Author's response to reviews

Title: Modulation of SOCS Protein Expression Influences the Interferon Responsiveness of Human Melanoma Cells.

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Version: 2 Date: 26 February 2010

Author's response to reviews: see over
February 26, 2010

Melissa Norton, M.D.
Editor In Chief
BMC Cancer

Dear Dr. Norton,

Enclosed please find a copy of our revised manuscript entitled: “Modulation of SOCS Protein Expression Influences the Interferon Responsiveness of Human Melanoma Cells” for re-submission as an article in BMC Cancer.

We believe that we have been able to adequately respond to the comments raised by the reviewers and have included a point-by-point response as part of our resubmission. We thank you in advance for your time and for considering this manuscript for publication.

The authors confirm this manuscript is original and is not under consideration elsewhere. In addition, none of the manuscript contents have been published previously except in abstract form. All authors of this manuscript have approved the final version and its submission to BMC Cancer. No authors of this paper have any conflicts of interest. All experimental research conducted in this manuscript was approved by The Ohio State University Institutional Biosafety Committee. This manuscript does not contain supplemental data or figures that will need to be in color, however we are willing to pay these charges if during the review process, these changes are deemed necessary by the editors or reviewers.

Sincerely Yours,

[Signature]

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Editorial Request:
Per editorial request, we have included a statement confirming that the work described in this manuscript was performed with the approval of an appropriate ethics committee.

Page 5, **Methods,**
“All studies were conducted with the approval of the Ohio State University Institutional Biosafety Committee.”

**Reviewer 1 (Dr. P. Lee)**
*Comment 1.* The major problem with this manuscript is the lack of novelty. It is already well established that SOCS1/3 negatively regulate IFN signaling pathways, and that SOCS1-3 expression is induced by STATs. Thus it is no surprise that over-expression of SOCS or knock-down of SOCS expression would alter IFN pathway responses in any cell type including melanoma cell lines.

We understand the reviewer’s concerns and are aware that the interaction between SOCS proteins and IFN response has been reported in other systems. However, we contend that attempts to define molecular pathways within individual tumor subtypes are deserving of publication. For example, recent studies have revealed that SOCS1 and SOCS3 gene expression are differentially regulated in breast carcinoma cells following stimulation with IFN-gamma and other growth factors (Evans MK *et al.*, Oncogene Mar22;26(13):1941-8, 2007). Contrary to findings in other systems such as melanoma, the authors showed that although IFN-γ induces SOCS1 expression in breast cancer cell lines, it actually down-regulated SOCS3 expression. Together these data caution that extrapolation of results across different tumor types can be misleading. Furthermore, our results on basal expression of SOCS3 protein in melanoma cell lines are novel as they are substantially different from those of another group (Kovarik *et al.*). These discrepancies are highlighted in our discussion and support the relevance and novelty of our studies. In addition, we have carefully acknowledged the previous work performed in this field on chronic myelogenous leukemia, T cell lymphoma and in murine models of cancer.

*Comment 2.* The authors conclude that SOCS expression may limit IFN effectiveness in melanoma cells. However, this is the normal function of SOCS proteins, and the data presented do not compare melanoma lines with other types of cells, or with melanoma lines from different stages, thus this conclusion is not well-supported. More solid evidence would be derived from assays that enhance IFN-resistance in cell lines or in vivo models coupled with demonstrations of the role of tumor-expressed SOCS proteins in such a setting.

We thank the reviewer for these suggestions. In response to these comments we have examined the expression of SOCS1 and SOCS3 proteins in human embryonic melanocytes and in the WM 793b (vertical growth phase) and WM 1552c (radial growth phase) melanoma cell lines. These new data indicate that SOCS1 and SOCS3 proteins were expressed at basal levels in each of these cell types. In contrast to our data obtained in metastatic melanoma cell lines (Figure 1a-b), these new data also revealed that IFN-α and IFN-γ did not upregulate SOCS1 or SOCS3 in melanocytes. SOCS3 was upregulated in response to IFN-γ in the WM 1552c melanoma cell line. These new results are
included as Figure 1c of the revised manuscript. We would also like to point out that Figures 3-4 of the original manuscript included studies in which SOCS proteins were over-expressed in human melanoma cell lines and led to enhanced IFN-resistance at both the level of signal transduction and gene expression. These studies validate the role of tumor-expressed SOCS in reducing the IFN-responsiveness of melanoma cell lines.

The following text has been added to the Results section (page 8):

“To determine whether SOCS protein expression might be restricted to melanoma cells at a particular stage of development, we conducted similar experiments in human embryonic melanocytes (HEM) and in the WM793b and WM1552c human melanoma cell lines. These two cell lines are derived from vertical and radial growth phase melanoma cells, respectively. SOCS1 and SOCS3 were expressed at basal levels in each of these cell types (Figure 1c). Treatment with IFN-α or IFN-γ did not upregulate SOCS1 expression in any of these cell lines, while SOCS3 was upregulated by IFN-γ only in the WM1552c radial growth phase cell line.”

Comment 3. The authors make the suggestion that targeting of SOCS proteins may be a therapeutic option for cancer patients in order to enhance the effectiveness of IFN therapy or endogenous IFN responses. However, as the authors mentioned in the discussion, it has been shown that “loss of SOCS1 expression is a critical event leading to elevated STAT3 signaling and over-expression of factors that promote cellular invasion and angiogenesis.” Thus, demonstrating the positive versus negative effects of SOCS inhibition in the cancer setting should be done before making such a suggestion, particularly since STAT3 activity in both the tumor and immune compartments plays a key role in tumor progression.

We appreciate the reviewer’s insightful comments. We have revised our discussion to acknowledge that the possible tumorigenic effects of SOCS1 inhibition must be considered if it is to be evaluated as a novel therapy. This comment has also prompted us to clarify to the reader that the focus of the present study is an examination of the role of SOCS protein expression in the tumor cell compartment. Conversely, other studies from Shen et al. and from our group (Zimmerer et al.) have also highlighted that SOCS1 inhibition in immune effector cells can lead to tumor regression in murine models of malignant melanoma. We have inserted this reference in our discussion and highlighted that our work is focused on the tumor cell compartment rather than the immune compartments. Finally, this suggestion from the reviewer prompted us to consider the role of STAT3 in our panel of cell lines and how it might influence our data.
Interestingly, we discovered that many human melanoma cell lines examined in this study have concurrent elevations in SOCS1/SOCS3 protein expression and basal phosphorylated STAT3 (at Tyr\(^{705}\)). These data indicate that the two events are not mutually exclusive.

![Figure 1. (d) Constitutive phosphorylation of STAT3 was evaluated in this panel of cell lines by immunoblot analysis. Antibodies directed against STAT3 protein and \(\beta\)-actin were included to control for variations in STAT3 across cell lines and equal loading, respectively.]

We have revised our results, discussion and conclusions sections as summarized below:

**Results (page 9):**

**Phosphorylated STAT3 expression in human melanoma cell lines.**

The STAT3 transcription factor promotes a metastatic phenotype and has been shown to be constitutively phosphorylated in human melanoma cell lines. Importantly, STAT3 is also sensitive to inhibition by SOCS proteins [33] and prior studies in melanoma brain metastases have suggested that loss of SOCS1 expression could promote metastasis via elevated STAT3 signaling. We therefore analyzed pSTAT3 levels in order to determine whether SOCS1 protein expression and basal STAT3 phosphorylation were associated in the panel of melanoma cell lines. These data indicated that basal STAT3 phosphorylation (at Tyr\(^{705}\)) and SOCS1 expression were present concurrently (Figure 1d). These data suggest that the presence of SOCS1 does not alter the level of basal pSTAT3.”

**Discussion (pages 14-15):**

“These data caution that modulating SOCS1 expression as a therapeutic strategy also has the potential to promote metastasis via STAT3 and this possibility should be carefully investigated in pre-clinical studies.”

“The inhibition of negative regulatory pathways to enhance the anti-tumor effects of cytokines represents a potentially novel approach against malignancy. Prior observations have primarily focused on inhibition of SOCS proteins in immune cells to allow for a greater anti-tumor effect. For example, Shen et al. have demonstrated that silencing of SOCS1 by siRNA in dendritic cells used as a therapeutic vaccine strategy resulted in superior anti-tumor activity in a murine B16F10 model of melanoma [44]. Our laboratory has also demonstrated that exogenously administered IFN-\(\alpha\) induced profound in vivo anti-tumor activity that
was immune-mediated (via CD8+ T cells) in SOCS1 and SOCS3 deficient mice [31]. Data from the present study have expanded our understanding of SOCS protein expression in melanoma and suggest that SOCS1 and SOCS3 proteins within the tumoral compartment represent a potential target that deserves investigation in future pre-clinical studies.”

Conclusions (page 16):

The present data suggest that SOCS1 and SOCS3 proteins represent a means by which melanoma cells can evade the direct effects of IFN-α and IFN-γ. The role for intratumoral SOCS1 and SOCS3 proteins as novel therapeutic targets remains deserves further evaluation in pre-clinical studies of melanoma or other malignancies.

Reviewer 2 (Dr. HM Johnson)

General Considerations. This study does not show a causal relationship between SOCS expression and the virulence of tumor cells. Thus the last sentence of the discussion that suggests that inhibition of SOCS proteins is a therapeutic strategy is not really addressed in this study. Further the authors state that others have not shown such a strategy. There is a study, however, that shows that SOCS1 siRNA treatment of dendritic cells results in potent anti-melanoma vaccine efficacy in the mouse melanoma model (Shen L et al. 2004, Nature Biotech 22: 1546).

We agree with the reviewer and revised our conclusions to state that our focus was on exploring the expression of SOCS proteins as they relate to melanoma cell responsiveness to clinically relevant cytokines, rather than the effect of SOCS proteins on the virulence of tumor cells (please see response to comment #3, reviewer 1 above). We also thank the reviewer for pointing out this reference and have cited the manuscript by Shen et al. in the revised discussion.

Comment 1. siRNA transfections are suggested to affect greater than 90% of cells (p. 10). It would seem that the knockdown in figure 5 would be more effective if this were so.

The experiments showing transfection efficiency were performed using a GFP-expressing plasmid vector and did indeed produce a GFP+ signal in approximately 90% of cells at the 24 hour time point. We were therefore confident in our ability to introduce genetic material in these melanoma cell lines with the transfection strategy employed. We understand the reviewer’s concern and are not certain the reason for the modest knockdown of SOCS1 and SOCS3 in the present study. Of note, we attempted to knock down SOCS1 and SOCS3 transcript using three separate published siRNA sequences (Ref # 31, Zimmerer et al., 2007; Ref # 44, Shen et al., 2004; Ref #41, Zittmann et al., 2007) and the siRNA sequence chosen produced the most reliable results with the greatest knockdown. Therefore, greater knockdown of SOCS expression proved to be technically challenging in these melanoma cell lines. These data led us to conclude that the reason for limited knockdown was possibly a function of the transient nature of siRNA silencing. However, it is important to note that even in spite of a modest reduction in SOCS1 or SOCS3, we did still observe an increase in gene expression following IFN-stimulation. We have modified our results section to acknowledge these limitations.
**Results** (page 10).

“Due to high basal expression, complete knockdown of SOCS1 and SOCS3 in these cells was difficult to achieve. However, their reduced expression following transfection of 1259 MEL cells with SOCS1 or SOCS3 siRNA led to consistent increases in IFN-α or IFN-γ-induced P-STAT1 as compared to cells transfected with control siRNA (Figure 5c).”

**Comment 2. No data are presented on non-melanoma cell lines for comparison.**

New data are included within the revised manuscript comparing human embryonic melanocytes and melanoma cell lines from vertical or radial growth phases (Figure 1c). Please see our response to comment # 2, reviewer 1.

**Comment 3. At the beginning of the results (p. 8), the authors indicate that “SOCS-specific peptide competitors eliminated SOCS3 specific immunoreactivity (Data not shown).” If the authors have a SOCS peptide inhibitor, it would seem that this has an important observation. It warrants more than ‘data not shown.’ More importantly, what do they mean by this statement?**

We apologize for the confusion. The inhibitor used was simply a commercially available peptide (Abcam, Inc. catalog # ab16199) which is used to confirm anti-SOCS3 antibody specificity in immunoblot experiments. As indicated in the discussion, prior reports from other groups did not observe basal SOCS3 protein expression in human melanoma cell lines (Kovarik et al.). Therefore, we were intrigued by this data obtained in our initial experiments (Figure 1a) demonstrating that human melanoma cell lines did indeed express SOCS3 protein via immunoblot analysis. To confirm the bands we detected by immunoblot were truly SOCS3-specific, we pre-incubated the SOCS3-specific Ab with the commercially available peptide inhibitor prior to probing the nitrocellulose membrane to compete out non-specific binding. We have revised the text in the **Results** (page 8) section to clarify our experimental data:

“Of note, pre-incubation of primary antibodies with commercially available SOCS3-specific peptide competitors (Abcam, Inc.) eliminated SOCS3 specific immunoreactivity (Data not shown).