mechanisms relevant to survival may be at play. Second, tumor evolution may dictate that tumors that express Stat6 protein in the original tumor may face selective pressures that result in loss of gene expression at the transcriptional level. While pilocytic tumors are of low grade and are generally not considered malignant, it is intriguing to speculate that those few pilocytic tumors that convert to higher grade might have been those with detectable STAT6. Unfortunately, the REMBRANDT data is not rich enough to calculate outcomes data associated with STAT6 expression in this subtype.

2) We apologize for not providing these details in the original manuscript. The Methods section has been expanded to include the requested information.

3) We have of course considered future assays for STAT6 function in vivo. Unfortunately, currently available model systems, which rely on implantation of human GBM cells into immuno-compromised mice, are unsuitable for assessing the potential therapeutic effects of STAT6 inhibition due to its well-documented role in the suppression of the host immune response against tumors (Sinha et al., 2005; Ostrand-Rosenberg et al., 2004). Immuno-competent STAT6 knockout mice, for example, are highly resistant against tumors, including both xenografts and spontaneous tumors. Any in vivo studies performed in immuno-compromised mice would fail to take this important, well-established role of STAT6 into account, and would not be representative of the potential outcome in humans. Confirmation of our present studies will thus necessitate development of an isogenic or orthotopic model of GBM for use in an immuno-competent model system.

4) Other than pointing out the lack of expression at both the mRNA and protein level, we cannot claim an understanding of the reason. We note that like U251, 16% of GBM tumors lack expression (Table 1).

5) In the modified text we have been careful to avoid reference to STAT6 as an oncogene or tumor suppressor except in citing other work that has made these claims.

6) We agree that it would be helpful to know whether STAT6 is expressed primarily in primary
or secondary GBM, and its correlation with EGFR status. Unfortunately, Rembrandt does not provide this information.

7) We thank the reviewer for pointing this out and have modified our comments on the effectiveness of GBM therapy.