Author's response to reviews

Title: Higher Proliferation of Peritumoral Endothelial Cells to IL-6/sIL-6R than Tumoral Endothelial cells in Hepatocellular Carcinoma

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Author's response to reviews: see over
First of all, we appreciate the opportunity to revise the manuscript (Higher Proliferation of Peritumoral Endothelial Cells to IL-6/sIL-6R than Tumoral Endothelial cells in Hepatocellular Carcinoma, MS1819091776170597). We carefully considered reviewers’ comments and questions, and made changes in this version, which have been highlighted in yellow. Point-by-point responses to reviewer comment are included with this letter.

We thank you for reviewing this revised manuscript and look forward to your reply.

Best regards.

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Reviewer #1
In this manuscript of “Higher proliferation of peritumoral endothelial cells to IL-6/sIL-6R than tumoral endothelial cells in hepatocellular carcinoma”, the authors demonstrated that PECs presented a much higher level of cell proliferation under co-culture with IL-6/sIL-6R by automatically up-regulating their surface gp130 proteins and increased phosphorylation of JAK2 and STAT3. This study suggests that PECs and associated peritumoral angiogenesis microenvironment may be a potential therapeutic target for anti-angiogenic
Q1. On page 9 line 13, how about the protein levels of IL-6, IL-6R and gp130 by western blotting?

As the reviewer kindly suggested, in our studies, we add western blot analysis of IL-6, IL-6R and gp130 in both tumor and peritumoral tissue, and we have revised accordingly in the present manuscript (paragraph 1 page 10, Figure 1F) and marked the text in yellow.

Q2. It has been reported that hypoxia could induce the expression of gp130, however, hypoxia had no direct effect on the expression of surface gp130 in PECs in your study, please give an explanation.

In our studies, we found that cobalt chloride-induced hypoxia of peritumoral endothelial cells markedly induced IL-6 expression confirmed by the RT-PCR and immunocytochemistry staining, however, hypoxia had no effect on the expression of surface gp130 in PECs, in terms of the up-regulation mechanism of IL-6 by hypoxia, it was not further investigated in our present study.

On the basis of previous studies by others, one likelihood is that hypoxia could effect mRNA synthesis of angiogenic factor via transcription regulation mediated via a hypoxia-responsive element (HRE) within their promoter which has been proved by previous studies[1, 2], thus we conclude that hypoxia could also
up-regulate IL-6 in PECs at the transcriptional level through HRE way which results in the increased mRNA level of IL-6, whereas in terms of gp-130, the signal transducer element, which is found in many cell types, could be up-regulated by exposure to hypoxia; however, in our present study, we found that hypoxia could not directly up-regulate the gp130 expression on PECs, and the controversial may be attributed to the fact that HIFs may differentially regulate the expression of the same angiogenic gene in different cell types;

In our present study, the mechanism of gp130 expression unmodulated in PECs under hypoxia condition may be due to its distinct cell surface markers: IL-6R(−) and gp130 (+). In vivo study, although we found peritumoral gp130 expression was positively correlated with peitumoral hypoxia, IL-6 and IL-6R, however in vitro study indicated that neither IL-6 nor sIL-6R could promote the gp130 expression, instead co-administration of both IL-6 and sIL-6R could lead to a synergistic increase in gp130 expression, which indicated that the up-regulation may be associated with effect of both IL-6 and IL-6R; therefore, although hypoxia could up-regulated the IL-6 expression on PECs, which could not alone up-regulate gp130 expression on PEC cell without IL-6R, and in vivo study, the elevated peritumoral sIL-6R deriving from the macrophages together with elevated peritumoral IL-6 could lead to the higher expression of gp130 in peritumoral tissue, which had no direct correlation with peritumoral hypoxia.

Q3. Please check your sentence structure and English grammar.
As the reviewer kindly suggested, we have checked our sentence structure and English grammar.

**Reviewer #2**

The authors explored interestingly the mechanism involved in HCC angiogenesis, taking into account the peritumoral endothelial cell (PEC) and the modulation of the important cytokines and factors involved in this type of cancer as IL-6, moreover, studies the IL6/sIL6R complex. They showed that these cell could be considered as potential target for anti-angiogenic treatment in HCC, where angiogenesis is critically associated with the hepatocarcinogenesis. Therefore, the question posed by the authors is very well defined and new.

The manuscript require essential revisions and following comments should be considered for manuscript publication in BMC:

1. **IHC results shown in figure 1A should include a representative tissue from one or two week after tumor injection in order to visualize difference observed in the 7th week.** Beside, should be include a representative IHC showing both tumor and peritumoral issue in order to visualize the curves indicated in part B, C and D in figure 1. A brief description of how tumor microarray was constructed from mouse. The reference [9] describe the method for human tumor, but the size from human tumor is different in mice. The authors should indicate the number of mice used in the experiment and How many experiments they were performed?

As the reviewer kindly suggested, in our studies, we add the representative images of IHC from 2wk, 5wk and 7wk after tumor injection showing both tumor and peritumoral issue in order to visualize difference observed in the 7th week (Figure
We are so sorry that we had not described the method of tissue microarray construction in our methods part of manuscript, that is, tissue microarray derived from mouse tissue was constructed in the same way as the tissue microarray of human tumor, and the method had nothing to do with tumor size, that is, after reviewing HE-stained slides for the location of tumor tissue and tissue adjacent to tumor (TAT) with the distance of 2 cm away from tumor, we constructed TMA slides (collaborated with Shanghai Biochip Company, Ltd, Shanghai, China). Two cores were taken from each formalin-fixed, paraffin-embedded HCC and TAT sample, respectively, by using punch cores that measured 1.0 mm in greatest dimension from the nonnecrotic area of tumor foci and TAT. We have revised accordingly in the second paragraph of the results (paragraph 2 page 6) and marked the text in yellow.

To study EC proliferation and angiogenesis-related genes in peritumoral tissue during tumor growth, 18 mice of the HCC tumor model was established by orthotopic implantation of a histologically intact tumor tissue derived from HCCLM3 cell line, and 6 mice were killed at the end of the 2, 5 and 7wk after tumor injection respectively. We have revised accordingly in the second paragraph of the results (paragraph 1 page 6) and marked the text in yellow.

2. The figure 2 A should be enlarged to distinguish the described increase of HIF-1a and IL-6. Figure 2D shown that IL-6 expression assayed by RT-PCR is
lower in hypoxia than normoxia, this right? It is not in concordance with the results in figure 2 B and C.

As the reviewer kindly suggested, we added the representative IHC images of HIF-1a and IL-6 with higher resolution in Figure 1A.

Firstly, we are so sorry that we have made the description mistake that we mistook △CT for −△CT in Figure 2D in our submitted manuscript, and we have revised in our present manuscript in Figure 2C. In our present study, the relative mRNA expression was normalized to that of β-actin. The relative amount of tissue mRNA was standardized by the amount of β-actin mRNA, and expressed as −△CT = CT(factor) − CT(β-actin). The ratio of the number of mRNA copies to the number of β-actin mRNA copies was then calculated as $2^{-\Delta CT} \times K$, where K is a constant, therefore, under hypoxia condition, the $-\triangle CT$ of IL-6 was much higher than its in normoxia condition (mean $-\triangle CT$, −0.01 ± 0.003 vs. −0.17 ± 0.052 for hypoxic and normoxic conditions, $P = 0.014$), and it means that the ratio of the number of IL-6 mRNA copies to the number of β-actin mRNA copies, that is the value of $2^{-\triangle CT} \times k$, was also much higher in hypoxia condition.

3. Western blot results require densitometry and statistical analyses. Since the authors have observed variations in proteins expression by this method, only this conclusion could be reached by performing a densitometric analysis and then determining by statistical significance, indicating the number of experiments performed.
Thanks for the kindly suggestion of the reviewer, and in our present study, specific protein expression levels were normalized to the GAPDH protein, and the results of densitometric analysis were conducted and all experiments were performed in triplicate. **We have revised accordingly in Figure 4.**

4. The authors could discuss the results about the differences observed within peritumoral and tumoral liver tissue after tumor implantation in mice. They should discuss about IL-6 function in liver tumor, taking into account the role of this cytokine in underlying cirrhosis that usually accompanies these tumors. Because a controversial result was found by the authors about gp130 expression unmodulated in PECs under hypoxia condition, they must discuss a possible mechanism.

Interleukin-6 (IL-6) plays a role in primary tumor progression [3]. Higher IL-6 could promote tumor growth, and the compression effect of tumor growth on peritumoral tissues, as induced by the primary tumor, could create the peritumoral hypoxia environment, which was in accordance with the peritumoral IL-6 expression, and furthermore, we found hypoxia can directly up-regulate the expression of IL-6 in PECs, therefore, IL-6 in tumor and peirtumoral tissue had cross-talk effect on both tumor growth and metastases.

In our present study, we found that hypoxia could directly up-regulate the expression of IL-6 in PECs, which may be associated with the enhanced transcription driven by the nuclear factor IL-6 site in the IL-6 promoter in cell under the hypoxia
condition; however, we found that hypoxia could not directly up-regulate the gp130 expression on PECs, which seems to be controversial that in vivo study that we found peritumoral gp130 expression was positively correlated with peitumoral hypoxia; In vivo study, peritumoral gp130 was also positively accompanied by peritumoral IL-6 and IL-6R, however, we found the neither IL-6 nor sIL-6R alone could promote the gp130 expression, and co-administration of IL-6 and sIL-6R lead to a synergistic increase in gp130 expression, which indicated that the up-regulation may be associated with both effect of IL-6 and IL-6R; therefore, the mechanism of gp130 expression unmodulated in PECs under hypoxia condition may be due to its distinct cell surface markers: IL-6R(–) and gp130 (+), the up-regulated IL-6 under hypoxia condition could not alone up-regulate the gp130 expression on PEC cell, and in vivo study, the elevated peritumoral sIL-6R which was derived from the macrophages together with elevated peritumoral IL-6 could lead to the higher expression of gp130 in peritumoral tissue.

We have revised accordingly in the discussion part (paragraph 1 page 16) and marked the text in yellow.

5. As suggestion in vitro studies could be performed in different hypoxia protocol in order to confirm the results observed by the authors in PECs.

The cobalt chloride (CoCl$_2$) was used to mimic hypoxia by inactivating the hydroxylase activity and preventing the interaction between HIF-α and pVHL,
thereby preventing the degradation of HIF-1α. To induce hypoxia, in our present study, PECs were preliminarily exposed to 50-200µmol/L CoCl₂ for 24h and 48h, and exposure to 100umol/L CoCl₂ was consequently selected as a condition to induce hypoxia by its minimal effect on cell viability. Our results showed that lower concentration of CoCl₂ (such as 50µmol/L) slightly induced the mRNA and protein expression of HIF-1α, and IL-6 was slightly up-regulated in PECs for 48 h (mean−ΔCT, −0.10 ± 0.004 vs. −0.17 ± 0.052 for hypoxic and normoxic conditions, P=0.0450) and gp130 was also not effected; and in our preliminarily study, PECs did not demonstrate good cell viability under exposure to higher concentration of CoCl₂ (200µmol/L), although the similar results was also observed for 48h that IL-6 expression was higher than that under lower concentration of CoCl₂ (mean −ΔCT, −0.0085 ± 0.001 for hypoxic, P = 0.0550) and gp130 expression was also not obviously effected by hypoxia condition. Therefore, in our present study, we selected 100umol/L CoCl₂ as a condition to induce hypoxia to conduct our subsequent hypoxia related study.

Minor revision

Several references are indicated as superscript in the text, must be corrected.

We have revised the reference in the present manuscript.
Reference

