Author's response to reviews

Title: Plexin-B1 silencing inhibits ovarian cancer cell migration and invasion

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Version: 3 Date: 27 July 2010

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Dear Sir or Madam:

We sincerely appreciate the editor and reviewer’s excellent suggestions. The concerns of the editor and reviewers and their suggestions for strengthening the manuscript have been addressed. Based on the comments, we have performed additional analyses and extensive revisions by supplementing new data necessary for providing compelling evidence in support of our conclusion. Below, we provide a point-by-point response to the comments (marked with tracked changes in the revised manuscript).
Referee 1:
Reviewer's report

Major compulsory revisions:

1) The authors have examined tumor tissues obtained from surgical specimens by immunohistochemical methods, and cell lines by RT-PCR and Western blot. This dichotomy can lead to a difficult interpretation of the results, especially in borderline tumors which show a faint immunohistochemical staining (see Figure 1C). For these reasons, the authors should support immunohistochemistry by RT-PCR (using routine material) at least in some cases of borderline tumors and carcinomas, to demonstrate the presence of Plexin-B1 mRNA.

In the present study, the Plexin-B1 protein expression was determined with immunohistochemistry performed on formalin-fixed, paraffin-embedded sections. As the study was carried out retrospectively, it’s really hard to test Plexin-B1 expression at mRNA level; therefore, we chose cell lines instead. Valente et al. has validated positive expression of Plexin-B1 in ovarian carcinomas using Western blot (Cellular Oncology, 31, 6; 2009). We are now collecting the fresh borderline and malignant ovarian tumour specimens for RT-PCR, however, it’s a time-consuming work, and we will show the results in future.

2) The WHO classification of ovarian tumors should be used to define, in the Methods and in the Results, the different histological types of tumors included in the study. The terms “borderline carcinoma” and “malignant carcinomas” (for instance in page 9 and in footnote of the table 1) are uncorrect. The authors should precise which histological grading system has been formulated.

All the borderline and malignant tumours in this study were histological diagnosed as serous lesions. Histological type and grade of ovarian tumors were diagnosed according to the World Health Organisation (WHO) criteria [31]. Staging was based on TNM classification of the International Union Against Cancer (UICC) [32] (Page 6). The terms of “borderline carcinoma” and “malignant carcinomas” in the manuscript, footnote of table 1, and figure legends were replaced by “borderline tumour” and “malignant tumours” respectively.
3) The authors have carried out a semiquantitative evaluation of the positive neoplastic cells, but they did not give any information about staining intensity. They should reconsider each case combining the percentage of positive cells and staining intensity, using the well-standardized Hybrid-Score system. Plexin-B1 immunoreactivity was determined using the well-standardized H-Score system \( (H = I \times P) \) [33]. \( I \) is the staining intensity which was evaluated according to the following criteria: 0 (no staining), 1 (weak immunostaining), 2 (moderate immunostaining), 3 (strong immunostaining), and \( P \) is the percentage of positively stained cells in each sample. A sample with an H score of \( \geq 20 \) was considered Plexin-B1 positive according to a recently report [30] (Page 7).

Minor essential revisions
1) Figure 1-C: an inset at higher magnification could be useful to better make out the reaction product
An inset at higher magnification was added in Figure 1-C to show membrane staining clearly.
2) Background page 4: ?this is the first report examining Plexin B-1 in ovarian carcinomas?: this sentence should be deleted, since at least one paper has been meanwhile published on this topic (Valente et al, Cell. Oncol. 31: 423-436, 2009).
As the reviewer’s suggestion, the sentence “this is the first report examining Plexin B-1 in ovarian carcinomas” has been deleted.
3) Several spelling mistakes are distributed in the text.
We have revised the spelling mistakes.

Referee 2:
Reviewer's report
This manuscript by Ye and coworkers addresses the functional role of Plexin-B1 (the receptor of Semaphorin 4D) in ovarian cancer cells. It is reported that Plexin-B1 is overexpressed in ovarian cancers with respect to normal ovary. Moreover, Plexin-B1 silencing experiments (by siRNA transfection) demonstrate that this receptor is required to mediate the migration and invasiveness of immortalized ovarian cancer
cells.
The function of Plexin-B1 in cancer cells appears to be controversial and these data contribute to the discussion bringing evidence to the hypothesis that this receptor may act as a promoter of tumor progression. On the other hand, the present work should be developed and improved with further experiments to provide compelling evidence in support of the authors?

Major Compulsory Revisions:

1) In certain experimental models, it has been shown that PlexinB1 stimulation by Sema4D leads to increased cell migration and invasive growth, while in other cellular contexts the opposite seems to be true (e.g. Giordano et al., 2002; Barberis et al, 2004; Basile et al., 2005; Swiercz et al., 2008). To further elucidate this important issue, the authors should test the functional activity of Sema4D in multiple lines of ovarian carcinoma cells, relative to the expression levels of PlexinB1 receptor, and also upon receptor knock-down.

We do appreciate the reviewer’s constructive comment and agree that additional experiments are required to provide compelling evidence for our conclusion. We have detected the expression of Sema4D, in both membrane-bound and secreted forms, in multiple lines of ovarian carcinoma cells. The data indicated that several cell lines including SKOV3 cells showed high expression of Sema4D as well as Plexin-B1. Furthermore, we have produced Sema4D fusion protein in vitro and further investigation is under way.

2) In order to confirm the role of PlexinB1 in cancer cell invasiveness, the authors should establish high expression of this receptor in ovarian cancer cells carrying low endogenous levels and test the functional outcome.

A recombinant plasmid vector encoding the gene Plexin-B1 is too long for us to construct technically that we have to ask for help from some researchers who had successfully constructed Plexin-B1. When we got Plexin-B1 cDNA, we will establish high expression of this receptor in OV2008 cells which have almost no endogenous expression of plexin-B1 and test the functional outcome. As this work is time-consuming, we are afraid that the data will be published in the future.
3) In order to elucidate the loss of function consequent to Plexin-B1 knock-down, cell viability/proliferation should be evaluated before cell migration/invasion.

We had carried out an experiment to evaluate viability/proliferation of SKOV3 cells after Plexin-B1 knock-down, which was described in the sections of abstract (Page 2-3), methods (Page 10), results (Page 14), discussion (Page 17), and figure legends. Viability/proliferation of SKOV3 cells were not affected by Plexin-B1 knock-down, as showed in Figure 3C.

4) It is not sufficient to show the effect of only one PlexinB1 siRNA transfected in ovarian cancer cells. Data should be shown concerning at least another siRNA (or shRNA) sequence, to establish specificity and rule out off-target effects.

We added cellular effect of Plexin-B1 knock-down by Plexin-B1 siRNA2 in Figure 3 and Figure 4 to confirm the specificity and efficiency.

5) Cytoskeletal changes observed upon sustained Plexin-B1 knock-down (Fig. 4) are explained here by implying that this signalling cascade normally elicits Rho activation in ovarian cancer cells. However, several other potential explanations can be put forward. Moreover, Rho regulation downstream to Plexin-B1 is a transient event and a rather controversial issue (e.g. see Barberis et al., 2005 and Swiercz et al., 2008). Thus, these data could be relevant if accompanied by a biochemical analysis of Rho activation in these cells (either upon Sema4D stimulation, or PlexinB1 knock-down).

We appreciate the reviewer’s comment of our insufficient explanations on the cytoskeletal changes observed upon sustained Plexin-B1 knock-down. We have added several other explanations in the discussion section (Page 18-19) as below:

On one hand, dimerization of Plexin-B1 is sufficient to induces a Rac-dependent activation of Rho and to regulate the remodeling of the actin cytoskeleton and alter cell movement in response to Sema 4D [22]. On the other hand, it has been proved that Plexin-B1 inactivates PI3K and dephosphorylates Akt and activates GSK-3β through R-Ras GAP activity. Activation of GSK-3β leads to inhibition of microtubule polymerization and stabilization. Furthermore, several other regulators of the actin cytoskeleton may be also implicated in Plexin-B1 induced reorganization of SKOV3
cells. For example, the MEK/ERK signalling pathway, which was reported to be constitutively activated in epithelial ovarian cancer cells [46], has been shown to restore stress fibers in transformed fibroblasts [47], and a recent study suggests that Plexin-B1 can utilize RhoA to stimulate ERK [48].

6) The last paragraph at the end of page 13 contains a wrong statement, since there is no evidence that Rho GTPases interact with the C-terminus of Plexin-B1 (only a PDZ-Rho-GEF).

We have deleted the sentence “On the one hand, Plexin-B1 inactivates R-Ras activity in response to Sema 4D, which requires the small GTPase Rnd1 interacting directly with the cytoplasmic domain of Plexin-B1”, and we elucidated the cytoskeletal changes observed upon Plexin-B1 knock-down with other explanations, please refer to the answer for question 5 above.

Minor Essential Revisions:

7) English should be revised since the text contains a number of mistakes. In addition, some of the references appear to be in the wrong format

We have thoroughly checked and corrected the errors in typos, including grammatical and spelling mistakes. The references were carefully revised as well.

Referee 3:

Reviewer's report

The authors of this manuscript have shown higher levels of plexinB1 protein expression in ovarian cancer tissue in comparison to normal ovarian tissue. The association between high levels of plexinB1 protein and ovarian carcinoma is statistically significant. PlexinB1 is also expressed in ovarian cancer cell lines. Knock down of plexinB1 by siRNA in one ovarian cancer cell line resulted in a reduction in Akt phosphorylation and a reduction in motility, invasive capacity and stress fibre formation.

This paper is interesting as it suggests that plexinB1 acts as an oncogene in ovarian cancer, as it may do in breast cancer and prostate cancer, in contrast to melanoma and
renal carcinoma where it appears to act as a tumour suppressor gene. The methods used are appropriate and well described and the data is sound.

Major Compulsory Revisions

1) Expression of plexinB1 in ovarian cancer has been reported previously by Valente et al. (Cellular Oncology, 31, 6; 2009) in a smaller series. PlexinB1 expression was found 9/15 ovarian serous carcinomas and 6/15 ovarian samples expressed both met and plexinB1 which was predictive for an unfavourable outcome. This should be discussed, and the statement “this is the first report examining plexinB1 in ovarian carcinomas?” (page 4) changed.

We added discussions on the data reported by Valente et al. as below:

Valente et al. [30] recently assessed the potential value of Plexin-B1 expression, either alone or in conjunction with Met, as a marker of tumour progression by analyzing their expression in a series of 50 malignant epithelial tumours (35 breast and 15 ovary). It showed that Plexin-B1 expression was found in 7/11 cases of ovarian serous adenocarcinomas and 6/15 ovarian adenocarcinomas expressed both Met and Plexin-B1, which was predictive for an unfavourable outcome. In the present study, immunohistochemical analysis was used to investigate Plexin-B1 expression in the ovarian serous tumours in a larger series. Consistent with data reported previously by Valente et al., our results revealed that elevated expression of Plexin-B1 was associated with increasing tumour malignancy in epithelial cells from 15.00% in normal and benign ovaries to 55.00% in malignant ovarian tumours (P<0.001), and 35.00% in borderline tumours, suggesting that Plexin-B1 might be an early factor in ovarian serous tumour development (Page 16). The sentence “this is the first report examining Plexin-B1 in ovarian carcinomas” was deleted.

2) The effect of knocking down plexinB1 is only shown for one ovarian cancer cell line. Ideally, to show whether or not this is a general phenomenon for ovarian cancer cell lines, the same experiments should also be done on other ovarian cell lines. It may be that the opposite effect is found, as in certain breast cancer cell lines (Swiercz et al 2008).

Work on investigating whether this oncogenic function of Plexin-B1 in SKOV3 cells
is a cell type–specific effect or universal in multiple ovarian cancer cell lines is ongoing. Data will be published in future.

3) A reduction in Akt phosphorylation is seen upon knockdown of plexinB1 and correlates with the remaining plexinB1 expression levels, suggesting that activated plexinB1 contributes to Akt phosphorylation in this cell line. Is the activation of plexinB1 in these experiments the result of: 1. sema4D expression in SKOV3 cells, acting via an autocrine/paracrine mechanism? and/or 2. does activation of plexinB1 result from overexpression and consequent clustering of the receptor? Ideally phosphorylation of plexinB1 in response to sema4D should be shown in this cell line? this may not be possible however if Akt is constitutively activated. These points should be discussed.

We discussed the points in the last paragraph of the discussion section (Page 19) as following:

However, in this report, it’s still uncertain that the decreased activation of AKT and the corresponding cellular reactions induced by Plexin-B1 inhibition is the result of Sema 4D in SKOV3 cells, acting via an autocrine/paracrine mechanism, or cell lines overexpression and consequent clustering of Plexin-B1 in SKOV3 cells leads to phosphorylation of its cytoplasmic region. We have produced Sema 4D fusion protein in vitro and further investigation is under way

4) Data should be shown for the effect of knockdown of plexinB1 with at least one of the other siRNAs used on migration and invasion

We added cellular effect elicited by Plexin-B1 knock-down with Plexin-B1 siRNA2 in Figure3 and Figure4 to confirm the specificity and efficiency.

Minor Essential Revisions

1) The authors state that the protein is translocated from the cell membrane to the cytoplasm in carcinoma cells. The term translocation implies that the protein is actively transported from the membrane to the cytoplasm. No evidence has been shown for this, therefore it would be more accurate to simply state that the protein is expressed in the membrane and cytoplasm in carcinoma cells.
We replaced the sentence “its immuno-staining trans-located from cell membrane to cytoplasm” with “its immunostaining was positive in the membrane and cytoplasm of the tumour cells” in the revised manuscript (Page 3).

2) Which santa cruz plexinB1 antibody was used?
The antibody of Plexin-B1 was commercially obtained from Santa Cruz Biotechnology, sc-28372 (Page 7).

3) There are a few errors in the references:
reference 31 should be referred to as Swiercz et al. rather than Jakub et al. In addition, the wrong reference is cited: Swiercz et al 2008 (concerning plexinB1 signalling via ErbB2 and Met) is reference 31 and should be cited instead of reference 44 in the discussion. Reference 44 is Korosteylev et al, (not Jakub/Swiercz et al.) (page 13). The reference cited at the beginning of the discussion should be a review of plexin action, rather than reference 33.
We have carefully checked the origins of references one by one, and revised the errors.

4) In the discussion (To date, it has been proved that???????) As far as I am aware, PlexinB1 has not so far been shown to bind cdc42 or directly with RhoA. Also, the reference cited for this (2) is wrong.
We deleted the sentence “Plexin-B1 interacts with Rho family-GTPase, including Cdc42, Rac1, and RhoA, by its C-terminus PDZ-domain–binding motif, resulting in migratory and proangiogenic response [2]”, and we provided several other explanations to elucidate cytoskeletal changes observed upon Plexin-B1 knock-down in the discussion section (Page 18-19).

5) Spelling ? phosphorylation page 11
Grammar ? some English grammar needs changing, eg. ?plexinB1 has been reported to implicate in the process?.(page 11)
“phosphostation” has been corrected as “phosphorylation”(Page 14). The sentence “Plexin-B1 has been reported to implicate in the process of cell mobility and cancer
metastasis” has been revised as “it has been reported that Plexin-B1 implicates in the process of cell mobility and cancer metastasis” (Page 14).

Referee 4:
Reviewer's report

I think this is a very interesting paper that shows some remarkable data and it can be published but there are some aspects than must be revised before it.

Major Compulsory Revisions:

1. In results section and in general in all the parts of the manuscript I think there is a problem with the terms the authors use that can be confusing. The term malignant carcinoma is not adequate because it is redundant. All the carcinomas by definition are epithelial malignant neoplasms. Probably, they would be better to use the term ovarian carcinomas when they discuss about the epithelial malignant cases.

   We have corrected “malignant carcinoma” as “malignant tumour” or “ovarian carcinoma” following the reviewer’s suggestion.

2. The figure 1B shows only blood channels and mesenchimal tissue and it seems like the ovarium hilia was the source of the photograph instead of a benign ovarian lesion as it is stated in the figure legend. Otherwise, in figure 1A a proliferation of epithelial tissue is shown. Is it possible that a mistake could occur between the two photographs?

   This is an aspect the authors must clarify before publishing. Moreover, the 1C figure is not clear because the quality of staining is poor. It would be better a higher magnification picture where the membrane staining was clearly demonstrated in the borderline tumor.

   We deeply respect your rigorous scientific attitude. In the revised paper, we provided pictures of typical pathologic morphology which had been confirmed by two professional pathologists in Tongji hospital. An insert was added to show the dashed line region at a higher magnification to demonstrate the membrane reaction product clearly in figure1C.

3. On the other hand the authors do not specify the benign conditions that they have
studied. It is of interest because they are discussing about epithelial neoplasms and it is not the same to evaluate an ovarian fibroma or a thecoma than a cystadenoma.

The benign ovaries were histological diagnosed as serous cystadenoma (Page 6). Work on investigating differences of Plexin-B1 expression in different histological types of tumours is under way.

4. Otherwise the authors do not mention the percentage of mucinous and serous carcinomas that are included in the series. It could be of interest to analyse the possible differences between the two histological types.

The ovarian samples, except for the normal ovary, were all histological diagnosed as serous lesions. We are about to investigate the possible differences following the reviewer’s good suggestion.

Minor Essential Revisions

1. In results, the authors have found that: Plexin-B1 expression level of SKOV3 cells was the highest, while negative expression was found in OV2008 cells and they present the figure 1 E and 1F where RT-PCR and western blot results can be analysed. In western blot, the cell line OV2008 seems to have some kind of protein expression although RT-PCR seems like negative.

We revised the sentence “while negative expression was found in OV2008 cells” in the results section as “while a faint expression was found in OV2008 cells” after carefully checking the figure 1F.

2. Typographical mistakes: Page 5: Immunohistochemistry: Line 4: "peroxidase". Also: "Furthermore". In figure 2B, lane 4: ?Plexin-B1 ?siRNA2? "peroxidise" was revised as “peroxidase”(Page 6). “Further more” was revised as "Furthermore" (Page 7). In figure 2B, lane 4 “Plxin-B1 siRNA2” was changed as “Plexin-B1 siRNA2”

3. In results section, the sentence: ?Although Plexin-B1 immunoreactivity evaluated gradually from stage I (45%) to stage IV (70%), there were no significant differences? must be changed in order to clarify its meaning.
The sentence “Although Plexin-B1 immunoreactivity evaluated gradually from stage I (45%) to stage IV (70%), there were no significant differences” was revised as “Although Plexin-B1 immunoreactivity increased gradually from stage I (45%) to stage IV (70%), there were no statistical differences” (Page 13).

EDITORIAL REQUESTS:
Authors' contributions - Please include an Authors' contributions section before the Acknowledgements and Reference list.

For the Authors' contributions we suggest the following kind of format (please use initials to refer to each author's contribution): AB carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. JY carried out the immunoassays. MT participated in the sequence alignment. ES participated in the design of the study and performed the statistical analysis. FG conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

An "author" is generally considered to be someone who has made substantive intellectual contributions to a published study. To qualify as an author one should 1) have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) have been involved in drafting the manuscript or revising it critically for important intellectual content; and 3) have given final approval of the version to be published. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Acquisition of funding, collection of data, or general supervision of the research group, alone, does not justify authorship.

All contributors who do not meet the criteria for authorship should be listed in an acknowledgments section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chair who provided only general support.

Authors' contributions have been added before the Acknowledgements and Reference list in the revised manuscript (page 20-21).