

JoVE58370 Response to Reviewers

We thank the Reviewers and Editors for their time and consideration given to our manuscript. Please find our point-by-point responses below.

Editorial Comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been carefully proofread by all authors and all noted errors and misspellings have been eliminated.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

A pdf file of the e-mail correspondence regarding the illustration of experimental results from our previous study (Mochizuki et al., *J Am Heart Assoc* 2017) has been added. Please note that this is not a modification of a previously published Figure in the proper sense, as we generated a new Figure from a set of experiments that is distinct from the one the results shown in our previous publication were retrieved.

3. Figures 1 and 3: Please line up the panels better. Some panels are off-set in Figure. Please ensure that the panels are of the same dimensions if possible.

The Figures have been revised accordingly.

4. Figure 2: Please include a space between numbers and their corresponding units (e.g., 2-3 days, 24 h, etc.).

Spaces have been added.

5. Figure 5: Please make the scale bar darker or thicker so it is easier to read.

The scale bars have been adjusted.

6. Table 2: Please combine the material information in Table 2 into the Table of Materials and Reagents.

The information of former Table 2 is now integrated in the Table of Materials and Reagents.

7. Please revise the Abstract to focus on the method being presented rather than the results of a specific experiment. Include a statement about the purpose of the method. A more detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate. Please focus on the general types of results acquired.

The abstract has been rewritten. Specifically, the purpose, advantages and limitations of the technique have been added, and possible applications are mentioned. The changes are highlighted in yellow in the tracked-changes version of the manuscript.

8. Please use SI abbreviations for all units: L, mL, μ L, h, min, s, etc.

The units have been adjusted.

9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Spaces have been added.

10. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Centrifuge speeds are now given in x g.

11. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: BioLegend #420301, lumox®, Sigma #L2020, Lonza #CC4132, Corning #354230, etc.

Commercial information has now been removed from the text body of the manuscript.

12. The Protocol should contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” Please revise lines 124-130 and 191-200, etc.

The indicated lines as well as the rest of the Protocol have been revised accordingly, and part of the information has now been incorporated as Notes where appropriate. These are highlighted in yellow in the tracked-changes version of the manuscript.

13. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

The Protocol has been thoroughly revised and details with respect to smaller experimental steps have been added. In addition, we created a new Figure (**Figure 2**) to depict the preparation of the different samples for staining before flow cytometry. Major changes are highlighted in yellow in the tracked-changes version of the manuscript.

14. 1.2: Please add more details to this step. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action. Please specify all surgical instruments used.

The previous step 1.2 is now described in more detail and divided into four action points (1.2-1.5). Action points 1.1-1.5 now read:

- 1) Inject each mouse with 200 mg/kg of pentobarbital i.p. and wait until it is fully anesthetized by checking the response to toe pinch.
- 2) Wipe the chest with 70% ethanol.
- 3) Cut the skin and thoracic wall with scissors to expose the thoracic cavity.
- 4) Lift the heart with forceps and cut it at the base using scissors.
- 5) Put the heart in a P100 with 25 mL (5 mL for P60) of cold PBS (3-5 hearts/P100 or 1-2 hearts/P60).

15. 1.5: What tool is used to remove the atria?

This is now action point 1.8. We used small scissors to remove the atria and 1.8 now reads:

- 8) Remove the atria using small scissors.

16. 1.6: What is used to wash, presumably cold PBS? What volume is used?

This is now action point 1.9, which now reads:

9) Cut the heart into 2 longitudinal pieces and wash them again in ice-cold PBS (25 mL/P100, 5 mL/P60).

17. 6.1.1: Please write the text in the imperative tense in complete sentences.

The Protocol text has been revised and action points are now written in the imperative tense.

18. 6.2.2: Please provide the conditions for trypsinizing cells. Please specify throughout.

More detail has now been added to the trypsinization steps in 6.II.2 and 6.III.2. These changes are highlighted in yellow in the tracked-changes version of the manuscript.

19. 6.2.4: How to collect supernatant? After centrifugation? Please clarify.

This is now step 6.II.5. The purpose of this step is to collect dead cells popping up from the culture plate and floating in the medium. This step now reads:

5) Collect the medium from the dish of step II.4 for collection of dead cells into a 15 mL tube after 2 days of culturing.

20. References: Please do not abbreviate journal titles.

Although importing the newest JoVE endnote file provided by JoVE with correct coding of bibliographic listing including full journal titles, we were not able to generate a correct bibliography on our computer using Endnote X8. The references have therefore been adjusted manually to our best of knowledge to comply with the journal requirements.

Reviewers' Comments:

Reviewer 1:

We thank the Reviewer for his/her time and thoughtful comments.

1. The authors should not just rely on the tube formation assay to show successful endothelial differentiation. I suggest they stain the cells/tubes for CD31 or vWF to show they are endothelial cells.

We agree with the Reviewer and have now added two additional Figure panels (new **Figure 7C and D**), which show that whereas vWF is expressed in virtually all cells after three weeks in endothelial differentiation medium, the intensity of immunoreactivity is substantially higher in cells differentiated on LN as compared to FN.

2. The authors need to include in Table 1, the references Vicinanza et al. 2017, Cell Death & Diff and Smith et al. 2014, Nat Prot, for ckit+/ CD45-/CD31- CPCs isolated from mouse heart.

The indicated References have now been included.

3. Table 1, the authors should state that the Isl+CPCs are found in the embryonic or fetal myocardium, and not in the adult.

A footnote stating "Isl-1 is found in the embryonic or fetal myocardium, not in the adult heart" has been added.

4. Line 88, remove the part of sentence about the controversy surrounding c-kit+ cells as it is not relevant to this manuscript. What would be good to write is that c-kit is expressed by a variety of cell types, including mast cells, endothelial cells and HSCs. The CPCs express c-

kit at a lower level than all these other cell types, and therefore are regarded as c-kit low (Vicinanza et al. 2017, CDD).

The paragraph has been rephrased accordingly and the changes are highlighted in yellow in the tracked-changes version of the manuscript.

5. Line 103, re-word what is written about CDCs, and delete the last sentence of this paragraph (line 104-105) starting with 'They mostly consist of undifferentiated....' as this is incorrect and was not shown in the Beltrami et al. 2003 Cell paper. The CDCs are a heterogeneous cell population composed of clusters of cells (mainly smooth muscle cells, fibroblasts, endothelial cells) which have outgrown from tissue biopsies/explants in culture.

We agree that the reference to the Beltrami paper was misplaced. The sentence has now been removed and replaced by the characterization of the cardiomyogenic fraction of CDCs as described by Cheng K et al., published in J Am Heart Assoc in 2014. This change is highlighted in yellow in the tracked-changes version of the manuscript.

6. Fig 2 and Line 300, it states that steps II and II of Figure 2, but they are no steps numbered in Figure 2.

This has now become Figure 3. The steps I and III have now been added to the Figure as appropriate.

7. Line 341, you cannot write here that 'These results indicate that a low cell density on LN with 0.1% serum may be suitable for induction of cardiac lineage commitment in CPCs' as a concluding sentence as you have not tested this yet. It would be better to write that a low cell density on LN with 0.1% serum decreases proliferation without affecting CPC viability.

These are now lines 376 and 377. We agree with the Reviewer and the sentence has been rephrased as suggested.

8. Line 349, use different words to describe the 'huge round shapes' cells in the culture. Perhaps write 'cells that were larger and different in morphology to the other cells were present at 7-14 days'. Also, why did these cells disappear towards the end of the differentiation phase? Could the authors perform some phenotyping to determine what type of cell these large cells are? They also appear in the high density LN condition and the FN high density condition at day 17.

We agree with the Reviewer that the wording was not ideal and rephrased the paragraph as follows:

“As shown in the white dashed circles in Figure 6A, within 7-14 days in endothelial differentiation medium, successful cultures contained cells that were larger and different in morphology to the other cells. Interestingly, these cells disappeared towards the end of the differentiation phase and also appeared in lower numbers and at later time points in high density cultures on LN and FN. Whereas we did not further characterize these cells, this protocol suggests...”

As stated in the revised paragraph, we did not perform any further phenotyping of these cells. The more round as opposed to the spindle-like morphology may suggest that some of these cells may adopt an epicardial-like phenotype after culturing under low serum conditions, which has previously been described for iPS cells cultured with low albumin (Bao et al., Nat Biomed Eng 2016) and such phenomenon may occur more robustly in cultures on LN as opposed to FN. Epicardial cells have a high propensity to differentiate into endothelial cells, which would also explain the disappearance of such cells over time.

We examined whether the CPCs used in our previous report might become epicardial cells under basal culture conditions, but the lack of expression of typical markers (except for Tbx18) did not support that notion (Supplemental information FigureS4 B in Mochizuki et al., 2018 JAHA). Similarly, the mouse SP-CPCs used in this study expressed Tbx18 but no other epicardial markers under basal experimental conditions (s. Figure provided to the Reviewers only), suggesting that these cells are not epicardial cells to begin with. This, however, does not preclude that these cells or some of them may transition through an epicardial-like phenotype during the endothelial differentiation process.

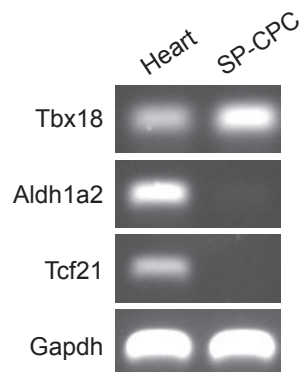


Figure: Gene expression of epicardial markers. mRNA was isolated from SP-CPC and mouse whole heart, then cDNA was generated and PCR was performed. Aldh1a2: aldehyde dehydrogenase 1 family member a2; Tcf21: transcription factor 21; Heart: RNA from adult mouse heart serving as positive control.

9. Line 361, it states 'the cells differentiated in high density cultures formed wider tubes' however when looking at Figure 6A and B this doesn't necessarily appear to be the case. Have the authors measured the width of the tubes?

We agree with the Reviewer that the term “wider tubes” is not based on a respective standardized assessment of the tubes. We rephrased the paragraph to describe the tube formation in a more qualitative rather than quantitative way. The paragraph now reads:

“...successful tube formation was consistently shown by cells plated at low density and differentiated on LN, whereas tube formation mostly failed by cells plated on LN at high density (**Figure 7A**). Similarly, tube formation was mostly unsuccessful in cells cultured on FN irrespective of cell density, although cells differentiated on FN at low density sometimes formed rudimentary tubes depending on the cell condition (e.g., isolate and/or passage number, **Figure 7B**).

10. Line 416, please provide a reference for CPCs from humans.

Reference of CPCs isolated from humans has been added.

11. Line 420, Please include original SCIPIO clinical trial reference for the sentence 'CPCs are currently in clinical testing for cell therapy'.

References to the SCIPIO and CADUCEUS trials have been added.

12. Line 450, it is the first time that incubation time is described as being critical for the protocol to be a success. Please can you explain in more detail.

Cell fate decisions are executed by dynamic shifts in the transcriptome that occur during specific phases of the cell cycle. Cell cycle lengthening allows for a fate-switch from proliferation to lineage commitment and differentiation during the prolonged gap phases and this has been shown to involve cell cycle regulatory genes (Skapek SX et al., Science 1995, Discher DE et al., Science 2009, Soufi A, Development 2016). Because we previously

identified downregulation of the cell cycle regulator Plk2 as a crucial step in early lineage commitment of CPCs, we sought for culture conditions, which slow down proliferation without, however, affecting cell viability. To achieve this goal, both serum concentration as well as incubation time under low serum conditions had to be fine-tuned to guarantee for maintained viability and successful induction of the cell fate switch. Interestingly, the use of low (or no) serum to induce differentiation has previously been advocated in other stem cells (e.g., Watanabe et al., Nat Neurosci 2005), but differences in cell robustness (or vulnerability) require adjustments in the length of serum-deprivation, i.e., in “low serum incubation time”.

13. Figure 1G - it is difficult to determine from the image that these are tubes wrapped in foil, perhaps a picture showing the tubes about to be wrapped in foil should be included.

Whereas we did not include a picture showing how the tubes are being wrapped, we added a more detailed description of what is depicted in the different panels to the Figure Legend of Figure 1. The description for Panel 1G now reads:

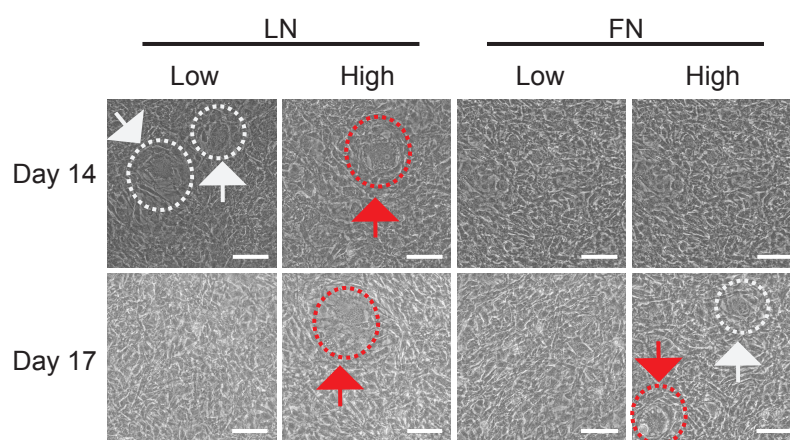
(G) Gentle reversal of a 50 mL conical tube containing the cardiomyocyte-depleted cell suspension and wrapped in tin foil for light protection after addition of Hoechst.

14. Figure 1I - Can the authors provide any data on what would happen if they took the Sca1⁺CD31⁺SP cells and placed them in the low density, low serum endothelial differentiation and tube formation assay conditions, would they behave as endothelial cells and make tubes? These would be a good cell control.

As per our experience, adherence of Sca1⁺CD31⁺-SP cells to culture plates upon isolation is extremely low and these cells cannot be successfully amplified *in vitro* as can the CD31⁻ fraction.

15. Figure 5 - please circle all the huge rounded cells of each picture. I can see them in LN day 14, high and LN day 17 High, FN day 17 high. Also, please increase in width the scale bar so it is more clear.

Figure 5 has been adjusted accordingly. However, please note that some of these cells (circled in red in the Figure to the Reviewers below) exhibit vacuolization and may undergo cell death and therefore might be of a different phenotype than the ones described in low density LN cultures. Nevertheless, for easier reading, all these cells have now been highlighted by white dashed circles in the original version of the manuscript.



Minor:

1. Line 76, re-word 'there are many different types of CPCs' as this is not necessarily true. It would be better to write, many different markers have been used to identify CPCs.

The sentence has been rephrased and CPCs identified based on different markers are now referred to as different “subpopulations”.

2. Lines 164 and 169, state why the 100um and 40um filters are used.

The 100 um filter has been used to remove undigested tissue residues and the 40 um filter to exclude larger cells including residual cardiomyocytes in order to obtain a cardiomyocyte-depleted cell suspension from the heart. This information has now been added to the protocol (highlighted in yellow in the tracked-changes version of the manuscript).

3. Line 183, does the centrifuge need to be on a brake?

No. This has now been specified: “Centrifuge the tube at 4000 g for 5 min at RT (without brake)”.

4. Figure legends for 3 and 4, need to state whether data is Mean +/- SD or SEM

We used SEM. This information has now been added to the Figure Legends.

Reviewer 2:

We thank the Reviewer for his/her time and thoughtful comments.

1. There are inadequate details on the 'trypsinization' of cells. What trypsin should be used and how is the reaction stopped? What are the volumes involved and what tube is used to spin cells down? The centrifugation description needs to include more than rpm and should include calculated Xg.

More detail has now been added to the trypsinization steps in 6.II.2 and 6.III.2, and rpm have now been converted into centrifugal force (x g). These changes are highlighted in yellow in the tracked-changes version of the manuscript.

2. There is inadequate characterization of the differentiated cell lineage from the protocol, presumably endothelial cells. The cells do not appear in culture as typical cobblestone endothelial cells. Furthermore, the tube formation assay does not show typical linear tubes, rather outgrows from cell clusters.

We agree with the Reviewer and more detail regarding the phenotype of these cells has now been added (s. below). We agree that our cells do not adopt a typical cobblestone pattern, but cell morphology is not necessarily a useful indicator of successful endothelial differentiation. Whereas SP-CPCs are a heterogeneous cell population, we also cannot exclude that there is indeed partial outgrowth of a subfraction of cells in the tube formation assay, in particular if cell aggregates are present at the time the cells are placed into matrigel. We have repeated the tube formation assay several times and revised the respective Figure (now **Figure 7**) to include results representative of all repeats, whereby negative outcome is depicted for conditions, which only inconsistently produced tubes. This new **Figure 7A and B** shows that there is tube formation of cells induced at low density on LN before differentiation, but not consistently in all other conditions, whereas there still might be some outgrowth of cells as well.

a. What percentage of the cells are CD31+ or positive for other markers as CD144 (VE-cadherin)?

After three weeks in differentiation medium, virtually all cells are immuno-reactive for von Willebrand Factor (vWF), whereby this again was much more pronounced in cells induced under low serum conditions on LN as opposed to FN. An additional Figure showing vWF expression after three weeks in endothelial differentiation medium has now been added (new Figure 7C and D).

b. Given that the tube formation assay is only one assay with questionable results, can the authors provide other functional assays such as uptake of acetylated LDL?

Whereas we tried to establish suitable culture conditions to perform the LDL uptake assay, we did not manage to establish a stable protocol to perform these studies within the time given for the revisions. This was mainly due to the fact that we plate our cells at low density (precluding for instance the use of 96-well plates for viability issues, s. results below) and that each differentiation step is taking 3 weeks a minimum, even if not counting the time for amplification and lineage induction. However, given that both, expression of endothelial markers (now shown for vWF) and tube formation, are standard assays and that the cells were differentiated in dedicated endothelial differentiation medium, we are confident that our cells exhibit endothelial cell properties.

The provided Figure shows that we were not able to maintain cell viability in culture settings traditionally considered suitable for LDL uptake assessment, and future attempts will have to aim at the use of larger plates and will require more time-consuming technical adaptations.

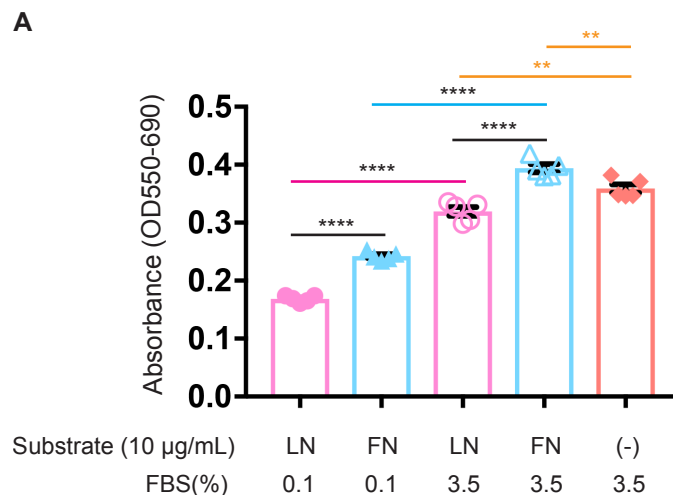
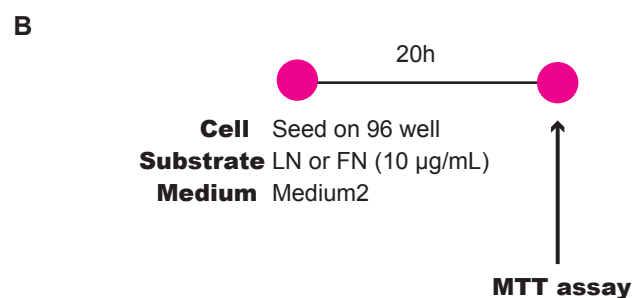


Figure: Viability of SP-CPCs under culture settings that are considered suitable to assess LDL uptake: (A) MTT assay with SP-CPCs. (B) Protocol. Cells were seeded at low density on a 96 well plate precoated with LN or FN with Medium 2 (including 0.1% FBS) for 20 h, then the MTT assay was performed. No substrate (-) and 3.5% FBS-containing medium were used as positive controls. Data are given as mean \pm SEM, ** $P < 0.005$, **** $P < 0.0001$ (t-test $n=5$).



Minor:

1. Line 57 states "After culturing for 2-3 days in medium including supplements that maintain pluripotency..." CPCs are typically regarded as multipotent but not pluripotent which is generally used to describe ES cells and iPS cells.

The term pluripotency has been replaced by multipotency.

2. Line 276 - need space between "2000rpm"

Rpm have now been converted in x g and spaces have been added between all numbers and units throughout the manuscript.

3. Line 327 - "we used FN and LN (both 10 ug/ml)..." Need to specify what size plate as ECM and culture surface area is key variable

Plate sizes have now been added to all protocol steps as appropriate. For this specific purpose we used 6-well plates (growth area 9.6 cm²/well), which is now indicated in the manuscript.