Object: Revision of manuscript NO: **65011** for **World Journal of Stem Cells Manuscript**

Dear Editor,

we are submitting the revised version of the manuscript 65011 entitled:

"Epigenetic modulators for brain cancer stem cells: Implications for anticancer treatment"

by Luana Abballe and Evelina Miele.

According to the Reviewers' and Editor's comments, the manuscript has been modified as follows:

Reviewer #1

Specific Comments to Authors: The manuscript by Aballe and Miele reviews the mechanisms of epigenetic modulation and its importance in the biology of brain cancer stem cells (CSCs). The manuscript is well written and provides to the reader basic information as well as cutting edge evidence. However, there are several points that need to be addressed before publication.

Authors' reply: We thank the Reviewer for appreciating our work. We have addressed all the points raised by the Reviewer and we believe that the additional explanations and modifications have improved our manuscript.

1. When listing the characteristics of brain CSCs, author incorrectly state that CSCs have "…the ability to give rise to new tissues (normal or tumoral)". CSCs cannot generate normal tissue since they carry oncogenic mutations.

   Authors' reply: We apologize for the mistake and we have corrected the manuscript according (line 87-88):

   "v) tissue regeneration: the ability to give rise to new tumoral tissues".

2. Authors highlight the fact that brain CSC surface markers cannot efficiently discriminate CSCs. Briefly discuss what strategies are better for this purpose and provide references for further reading.

Authors' reply: We apologize to the Reviewer for the unclear data presented about brain CSCs' detection methods. We have expanded the discussion about new available strategies (lines 110-128): "As already described by Abbaszadegan et al., the gold standard stratey to efficiently identify brain CSCs is to test their in vivo tumorigenicity. Limiting dilution assay (LDA) is the best tumorigenicity method that is commonly used for evaluation of CSCs frequency. However, this method presents some critical points, being influenced by the number of the cells, the implantation site and growing time of incubation. Moreover, it is not feasible on large scale studies. Complementary in vitro functional assays could be used to identify CSCs based on i) their intrinsic properties (e.g. self-renewal, asymmetrical division, slow proliferation phenotype, and aldehyde dehydrogenase 1 expression); and ii) their survival pathways (e.g. Wnt/β-catenin, Hedgehog and Notch signaling pathway), in term of expression of transcription factors/key proteins/ microRNAs. Among the recently developed approaches to isolate CSCs, there are Next Generation Sequencing (NSG) technologies. For example, Joasson et al. isolated the stem-like subpopulation using a functional cellular assay, that enriches for cells that can self-renew and differentiate, combined with NGS technologies (single-cell RNA sequencing) to identify CSCs[22,23,24]. Moreover, Rodriguez-Meira et al., in their scientific work, developed an NGS platform that combines single-cell RNA-seq with mutational analysis allowing the identification of distinct subclones of cancer cells [25,26]. This evidence suggests that a combination of cell surface markers and functional assays provide an efficient tool for their identification."

3. Authors have selected some examples to show that epigenetic changes occur in brain CSCs. Please provide details of the studies (not just the conclusion). For example: what methods were employed? how many patients/cell lines were studied?

   Authors' reply: We thank the Reviewer for the suggestion. We have provided, in the "epigenetic modulators" section, more details about the cited studies.

4. Considering what is mentioned in points 2 and 3 above: are the epigenetic changes the same in CSCs and in tumor bulk cells? If the studies report specific analysis in the CSC pool, were the methods for isolation/characterization adequate?

Authors' reply: We thank the Reviewer for the suggestion. We have better explained the points raised by the Reviewer (lines 167-175;245-247):

"Abnormalities of DNA methylation are early events in pre-malignant transformation, and are maintained in the global tumor population. However, the epigenome is in continuous evolution and some of the changes are detectable in later steps of tumorigenesis, as a result of positive selection. In this way, epigenome contributes to tumor heterogeneity and plasticity, which gives rise to a heterogeneous tumor composed of different cell subpopulations, one of them could have "stem-like" features. Additionally, compared to the bulk tumor, CSCs could acquire further epigenetic alterations in response to stress of different nature (e.g. chemotherapy/radiotherapy, chronic inflammation and environmental exposures), contributing to tumor relapse[40]".

"CSCs could acquire mutations in epigenetic marks or changing in methylation/acetylation status, or again in miRNAs signature, that make them sensitive to epigenetics-based drugs' approaches".

Regarding the second point raised by the Reviewer, the studies cited in this review have used adequate techniques for the isolation of CSCs' pool. Particularly, most of them used a combination of multiple identification methods, such as functional assays and surface markers.

5. Compare the effects of the drugs in tumor-bulk cells vs. CSCs. For example, you state that HDACi induce cell cycle arrest in CSC, but previously you mentioned that quiescence is a characteristic of CSCs. This comparison is crucial to understand the potential clinical importance of the drugs.

Authors' reply: The effects of epidrugs in tumor-bulk cells vs. CSCs depending on specific epigenetic alterations (please see the answer to comment 4). If tumor-bulk cells and CSCs share the same epigenetic alteration, epi-drugs will target both, instead if CSCs showed a peculiar epigenetic alteration, the epi-drug (directed towards that epigenetic change) will target only CSCs. For this reason, epidrugs are designed to be used both as a single therapy or in combinatorial treatments.

Regarding data mentioned about the action of HDACi in inducing cell cycle arrest in CSCs, we apologize to the Reviewer for the unclear concept, we rephrase the sentence (line 277):

"HDACi target the escape mechanism of CSCs, reversing chemo-radio-therapy resistance by inducing cell differentiation, apoptosis, inhibition of angiogenesis, and upregulation of tumor suppressor genes[60]".

However, cell cycle arrest is not in contrast with the quiescence of CSCs, because another characteristic of CSCs is the asymmetrical division, which gives rise to a daughter stem cell and a daughter progenitor cell (directed towards the differential fate). Cell cycle arrest probably refers to progenitor daughter cells, that are more differentiated than the parent stem cell, and can actively proliferate.

6. In the "Traslational significance…" section, the examples provided require further detail (see comment 3).

Authors' reply: We thank the Reviewer for the suggestion. We apologize for the missing information and have added more details in the "translational significance of epigenetics: epidrugs section.

7. What are the underlying mechanism of drugs´ toxicity? Are they caused by "on-target" effects?

Authors' reply: We thank the Reviewer for this comment, and we have modified the manuscript, accordingly, emphasizing the theme of epidrugs' toxicity (lines 314-320):

"There are several reasons behind the epi-drugs' toxicity. It is partly due to "on-target" effects, which could be explained with the concept of "pleiotropy". Specifically, a single target gene could be involved in different signaling and controls multiple phenotypic effects. Another reason is "off-target" effects. Epi-drugs are designed to inhibit aberrant epigenetic enzymes, but it is known that they could also affect other classes of substrates belonging to unintended cellular pathways, at both intracellular and extracellular levels[71]".

8. Conclusion needs to be restructured: a) provide your own point of view of how the field is evolving (which should be supported by the evidence presented); and b) you mention that the microenvironment as a key regulator of epigenetics, but the previous text does not elaborate on that. Drugs, although are external factors, cannot be considered part of the tumor microenvironment, nor the CSCs´ niche.

   Authors' reply: We apologize for not providing details regarding tumor microenvironment as a key regulator of epigenetics, we have modified the text accordingly in the revised version of the manuscript (235-239):

   "The tumor microenvironment (TME) also acts as an epigenetic regulator for cancer cells. TME communicates with cancer cells through extracellular vesicles (EVs) secreted by many TME's cell types that contain various mediators including proteins and nucleic acids. Also microRNAs can be charged in the EVs and thereby alter the epigenome of the recipient cancer cell[52]".

   We rewrite the conclusions.

Science Editor:

5 Issues raised:

1. The authors did not provide the approved grant application form(s). Please upload the approved grant application form(s) or funding agency copy of any approval document(s).
   Authors' reply: We thank the Science Editor for his/her comments. We have uploaded the approved grant application form(s).

2. The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor.

Authors' reply: We prepared the figure using PowerPoint as required, but the figure was created with BioRender.com. and the export file is in non-editable format.

3. If an author of a submission is re-using a figure or figures published elsewhere, or that is copyrighted, the author must provide documentation that the previous publisher or copyright holder has given permission for the figure to be re-published; and correctly indicating the reference source and copyrights. For example, "Figure 1 Histopathological examination by hematoxylin-eosin staining (200 ×). A: Control group; B: Model group; C: Pioglitazone hydrochloride group; D: Chinese herbal medicine group. Citation: Yang JM, Sun Y, Wang M, Zhang XL, Zhang SJ, Gao YS, Chen L, Wu MY, Zhou L, Zhou YM, Wang Y, Zheng FJ, Li YH. Regulatory effect of a Chinese herbal medicine formula on non-alcoholic fatty liver disease. World J Gastroenterol 2019; 25(34): 5105-5119. Copyright ©The Author(s) 2019. Published by Baishideng Publishing Group Inc[6]". And please cite the reference source in the references list. If the author fails to properly cite the published or copyrighted picture(s) or table(s) as described above, he/she will be subject to withdrawal of the article from BPG publications and may even be held liable.

   Authors' reply: The figure was created with BioRender.com. and we have cited the figure with "created with BioRender.com." in the figure caption, as indicated by the site.