Author's response to reviews

Title: The Hypoxic Microenvironment Upgrades Stem-like properties of Ovarian Cancer Cells

Authors:

Dongming Liang (liangdmnmng@gmail.com)
Yuanyuan Ma (yuanma@rr-research.no)
Jian Liu (jianliu@163.com)
Claes Goran Trope (c.g.trope@medisin.uio.no)
Ruth Holm (ruth.holm@ous-hf.no)
Jahn M. Nesland (j.m.nesland@medisin.uio.no)
Zhenhe Suo (zhenhes@medisin.uio.no)

Version: 2 Date: 16 March 2012

Author's response to reviews: see over
Dear Editor Chap,

Thank you very much for your effort in evaluation of our manuscript “The Hypoxic Microenvironment Upgrades Stem-like properties of Ovarian Cancer Cells”. After we received the comments and suggestions from you and the reviewer, we have organized several meetings within our group, and gone through all of the original data. We apologize for some mistakes in our previous manuscript. However, we have made corresponding changes and answered all the questions one by one. We list all the questions and corresponding answers as following:

Major comments:
(1) The authors attribute the stem-like properties to the expressions of Oct3/4 and Sox2. However, cancer stem properties indicate heterogeneity in the tumor, so authors should show CD144dim cells in the sphere and colony derived from CD144bright cells by immunohistochemistry and FACS.

Our answer: We appreciate the comment very much. Yes, we consider the induction of Oct3/4 and Sox2 expression in these cells as an indication of upregulation of cell stemness, but not limit to this. Besides of these transcription factors, we have examined the status of SP, cell cycle analysis, sphere/colony formation efficiency and the typical stem cell markers CD44 and CD133 in these cell lines in consideration of hypoxia impact. All these data pinpoint to a similar direction, i.e. the cells’ stemness is upregulated. Yes we understand the suggestion to further analyze the CD44dim cells in the CD44bright cell population, especially after a period of colony formation or sphere formation experiment. However, this is beyond our current study, since such experiment is going to examine whether CD44bright and CD44 dim cells are pure stem and non-stem cell population. We have experienced in our lab with CD133bright and CD133dim cells, and we know that these subpopulations will change under conventional culture condition. Moreover, it does not change our present finding that hypoxia upregulates cell stemness in vitro, no matter what results we may obtain if we carry out further experiments of CD44dim and CD44bright cells.

(2) The authors suggested that the effects of hypoxic pretreatment on cancer cells depend on the increasing levels of HIF-1α and HIF-2α. However, the significance of these data in the present study is hard to understand since HIFs elevation under hypoxia is well-known. I believe that during the colony and sphere formation for 14 days under normoxia, hypoxic pretreated cancer cells utilize an independent pathway of HIFs.
Our answer: Soon after we placed the hypoxia pretreated cells back into normoxia, these cells met the same condition as the control cells. After this moment, the influence of hypoxia would gradually disappear. However, we still observed the difference in colony formation and sphere formation efficiency, which must be due to the hypoxia pretreatment. We do not mean that this difference is due to the 14 days cultivation condition, but due to the already induced cell stemness during the hypoxia pre-treatment which is the main point of this study.

(3) The authors should clarify the significance of hypoxic pretreatment of cancer cell lines from the viewpoints of physiological cancer biology.

Our answer: Yes, we linked this observation to clinical situation and have discussed this issue. The last paragraph of page 16 in the original version should be relevant to this question, and we have modified a little this time. We write now: These results indicate that cancer cells may switch into a more stem-like status when meeting with hypoxic stress, and develop more aggressive phenotype in a manner of selection in a suddenly higher oxygen environment, such as when tumor cells penetrate into blood stream or when the “dormant” metastatic solid tumor cells are mobilized out of bone marrow by till now un-clarified mechanisms. This may be useful to explain why hypoxia not only accounts for tissue necrosis, but also a strong impact on tumor cell biology, with a decreased sensitivity to apoptotic and other cell-death signals, and an increased signaling to promote angiogenesis, proliferation and systemic metastasis capacity when the niche permits [43-46].

(4) In vivo studies, such as tumorigenicity and metastatic ability, would strongly confirm this research.

Our answer: Yes, this is a very good suggestion. However, it is beyond the work of our present PhD student who is preparing dissertation. Our group has been working in different tumor cell lines, and we will perform such experiments in consideration of hypoxia impact and other cytokines. We realize now that only hypoxia may not be enough to significantly change the stemness of tumor cells, so that these cells may be used for further basic and clinical orientated studies. But we have to finish this study first, mainly focusing on the stemness effect, and then based on this study we will extend to other niche factors as well.

(5) Under conventional normoxia condition, it is hard to understand that so many cells express CD44 and/or CD133, since these cell lines are probably homogeneous (In Figure 4, ES-2 data seems to be reasonable, that is all ES-2 cells are CD44 positive and CD133 negative independent of oxygen concentration).

Our answer: Yes, we understand this comment. We had the same feeling before. But indeed, more cells in some cell lines express such proteins than other cell lines. This is true. However, hypoxia treatment could result in even higher levels of expressions of these factors in these cells. In another study of lung cancer cell lines which were developed in our hospital, we discovered only one cell line highly expressing CD133 under normoxia, but other cell lines only weakly positive for CD133 expression (manuscript in preparation).

Minor comments:
(1)To obtain accurate oxygen tensions in different incubators, it would be better to measure the oxygen tensions of medium directly because there could be a gradient of the oxygen tension in medium.

Our answer: Yes, it is known that oxygen gradient exists in the medium in such cell culture system. Further characterization of the impact of oxygen gradient on cell stemness may shed new light in this field. However, it does not change our current conclusion that hypoxia upregulates cell stemness of ovarian cancer cells in vitro.

(2)In Fig.3D & Fig.5C, “colonies numbers” should be “colony numbers”.

2
Our answer: We appreciate very much for the corrections, and we have made corresponding changes.

(3) page 13, line 7, “are opposite than those in Fig 1A” is incomprehensive expression.
Our answer: We apologize for this uncleanness. We have changed as following:
As shown in Figure 2A, all the hypoxia pretreated cells grow faster than those control cells always cultivated in normoxia. When the cells were pretreated under hypoxia for 48 hrs and then cultivated under normoxia, MTT values of both cell lines were significantly higher than those cells always kept under normoxia ($P < 0.05$).

(4) In Fig 4, I do not understand why isotype control shows CD44 positive and CD133 positive peak. I think FACS compensation was not performed adequately.
Our answer: We have checked our data base together with our flowcytometry core facility people, and found that the Figure 4A and B were wrong. These figures were taken from from our optimizing tests before we started the examinations. We have changed these figures with representative figures of such analyses, and all the flowcytometry data (in the results) were obtained from three independent experiments. We apologize for this mistake and do appreciate for such a meaningful recognition.

(5) The last paragraph in the Introduction is the same as Results in Abstract, rewrite briefly.
Our answer: We agree with this suggestion. We have deleted those repeated statements, so that it is clearer and concise now.

(6) Technically speaking, the spheres and colonies in the Figure seem to consist of a few thousand of single cells. How do you count 30 cells?
Our answer: As we stated in the methods, we count only those spheres or colonies with more than 30 cells as qualified spheres or colonies. The figures showing with many cells are just examples, does not mean all the spheres and colonies with so many cells. As long as we were sure that there were more than 30 cells in a cell mass, we counted this as a colony or sphere, depending on the method.

We have all read these answers and the final version manuscript. We all agree to submit this revised manuscript to BMC Cancer again, hoping to receive your decision in your earliest convenience.

Sincerely yours,

Zhenhe Suo MD PhD
Professor
Principal Investigator
Dept of Pathology
Oslo University Hospital
University of Oslo
Montebello, N-0310
Oslo, Norway
E-mail: Zhenhes@medisin.uio.no
Phone: 004722934215
Fax: 004722730164