Author's response to reviews

Title: Pluripotency-associated genes in human nasopharyngeal carcinoma CNE-2 cells are reactivated by a unique epigenetic sub-microenvironment

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Author's response to reviews: see over
Dear Professor Graham,

We would like to give our sincere appreciation to you and the two reviewers for your valuable time and effort reviewing our work. Now we submit our revised manuscript in your prestigious Journal. We declare that we have no competing interests.

As suggested by the two reviewers, we have made the following revision point by point:

To Reviewer 1:

1. Page 4 “The markers and phenotype of CSCs were similar to that of normal neural stem cells.” Generalized statement - obviously not true for all CSCs. Do the authors mean? - “The markers and phenotype of CSCs were similar to that of normal stem cells.”

Answer: Yes. You are absolutely right. We have corrected it as “Some markers and phenotype of CSCs were similar to that of normal neural stem cells.” (Page 4, line 14)

2. In material and methods the origin of THP-1 should be identified (human acutemonocytic leukemia cell line)
Answer: We have added the origin of THP-1 in material and methods according to your advice. (Page 5, line 16)

3. It is not clear how single cell clones were obtained. The authors write: ”Cells were seeded in a 24-well plate with 1 cell/ml and cultured until there is single clone in one well.” Do the authors mean: … single cell clones were isolated from wells with only a solitary colony…?

Answer: Yes. We have changed the statement according to your suggestion. (Page 6, line 1)

4. Page 12 PMA induced THP-1 cells are not bone fide macrophages, these cells are differentiated tumor cells with macrophage phenotype. The term macrophage should only be used for primary macrophages isolated from healthy human donors.

Answer: Sure. We agree with you that PMA is able to induce THP-1 cells to a macrophage-like phenotype, which mimics bone fide macrophages. Therefore we have amended macrophages to macrophage-like cells in the whole text.
5. The authors use the expression of “coculture” as a synonym for incubation with conditioned medium. E.g. “…the CNE-2-2 cell clone contained 9.8% SP cells after co-cultured with macrophage,…” This is not correct. Coculture means mixed cell cultures where two or more cell types are present simultaneously in the same cell culture.

Answer: We appreciate your comment regarding the definition of co-culture and amended co-culture to conditioned culture in the whole draft accordingly. The reason for us to perform tumour cell culture with conditioned medium of macrophage-like cells is that we would like to consider specifically the paracrine loop using a simplified model rather than the physical interaction of the two cell types.

6. “we selected another human neuroblastoma cell line SK-N-CH” – Do the authors mean? .. we selected an other cell line, the human neuroblastoma cell line SK-N-CH”?

Answer: Yes. We have corrected the sentence according to your advice. (Page 12, line 16)

7. “the express of CD133,” – should not it be “the expression of
CD133,”?

Answer: Yes. We have corrected it. (Page 12, line 18)

8. the authors state that “Propidium iodide-positive dead cells (<15%) were excluded from the analysis.” However Figure 3 reveals a large amount of apoptotic cells in the control and proportionally less in the “coculture” population” The authors should consider and discuss the possible anti-apoptotic effect of the differentiated THP-1 conditioned media especially as ARMER was one of the most intensely upregulated gene in the “coculture” sample.

Answer: We have made further discussion regarding it. The possible anti-apoptotic effect of ARMER may be induced when CNE-2 cells are cultured with the differentiated THP-1 conditioned medium. (Page 15, line 19-23)

To Reviewer 2:

1. There are several NPC cell lines, CNE-1, CNE-2, CNE-3,… Why authors select CNE-2 for this study? The authors have cited the previous work by Wang et al.(2007), but there is no reference.
Answer: Yes. CNE-2 and CNE-3 are more poorly undifferentiated carcinoma cell lines than CNE-1. We studied the cancer stem cell of nasopharyngeal carcinoma so we chose the CNE-2 cell line. We have added that particular reference. (Page 5 and 20, References 28)

2. It is good that authors used neuroblastoma for validation of lab system and showed the effect from macrophage interaction, although is an indirect evidence to CNE-2.

Answer: Yes. Thanks. We only want to validate the technique system.

3. What is the reason to have such big range of SP cells from 0.1% to 73.7%? How to exclude the introduced errors due to changes of cell culture condition or manipulation?

Answer: We think one reason for such big range of SP cells is the tumor heterogeneity. The fraction of SP was identified by its characteristic fluorescent profile in dual-wavelength analysis with Hoechst 33342. In order to exclude the introduced errors due to changes of cell culture condition or manipulation, we repeated the screening by same methods to confirm the stability of SP content in each single cell clone. After further
comparison the results of SP fraction, we treated cells with verapamil in each case as control, the SP population was markedly blocked for exclusion of the Hoechst dye, indicating that that population was truly SP.

4. The present result is from a single NPC cell line, CNE-2 and it would be interest to know if the results are producible and represent a common character of NPC from several NPC cell lines, whereas if these involved genes were found in other NPC cell lines as well.

Answer: Yes. Thanks. It’s a good idea. We are going on to test more NPC cell lines and even in tumor tissues afterwards.

5. From SSH, there are so many genes involved, why those gene are selected from up-regulation and what is the evidence of these gene linked to cell growth and metastasis of NPC?

Answer: Yes. From SSH, we can get the gene information about up-regulation and down-regulation at the same time. In this paper, we pay more attention to the mechanisms that sub-microenvironment is involved in the changes from non-SP to SP, so we sequenced the particular genes with huge changes in the presence of conditioned medium from
macrophage-like cells. Following culture with conditioned medium, non-SP cells showed more aggressive characters, for example increasing cell migration. Meanwhile, expression levels of many pluripotency-associated genes are changed. Therefore, the data suggest those gene regulation may be associated with tumour cell growth or metastasis of NPC.

6. NPC is 100% association with EBV virus. It is more interesting to see if CNE-2 cell (EBV negative) was infected with EBV.

Answer: Thanks for this point. It could be a very interesting project. We hypothesize that CNE-2 EBV negative cells may possess different cancer stem characters compared with EBV positive cells.

Minor comment:

1. What is the difference of human nasopharyngeal cancer cell line THP-1 from original CNE-2?

Answer: THP-1 is a human acutemonocytic leukemia cell line.

2. CNE-2-2 cell line should be described in Materials and Methods.
Answer: Yes. We have added the definition of CNE-2-2 in Materials and methods. (Page 6, line 5)

3. Clone ID 2 in text was not showed in Fig. 1C.

Answer: We have corrected Clone ID 2 to Clone 2 in the text. (Page 12, line 3)

4. There are no (D) in Fig. 4 legend

Answer: Yes. Thanks. We have added D in the Fig. 4 legends. (Page 24, line 5)

5. There are several misspellings (from to form, page 7, line 3), and un-properly term (lysed cells in Trizol, not homogenized cells, page 8, line 4 from bottom)…..

Answer: Thanks. We have amended the misspelling and un-properly term according to your advice and done proof-reading of the whole text.

Again, we appreciate your time and effort for examining our work. We
look forward to your favorable decision.

Sincerely yours,

Quentin

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