Author's response to reviews

Title: The PI3K/ Akt pathway upregulates Id1 and integrin alpha4 to enhance recruitment of human ovarian cancer endothelial progenitor cells

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Author's response to reviews: see over
Dear Managing Editor:

Thank you for your e-mail. Our manuscript (MS: 8569396573741498) has been revised according to editor and reviewer’s comments. Now, we will answer all questions raised by reviewer.

Reviewer #1: Minor essential revisions:

Comment 1. The actual values of figures 2C and 4B, including numbers for standard error and number of analyses conducted (n), should be stated in the results section or in the legend.

Response: According to your comment, we have revised it in results section.

To what do the relative protein levels relate to (what is set “1”)?

Response: Generally speaking, as first step, we assessed concentration of total protein in the sample using commercial kit. Next step, internal control protein were detected by means of western blotting analysis in the same amount of loading sample. According to the results, we normalized amounts of each sample loading with expression level of internal control for differences in sample concentration and loading. Thereafter, target proteins and internal control were examined by western blotting. Finally, relative expression amount of each target protein was calculated by the ratio of optical density (OD) of corresponding target protein to OD of the internal control.

Which statistical method was used to determine the p-values?

Response: Multiple comparisons were analyzed by Anova followed by post-hoc analysis to adjust the significance level.

Comment 2. The quality of figure 3B should be improved. Enhancing the resolution might help. A smaller image with a higher magnification could be inserted into each panel.

Response: According to your comment, we have revised it in figure 3B.

Comment 3. The panels of figure 3B have to be labeled.

Response: According to your comment, we have revised it in figure 3B.

Comment 4. The authors should be more accurate with the phrasing.

- By example in the second sentence of the abstract, the term “EPCs with ovarian
cancer” should be replaced by “EPCs of patients with ovarian cancer”. • In the first sentence of the introduction, the authors state that angiogenesis is “chiefly mediated” by endothelial progenitor cells. While this can be discussed for the adult organism, this can certainly not be stated for angiogenesis in general.

• In the last sentence of the introduction, the authors suggest that inhibiting Id1 might “disrupt” ovarian cancer cells. What exactly do they mean with this? These are just examples of inaccuracies in phrasing and the usage of the language. I strongly recommend the manuscript to be checked again carefully for accurate phrasing.

Response: The paper have been checked carefully and corrected some mistakes by a native English speaker.

Comment 5. Regarding the description of the material used for the Western blot:

• There are various antibodies commercially available from Cell Signaling against P-Akt (phosphorylated AKT). Please indicate the exact reference. This is specifically important since some of these antibodies recognize different phosphorylation sites with different biological relevance.

• What is “T-Akt”?

Response: According to your comment, we have revised it in Materials and methods section (Page 6).

Discretionary revisions:

Comment 6. The quality of figure 4 A should be improved.

Response: According your comment, we have replaced figure 4 A with high quality of images in the revised version of manuscript (see Fig.4A).

Comment 7. Figure 1: The panel in the lower right corner is labeled as “overlay”, but it is not clear what is overlaid here. Please indicate this in the legend, in the text, or in the figure.

Response: We are sorry that we did not make it clear. We have revised it in the Figure 1 legend section ( page 20).

Reviewer #2:
Comment 1 Fig. 1 EPCs were cultured from the mononuclear fraction derived from peripheral blood. EPCs were defined ac-LDL+ lectin+ and expressed vWF, VEGFR2, and CD31.

To call these progenitors the authors must use a progenitor marker such as c-kit. Are these cells negative for classical hematopoietic markers such as CD11b, CD45, macrophage markers.

Response: Regarding to the problem you have raised, we considered it during performing this study. However, it is a pity that no unique identifying marker for EPCs has been reported and functional characterization of the rare putative EPC population. Therefore, our descriptions of these cells may not be universally applicable and makes comparisons with other published work difficult. According to previous reports, most groups define EPCs as peripheral blood- or bone marrow-derived mononuclear cells that adhere to matrix molecules such as fibronectin and demonstrate dual positivity to acLDL and UEA-1 lectin. Increasingly, FACS analysis using combined endothelial and hematopoietic cell markers has been utilized to characterize EPCs. CD34, CD133 and VEGFR2 are commonly used for this purpose. We therefore analyzed EPCs expressing CD34, VEGFR2 by flow cytometry. We found that VEGFR2+ cells percentage was about 80%, and CD34+ cells percentage was about 10% (figure 1, data not shown in manuscript). They express different surface markers depending on their state of differentiation in vitro. Early immature EPCs express hematopoietic cell markers such as CD133, CD34 and CD117 (c-kit) and monocytic/macrophagic cell markers such as CD11 and CD14. In a more differentiated state, these hematopoietic markers are lost and there is increased expression of endothelial markers such as VEGFR2, VE-cadherin, vWF and CD31. Our study found that EPC express CD31, VWF, and VEGFR2 but do not express CD45 and CD14. Some samples cells express CD34.

Figure 1  Detection of cell markers on EPCs by flow cytometry. A, C, isotype negative control. B, the percentage of VEGFR2 positive cells. D, the percentage of CD34 positive cells.

What about their functional attributes. Do they form endothelium or incorporate into nascent vessels?

Response: EPCs are highly clonogenic and can be mobilized to the peripheral circulation as circulating EPCs in response to angiogenic stimuli. Circulating EPCs then participate in organ and tumor neovascularization by incorporating into newly-formed blood vessels. The extent of vascular incorporation of EPCs may depend on the mouse strain, the tumor type and its developmental stage. Because it is not clear whether EPCs originate from haematopoietic, mesenchymal or haemangiocompetent stem cells.

Comment 2. The authors claim that pharmacological inhibition of the PI3K pathway blocks EPC functions in a Id1 and integrin a4 dependent manner. Will overexpression of Id1 or integrin rescue the EPC defects following PI3K inhibition (migration, adhesion).

Response: We would like to thank you for your valuable suggestion, because you provide a very interesting idea for us. Our study show that pharmacological inhibition of the PI3K pathway blocks EPC functions in a Id1 and integrin a4 dependent manner.
Based on our results, we speculate that overexpression of Id1 or integrin may partly rescue the EPC defects following PI3K inhibition, it may depend on intensity of overexpression and PI3K inhibition. It is obvious that the answer of the question need confirm by means of experiments. Due to the mechanism is very complicated, so Id1 and integrin a4 pathway is only one of many pathways associated with PI3K/Akt.

Thanks for Editor’s help again. We wish that our reply will be satisfied for you and reviewers. By the way, we have corrected our manuscript according to format suggestion.

Please keep in touch with us.

With our best regards!

Yajuan Su, et al.

07/11/2010