Response to reviewers:

Reviewer 1: This is a very interesting study describing the maturation of human induced pluripotent stem cell-derived cardiomyocytes. The descriptions about the maturation of hiPSC-CMs by co-incubation with mesenchymal stem cells may be revised to include the factors secreted from MSCs.

We thank the reviewer for their kind comments. We have added information about the co-incubation of hiPSC-CMs with hMSCs to promote maturation on page 12.

"This study then implicated VEGF, bFGF, SDF-1, and GM-CSF as secreted factors from hMSCs that are key in hiPSC-CM maturation. It is thought that secretion of these factors into hiPSC-CM cultures is able to induce maturation through upregulation of crucial adult cardiomyocyte gene *MYH7*."

Reviewer 2: The manuscript illuminates the difference between adult cardiomyocytes and hiPSC-CMs in 4 important ways, including the expression of specific genes, differing structural features, altered metabolism and contractile function. In addition, the manuscript introduces systematically the approaches for the maturation of hiPSC-CMs from three aspects. The combination of several of these approaches may lead to the optimal maturation conditions. However, there are several issues that should be addressed.

1. Please add some information concerning the mechanisms of improvement in the maturation of hiPSC-CMs in the approaches introduced.

For each approach for the maturation of hiPSC-CMs we have added information about the known or hypothesized mechanism of action.

"The mechanism of T3 in hiPSC-CM maturation is not completely understood; however, T3 has been shown to have an important role in cardiomyocyte differentiation through transcriptional regulation. Interestingly, blocking the action of T3 results in lower cardiomyocyte yield. It is hypothesized that downstream effects of T3 signaling may be responsible."

"Immature hiPSC-CMs show remarkable flexibility in adapting to growth conditions. As such, incubating these cells with fatty acid-rich, glucose-free medium seems to be altering the transcriptional signature of these cells towards a more mature phenotype. As the immature hiPSC-CMs suddenly face glucose starvation, they may be pushed towards increasing transcription of genes key in metabolizing fatty acids in order to survive. As mentioned, fatty acid metabolism is characteristic of adult cardiomyocytes."

"The mechanism of hiPSC-CM maturation through the addition of mechanical and electrical cues is yet to be completely understood. However, it is hypothesized that conditional cues may upregulate the expression of key genes involved in

establishing proper cardiomyocyte structure and contractility. For example, expression of calcium handling genes *SERCA2* and *RYR2* is increased following administration of static stress."

2. What are limitations and questions in 3D cardiomyocyte cultures?

In the 3D approaches section, on page 16 and in the conclusion, we have added paragraphs which highlight the limitations and ongoing issues in 3D hiPSC-CM cultures.

"While promising, 3D hiPSC-CM models display some key disadvantages in disease modelling. First, many disease models require the use of single-cells to characterize disease phenotypes. Efficient dissociation and re-plating of 3D hiPSC-CMs is a known problem as many cells do not survive post-dissociation. This also poses a problem for potential clinical applications as typically, protocols involve the use of single cardiomyocytes for injection into a recipient animal myocardium [14,15]. Second, unless organoid cell numbers and aggregate size are not carefully optimized, drug testing may be inaccurate as organoids may not be exposed to the same dose of drugs. Further, routine cell sorting may be required to ensure the cellular homogeneity of cultured organoids. The recent development of tissue-culture plates such as AggreWell (STEMCELL Technologies), and cardiomyocyte recovery/dissociation medium (STEMCELL Technologies) may prove useful in regulating cardiac organoid cell size and optimizing cell recovery; however, further research in this area must be done to definitively address these concerns."

"Current protocols for the derivation of cardiomyocytes from iPSCs are highly efficient; however, hiPSC-CM culture conditions have not been adequately understood. Addition of various cytokines, environmental cues, and mechanical/electrical stimulation have yet to be optimized. As a result, current protocols result in cardiomyocytes that are most consistent in their properties with fetal cells which potentially limits their use for disease modelling and clinical translation of adult diseases."

3. What are causes of the hiPSC-CMs being qualitatively and quantitatively immature?

We have now added two additional paragraphs in the Introduction (page 7) detailing some of the causes of hiPSC-CMs being immature.

"Given that hiPSC-CMs are being derived from pluripotent cells, it is not unexpected that the initial differentiated cells generated will be immature or fetal in their characteristics. It is therefore reasonable to expect that an additional maturation protocol (Figure 2) will be necessary to generate cells that truly reflect the *in vivo* tissue."

“Current protocols for hiPSC-CM production have failed to mature these cells due to a lack of knowledge regarding the mechanisms of heart maturation *in vivo.* At present, the field of cardiac regenerative medicine does not know the correct secretory factors, environmental cues, and external stimulation necessary to achieve proper adult-like cardiomyocytes.”

Reviewer 3: This review is very well written, compactly summarizing the recent methodological progress in inducing the differentiation of hiPSC into adult-type cardiomyocytes. This review will contribute to further technological advancement of the provision of maturated cardiomyocytes that are useful in drug discovery and transplantation therapy.

We thank the reviewer for their kind comments.

Reviewer 4: The manuscript entitled “Current Methods for the Maturation of Induced Pluripotent Stem Cell-Derived Cardiomyocytes” addresses very important issue of differentiation approaches to obtain adult specific functional cell types, particularly cardiomyocytes. Although spontaneously beating cells are rather easy to differentiate from human pluripotent stem cells, they are not correspond to their natural analogues from adults heart largely representing some embryonic or fetal cardiac cells. Thus, developing of some maturating procedures in vitro is of importance. Title and abstract reflect main subject of the review, introduction brings to the importance of the topic. Main difference between adult cardiomyocytes and stem cells derived is in their gene expression pattern, which in turn leads to the structural, metabolic, and functional differences. Authors revise recent achievements in the field using modern literature and discuss biochemical, environmental, and structural approaches for cardiac cells maturation. The paper is well written and interesting. I would advise to include some more illustrative material for environmental and 3D approaches for cardiac cells maturation.

We thank their reviewer for their thoughtful comments. We have added two figures (figure 2 and figure 3) illustrating methods of maturation and an example of hiPSC-CMs cultured in 3-dimensions.