

**Dear Editor,**

I enclose herewith a revised manuscript entitled, “**Microfluidic 3D cell culture of stem cells for high-throughput analysis**” for consideration as a paper in *World Journal of Stem Cells*.

We would like to thank the editor and reviewer for very helpful comments and valuation, which have made our manuscript better. We have addressed the reviewer’s comments and revised the manuscript according to the reviewer’s suggestions.

Sincerely,

**Rhee, Won Jong**

Division of Bioengineering

Incheon National University

119, Academy-ro, Yeonsu-gu, Incheon, South Korea, 406-772

E-mail) [wjrhee@inu.ac.kr](mailto:wjrhee@inu.ac.kr) Phone) +82-32-835-8299 Fax) +82-32-835-0804

## Response to reviewer #1

**Reviewer's comments:** A well written good conceived manuscript introducing the role and use of three-dimensional microsystems for in vitro cell culture of stem cells aiming compound screening for drug discovery. Comments In the chapter, the paragraph starting from “therefore 2D cell cultures and ending in “for these reasons” could use a rephrasing (especially of the phrase starting with however that reads somehow unclear). In the chapter 3D microfluidics for stem cell engineering, I miss an initial display of most important methods used for this purpose. Reader is referred to Table 1, however a phrase stating which exactly are this modality before proceeding to describe them would be beneficial, or at least stress them out within the text when describing them. What is that authors call repetitively within the manuscript “stem cell engineering”? Do they refer to an eventual process of modifying stem cells or they rather mean tissue engineering? Please describe as the terminology is not common in the literature. In the chapter 3D TISSUE MODEL FOR STEM CELL ENGINEERING the introductory part is rather confusing. In which way the basic stem cell proprieties (proliferation and differentiation) are more functional and ideal” than immortalized cells and how stem cells readily mimic the architecture and specific function of human organs? Regarding IPSC based modeling of disease it is useful to mention this modeling function well for disease that involve inherited or acquired gene expression but this does not mean all the diseases can be modeled using this method. Interesting denomination of top bottom and bottom top approaches in microfluidic modeling. In this line it would be even better to further remain consequent with this idea and explain in which category the further described technology fall (organ on a chip and organoid on a chip) The authors make a very well documented and supported case (even though the organization could be improved) for the use of microfluidic devices and high-throughput methods of investigation. It is understandable that for drug screening this is a remarkable modality of accelerating drug development with potentially reducing costs. However, due to the challenges the authors themselves describe regarding the “sensitivity “of stem cells to the unnatural conditions posed by a microfluidic device, how can tests about stem cell propriety be reliable? Are the authors aware of comparative studies describing in parallel results obtained from a classical modality of investigating certain stem cell feature and function compared to results obtained with microfluidic devices?

**Response:** We appreciate the reviewer's valuable comments on our manuscript. We have made every effort to address the issues raised by the reviewer one by one and have revised the manuscript accordingly, as follows.

1. In the chapter, the paragraph starting from “therefore 2D cell cultures and ending in “for these reasons” could use a rephrasing (especially of the phrase starting with however that reads somehow

unclear).

**Response:** We agree with this comment. The paragraph the reviewer mentioned above was unclear, so we have slightly modified it to avoid confusing readers, as follows.

“On the other hand, *in vivo* animal tests have traditionally been the gold standard models for preclinical efficacy tests in the drug discovery process; however, various issues still exist, such as ethical issues and genetic differences between species. In addition, animal models have many drawbacks, such as high cost and uncertainties in the interpretation of the results in many pathological studies. Due to these weaknesses of the traditional models, an alternative cell culture model that corresponds to *in vivo* system is required in order to obtain better predictions of the preclinical response to drugs.”

2. In the chapter 3D microfluidics for stem cell engineering, I miss an initial display of most important methods used for this purpose. Reader is referred to Table 1, however a phrase stating which exactly are this modality before proceeding to describe them would be beneficial, or at least stress them out within the text when describing them.

**Response:** We agree with this comment. We have added the several important references relevant to the initial microfluidic technology used in stem cell research, along with the following sentences.

The following sentence was added to the chapter “3D MICROFLUICS IN STEM CELL ENGINEERING”.

“Since some groups started to use a microfluidic technology for patterning or capturing stem cells in the early 2000s<sup>[30-32]</sup>, the use of this technology in stem cell research has increased significantly.”

The following references have been added to the REFERENCE list.

30 **Chung BG**, Flanagan LA, Rhee SW, Schwartz PH, Lee AP, Monuki ES, Jeong NL. Human neural stem cell growth and differentiation in a gradient-generating microfluidic device. *Lab Chip* 2005; **5**: 401-406 [PMID: 15791337 DOI: 10.1039/b417651k]

31 **Khademhosseini A**, Yeh J, Jon S, Eng G, Sah KY, Burdick JA, Langer R. Molded polyethylene glycol microstructures for capturing cells within microfluidic channels. *Lab Chip* 2004; **4**: 425-430 [PMID: 15472725 DOI: 10.1039/b404842c]

32 **Tan W**, Desai TA. Microfluidic patterning of cells in extracellular matrix biopolymers: Effects of channel size, cell type, and matrix composition on pattern integrity. *Tissue Eng* 2003; **9**: 255-267 [PMID: 12740088 DOI: 10.1089/107632703764664729]

Also, as the reviewer suggested, the “3D MICROFLUICS IN STEM CELL ENGINEERING” chapter has been modified using the same phrase mentioned in Table 1 to enable readers to follow each example easily. All changes are marked in red in the manuscript.

“For patterning cells or ECMs in desired locations, the~”.

“In particular, the chemical gradient in a microfluidic channel is one of the unique features that allows for this heterogeneous microenvironment. ~”.

“To study cell-cell or cell-ECM interactions, the~”.

“Flow is one of the most important stimuli since some organs are affected by shear flow induced by the blood stream. Microfluidic ~”.

“Optical tweezers, electrical impedance, and dielectrophoresis (DEP) techniques combined with microfluidic technologies can be used to sort or separate cells. For instance, ~”.

“Recent advances in microfluidic technology using hydrodynamic trapping ~”.

“(e.g., integration and automation<sup>[47-50]</sup>, mechanical and electrical actuator<sup>[51, 52]</sup>)”.

3. What is that authors call repetitively within the manuscript “stem cell engineering”? Do they refer to an eventual process of modifying stem cells or they rather mean tissue engineering? Please describe as the terminology is not common in the literature.

**Response:** In this manuscript, we intended for "stem cell engineering" to be one of the research fields. According to the one literature (Nerem, 2014), stem cell engineering" is the interface of engineering with the world of stem cells, and has emerged as an important field of research and application over the last decade. The field ranges from basic stem cell research to the development and application of stem cell models, tools, and various technologies. There are a variety of ways that engineers and engineering approaches can contribute to the development of stem cell research. We believe that this terminology should be familiar with the readers; however, we have added further information about “Stem cell engineering” to the manuscript along with the following reference. Furthermore, some repetitive and overused terms of “Stem cell engineering” were changed to “stem cell research” to make text clear.

Abstract: “Stem cell engineering” was changed to “stem cell research”.

Core tips: “Stem cell engineering” was changed to “stem cell research”.

Introduction: “Stem cell engineering, the interface of engineering with the world of stem cells, has emerged over the last decade and covers fields from basic science to engineered approaches<sup>[1]</sup>.” was added.

In reference list, the following reference was added.

1 **Nerem RM.** Stem cell engineering. *Tissue Eng Part A* 2014; **20**: 983-894 [PMID: 24527967 DOI: 10.1089/ten.tea.2013.0764]

4. In the chapter 3D TISSUE MODEL FOR STEM CELL ENGINEERING the introductory part is rather confusing. In which way the basic stem cell proprieties (proliferation and differentiation) are

more functional and ideal than immortalized cells and how stem cells readily mimic the architecture and specific function of human organs? Regarding iPSC based modeling of disease it is useful to mention this modeling function well for disease that involve inherited or acquired gene expression but this does not mean all the diseases can be modelled using this method.

**Response:** As the reviewer commented, the patient-specific stem cells and organoids that have been developed are usually used to model the genetic disease. Although some studies have investigated the relationship between genetic and epigenetic variations, these models have still limitations for modeling all types of diseases. Therefore, we modified this sentence to narrow the claim to a specific disease model so as not to be misleading. The modified sentences are marked in red.

In the first paragraph of the “3D TISSUE MODEL FOR STEM CELL ENGINEERING” chapter:  
“Immortalized cell lines are capable of extended proliferation but exhibit fewer organ-specific activities than primary cells or stem cells. Moreover, primary cells are functional, but have limited cell number and a finite lifespan. Therefore, stem cells that are able to differentiate into specific organs are considered to be more functional, and an ideal source to mimic the architecture and specific activity of human organs, and are more likely to be accurate with respect to human bodies. As a more reliable and sustainable human source that represents the phenotypical characteristics of the inherited disease or genetic disorders, patient-specific cells are needed”.

5. Interesting denomination of top bottom and bottom top approaches in microfluidic modeling. In this line it would be even better to further remain consequent with this idea and explain in which category the further described technology fall (organ on a chip and organoid on a chip) The authors make a very well documented and supported case (even though the organization could be improved) for the use of microfluidic devices and high-throughput methods of investigation.

**Responses:** We believe that this section lacks an explanation of the organoid-on-a-chip, and making the text misleading. We described the organ-on-a-chip as a top-down approach and the organoid as a bottom-up approach in the text; however, this chapter only covers the microfluidic approaches, i.e. organ- or organoid-on-a-chip. By combining the strengths of both the organ-on-a-chip and organoid technologies through a synergistic strategy, the organoid-on-a-chip platform has emerged as a new model to mimic the *in vivo* tissue microenvironment (Skardal *et al.*, 2016; Takebe *et al.*, 2017). With the aid of microfluidic technology, the organoid can be developed in a microfluidic device with enhanced nutrition exchange under automatic control. Currently, the boundary between organoid-on-a-chip and organ-on-a-chip approaches using stem cells is unclear. Therefore, we attempted to describe these two technologies in the same chapter by specifying them in Table 3. To avoid confusing the readers, we have added the following sentences to the manuscript for clarification.

In the last paragraph of the “3D TISSUE MODEL FOR STEM CELL ENGINEERING” chapter:

“However, both approaches have their own limitations. For instance, organoid systems have low controllability for recreating the biochemical and biophysical microenvironment of 3D organoids, while organ-on-a-chip systems have limitations when reconstituting the biological complexity of tissue development. Thus, by combining the strengths of both approaches, the organoid-on-a-chip platform has emerged as a synergistic approach to recapitulate both the physiological and biochemical features of *in vivo* tissue<sup>10,14</sup>. In this section, we introduce examples of stem cell-based organ-on-a-chip and organoid-on-a-chip systems using microfluidic technologies for high-throughput analysis.”

6. It is understandable that for drug screening this is a remarkable modality of accelerating drug development with potentially reducing costs. However, due to the challenges the authors themselves describe regarding the “sensitivity “of stem cells to the unnatural conditions posed by a microfluidic device, how can tests about stem cell propriety be reliable? Are the authors aware of comparative studies describing in parallel results obtained from a classical modality of investigating certain stem cell feature and function compared to results obtained with microfluidic devices?

**Responses:** Perfusion systems using microfluidic techniques are very useful for culturing organoids derived from stem cells for long-term period, but shear stress might affect cells and cause unwanted phenotypic changes. For instance, Toh, *et al.* reported that a perfusion system induced shear stress that affected the cell growth and differentiation of embryonic stem cells. They varied the shear stress level based on microfluidic flow, and demonstrated that phenotypic cell changes occurred under the high shear stress condition compared to the mild shear stress or static condition. This result suggested that flow could be an important factor to consider when cultivating cells in microfluidic systems and mild manipulation might be needed in some cell types. However, mechanical stress is not always a harmful factor. In some cases, this stimulus can bring about positive effects on differentiation and enhance the functionality of cells since some organs, (e.g. bones and vessels), are naturally affected by this physical stimulus *in vivo*. We have slightly modified these sentences in the manuscript and have added a reference (Toh et al .2011), as follows.

In the first paragraph of the “CHALLENGES AND FUTURE PROSPECTS” chapter:

“Under such physical conditions, certain types of stem cells can be very sensitive to the excessively high shear stress induced by flow, which might cause phenotypic changes or adversely affect cell viability in microfluidic devices during long-term cultures<sup>[106]</sup>”.

The following reference has been added to the REFERENCE list.

106 **Toh YC**, Voldman J. Fluid shear stress primes mouse embryonic stem cells for differentiation in a self-renewing environment *via* heparan sulfate proteoglycans transduction. *FASEB J* 2011; **25**: 1208-1217 [PMID: 21183594 DOI: 10.1096/fj.10-16897]