Author’s response to reviews

Title: Sema3A Drastically Suppresses Tumor Growth in Oral Cancer Xenograft Model of Mice

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Author’s response to reviews:

Dear chief editor,

Thank you for giving us an opportunity to revise this manuscript. We sincerely appreciate the critiques, comments and suggestions of the reviewers.

In response to the reviewers’ comments, we have revised the manuscript extensively.

The places revised were marked with underline. We believe that we have addressed all the issues raised by the reviewers and that the revised manuscript is significantly improved. Below please find our “point-by-point” reply to the reviewers’ critiques. We appreciate your re-evaluation of the revised manuscript for publication in the BMC Pharmacology and Toxicology.

Sincerely,

Jian-Hua Huang, M.D., Ph.D
Response to Reviewer 1:

Major issues

* In the Methods section, the vector system used for lentiviral production should be stated, i.e. what are the gag/pol and env encoding vectors? Second or third generation system?

Response: We added the description of the vector system used for the lentiviral production in the methods section at page 5, line 10-18.

* The resolution of some plots and images in the Figures are very poor. Higher resolution images should be used. Also, Fig. 3B appears out of focus.

Response: We improved the resolution of plots and images in the figures. We replaced the pictures unfocused in Fig. 3B with the pictures in focus (please see Fig. 3E).

* Statistical Student t-tests should be performed to confirm significance of pairwise comparisons of vector control versus SEMA3A expressing cells.

Response: We did Student t-tests to confirm significance of pairwise comparisons of vector control versus Sema3A expressing cells (please see page 10, line 3-7).

* It is not clear whether the tumor suppressive effects of SEMA3A are cell autonomous or non-cell autonomous or a combination. SEMA3A blocks VEGFR2 activity in tumor cells, so could have an anti-proliferative effect in addition to the effects on endothelial cells. These alternatives should be discussed in more detail.

Response: We added some discussion about the effects of Sema3A on tumor cells which indicated that Sema3A have an anti-proliferative effect of tumor in addition to the effects on endothelial cells (please see page 15, line 5-11).

Response to Reviewer 2:

Major issues:

* Fig 3A: In the tube formation experiments and also in the methods is not described whether the HUVECs have been previously starved (only that the cells were in GF reduced
matrigel). Starvation should be clearly mentioned, so that is clear whether the basal angiogenesis levels are due to autocrine effect or due to FBS growth factors.

Response: the HUVECs have been previously starved in our experiments. We are sorry no to clearly describe it in the manuscript last time. This time we clearly described it in the manuscript. Please See page 8, line 7.

* In Fig 3A it is clear that Sema3A blocks angiogenesis. However, should starvation occur it would be better to add VEGF as a stimulating growth factor, thus two more groups in the experiment: VEGF and VEGF + Sema3A combination. This would verify the specific role of Sema3A on VEGF signaling.

Response: According to the reviewer’s suggestion, we added 2 more groups in the experiment: VEGF and VEGF+Sema3A combination. Please see page 8, line 4-7; Fig3C and 3D.

* According to current knowledge tumor angiogenesis is stimulated from cancer cell-derived growth factors acting to the adjacent endothelial cells. On this regard, an interesting complementary experiment would be to treat starved HUVEC in the matrigel assay with starvation medium from SSC-9 with and without Sema3A overexpression.

Response: According to the reviewer’s suggestion, we performed the matrigel assay with starvation medium from SSC-9 with and without Sema3A overexpression. Please see page 8, line 5-9; Fig3A and 3B.

* At the CAM angiogenesis assay in Fig 3B: Measurements of the control and the Sema3A group should be included. The same goes for the picture of the Sema3A alone. This would provide information whether Sema3A just blocks VEGF-induced angiogenesis in this model or angiogenesis blockade goes below the control levels.

Response: According to the reviewer’s suggestion, we measured angiogenesis of the control group and Sema3A group in CAM assay. Please see page 8, line16-17; Fig3E and 3F.

* In Fig.5 the authors discuss the "interaction between Sema3A and VEGFR2", however there are no experiments showing interaction (i.e. immunoprecipitation, colocalization) included in the manuscript. Such experiments and data should be included to discuss a possible interaction between Sema3A and VEGFR2. Therefore I believe the authors should change
the description from "interaction…" to "effect of Sema3A on VEGFR2 phosphorylation levels". Alternatively, they can show interaction data.

Response: According to the reviewer’s suggestion, we change the description from "interaction…" to "effect of Sema3A on VEGFR2 phosphorylation levels". Please see page 12, line 12.

Minor issues:

* Fig.5: The authors show phosphorylation levels of VEGFR2, Src and FAK without or with Sema3A overexpression. However, it is not clear how Sema3A overexpression leads to this phosphorylation of the VEGFR2 signaling pathway. A nice complementary approach would be to include VEGF stimulation in the SSC-9 cell line and show increase of phosphorylation levels on the VEGFR2 pathway with/without Sema3A overexpression.

Response: According to the reviewer’s suggestion, we did the VEGF stimulation in the SSC-9 cell line with/without Sema3A overexpression. We found that Sema3A inhibited the phosphorylation of VEGFR2 of SSC-9 cells with VEGF stimulation. Please see Fig 5A.

* On Fig 2A should be mentioned on the figure which of the western blots shows Sema3A and which actin.

Response: We marked the Sema3A and Actin in original Fig 2A. Please see Fig 1A

* Fig.2A has to be before Fig.1, either in the same figure or separate. Alternatively, Fig.1 could go as a supplement.

Response: we did it according to the reviewer’s suggestion. please see Fig 1A.

* Page 12, line 15: there is no Fig 4G. Instead the authors mean Fig. 4E I guess.

Response: We corrected it with Fig.4E. Please see page 12, line8.

* SSC-9 is a tongue cancer cell line. Although Xenograft models are still used, it is important to mention in the manuscript the importance of the orthotopic model which has been
developed and used. In this model, cells are inoculated in the tongue. We have seen different outcomes of the same cell lines in the Xenograft and orthotopic models.

Response: We added some discussion on the limitation of the Xenograft models of this experiment, compared with the orthotopic model. Please see page 15, line 12-16.

* Title has to be corrected: It is a "Mouse Oral Cancer Model"

Response: We changed the title as: “Oral Cancer Xenograft Model of Mice”, Please see the title at page 1.

Response to Reviewer 3:

In their manuscript authors investigate the effects of lentivirus-mediated Sema3A overexpression in the human tongue cell carcinoma cell line SCC9. As conceivable, based on a wide body of literature (for review see Neufeld et al., Cold Spring Harb. Perspect. Med., 2012, 2:a006718 doi: 10.1101/cshperspect.a006718; Worzfeld & Offermans, Nat. Rev. Cancer, 2014, 13:603-621) authors observed that Sema3A inhibits: i) endothelial tube formation on matrigel; ii) in vivo angiogenesis in the chicken chorioallantoic membrane and in subcutaneous SCC9 xenograft in mice; iii) growth of SCC9 xenografts in mice; iv) VEGF signaling. This is a largely confirmatory manuscript that does not add any new knowledge to the field of semaphorins in angiogenesis and cancer.

Response: As reviewer mentioned, there are a wide body of literature

Showed that the Sema3A inhibits the angiogenesis of tumor, however, there are few reports that studied the relationships between Sema3A and oral cancer, especially Sema3A and angiogenesis in oral cancer.

The general quality of data is poor; for example the quality of pictures documenting in Figure 3 the results of in vitro tube formation and chicken chorioallantoic membrane assays are out of focus and extremely difficult to evaluate.

Response: This time we improved the quality of data.