**Journal title:** World Journal of Stem Cells

**Manuscript NO:** 76157

**Title:** Changes of cell membrane fluidity for mesenchymal stem cell spheroids on biomaterials surface

**Authors:** Chui-Wei Wong, Hao-Wei Han and Shan-hui Hsu

EDITORIAL OFFICE'S COMMENTS

Authors must revise the manuscript according to the Editorial Office's comments and suggestions, which are listed below:

(1) Science editor:

This manuscript investigate the linkage between cell membrane fluidity and cell morphological change of MSC spheroids on the surface of biomaterials. In general, the article is well organized, and the topic is interesting. Though the study have some innovative, there are still some concerns need to pay attention. Some detail information about the study need to be added, such as provide some evidence whether the vesicle-like bubbles represent a new form of exosomes. More quantitative descriptions about the results is needed. Also, the language of this paper need further revise though the author stated in the file "Non-Native Speakers of English Editing Certificate" that the manuscript is edited for proper English language, spelling, grammar, and punctuation.

Language Quality: Grade B (Minor language polishing)

Scientific Quality: Grade B (Very good)

Answer:

Thank you for the suggestion. We have provided details on the sources of cell lines, reagents, and instruments in the Materials and Methods section. Based on our

observation, we suggest that the vesicle-like bubbles may not be a new type of exosomes. First, the diameters of exosomes and vesicles are in general 30–1000 nm. However, the scale of the vesicle-like bubbles shown in this study was much larger (>7 μm). Furthermore, the forming process of vesicle-like bubbles demonstrated by time-lapse recording revealed that these bubbles were rapidly recycled rather than secreted by the cells after formation. Thus, it is rather difficult to collect these vesicle-like bubbles for further investigation on if the vesicle-like bubbles represent a new form of exosomes or giant plasma membrane vesicles. In our hypothesis, some membrane proteins of MSCs might interact with the current hydrophilic materials during spheroid formation, and then the crosstalk would lead to the generation of vesicle-like bubbles depending on the dynamic remodeling of the cell membrane. We have added the corresponding quantitative description of the results in the Results section. We also invited the Native Speakers of English to revise the manuscript, and the manuscript was edited for proper English language, spelling, grammar, and punctuation.

(2) Company editor-in-chief:

I have reviewed the Peer-Review Report, the full text of the manuscript, and the relevant ethics documents, all of which have met the basic publishing requirements of the World Journal of Stem Cells, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office's comments and the Criteria for Manuscript Revision by Authors. Before final acceptance, uniform presentation should be used for figures showing the same or similar contents; for example, "Figure 1Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...". Please

provide decomposable Figures (in which all components are movable and editable), organize them into a single PowerPoint file. Please check and confirm whether the figures are original (i.e. generated de novo by the author(s) for this paper). If the picture is 'original', the author needs to add the following copyright information to the bottom right-hand side of the picture in PowerPoint (PPT): Copyright ©The Author(s) 2022.

Answer:

Thank you for the revision. The figures presentation has been unified as required. The decomposable Figures were provided, organized, and added copyright information into a single PowerPoint file.

**List of changes made/ replies to reviewers' comments**

We thank the reviewers for their valuable comments. We have revised the manuscript by following the comments carefully. Our replies to their comments and changes made are listed below and highlighted as blue in the main text.

**Reviewer #1:** The authors submitted a manuscript investigating the effect of different biomaterials substrates on the cell membrane fluidity and cell morphological changes of MSCs spheroids. Cell membrane is made up of a complex structure of lipids and proteins that diffuse laterally giving rise to what we call membrane fluidity. Membrane fluidity is a key property for maintaining cell functionality, and depends on lipid composition and cell environment. The manuscript's perspective is somewhat innovative for the research on cell membrane fluidity. Overall, this manuscript is better designed, executed, written, and the logic is relatively clear. Nonetheless, there are a number of issues that need to be noted.

1. In the Results section, the authors should add more quantitative descriptions as appropriate.

Answer:

Thank you for your kind suggestion. We have added the corresponding quantitative description of the results in the Results section.

2. The author should give a reasonable explanation for the cause of the vesicle-like bubbles in the Discussion section.

Answer:

Thank you for the suggestion. Cytoskeleton-associated signal transduction is affected by the interaction between cells and culture substrates, which further influences the morphology and migration ability of cells [1]. Therefore, the final shape of cellular spheroids on the surface of cell culture substrates may determine their mobility. In the current study, we cultured stem cells on the hydrophilic substrates to generate 3D spheroids. Stem cells could form a compact cellular spheroids on chitosan-HA substrates [2, 3], while the spheroidal cancer cells displayed relatively less compacted structure and required a longer period of time to stabilize the spheroidal morphology [4, 5]. Besides, two types of cells were assembled into the co-spheroids with distinct patterns of cell organization on various culture substrates [5]. During the spheroid formation, the hydrophilic materials may interact with some structural proteins

anchored on the cell membrane, leading to the cell membrane remodeling. Hence, cell-substrate interactions may change the morphology and compactness of the spheroids as well as the distribution of cells within the co-spheroids. In the present study, the membrane fluidity of stem cells increased more significantly than that of cancer cells when assembled into cellular spheroids. These results suggested that stem cells had more interaction with chitosan-HA substrates to affect the cell morphology than cancer cells. The different rigidity of cell membrane and alternative expression of surface receptors are both the possible reasons for the difference in cell-substrate interactions. Together with the phenomenon of cytoskeletal rearrangement during the formation of 3D spheroids, we believe that the vesicle-like bubbles were derived from the dynamic remodeling of cell membrane and cytoskeleton in MSCs. This description has been included in the Discussion section (page 26).

References


1 Moujaber O, Stochaj U. The cytoskeleton as regulator of cell signaling pathways. Trends. Biochem. Sci. 2020; 45: 96-107 [PMID: 31812462 DOI: 10.1016/j.tibs.2019.11.003]

2 Huang G-S, Dai L-G, Yen BL, Hsu S-h. Spheroid formation of mesenchymal stem cells on chitosan and chitosan-hyaluronan membranes. Biomaterials 2011; 32: 6929-6945 [PMID: 21762982 DOI: 10.1016/j.biomaterials.2011.05.092]

3 Huang G-S, Hsieh P-S, Tseng C-S, Hsu S-h. The substrate-dependent regeneration capacity of mesenchymal stem cell spheroids derived on various biomaterial surfaces. Biomaterials Science 2014; 2: 1652-1660 [PMID: 32481946 DOI: 10.1039/C4BM00053F ]

4 Huang Y-J, Hsu S-h. Acquisition of epithelial–mesenchymal transition and cancer stem-like phenotypes within chitosan-hyaluronan membrane-derived 3d tumor spheroids. Biomaterials 2014; 35: 10070-10079 [PMID: 25282622 DOI: 10.1016/j.biomaterials.2014.09.010]

5 Wong C-W, Han H-W, Tien Y-W, Hsu S-h. Biomaterial substrate-derived compact cellular spheroids mimicking the behavior of pancreatic cancer and microenvironment. Biomaterials 2019; 213: 119202 [PMID: 31132644 DOI: 10.1016/j.biomaterials.2019.05.013 ]

3. It is debatable whether the experimental results in the manuscript can be used as a direct indicator of cell membrane fluidity.

Answer:

In the current study, we employed the PKH fluorescent reagent to label cells, following the standard labeling protocol provided by the manufacturer, and such PKH dye is theoretically restricted to the cell membrane. Cell membrane fluidity is essential for membrane exchange when cells contact with each other in the spheroids. Therefore, we propose that translocation of the membrane-labeled PKH dyes observed in this study indicates the increased membrane fluidity in 3D MSC spheroids.

4. In the Materials and Methods section, the writing format of the source of the reagents and instruments used should be consistent. The source of some reagents and the model of some instruments should be specified. For example, what is the source of MSCs?

Answer:

Thank you for pointing out the negligence. We have provided details on the sources of cell lines, reagents, and instruments in Materials and Methods section.

5. There are some errors in grammar and format in the whole manuscript: inconsistencies; spelling mistakes; single and plural expressions; the use of prepositions and definite/indefinite articles. For examples: p(in italics), p; ’s, 's; “the HA power” should be changed into “the HA powder”; The definite article “the” and the indefinite article “a” are missing from some sentences.

Answer:

Thank you for pointing out this error. We have corrected all the grammatical errors in the manuscript. We have corrected “the HA power” on page 9 to “the HA powder”. The lack of the definite article “the” and the indefinite article “a” in some sentences has been corrected in the manuscript.

==========

**Reviewer #2:** The present study aims to investigate the linkage between cell membrane fluidity and cell morphological change of MSC spheroids on the surface of biomaterials. The authors have used different materials as substrates, they have cultured the cells on the surface of each substrate to create spheroids and investigated the linkage between cell membrane fluidity and cell morphological change. Moreover, they have co-cultured MSC with other types of cells to generate cell co-spheroids. Through these approaches, they have investigated cell-cell interactions and cell membrane exchange in the various spheroids obtained. Their results indicated the appearance of some vesicle-like bubbles on the outer layer of MSC spheroids. Be deeper examination of bubbles formation they have found that these bubbles were originated from the dynamic movement of cell membrane during the formation of spheroids. They have finally concluded that this phenomenon may explain the various

complicated physiological alterations of cells during spheroid formation. The work is interesting. The text is well-written. However, there are some points requiring correction and/or clarification, as follows.

1. The text needs minor language revision.

Answer:

Thank you for the comment. We have corrected all grammatical mistakes and typos in the manuscript.

2. Since the abbreviation CS is largely used in the case of chondroitin sulfate, a common constituent of ECM, it is suggested to use a different abbreviation for chitosan, or, better, no abbreviation.

Answer:

Thank you for the suggestion. The abbreviation "CS" in the manuscript have been replaced by "chitosan".

3. Give information on the supplier of the various cells and describe the MSCs (page 10, line 7).

Answer:

Thank you for the suggestion. The detailed information on various cells is provided in the Materials and Methods section. Human bone marrow mesenchymal stem cells (MSCs) were obtained from the Tulane Center for Preparation and Distribution of Adult Stem Cells [1]. Lung cancer cells (A549) and murine fibroblasts (NIH/3T3, 3T3) were obtained from the American Type Culture Collection (ATCC). (page 10)

Reference:

1. C.-Y. Fu, W.-T. Chuang and S.-h. Hsu, ACS applied materials & interfaces, 2021, 13, 9702-9713.

4. It is suggested to the authors to examine or to provide some evidence whether the vesicle-like bubbles represent a new form of exosomes or of giant plasma membrane vesicles.

Answer:

Thank you for the comment. Based on our observation, we suggest that the vesicle-like bubbles may not a new type of exosomes. First, the diameters of exosomes and vesicles are in general 30 – 1000 nm. However, the scale of the vesicle-like bubbles shown in this study was much larger (>7 μm). Furthermore, the forming process of vesicle-like bubbles demonstrated by time-lapse recording revealed that these bubbles were rapidly recycled rather than secreted by the cells after formation. Thus, it is rather difficult to collect these vesicle-like bubbles for the further investigation on if the vesicle-like bubbles represent a new form of exosomes or giant plasma membrane vesicles. We hypothesized that some membrane proteins of MSCs might interact with the hydrophilic substrates during the spheroid formation, and such crosstalk would lead to the generation of vesicle-like bubbles depending on the dynamic remodeling of cell membrane.

5. Describe in detail the conditions for cross-linking of HA-coated CS membranes (page 10, 1st line).

Answer:

Thank you for the suggestion. The detailed conditions for the crosslinking procedure of HA-coated chitosan membranes have been provided on page 9.