Author’s response to reviews

Title: IL-10Rα expression is post-transcriptionally regulated by miR-15a, miR-185, and miR-211 in melanoma

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miRNA dysregulation in melanoma cell responsiveness to IL-10

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Reviewer #1: The manuscript "miRNA dysregulation in melanoma cell responsiveness to IL-10" by Venza et al. investigates the expression of IL-10 and its receptors regulated by miRNAs in melanoma cells and the effects of IL-10 on melanoma cell growth. The authors proposed that the growth of melanoma cells was influenced by IL-10 levels in the tumor microenvironment. The different responsiveness of various melanoma cell lines to IL-10 is affected by IL-10a receptor expression, which is post-transcriptionally inhibited by several microRNAs including miR-15a, miR-185, and miR-211. Overall, the data is novel and provides an interesting model of how specific microRNAs regulate cell growth by modulating immunoregulatory cytokine receptor levels in the tumor microenvironment.

There are a number of points the authors need to address before this article can be considered for publication.
Major points:

1. There is a conceptual mistake in this article: microRNAs are considered to regulate gene expression post-transcriptionally. They may confer epigenetic modulations by regulating genes involved in histone and DNA methylation, which is considered as "epigenetic" effects. In this article, it mentioned several times in the text that genes are regulated epigenetically by miRNAs but all the data shown is just posttranscriptional regulation. Please edit the text accordingly.

We have now edited the text according to the reviewer’s suggestions by replacing the word “epigenetic” with the word “posttranscriptional” (Abstract: pag. 2, line 4; Introduction: page 5, line 6; Results: page 8, line 19; Discussion: page 13, line 19; Conclusion: page 13, line 24).

2. Both G361 and GR-M are cutaneous melanoma lines, but they are very different considering the expression of IL-10Ra and IL-10Rb based on the data presented in this manuscript. Why is G361 more similar to the uveal melanoma cell line OCM-1 with respect to IL-10Ra expression, miRNAs expression and responses to IL-10? Is there any physiological relevance of this difference? As rightly suggested by the referee, we have now argued this issue in the Discussion by emphasizing how previous studies showed that the two lines of cutaneous melanoma, G361 and GR-M, exhibited different expression patterns and different responses to the same stimuli. Moreover we reported that the cutaneous melanoma line G361 displayed the same DcR1 and DcR2 expression profile than that of uveal melanoma cell lines as a result of promoter hypermethylation and a likewise identical responsiveness to the demethylating agent 5-aza-dC (page 11, lines 17-25).

It is necessary to include data from primary melanoma tumors (cutaneous and uveal) to evaluate the expression of IL-10, IL-10 receptors and miR-15a, miR-185, and miR-211. We agree with this comment and have now evaluated the expression levels of IL-10, IL-10 receptors and miR-15a, miR-185, and miR-211 also in cutaneous and uveal melanoma specimens (Figure 4; Abstract: page 2, lines 4 and 20,21; Material and methods: page 5, lines 18-23; page 6, lines 1,2; Results: page 10, lines 1-7; Discussion: page 12, lines 21-23; Figs. 4 legend).

3. miR-15a, miR-185, and miR-211 are all upregulated in G361 and OCM-1, however, the effect of these miRNAs was evaluated individually by luciferase, WB, and growth assays. It would be interesting to know whether there are synergistic or additive effects from all three miRNAs.

As rightly suggested by the referee, in addition to the effect of individual miRNA mimic or inhibitor, we have also evaluated the impact of their combination on IL-10 transcription, protein levels, and cell growth (see Figures 3 and 5; Abstract: page 2, lines 20-25; Material and methods: page 6, lines 17,18 and page 7, lines 10 and 13; Results: page 9, lines 14,15, 18,19, 22-25; Results: page 10, lines 20-25; Discussion: page 13, lines 6-13; Figs. 3 and 5 legends).
4. In Fig. 4A, how is the concentration of IL-10 (50, 100, or 500 U/ml) determined? Is it based on the IL-10 concentration measured in the tumor microenvironment? Please provide reference or justification. Why does 100 U/ml have the best effects on promoting cell growth?

As rightly suggested by the referee, we have now provided the reference relative to the concentration of IL-10 (50, 100, or 500 U/ml) employed in our experiments (see Material and methods, page 8, lines 2,3) and have specified that, accordingly to other reports, IL-10 yet at 100 U/ml was able to induce the optimal proliferative effect that was also retained at 500 U/ml (see Results, page 10, lines 13,14, 16-18).

5. Transfecting miR-15a, miR-185, and miR-211 mimics into GR-M line inhibits cell growth; it must be shown that these miRNA mimics reduce IL-10Ra levels.

We have now evaluated the effects of miRNA mimics on IL-10Rα levels also in GR-M cell line (see Fig. 3; Results, page 9, line 11)

It would be optimal to test the effects of IL-10 on cell growth treated with miRNA inhibitors or mimics to formally test the model that miRNAs regulate IL-10Rα levels to confer response to IL-10 in melanoma cells.

We have now performed proliferation experiments on IL-10-treated cells transfected with miRNA inhibitors and mimics regulating IL-10Rα expression (see Fig. 5; Results, page 10, lines 20-25).

Minor points:

6. There is some mislabeling. For example, the Fig. 2B referred in the text does not match the Figure 2B, which is miRNA array data instead of qPCR data. In Fig. 4A, the labeling should be IL-10 instead of IL-10Ra.

Thank you for making us aware of these mistakes which have now been corrected (see Results: page 8, line 24 and Fig. 5A)

Reviewer #2: This manuscript focuses on the role of IL-10 in melanoma. Specifically this group focuses on regulation of IL-10 signaling by microRNAs. I think the possibility that melanoma cells rely on IL-10 signaling for proliferation is very interesting. However, I do not feel the authors have sufficiently demonstrated the mechanism by which this occurs. The miRNA data is interesting, but requires some additional controls.

1. The title of the article is slightly confusing.
As proposed by the referee the title of the article has been now changed to “IL-10Rα expression is post-transcriptionally regulated by miR-15a, miR-185, and miR-211 in melanoma”.

2. Should look at protein levels or IL-10, IL-10Ra, IL-10Rb to see if there levels correlate - especially bc looking at effect of miRNAs.

We have now determined the effects of miRNA mimics (alone or in combination) on the protein levels of IL-10, IL-10Ra, IL-10Rβ in the three cell lines examined (Material and methods, page 7, lines 2-3 and page 8, lines 7,8; Results, page 10, lines 6,7; Discussion, page 12, lines 20,21; Fig. 3 legend).

3. "The expression levels of these miRNAs were confirmed by qPCR (Fig 2B.)" - this data does not appear to be qPCR data.

We thank the referee for this remark. We have now corrected the text (see Results, page 9, line 24).

4. Figure 2C is not referenced in the text

It has been now done (Results, page 8, line 20).

5. At the resolution provided in this draft, the reader cannot tell the difference between the different conditions in Figure 4A.

As rightly suggested by the referee, we have now improved the resolution of this previously labelled Figure 4A (now labelled Fig. 5A)

6. The magnitude of the effect of the miRNA inhibitors on proliferation in 4B and mimics in 4C seems dramatic compared to the magnitude of the effect of IL-10 in 4A. To me this suggests that the effect of miR-15a, 185, and 211 on proliferation may be through effects other than IL-10. I think that an siRNA approach (or analagous) to specifically knock down IL-10 signaling is a critical control and the magnitude of this effect should be compared to the magnitude of the miRNA experiments. Another approach to show the effect of the miRNA depends on knockdown of IL-10 would be appropriate.

Although the magnitude of the effect of the miRNA inhibitors on cell growth is more or less of the same order as the magnitude of the effect of IL-10 (~ 5 and ~ 6 folds, respectively), we have now evaluated the impact of IL-10Rα silencing on the proliferative effects exerted by miR-15a, miR-185, and miR-211 inhibitors (see Fig. 5C; Abstract, page 3, line 25; Material and methods, page 7, lines 19-22; Results, page 10, line 25; Discussion, page 10, lines 23-25; Fig. 5 legend).
7. The statement: "...relative to normal melanocytes, any significant change in IL-10 amounts occurred in the melanoma cell lines studied (Fig. 1)" is confusing.

We agree with this comment and have now replaced the sentence “...relative to normal melanocytes, any significant change in IL-10 amounts occurred in the melanoma cell lines studied” with “There were no differences in IL-10 mRNA levels between any of the 3 melanoma cell lines tested and normal melanocytes” (Abstract, page 2, lines 10,11).

8. The last paragraph of the discussion is confusing and does not seem to reconcile the difference in findings mentioned in Ref 14.

We have now made more clear the last paragraph (see Discussion, page 13, lines 16-19).