Response to Reviewer 1 Comments

Comments and Suggestions for Authors

In manuscript "GDNF promotes glioblastoma cell migration and invasion via SMAD2/3-SERPINE1 signaling axis", the authors explored the promotion effect of GNDF on GBM progression, especially migration and invasion and uncovered the mechanism. They found that GDNF enhances GBM cell invasion and migration through SMAD2/3-SERPRINE1 pathway. This study is interesting and significant. However, it can be further improved with the following comments:

1. Summary

Thank you very much for your valuable comments concerning our manuscript entitled "GDNF promotes glioblastoma cell migration and invasion via SMAD2/3-SERPINE1 signaling axis" (ID: ijms-3169672). These comments help us to improve our manuscript, and provide significant guidance for our study. We have carefully studied these comments and made corrections. Revised portion are marked in red in the manuscript. The main corrections in the manuscript and responses to the comments are as followed.

2. Questions for General Evaluation	**Reviewer's Evaluation**	**Response and Revisions**
Does the introduction provide sufficient background and include all relevant references?	Yes	
Is the research design appropriate?	Can be improved	Agree. We have revised it.
Are the methods adequately described?	Yes	
Are the results clearly presented?	Yes	
Are the conclusions supported by the results?	Can be improved	Agree. We have revised it.

3. Point-by-point response to Comments and Suggestions for Authors

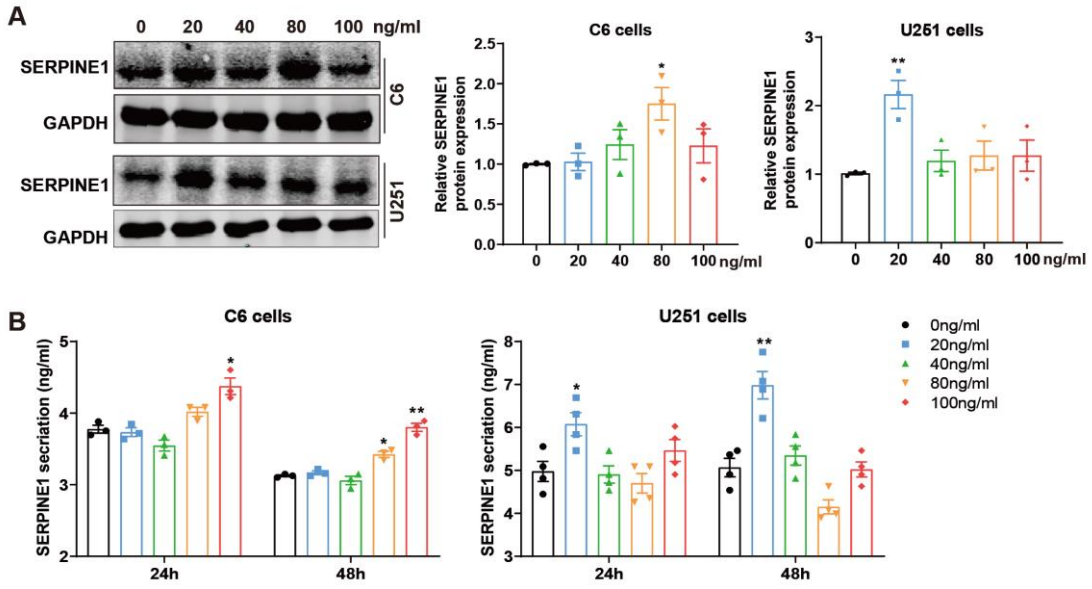
Comments 1: In figure 1E, are those normal tissue and GBM tissues paired samples which are from the same person?

Response 1: Thank you very much for your comments. In figure 1E, normal brain (NB) tissues and GBM tissues are not paired samples from the same person. Sorry. It is an oversight in our manuscript. We have clearly stated it in the section of "Materials and methods-4.1" (page 9, line 282-285). The details are as followed.

-GBM and NB tissues were acquired from Affiliated Hospital of Xuzhou Medical University. Three GBM tissues were collected from the surgical specimen archives, and three NB tissues were obtained from the patients who suffered acute brain damage and underwent intracranial decompression.

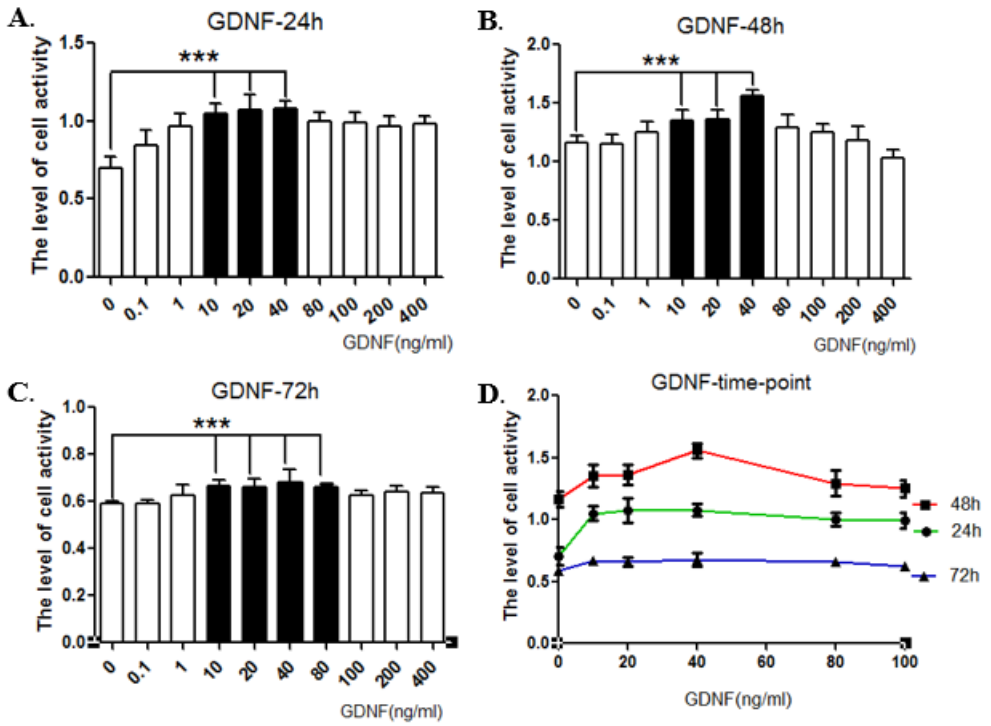
Comments 2: For the western blot data, the GAPDH bands are over exposed. Please post a short time exposure one.

Response 2: Thanks for your valuable suggestions. The GAPDH band has been changed to a short time exposure one. Therefore, a new Figure 2 were provided. (page 4, line 118)



Comments 3: In figure 2, for U251 cells, it doesn't show a good dose response. 5 and 10 ng/ml treatment may be needed.

Response 3: Thanks for your valuable comments. We agree with your perspective. The data will be more elaborate if more GDNF concentrations were used. In the present study, the concentrations of GDNF were selected based on our published article (PMID: 29088765, title: Neuropilin-1 is a glial cell line-derived neurotrophic factor receptor in glioblastoma). This paper has been added to the reference in the revised manuscript (page 3, line 109). In this paper, C6 cells were treated with various concentrations of GDNF (0, 0.1, 1, 10, 20, 40, 80, 100, 200 and 400 ng/ml) and grown for 24, 48 or 72h. The detailed results are shown in the figure below. To determine the most optimal concentration of GDNF to promote SERPINE1 expression in the present work, the protein and secretion levels of SERPINE1 were separately examined by western blotting and ELISA, after cells were treated with various GDNF concentrations (0, 20, 40, 80, 100 ng/ml) for 48 h.



Comments 4: In figure 2, for C6 cells, the western blot data don't match with ELISA data. 80 ng/ml GDNF induced the highest response in western blot data, while 100 ng/ml for ELISA data.

Response 4: Thank you for your valuable comments. We also noticed this phenomenon. The possible reason is that SERPINE1 expressed inside the cells and secreted outside the cells peak at different time. In this study, the results of western blotting and ELISA were combined to determine the most optimal GDNF concentration.

Comments 5: In figure 3 and 4, cell migration protein markers should be characterized.

Response 5: Thanks for your good suggestions. We agree with you. Detection of proteins associated with cell migration and invasion is a good complement to the results of *in vitro* functional experiments. *In vivo* experiments, we used IHC staining to detect the expression of migration protein markers such GFAP, MMP2 and MMP9. Therefore, this study indicated that GDNF promoted GBM cell migration and invasion by SERPINE1 according to the results of in vitro and in vivo experiments. Additionally, this suggestion is valuable for our future researches. At present, there are two ongoing studies on GDNF promoting malignant progression of GBM. We will add the detection of protein markers to enrich our results.

Comments 6: In figure 5, the authors tested the acceleration of GDNF on GBM growth using a xenograft mouse model. The invasion model could be better, given that this paper focused on migration and invasion.

Response 6: Thanks for your good proposal. We agree with you. In the present study, a mouse subcutaneous xenograft glioma model was established to assess the effect of GDNF on GBM growth. This model clearly determined the influence of GDNF and

SERPINE1 on the volume and weight of GBM tumors (PMID: 33278485, title: Enhancer II-targeted dsRNA decreases GDNF expression via histone H3K9 trimethylation to inhibit glioblastoma progression). To uncover the roles of GDNF in GBM cell migration and invasion using this model, IHC assay was performed to detect the expression of migration protein markers such GFAP, MMP2 and MMP9. Indeed, the invasion model is better for studying cell migration and invasion. We have pointed out this deficiency in the "Discussion" section (page 8, line 232-236). In our following researches, we will utilize more animal models to explore the effects of GDNF on GBM progression. In fact, our another manuscript entitled "Hypoxia-driven GDNF-HIF-1α loop reinforces glycolysis in glioblastoma" will use the orthotopic GBM model to identify the roles of GDNF (unpublished).

The newly added discussion is as followed.

-To verify the results of in vitro experiments, mouse subcutaneous xenograft glioma model was established to assess the effects of GDNF and SERPINE1 on GBM growth in this study. However, the orthotopic GBM models could assess tumor behaviors more accurately. In the future researches, we will utilize more animal models to explore the effects of GDNF on GBM progression.

4. Response to Comments on the Quality of English Language

Point 1: It can be improved.

Response 1: Agree. Although we have tried our best to improve the English Language, this manuscript still requires improvement to the language. We accept MDPI Author Services.

Once again, special thanks to you for your valuable comments.