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Communication between host organism and cancer cells is transduced by systemic sphingosine kinase 1/sphingosine 1-phosphate signaling to regulate tumor metastasis

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 02 February 2012

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript.

You will see that they find the topic of your manuscript interesting and the experiments overall well executed. However, they feel that the data need to be strengthened, particularly on a mechanistic level (referee #1 point 1 and referee #2 point 4) and they make constructive suggestions for that.

Should you be able to address these criticisms in full, we would be willing to consider a revised manuscript.

Please note that it is EMBO Molecular Medicine policy to allow only a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor. Also, the length of the revised manuscript may not exceed 60,000 characters (including spaces) and, including figures, the paper must ultimately fit onto optimally ten pages of the journal. You may consider including any peripheral data (but not methods in their entirety) in the form of Supplementary information.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The authors have used all the right models in this study, including an anti-S1P antibody that might be useful for inhibiting metastasis.

Referee #1 (Other Remarks):

This is a nice paper that distinguishes between the roles of systemic S1P and locally-produced S1P (in tumors) in lung colonization/metastasis. The experiments are performed well, and the authors have included all of the right controls to validate their data. I only have a few comments:

1. What is the mechanism by which Brms1 is regulated by SK1/S1P? While experimental evaluation of this issue might be beyond the scope of the current paper, the authors should at least discuss how they think this might occur. In this regard, did they examine the role of any of the other genes (i.e. Mdm2 and Mmp10) that were also up-regulated in their arrays?
2. The effects that the authors see are often 'partial' (i.e. Fig. 1A), but this is not always obvious from the description in the text. This should be addressed. Ditto 'novel', which is used unnecessarily in the legend of Fig. 9.
3. How specific are the S1P2 and S1p1 antagonists (Fig. 5)?
4. Since Dr. Obeid is an author, she does not need to appear in the Acknowledgments.
5. n values are often missing in the figure legends`

Referee #2 (Other Remarks):

This manuscript convincingly demonstrates that metastasis is promoted by systemic, as opposed to tumor cell-derived, sphingosine-1-phosphate (S1P). In this system S1P, acting specifically through the tumor cell S1P2 receptor, inhibits expression of Brms1, a suppressor of metastasis. This is a novel finding that advances the understanding of metastasis by:
1. Demonstrating that is systemic, rather than tumor derived, S1P that enhances metastasis as well as primary tumor growth.
2. Identifies a specific signal transduction/effector pathway that enhances metastasis.
Minor alterations to the manuscript are requested:
1. Figure 3, Panel C. The number of metastasis (rather than weight as in Panel D) should be quantitated.
2. Figure 4. Panel A. It is not clear from either the methods or the figure legend what is being presented in this figure. It looks like a scan of fluorescence, but this reviewer is not familiar with this form of presenting qPCR data.
3. Figure 4. Panel D. The Westerns used to derive this data should be shown.
4. Figure 5. In determining the S1P receptor subtype responsible for inhibition of BRMS1 expression, the authors test A549 cells and MEFs derived from S1P2 knockout mice. At the very least the authors should test MB49 cells with the pharmacological inhibitors, siRNA depletion of S1P2 would be even better.
5. Figure 5, Panel E.
a. The authors are using medium as a source of S1P, and controlling this by using medium from SK knockout cells. It would be more straightforward to use purified S1P. The authors need to provide an explanation for using medium instead, and a more convincing control for depleting S1P (use of the S1P antibody?) must be provided if there is a compelling reason to use medium.
b. The authors demonstrate that S1P2 depletion by itself, even in the presence of medium that should not contain S1P, enhances BRMS1 expression. The authors should provide an explanation for this observation.
6. Figure 8, Panel B. The sphingomab-treated animals should have decreased metastatic colony

formation, according to Figure 3F, yet it does not seem to be significant in this experiment. The authors need to explain this.

1st Revision - Authors' Response 22 March 2012

Thank you for your letter of Feb. 20, 2012, regarding the review of our manuscript (EMM-2012-01241). We would like to thank the Reviewers for their careful and positive review. We are pleased that the manuscript was viewed as significant, and the revision was invited for further consideration.

We have revised the manuscript to address the reviewers' comments, and are now submitting the amended manuscript entitled "Communication between host organism and cancer cells is transduced by systemic sphingosine kinase 1/sphingosine 1-phosphate signaling to regulate tumor metastasis" to be considered for publication in *EMBO Molecular Medicine*.

Please find below our point-by-point response to the comments raised by the Reviewers:

Reviewer 1:

We thank the Reviewer 1 for stating that "This is a nice paper that distinguishes between roles of systemic S1P and locally produced S1P (in tumors) in lung colonization/metastasis". We are also pleased that the Reviewer thought that the experiments were performed well, and right controls were included to validate data. We have addressed the points raised by the Reviewer as follows:

1. The Reviewer pointed out that the possible mechanisms involved in Brms1 regulation by SK1/S1P should be discussed. We agree with the Reviewer, and although this point is not within the scope of this study, our data suggest that SK1/S1P inhibits Brms1 transcription via S1PR2 signaling by an as yet undefined mechanism. Recently, it was shown (Liu et al, Oncogene 31:1143-1154, 2012) that p65/RelA subunit of NFkB mediates the repression of Brms1 transcription via recruitment of DNMT-1 (DNA [cytosine 5]-methyltransferase-1) in response to tumor necrosis factor (TNF). It is also known that SK-1/S1P signaling is associated with NFkB activation (Billich et al, 17:1203-1217, 2005). Therefore, it is possible that SK1/S1P/S1PR2 signaling inhibits Brms1 expression through activation of NFkB, and that inhibition of SK1/S1P induces Brms1 via alterations of NFkB activation. This however needs to be further evaluated in future experiments. These points are now discussed in the revised text (p. 17, lines 6-14), and references are included also.

Moreover, it was asked whether Mdm2 and Mdm10, which were also upregulated in response to genetic loss of SK1, play any roles in the regulation of metastasis by systemic SK1/S1P. We agree that the alterations of these genes might also play a role, however, because both Mdm2 and Mmp10 are known to be positive regulators of metastasis (as reported by Shi et al, *Mol Cell Biochem*, 2011, and Liu et al, *Dis Esophagus*, 2011, respectively), and their expression was higher in SK1-/-lungs in which metastasis was decreased, we did not evaluate their involvement in our model.

1. As suggested by the Reviewer, description of partial effects (i.e. Fig. 1A) and over use of "novel" (i.e. Fig. 9 and in the overall text) are now corrected in the revised manuscript.
2. The Reviewer asked "how specific are S1PR2 and S1PR1 antagonists used. JTE-013 is a well known specific inhibitor of S1PR2 (reviewed by Pyne and Pyne, *Nature Reviews Cancer,* 2011). FTY720 is known to inhibit S1PR1 (as well as S1P3-5), but not S1PR2 (see reference above). In addition, our new data in which S1PR2 expression was knocked down using siRNAs (to address the point raised by the Reviewer #2, see point #4 below), which increased Brms1 mRNA, similar to the effects of JTE-013 (see Supplemental Fig. S4A-B), support the involvement of S1PR2 in this process. These data are now shown in Fig. 5C.

4-5. The acknowledgement and n values in the legends are now corrected/included, as suggested.

Reviewer 2:

We thank the Reviewer 2 for stating that "This manuscript convincingly demonstrates that metastasis is promoted by systemic, as opposed to tumor cell-derived S1P". We are also pleased that the Reviewer found this study novel that advances the understanding of metastasis. We have addressed the points raised by the Reviewer as follows:

1. In Fig. 3C, the number of metastatic tumors are now reported, as suggested.
2. Data and methodology regarding laser capture microdissection (LCM) for isolation of total RNA from tumor tissues shown in Fig. 4A are now described clearly, and confusing quality control RNA gel quantification data obtained during LCM are now removed, as suggested.
3. In Fig. 4B-C, a Western blot representing the data and quantification are now shown, as suggested.
4. As suggested by the Reviewer, we performed additional experiments, and effects of S1PR2 knockdown on Brms1 expression in MB49 cells are now shown in Fig. 5C. The efficacy of siRNA-mediated S1P2 knock down was also confirmed using Q-PCR, which is shown in Supplemental Fig. S4C.

5a. As suggested by the Reviewer, we performed additional experiments in which purified S1P was used, and its effects on Brms1 down-regulation was determined in MB49 cells. These data are now shown in Fig. 5D.

Moreover, in addition to using S1P-depleted media (obtained from SK1-/-MEF growth media), we performed additional experiments for determining the effects of the

depletion of S1P in control media (containing S1P) using Sphingomab, which induced Brms1 expression in WT-MEFs, but not in S1PR2-/-MEFs as suggested by the Reviewer. These data are now shown in Supplemental Fig. S4D .

5b. The Reviewer pointed out correctly that S1PR2 depletion by itself, even in the presence of medium that should not contain S1P, enhances Brms1 expression (in Fig. 5E). This is an important point, and it should be noted that conditioned media obtained from SK1-/-MEFs contains reduced S1P, but not completely depleted S1P (about 50% reduction, as shown in Fig. 1C) compared controls. To this end, genetic loss of S1PR2 induced Brms1 slightly more when S1PR2-/-cells were grown in serum with reduced S1P compared to control media, as expected. In fact, addition of exogenous S1P did not have any significant effect on Brms1 expression in S1PR2-/-MEFs, but it decreased Brms1 in WT MEFs. These data are now shown in Fig. 5D. Moreover, addition of Sphingomab increased Brms1 in WT cells grown in control media (as mentioned above under point 5a).

6. The Reviewer mentioned that Sphingomab-treated animals should have decreased metastatic colony formation (according to data shown in Fig. 3F), which was not very clear in Fig. 8B. This is an important point, and it should be noted that there is about 40% decrease ($p<0.005$) in tumor metastasis in response to Sphingomab compared to controls in these data, which are shown in Fig. 8B. We apologize that the significance of these data was not mentioned, which is now corrected. It should also be noted that the data presented in Fig. 3F were obtained after treatment with 7.5 mg/kg/day for 16 days of Sphingomab, whereas data shown in Fig. 8B were obtained using 20 mg/kg at every 3 days of the antibody for 22 days. We believe that slight differences of the effects of Sphingomab on the inhibition of metastasis in Fig. 3F and 8B (about 60% versus 40% decrease in metastasis, respectively) might be due to these dose/treatment schedule differences.

In summary, we thank the Reviewers and editorial team for their positive review and constructive comments. We are very excited about our novel data presented in this manuscript. We hope that the revised manuscript will meet the criteria for publication in the *EMBO Molecular Medicine*.

Authors would like to disclose that Dr. Roger Sabbadini is a founder of Lpath, and a member of its scientific advisory board.

Thank you for your consideration.

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2nd Editorial Decision 29 March 2012

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referee asked to re-assess it. As you will see the reviewer is now supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1/ The quality of the current figure images is a bit low (the text in the figures is rather blocky/blurry, as well as the bars of plots).
Please provide higher resolution versions, and check to make sure that text/line-art remains clear even when zooming in. You may find that saving the images as EPS or PDF will better preserve the text and line-art resolution. If this does not help, you may need to remake the figures in a quality vector graphics program like Illustrator or the free opensource, alternative Inkscape.
Please make sure that all text will remain readable even if the figure is reduced to fit the needs of the typeset article.

2/ Please provide a Table of content as the 1st page of your Supplementary Information

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #2 (Comments on Novelty/Model System):

The authors have completely addressed the previous critiques.