Author's response to reviews

Title: Upregulation of Wnt5a promotes epithelial-to-mesenchymal transition and metastasis of pancreatic cancer cells

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Author's response to reviews: see over
Responses to the comments on our manuscript (MS: 1734429067959864)

We thank reviewers for critical review of our manuscript, and highly appreciate their comments and suggestions that greatly contributed to improving the quality of the manuscript. Our point-by-point responses to the reviewers’ comments are provided below. The major changes to the manuscript have been highlighted in red.

Referee 1:

Discretionary Revisions:

I would suggest the Authors evaluate the significant prognostic variable of Wnt5a in multivariate analyses, using Cox’s proportional hazards model to estimate the magnitude and the direction of the effect.

Response: The Kaplan-Meier analysis revealed that the patients with Wnt5a-positive tumors had a slightly higher median cancer-specific survival than those with negative Wnt5a expression (8.7 vs. 6.8 months); however, the difference was not statistically significant ($P > 0.05$). The present data suggest that tumoral expression of Wnt5a may have a limited prognostic value in pancreatic cancer. Therefore, we did not perform Cox multivariate analysis to determine whether Wnt5a was significant in independently predicting duration of survival.
Other revisions in the text include:

Material and methods:

- Remove one of the “according to” from “according to according to the World Health Organization classification”

Response: Done.

- Change “.” with “,” in the following sentence “PANC-1 and BXPC-3 were obtained from Institute of Cellular Research, Chinese Academy of Science, Shanghai, China.”

And cultured”

Response: Done.

- please provide the name of company, city and county of the following material RPMI-1640, FBS, penicillin/streptomycin, Lysis-buffer in Western blot, CIAP, polyvinylidene difluoride membranes. …and also explain the incubation conditions of your cell lines.

Response: Done.

Discussion:

- please provide at least one reference for the following sentence: “Induction of EMT has been associated with increased cancer metastasis and aggressive clinical behaviors”

Response: Done.
Figures:

Figure 3: please quantify the wound healing area in all the conditions in order to assess the percentages of cellular migration of the PANC-1 and PXPC-3 into the wound area. Moreover, I would also suggest the Authors to change the color of the wound healing area.

**Response:** As you suggested, cell migration was quantified and the results are added in Fig. 3. The figure has been improved and changed to white and black.

Figure 4: bar graphs in the bottom panel are missing from figures 4!, please provide this information as a quantification of the invaded cells into the lower surface of the filter.

**Response:** Thank you very much for bringing this point to our attention. It has been added.

Figure 5: I would suggest the Authors to show the representative images of H&E and immune staining of the mice injected with empty vector-transfected cells as control.

**Response:** As you suggested, representative images of H&E and immune staining from mock mice are shown in Figures 5 and 6.
Referee 2:

The article “Up-regulation of Wnt5a promotes epithelial-to-mesenchymal transition and metastasis of pancreatic cancer cells” by Haiji Bo et al. deals with the investigation of the role of Wnt5a in EMT regulation. The topic is interesting, but not particularly original, innovative or completely well performed. Although in dissimilar forms, a number of aspects have already been suggested by literature (see for example, Ripka S et al., Carcinogenesis 2007; Kikuchi A et al., Acta Physiol 2012; Yingzi Yang, Cell & Bioscience 2012). Moreover, despite the importance of exploring new pathways and signals driving EMT, the paper describes only a partial event connected to this phenomenon, not deeply reporting (or at least suggesting) the complexity of the regulatory networks that control Wnt5a and therefore EMT. There are a number of genes and networks that control EMT. What would be really interesting is to know those that are most important and why. This paper describes only one of the possibilities and therefore its importance remains severely limited.

Response: As described in the references you mentioned above, Wnt5a is implicated in cancer development and progression. Ripka et al (Carcinogenesis 2007;28:1178-87) reported that knockdown of Wnt5a reduces migration and invasion in a panel of pancreatic cancer cells, whereas overexpression of Wnt5a promotes pancreatic cancer cell migration and invasion. Our present data validate and extend the in vitro studies, and reveal that enforced Wnt5a expression facilitates cancer invasion and metastasis in an orthotopic mouse model of pancreatic cancer. It has been widely accepted that
induction of EMT contributes to aggressive tumor phenotypes. Interestingly, we found that Wnt5a overexpression leads to an EMT of pancreatic cancer cells, which involves activation of β-catenin signaling. Silencing of β-catenin blocked Wnt5a-induced EMT and invasion in pancreatic cancer cells. Taken together, our data confirm the favorable effects of Wnt5a on pancreatic cancer progression and shed light on the involvement of EMT in Wnt5a-mediated aggressiveness.

Additionally, to the best of knowledge, it is the first work to explore the prognostic significance of Wnt5a in pancreatic cancer. Our data indicate that the median cancer-specific survival was comparable between patients with positive versus negative expression of Wnt5a. This result suggests that tumoral Wnt5a expression alone may not be suitable for predicting survival in patients with operable pancreatic cancer.

In conclusion, we provide evidence for the metastasis-promoting role of Wnt5a in pancreatic cancer through activation of the β-catenin signaling and induction of EMT. Therefore, this study has significant biological relevance.

Major Compulsory Revisions

- Authors did not show any indication of one essential aspects of EMT, i.e. cell morphological changes; they have to demonstrate this aspect;

Response: As you suggested, morphological changes of pancreatic cancer cells
transfected with Wnt5a-expressing plasmid were examined. The results demonstrate that

• they are not clear in describing how they reach their aims; they should be more clear and concise;

Response: In this study, we sought to determine the clinical and biological significance of Wnt5a in pancreatic cancer. Immunohistochemistry demonstrated that the percentage of Wnt5a positive expression showed a bell-shaped pattern in pancreatic cancer tissues, peaking in well-differentiated carcinomas. The Kaplan-Meier analysis showed that the median cancer-specific survival was comparable between patients with positive versus negative expression of Wnt5a. Functional studies revealed the favorable role of Wnt5a in pancreatic cancer invasiveness and metastasis, which was associated with induction of EMT. Targeted reduction of β-catenin antagonized exogenous Wnt5a-induced EMT and invasiveness in pancreatic cancer cells, suggesting an involvement of the β-catenin signaling in Wnt5a-mediated tumor phenotype. Taken together, our data provide evidence for the metastasis-promoting role of Wnt5a in pancreatic cancer through activation of the β-catenin signaling and induction of EMT. The Discussion section has been revised to provide a more accurate interpretation of the results.

• the clinical significance is not clearly suggested by results (see later)

More specifically:
1 – (Paragraph “Wnt5a expression negatively correlates with histological grade of tumors”) Patients included in the different tumor grade classes are not balanced; although the result is statistically significant, authors cannot be so sure their results are absolutely true and therefore they have to be less categorical in stating their assumptions. Second, from what I can understand, authors assert Wnt5a has a negative role: it promotes EMT and therefore metastatic spread. But at the same time they observed patients with Wnt5a-positive tumors had a slightly higher median cancer-specific survival than those with negative Wnt5a expression although not statistically significant. How they explain this? They should make some suggestions.

Response: In terms of the relationship between Wnt5a and tumor grade, the statement has been changed to “Wnt5a expression tended to be negatively associated with tumor histological grade ($P < 0.001$)”. In terms of the inconsistency between the biological and clinical findings, it may be explained by that Wnt5a exerts its metastasis-promoting effects largely through activation of the β-catenin signaling, which can be regulated by multiple factors other than Wnt5a in vivo. In the absence of Wnt5a, other activators such as ATDC [ref. 27] and MUC1 [ref. 29] may be responsible for triggering the β-catenin signaling and, in turn, promote the Wnt5a-independent growth and metastasis of pancreatic cancer. Accordingly, tumoral Wnt5 expression is not regarded as a determining factor for the duration of survival of patients with pancreatic cancer. The above comments have been added to the 1st paragraph, page 19.
2 – In vitro results seem to diverge strongly from in vivo results: from the presented data, in pancreatic CELLS in vitro, Wnt5a seems to be a master regulator of EMT, BUT from in vivo results presented by authors it seems not particularly important (it is expressed almost in every pancreatic cancer and shows no influence on survival). In my opinion, the paper needs a complete ‘overhaul’ before being considered for publication in BMC Cancer.

**Response:** Mounting evidence indicates a critical role for the β-catenin signaling in the pathogenesis of pancreatic cancer. Our in vitro data demonstrated that targeting β-catenin antagonized Wnt5a-induced EMT and invasiveness, indicating an essential role for the β-catenin signaling in Wnt5a-mediated tumor aggressiveness. Although the in vitro evidence indicated a master role of Wnt5a in inducing aggressive tumor phenotypes, Wnt5a expression showed little prognostic significance in pancreatic cancer. We found that there was no significant difference in the cancer-specific survival of patients with Wnt5a positive vs. negative pancreatic tumors. Such inconsistency between biological and clinical findings may be explained by that Wnt5a exerts its metastasis-promoting effects largely through activation of the β-catenin signaling, which can be regulated by multiple factors other than Wnt5a in vivo.

As per your suggestion, the manuscript, especially the Discussion section, has been thoroughly revised and improved. Thank you very much for your valuable comments.