CD44 modulates cell migration and invasion in Ewing sarcoma cells

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Answers to Reviewer 2

We greatly appreciate the reviewers' comments, thanks to which the manuscript has improved in understanding and interest. The comments of each one of the reviewers have been answered point by point, indicating in each case the modifications made to the manuscript. Changes were marked in red in the text and deleted words/phrases/paragraphs were marked with red strikethrough text.

The experimental pathway of the study is well planned and the results on the correlation of CD44 upregulation with cell migration and invasiveness in the EWS in vitro model appoint CD44 as marker of the EWS1::FLI1 low phenotype. However it remains to clarify CD44 role on EWS progression respect/with other dysregulated genes to select CD44 as potential target for treatment.

Specific criticisms

Introduction *(page2 lines 57-62): the paragraph should be reworded as the sequence of the milestone discoveries is mixed up. After citing Franzetti, 2017 (Ref 4) and Aynaud, 2020 (ref 5) on the composite landscape of Ewing Sarcoma the Authors say that in "recent" years they looked for genes positively or negatively expressed in EWS cell models (ref 6-11): ref 7 and 8 are dated 2006 and 2007, respectively!*

We apologize for the misunderstanding. In order to provide a clearer explanation, the sentence has been rewritten (**page 2, line 60**).

Page 2, line 81: their possible role should be "its" possible role –

We appreciate the reviewer's positive feedback, the word "their" has been replaced by "its". **Page 2, line 83**.

RESULTS Page 4 line 147 : "additionally" can be deleted –

We appreciate the reviewer's positive feedback, the word "additionally" has been removed from the final version of the manuscript (**page 5, line 149**).

Page 4 lines 130-145 this paragraph should be summarized to be appropriate to a Results section.

We agree with the Reviewer. The paragraph has been summarized in the latest version of the manuscript (page 4, lines 132-138).

Discussion *Page 14 368-385 There are concepts already expressed in the Introduction which might be slimmed. Based on the obtained findings could the Authors discuss the coherence of EWSR1::FLI1low expression program, upregulation of CD44 and the mechanisms of epithelial-mesenchymal transition, extracellular matrix interaction and degradation?*

We appreciate the reviewer for providing this comment. We agree that discussing the different processes involved in the context of CD44, EMT, and ECM would be enriching for the manuscript. Consequently, a new paragraph discussing their interconnection in the context of Ewing sarcoma has been added (including new references) to the final version of the manuscript (**page 14, lines 416-428**, and **page 16, lines 508-517**). Additionally, the concepts already expressed in the introduction have been removed (**page 14, lines 385-390, and lines 393-398**).

Though CD44 has been cataloged as a pro-metastatic marker in several malignancies, this is the first evidence in EWS, accounting for the Authors' statement that CD44 is a potential driver of cell migration and invasion in this tumor. However, CD44 intersection with the overall EWS transcription profiling is completely unknown. To this regard a comment on other genes upregulated upon EWSR1::FLI1 knockdown would be appropriate: but with the exception of CCK no other gene is mentioned as potential player and target for treatment action.

We appreciate the reviewer for highlighting these interesting concepts. We agree with the reviewer's suggestion that discussing other genes upregulated upon EWSR1::FLI1 depletion would be both interesting and enriching for the manuscript. Consequently, a paragraph discussing these concepts has been added to the final version of the manuscript (**page 16, lines 518-527**).

Conclusions *The results obtained in this study point to the relevance of CD44 in the EWSR1::FL1 knockdown and predict CD44 might concur with other genetic and epigenetic determinants to the progression of EWS. The Authors should underline that this information is acquired using an in vitro model that simplifies the in vivo composite and dynamic situation of the coexistence of two EWS cell populations. This is one limitation of the study. In addition, even if a new challenging plug is disclosed to understand the biology of EWS, the mechanistic trajectory remains to be delineated to raise translational opportunities.*

We appreciate the reviewer's comment. We agree with the reviewer that the limitations of this study are inherent to *in vitro* experimentation, and further research is needed to fully describe the complete tumor scenario in which CD44 exerts its function. Consequently, an additional paragraph addressing these limitations has been incorporated into the final version of the manuscript (**page 17, lines 543-547**).