May 7, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: manuscript_revised_.doc) and our answers concerning the various points raised by the 3 reviewers.

Title: Nutrient supplemented serum free medium increases cardiomyogenesis efficiency of human pluripotent stem cells.

Author: Sherwin Ting, Marti Lecina, Yau-Chi Chan, Hung Fat Tse, Shaul Reuveny, Steve K.W. Oh

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The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated according to Journal guidelines

2 Revision has been made according to the suggestions of the reviewer

Reviewer 1

First of all it would be very interesting if the authors also do apoptosis tests of all improved culture conditions to distinguish between high cardiomyocyte number from improved and normal/control culture conditions. It must be done at the time zero (with stem cells, before induction of cardiomyocyte differentiation), and in the middle time (when stem cells get cardiomyocyte fate).

We have measured cell yields and apoptosis during the differentiation process using nuclei counts and Annexin V expression marker respectively (details in page 7, Materials and Methods). We found high levels of apoptosis (60%) during the first day of growth which correlated with some cell death probably as a result of mechanical manipulation of the culture during seeding. Thereafter, gradual increase in cell yields and down regulation of apoptotic markers were observed. These data were added on page 10 and Supplementary Figure 2.

Second, they must also perform experiments during cardiomyocyte differentiation testing for proliferation rate. It must be done at the time zero (with stem cells, before induction of cardiomyocyte differentiation), and in the middle time (when stem cells get cardiomyocyte fate).

Cell proliferation was evaluated by measurement of Ki-67 expression (proliferation marker) (See page 7 in materials and methods). At Day 2 of differentiation, 12% increase in Ki-67 expression was observed in cultures differentiated in SupSB medium compared to SB medium. By day 4 onward, Ki-67 expression was similar in both cultures. This sentence is added on page 11-12.

Third, it would be interesting if they also repeat real time PCR for another cell differentiatied markers as for neuronal cells, fibroblast, adipocyte, and include ANP, BNP and MYH7, to verify cardiomyocyte normal physiological conditions.

In Figure 4, using electrophysiology methods, it was shown that cardiomyocytes cultured in SupSB medium present the three sub phenotypes (nodal-, atrial- and ventricular-like) indicating cardiomyocytes with normal physiological conditions.

Moreover, cells grown in SupSB medium were used in the analysis of electrical stimulation effects on cardiomyocyte maturation by Prof. Hung Fat Tse's group. In this work, qRT-PCR was done using cardiomyocyte maturation markers such as SCN5A (sodium channel), Kv4.3 (potassium channel), MLC2v (Myosin Light Chain Ventricular), and SERCA (Ca^{2+} ATPase). Their results shows that-cells grown in SupSB medium can develop into mature cardiomyocytes and has been submitted as a separate paper.

I also inquire to the authors to characterize the cell differentiated karyotype, as stem cells after so many passages can present cell cycle arrest (it can be answered with cell apoptotic and proliferation assays) and aneuploidy.

Differentiated HES-3 cell show normal karyotype (see supplement Figure 5)

Reviewer 2

In the Results section, Identifying nutritional supplements that can improve cardiomyocyte differentiation subsection, the authors mention >85% viability with the supplements but only 65% under control conditions. How was viability measured? It would be worth doing apoptosis assays here, such as TUNEL staining or flow cytometry for annexin V.

The viability was measured by nuclei count (see page 7, Materials and Methods). Moreover, we have evaluated apoptosis using Annexin V expression and cell proliferation using Ki-67 expression (by flow cytometry). This issue has been addressed in reviewers 1 response (comment no.1).

In the Results section, Kinetics of cell growth and marker expression during HES3 differentiation subsection, the authors have not looked at expression of Isl1, an established marker of cardiac progenitors. It would be worth looking at that to help establish a mechanism.

We have used Nkx2.5 as a cardiomyocyte progenitor marker (Figure 2). This marker has been used by many other groups for identification of cardiomyocyte progenitors (see also refs 2,4,5,6,8).

In Table 1, there are substantial discrepancies between different human pluripotent stem cell lines regarding cardiomyocyte yields. I was puzzled by the fact that the H1 line in SB media gives 20% SA+ cells but only 5% MHC+ cells. What does that mean? What is the difference between SA+/MHC+ double positives and SA+/MHC- cells? Is there an electrophysiological or contractility difference? It is clear that SupSB in this line makes essentially all SA+ cells also + for MHC. Here are some potential mechanisms for this:

- **SupSB turns on MHC promoter**
- **SupSB facilitates or speeds up cardiomyocyte maturation.**

What's even more puzzling is that SupSB does not have this effect on the MHC population in HES3 and Siu1-hIPSC lines. A discussion of these issues would be helpful.

During the early stages of myocyte differentiation, all of the sarcomeric proteins are accumulated diffusely in the cytoplasm. This includes the sacromeric a-actinins and together the sarcomeric proteins form the Z lines, which are hypothesized to be the organizing structures during myofibrillogenesis. The myosins appear at a later stage of differentiation and form the myosin thick filaments (Gregorio et al. 2000). Thus, cells expressing higher levels of a-actinin protein as compared to myosin(a ratio of 16:1 compared to 1:1, SA:MHC) indicates cells in an earlier differentiation stage These different ratios of expression levels of a-actinin and myosin in our cultures demonstrate different stages in the development of sacromeres.

This issue has been described in page 13, Discussion.

***Gregorio CC**,Antin PB. To the heart of myofibril assembly. Trends Cell Biol 2000; 10: 355-362 [PMID: 10932092]*

In the introduction, 2nd paragraph, a quick explanation of the mechanism of action of MAPK inhibitors in cardiac differentiation would help the general audience.

The suggested mechanism of p38 inhibitor was added in page 4.

In the Results section, *Additive effect of HySoy and BSA supplements…* subsection, the increases in cardiomyocyte yield (16.5% vs 10-12%) do not much the data presented in the Table.

In Table 1, the combined effect of HySoy and BSA is demonstrated. The effect of individual supplement is presented in Figure 2. From this figure, it can be seen that addition of either resulted in a 10-12% expression of MHC but the combined effect of HySoy and BSA resulted in 16.5% expression of MHC.

In the Results section, *Additive effect of HySoy and BSA supplements…* subsection, the population statistics with different batches of BSA and HySoy are not presented.

Results presented in Table 1 for HES-3 cells were done using different batches of HySoy and BSA (n=9).

In the Results section, *Kinetics of cell growth and marker expression during HES3 differentiation* subsection, the increase in MHC gene expression is not significant. Would a bigger n fix that problem?

It is true that using triplicate measurements we did not see significant increase in the late gene expression MHC. However, we observed significant increase in early cardomyocyte gene marker Nkx2.5.

Reviewer 3

The manuscript must be revised to avoid grammatical errors.

The paper was once again reviewed by 2 additional readers and the English improved where needed.

3 New references have been added and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Stem Cells.*

Sincerely yours,

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