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Induction of endothelial differentiation using cardiac progenitor cells under low serum conditions

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Cover Letter

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Indrani Mukherjee, PhD
Science Editor
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Basel, August 30, 2018

Revised manuscript 58370-R1 "Induction of endothelial differentiation using cardiac progenitor cells under low serum conditions"

Dear Dr. Mukherjee,

We would like to thank you and the Reviewers for their time and helpful comments to improve our manuscript. The manuscript has been thoroughly revised based on the Reviewers' and Editors' suggestions.

Major changes include:

- We now provide more extensive characterization of the cells after endothelial differentiation. In particular, immunocytochemistry for von Willebrand factor has been performed and representative images are provided in a new Figure 7C and D. In addition, all differentiation experiments were repeated multiple times and more representative results from the tube formation assay are given, whereby in cases of inconsistent results (or less than 100% successful tube formation) the negative outcome data are now provided.
- Important technical details for minor experimental steps have now been added and the workflow leading up to flow cytometry is now illustrated in an additional Figure (new Figure 2). Furthermore, a new Figure Legend to Figure 1 describes each step illustrated in more detail, and in Figure 3 (former Figure 2) the respective experimental steps are indicated.
- The Abstract has been revised to include the purpose, advantages and limitations of the protocol as well as possible applications.
- All protocol steps have been rephrased in the imperative tense and comments that could not be put in imperative tense have been included as "Notes".

Please note that the illustration of results (now Figure 8) that were obtained within the scope of experiments conducted for the previous paper (Mochizuki et al., J Am Heart Assoc 2017), which was published under a Creative Commons Attribution-NonCommercial-NoDerivs License, was approved by JAHA staff in an e-mail correspondence, which is enclosed as a pdf-file.

The revised version of the manuscript has been approved by all authors, and we hope that it is now acceptable for publication in JoVE.

Thank you very much and kind regards,

Gabriela M. Kuster Pfister, MD

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Basel, September 13, 2018

Revised manuscript 58370-R1 "Induction of endothelial differentiation using cardiac progenitor cells under low serum conditions"

Dear Dr. Mukherjee,

Please find enclosed our manuscript as per August 30 including now the additional formatting and specifications requested by the Editors and detailed in our Response to Editorial Comments.

We thank you for your effort and hope that the manuscript is now acceptable for publication in JoVE.

Thank you very much and kind regards,

Gabriela M. Kuster Pfister, MD

TITLE:

Induction of Endothelial Differentiation in Cardiac Progenitor Cells Under Low Serum Conditions

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KEYWORDS:

Cardiac side population cells, cardiac progenitor cells, endothelial differentiation, low serum condition, supplement-free culture medium, low cell density culture, differentiation medium, extracellular matrix

SUMMARY:

This protocol describes an endothelial differentiation technique for cardiac progenitor cells. It particularly focuses on how serum concentration and cell-seeding density affect the endothelial differentiation potential.

ABSTRACT:

Cardiac progenitor cells (CPCs) may have therapeutic potential for cardiac regeneration after injury. In the adult mammalian heart, intrinsic CPCs are extremely scarce, but expanded CPCs could be useful for cell therapy. A prerequisite for their use is their ability to differentiate in a controlled manner into the various cardiac lineages using defined and efficient protocols. In addition, upon *in vitro* expansion, CPCs isolated from patients or preclinical disease models may offer fruitful research tools for the investigation of disease mechanisms.

Current studies use different markers to identify CPCs. However, not all of them are expressed in humans, which limits the translational impact of some preclinical studies. Differentiation

protocols that are applicable irrespective of the isolation technique and marker expression will allow for the standardized expansion and priming of CPCs for cell therapy purpose. Here we describe that the priming of CPCs under a low fetal bovine serum (FBS) concentration and low cell density conditions facilitates the endothelial differentiation of CPCs. Using two different subpopulations of mouse and rat CPCs, we show that laminin is a more suitable substrate than fibronectin for this purpose under the following protocol: after culturing for 2 - 3 days in medium including supplements that maintain multipotency and with 3.5% FBS, CPCs are seeded on laminin at <60% confluence and cultured in supplement-free medium with low concentrations of FBS (0.1%) for 20 - 24 hours before differentiation in endothelial differentiation medium. Because CPCs are a heterogeneous population, serum concentrations and incubation times may need to be adjusted depending on the properties of the respective CPC subpopulation. Considering this, the technique can be applied to other types of CPCs as well and provides a useful method to investigate the potential and mechanisms of differentiation and how they are affected by disease when using CPCs isolated from respective disease models.

INTRODUCTION:

Recent studies support the existence of resident cardiac progenitor cells (CPCs) in the adult mammalian heart[1-3], and CPCs could be a useful source for cell therapy after cardiac injury[4,5]. In addition, expanded CPCs may provide a fruitful model for drug screening and the investigation of disease mechanisms when isolated from patients with rare cardiomyopathies, or from respective disease models[6,7].

CPCs isolated from the adult heart possess stem/progenitor cell characteristics[1-3,8] as they are multipotent, clonogenic, and have the capacity for self-renewal. However, there are many different (sub)populations of CPCs exhibiting different surface marker profiles, including, for instance, c-kit, Sca-1, and others, or retrieved by different isolation techniques (**Table 1**). Several culture and differentiation protocols have been established[1,2,8-18]. These protocols vary mostly with respect to the growth factor and serum content, which are adjusted according to the purpose of the culturing and which can lead to differences in results and outcomes, including differentiation efficiency.

Marker-based Isolation Techniques:

CPCs can be isolated based on a specific surface marker expression[1,2,8-18]. Previous studies suggest that c-kit and Sca-1 may be the best markers to isolate resident CPCs[1,11,14,19,20]. Because none of these markers is truly specific for CPCs, combinations of different markers are usually applied. For example, whereas CPCs express low levels of c-kit[21], c-kit is also expressed by other cell types, including mast cells[22], endothelial cells[23], and hematopoietic stem/progenitor cells[24]. An additional problem is the fact that not all markers are expressed across all species. This is the case for Sca1, which expresses in mouse but not in human[25]. Therefore, using protocols that are independent of isolation markers may be advantageous in view of clinical trials and studies using human samples.

Marker-independent Isolation Techniques:

There are several major techniques of CPC isolation which are primarily independent of surface marker expression but which can be refined by the consecutive selection of specific marker-positive subfractions as needed (see also **Table 1**). (1) The side population (SP) technique has originally been characterized in a primitive population of hematopoietic stem cells based on the ability to efflux the DNA dye Hoechst 33342[26] by ATP-binding cassette (ABC) transporters[27]. Cardiac SP cells have been isolated by different groups and reported to express a variety of markers with some minor differences between reports[2,8,13,14]. (2) Colony-forming unit fibroblast cells (CFU-Fs) have originally been defined based on a mesenchymal stromal cell (MSC)-like phenotype. Isolated MSCs are cultured on dishes to induce colony formation. Such colony-forming MSC-like CFU-Fs can be isolated from the adult heart and are capable to differentiate into cardiac lineages[15]. (3) Cardiosphere-derived cells (CDC) are single cells derived from clusters of cells grown from tissue biopsies or explants[28-31]. It was recently shown that mostly the $CD105^+/CD90^-/c\text{-}kit^-$ cell fraction exhibits cardiomyogenic and regenerative potential[32].

Here, using SP-CPCs isolated from mice, we provide a protocol for the efficient induction of endothelial lineage based on a previous study in rat CPCs and mouse SP-CPCs[33]. The protocol contains specific adaptations to the culture and expansion technique with respect to the cell density, the serum content of the medium, and the substrate. It can be applied not only to mouse SP-CPCs but to different types of CPCs for the purpose to induce a fate switch from an amplifying to an endothelial-committed CPC, be it in view of transplantation of these cells or their use for mechanistic *in vitro* studies.

PROTOCOL:

The use of mice for cell isolation purpose was in accordance with the Guide for the Care and Use of Laboratory Animals and with the Swiss Animal Protection Law and was approved by the Swiss Cantonal Authorities.

NOTE: The isolation of $Sca1^+/CD31^-$ SP-CPCs from the mouse heart was essentially done as previously described[34] with some modifications. For the materials and reagents used, see **Table of Materials**. For all experiments, cardiac SP-CPCs isolated from mice were amplified, passaged, and used in a cell line-like manner. Passages 7 - 20 were used for this study.

1. Tissue Preparation

NOTE: All experiments using mice must be carried out according to the guidelines and regulations. This protocol uses four mice. Culture plates that are 100 mm in diameter are described as P100 and culture plates that are 60 mm in diameter are described as P60 for the following parts of the protocol.

1.1) Inject each mouse with 200 mg/kg of pentobarbital intraperitoneally (i.p.) and wait until it is fully anesthetized by checking its response to toe pinching.

1.2) Wipe the chest with 70% ethanol. Cut the skin and thoracic wall with scissors to expose the thoracic cavity.

1.3) Lift the heart with forceps and cut it at the base using scissors. Put the heart in a P100 with 25 mL (5 mL for P60) of cold phosphate-buffered saline (PBS) (three to five hearts per P100 or one to two hearts per P60).

1.4) Pump the heart with forceps to eject the blood out of the cavities (**Figure 1A**).

1.5) Put the heart in a P100 with 25 mL (5 mL for P60) of cold PBS for washing. Remove the atria using small scissors. Cut the heart into two longitudinal pieces and wash them again in ice-cold PBS (25 mL/P100, 5 mL/P60).

1.6) Transfer the pieces to a new P100 with 25 mL (5 mL for P60) of cold PBS. Cut the pieces into smaller pieces using small scissors (**Figure 1B**). Add a drop of 1 mg/mL collagenase B diluted in Hank's balanced salt solution (HBSS) and mince the small pieces thoroughly with a sterile razor blade (**Figure 1C**).

2. Digestion

2.1) Add 10 mL (2.5 mL for P60) of the 1 mg/mL collagenase B solution to the dish from step 1.6.

2.2) Put the collagenase B solution containing the minced heart pieces in a tilted (about 30°) P100 (or P60) in a 37 °C incubator (**Figure 1D**).

2.3) Incubate the minced heart pieces for a maximum of 30 min; homogenize by repeatedly passing them through a Pasteur pipette every 10 min during the incubation (**Figure 1E**).

NOTE: It is important to not exceed 30 min of incubation in total for step 1.3.

3. Filtration

3.1) Add 10 mL (5 mL for P60) of cold HBSS supplemented with 2% FBS to the minced and homogenized heart pieces.

NOTE: HBSS supplemented with 2% FBS quenches collagenase B activity.

3.2) Filter the heart pieces through 100 µm filter to remove undigested tissue and centrifuge at 470 x *g* for 5 min at room temperature (RT) (**Figure 1F**, yellow filters).

3.3) Discard the supernatant and resuspend the pellet in 5 mL (3 mL for P60) of red blood cell lysis buffer, and incubate the pellet for 5 min with occasional shaking on ice.

3.4) Add 10 mL (5 mL for P60) of PBS (to stop the lysis reaction) and filter the sample through a 40 µm filter to exclude larger cells, including residual cardiomyocytes (**Figure 1F**, blue filters).

3.5) Centrifuge the tube at 470 x *g* for 5 min at RT (without brake). Discard the supernatant and resuspend the pellet in 1 mL of DMEM including 10% FBS.

3.6) Count the cells in an aliquot with a hemocytometer. Resuspend the cells with DMEM including 10% FBS, aiming at a final cell concentration of 1 x 10^6 cells/mL.

NOTE: Roughly 5 x 10^6 cardiomyocyte- and erythrocyte-depleted cells can be estimated per mouse.

3.7) Distribute the cells into two tubes (**Figure 2**): in **tube A**, add 1.5 mL for Hoechst 33342 staining with verapamil; in **tube B**, add 17.5 mL for Hoechst 33342 staining and proceed to stain and sort (steps 4.1 and 4.2) cardiac SP cells by flow cytometry.

4. Sorting of Cardiac SP Cells by Flow Cytometry

NOTE: Verapamil inhibits Hoechst efflux by blocking multidrug resistance (MDR) ABC transporter activity. Hoechst 33342 is a DNA-binding dye that can be used in living cells to detect the cell cycle as it correlates with the DNA content. Hoechst-33342-extruding cells appear in the Hoechst low part of both emission channels (450 nm, Hoechst blue; 650 nm, Hoechst red), that is, aside of the Hoechst-retaining "main population", giving them their name "side population". SP cells are enriched in cells with progenitor properties and show a high expression of multidrug-resistant ABC transporters (such as MDR1 and ABCG2). Hoechst 33342 is written as Hoechst for the following parts of the protocol. It is important to protect light-sensitive materials for ideal results.

4.1) Staining with Hoechst in the presence and absence of verapamil

4.1.1) Add verapamil (with a final concentration of 83.3 µM) and Hoechst (with a final concentration of 5 µg/10^6 cells) to the cell solution. For **tube A**, use Verapamil and Hoechst; for **tube B**, use Hoechst only. Incubate in a water bath (at 37 °C) for 90 min and revert the tubes every 20 min (**Figure 1G**).

4.1.2) Centrifuge the tubes at 470 x *g* for 5 min at RT. Discard the supernatant and resuspend each pellet in HBSS (1 x 10^6 cells/mL).

4.1.3) Take a 1.5 mL aliquot for single stainings and negative control from **tube B** (**Figure 2**): (i) fluorescein isothiocyanate (FITC)-conjugated anti-Sca-1; (ii) allophycocyanin (APC)-conjugated anti-CD31; (iii) nonstained cells (negative control).

NOTE: Isotype controls are used here for setting up the isolation protocol.

4.1.4) Centrifuge **tubes A and B** and the single-staining and negative control aliquots at 470 x *g* for 5 min at RT for washing out Hoechst and verapamil.

4.1.5) Discard the supernatant and resuspend the pellets of **tube A** and (iii) the negative control in 250 µL of HBSS and keep them on ice in the dark until sorting.

4.1.6) Resuspend the pellet of **tube B** in 200 µL of HBSS and the pellet of (i, ii) the single-staining aliquots in 100 µL of HBSS. Add FITC-conjugated anti-Sca-1 (0.6 µg/10^7 cells) and APC-conjugated anti-CD31 (0.25 µg/10^7 cells). Incubate the pellets for 30 min on ice and shake them from time to time in the dark.

4.1.7) Add 2 mL of HBSS to the tubes. Centrifuge the tubes at 470 x *g* for 5 min at RT.

4.1.8) Discard the supernatant and resuspend all pellets with 1 mL of HBSS and centrifuge as above. Discard the supernatants. Resuspend the pellet of the single-staining aliquots in 200 µL of HBSS. Resuspend the pellet of **tube B** in HBSS (20 x 10^6 cells/mL).

4.1.9) Keep the samples on ice and protected from light until sorting.

4.2) Sorting with flow cytometry

4.2.1) Prepare sterile 1.5 mL sorting tubes with 500 µL of HBSS including 2% FBS.

4.2.2) Stain the cells with 7-aminoactinomycin D (7-AAD) (0.15 µg/10^6 cells) on ice for 10 min to exclude dead cells.

4.2.3) Sort the cardiac SP using the following settings: excite Hoechst using 350 nm (UV) excitation, collect fluorescence emission with a 450/50 nm band-pass filter (Hoechst Blue) and a 670/30 nm band-pass filter (Hoechst red), and use a nozzle size of 100 µm and pressure of 15 psi.

4.2.4) For analysis, record 5 x 10^5 events for the sorting samples and 1 x 10^5 events for the negative control, verapamil control, and single-staining samples.

NOTE: The cardiac SP is around 0.5% - 2% (**Figure 1H**) but may vary between laboratories and isolates. The $Sca1^+/CD31^-$ fraction is around 1% - 11% of the total cardiac SP (**Figure 1I**) but may vary between laboratories and isolates. $Sca1^+/CD31^-$ SP-CPCs are written as SP-CPCs for the following parts of the protocol.

5. Primary Culture of Isolated SP-CPCs

NOTE: Three different types of media were used in this protocol. They are referred to as Medium 1 (according to Noseda *et al.*)[8], Medium 2, and Medium 3 and are described in the **Table of Materials** regarding their composition.

5.1) Warm up Medium 1 to 37 °C before use and cool down the centrifuge to 4 °C. Centrifuge the sorting tubes at 4 °C and 470 x *g* for 6 min and resuspend the cells in Medium 1.

5.2) Put the cells on a gas-permeable P60 dish with 4 mL of Medium 1. Change the medium every 3 d until the cells have reached 70% - 80% confluence.

NOTE: Step 5.2 may take around 2 - 3 weeks.

6. Expansion and Differentiation of SP-CPCs

6.1) Cell culture

6.1.1) Culture 3 x 10^5 SP-CPCs in 8 mL of Medium 1 in a T75 flask.

6.1.2) Incubate SP-CPCs at 37 °C with 5% CO_2 until 70% - 80% confluence, changing the medium every 2 - 3 d.

NOTE: The cell number should be modified according to the type of CPCs used, the doubling time, and the cell size.

6.2) SP-CPC growth and viability under different serum concentrations

6.2.1) Culture SP-CPCs for 2 - 3 d in T75 flasks with 8 mL of Medium 1. Carefully aspirate the medium and rinse gently with 5 mL of warm (37 °C) HBSS.

6.2.2) Treat the cells with 5 mL of Trypsin-EDTA for 5 min in the cell incubator, add 5 mL of Medium 1 to stop the Trypsin activity, and transfer the cell suspension to a 15 mL tube.

6.2.3) Centrifuge the cells at 470 x *g* for 5 min at RT.

6.2.4) Resuspend the cells in Medium 1 or Medium 2 (lineage induction medium) and plate 2.5 x 10^5 SP-CPCs on P60 dishes with 3 mL of medium containing different serum concentrations.

6.2.5) Collect the medium from the dish of step 6.2.4 for the collection of dead cells into a 15 mL tube after 2 d of culturing in Medium 1 or Medium 2.

6.2.6) Trypsinize adherent cells as in step 6.2.2 and collect the cell suspension into the 15 mL tube of step 6.2.5. Centrifuge the cells at 840 x *g* for 5 min at RT.

6.2.7) Aspirate the supernatant and add 1 mL of Medium 1 or Medium 2 for cell counting. Stain SP-CPCs with trypan blue (0.4%) and count trypan blue-positive (dead) and trypan blue-negative (viable) cells.

NOTE: The cell viability (step 6.2.7) is given as the trypan-blue negative cell number in relation to the total cell number.

6.3) Induction of endothelial differentiation

6.3.1) Precoat a (6-well) culture plate with 10 µg/mL of laminin (LN) or fibronectin (FN).

6.3.1.1) Make a substrate solution containing 10 µg/mL of LN or FN with F12 medium (or PBS). Add 2 mL of substrate solution to each well. Maintain the plate for 30 min at 37 °C.

6.3.1.2) Aspirate the solution from the plate and add 2 mL of PBS to each well until using it.

6.3.2) Aspirate the medium from the cells from step 6.1.2, rinse them gently with 5 mL of warm (37 °C) HBSS, treat them with 5 mL of Trypsin-EDTA for 5 min in the cell incubator, add 5 mL of Medium 1 to stop the Trypsin activity, and transfer the cell suspension to a 15 mL tube.

6.3.3) Centrifuge the cells at 470 x *g* for 5 min at RT. Aspirate the supernatant and add Medium 2 for cell counting.

6.3.4) Seed 8 x 10^4 cells per well on the coated plate with 3 mL of Medium 2 and keep it at 37 °C for 20 - 24 h. Change the medium to 3 mL of Medium 3.

NOTE: We recommend using medium containing a low serum concentration—and without supplements—for the first 20 - 24 h. We recommended using <60% cell confluence (*i.e.*, the cell number of step 6.3.4 has to be adjusted depending on the cell size and growth rate).

6.3.5) Culture the cells for 21 d and change the medium every 3 d.

6.3.6) Verify the endothelial nature of differentiated cells by staining them with an endothelial marker such as von Willebrand Factor (vWF) and performing fluorescence microscopy.

6.3.6.1) Wash the cells with 1 mL of PBS and fix the cells in 3.7% formaldehyde for 2 min at RT.

6.3.6.2) Permeabilize the cells with 0.1% Triton X in ddH_2O for 30 min and block it with 10% goat serum for 1 h at RT.

6.3.6.3) Incubate the cells with anti-von Willebrand factor antibody (1:100) for 48 h at 4 °C.

6.3.6.4) Wash the cells 3x with 1 mL of PBS, for 10 min each time. Incubate them with Alexa Fluor 546 goat anti-rabbit secondary antibody (1:500) for 1 h at RT in the dark.

6.3.6.5) Wash again 3x, for 10 min each, with 1 mL of PBS. Stain the cell nuclei with 4'6-diamidino-2-phenylindole, dihydrochloride (DAPI; 1:500) for 5 min at RT in the dark.

6.3.6.6) Wash the cells 3x, for 5 min each, with 1 mL of PBS. Mount the cells and store them at 4 °C until the fluorescence microscopy. Verify the staining using standard fluorescence microscopy.

NOTE: The culturing conditions (substrate, medium, supplemental reagents, and FBS concentration) of steps 6.1 and 6.3 are described in **Figure 3**.

6.4) Tube formation assay

6.4.1) Prepare a basement membrane matrix (*e.g.*, Matrigel, henceforth referred to as matrix) plate.

6.4.1.1) Thaw the matrix at 4 °C overnight.

6.4.1.2) Coat a 96-well plate with 100 µL of the matrix on ice.

NOTE: It is important to avoid any bubbles in the matrix. The plates have to be coated on ice to avoid the jellification of the matrix.

6.4.1.3) Maintain the plate at 37 °C for 30 min.

6.4.2) Aspirate the medium from the cells (after the completion of step 6.3.5), gently rinse the cells with 5 mL of warm (37 °C) HBSS, and treat them with Trypsin-EDTA for 5 min in the cell incubator. Add Medium 3 to stop the Trypsin activity and transfer the cell suspension to a 15 mL tube.

6.4.3) Centrifuge the cells at 470 x *g* for 5 min at RT. Aspirate the supernatant, add 1 mL of Medium 3, and pipet gently.

6.4.4) Filter the cells with a 35 µm cell strainer, if the cells are aggregated.

6.4.5) Count the cells and seed 2 x 10^3 to 4 x 10^3 cells in 100 µL of Medium 3 in each matrix-coated well. Keep the plate at 37 °C in the cell incubator for 16 h.

6.4.6) Take a picture with a bright-field microscope at 2X magnification.

NOTE: In the case of an incomplete trypsinization, prolong the exposure time to Trypsin-EDTA to a maximum of 7 - 8 min.

REPRESENTATIVE RESULTS:

Mouse SP-CPC Isolation:
In this study, we used mouse CPCs isolated according to the SP phenotype, whereas results from rat CPCs are modified and added from a previous report with permission (**Figure 8**)[33].

Cell Proliferation Under High and Low Cell Densities and with Different Serum Concentrations:
Our previous study showed that the mRNA expression of cardiac lineage markers changed within the first 24 h culturing step. It is known that the extracellular matrix affects cell fate decisions, including endothelial differentiation[35]. To explore suitable conditions for the facilitation of endothelial lineage commitment of CPCs, we used FN and LN (both 10 µg/mL) on a 6-well plate (growth area: 9.6 cm^2/well) in this study. Because cell cycle and cell fate decisions are closely

linked, we are seeking the condition that exhibits a low cell proliferation rate in the absence of cell death, as such a condition may reflect the transition from cell proliferation to differentiation. We, therefore, applied the following conditions and compared cell proliferation rates: for cell density, high (80% - 90%) confluency and low (<60%) confluency, and for serum concentration, normal culture conditions (in this study. 3.5% FBS) and low serum conditions (≤0.1% FBS). First, we tested the low cell density condition. There were no significant differences of cell viability and proliferation in the low cell density with 3.5% FBS between LN and FN, whereas a low cell density with 0.1% FBS showed a decreased cell proliferation on LN compared to FN but no increase in cell death (**Figure 4**). In contrast, under high cell density conditions, serum concentrations of both 3.5% and 0.1% showed no differences in cell proliferation and in cell death between the two substrates (**Figure 5**).

These results indicate that a low cell density on LN with 0.1% serum decreases proliferation without affecting the CPC viability.

Changes in Cell Shape in the Endothelial Differentiation Medium:

Although requiring further studies to understand the significance and underlying mechanisms, changes in the cell shape appear to be an indicator of suitable culture conditions as specific changes can be observed early on in cultures, in which endothelial differentiation is going to be successful (**Figure 6**). As shown in the white dashed circles in **Figure 6,** within 7 - 14 d in the 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 tracking the cell shape every 2 - 3 d until around day 14. If no such cells appear, the number of cells seeded should be decreased.

Evaluation of the Endothelial Ability with a Tube Formation Assay:

The tube formation assay is a useful technique to evaluate the efficiency of endothelial differentiation of CPCs by measuring the tube formation capacity of differentiated cells. We, therefore, performed the tube formation assay with cells differentiated according to the described conditions. Interestingly, successful tube formation was consistently shown by cells plated at low density and differentiated on LN, whereas tube formation mostly failed in cells plated on LN at a 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 a low density sometimes formed rudimentary tubes, depending on the cell condition (*e.g.*, isolate and/or passage number, **Figure 7B**). To confirm the endothelial nature of the cells, cells plated on coverslips were differentiated according to the described protocol and stained for vWF. Again, the vWF expression was more homogeneous and more pronounced in CPCs differentiated on LN compared to FN (**Figure 7C,D**). **Figure 8** shows the results from the tube formation assay as performed for previous studies using rat CPCs under the same protocol as here described[33]. These results show that tube formation is more efficient in cells differentiated on LN as compared to FN when plated under low cell density conditions and with low serum (0.1% FBS) for 20 - 24 h

before differentiation in endothelial differentiation medium. These results suggest that this protocol could be useful for various cell types and independent of species.

FIGURE AND TABLE LEGENDS:

Figure 1: Illustration of specific isolation steps to obtain a cardiomyocyte-depleted cell suspension from isolated mouse hearts and representative flow cytometry readouts of the cardiac SP. (**A**) This panel shows the ejection of residual blood from the heart cavities through repeated slight pressure applied using small forceps. (**B**) This panel shows the cutting of the hearts into small pieces using small scissors. (**C**) This panel shows the mincing of the heart pieces using a razor blade. (**D**) This panel shows the incubation of the minced hearts with collagenase B in tilted plates at 37 °C. (**E**) This panel shows the homogenization of the minced hearts using a Pasteur pipette during the incubation step. (**F**) This panel shows the filtering of the digested tissue through a 100 µm filter (yellow) and a 40 µm filter (blue) for the removal of undigested tissue residues and cardiomyocytes. (**G**) This panel shows the gentle reversal of a 50 mL conical tube containing the cardiomyocyte-depleted cell suspension and wrapping in tin foil for light protection after the addition of Hoechst. (**H**) This panel shows representative flow cytometry readouts of Hoechst-stained cells in the presence and absence of the ABC transporter inhibitor verapamil for the identification of the SP. (**I**) This panel shows a representative dot plot of SP cells according to CD31 and Sca-1 positivity for the identification of the Sca-1^{+}/CD31^{-} subfraction.

Figure 2: Schematic overview of the sample preparation leading up to the cardiac SP sorting.

Figure 3: Protocol for endothelial lineage induction and differentiation.

Figure 4: Mouse SP-CPC proliferation when plated at a low cell density under different serum concentrations on LN and FN. (**A** and **B**) These panels show the cell numbers at day 2. (**C** and **D**) These panels show the viability at day 2. The data are shown as the mean ± the standard error of the mean (SEM); N = 5; different passages; * $p < 0.05$ by Student's *t*-test.

Figure 5: Mouse SP-CPC proliferation when plated at a high cell density under different serum concentrations on LN and FN. (**A** and **B**) These panels show the cell numbers at day 2. (**C** and **D**) These panels show the viability at day 2. The data are shown as the mean ± the SEM; N = 5; different passages.

Figure 6: Cell morphology at 14 and 17 d during the differentiation process. This figure shows bright-field (BF) images of mouse SP-CPCs seeded on LN- or FN-coated dishes with a low (Low) or a high (High) cell density and with Medium 2 for 20 h, followed by Medium 3 for 14 or 17 d. White dashed circles mark the areas containing round-shaped cells with a larger cell size. The imaging was performed with bright-field microscopy. The magnification = 2X; the scale bar = 100 µm.

Figure 7: Tube formation and vWF staining after endothelial differentiation of SP-CPCs on LN and FN. (**A** and **B**) These panels show the tube formation. (**C** and **D**) These panels show the vWF staining. Cells were seeded on 10 µg/mL of LN- or FN-coated dishes with a low or high cell density

and with Medium 2 for 20 h, followed by Medium 3 for 21 d, and then harvested with Trypsin and seeded on the basement membrane matrix. All pictures were taken after 16 h. The imaging was performed with bright-field microscopy and the magnification = 2X for panels **A** and **B**. The imaging is performed with fluorescent microscopy and the magnification = 10X for panels **C** and **D**. Panels **A** and **C** show LN-coated dishes. Panels **B** and **D** show FN-coated dishes. The scale bar = 100 µm.

Figure 8: Tube formation after the endothelial differentiation of rat CPCs. Rat CPCs were seeded at a low cell density on 10 µg/mL of LN- or FN-coated dishes with 0.1% FBS-containing F12 medium for 20 h, followed by another 21 d in Medium 3, and then harvested with Trypsin. On the basement membrane matrix on a 96-well plate, 4 x 10^4 cells were seeded in 100 µL of Medium 3. The imaging was performed with bright-field microscopy. The magnification = 2X; the scale bar = 50 µm. This figure is modified based on a previous study[33].

Table 1: Isolation techniques and markers of cardiac progenitor cells.

DISCUSSION:

Advantages of this Protocol:

This protocol provides an endothelial differentiation technique of CPCs. We found that a low serum concentration and low cell density could improve the efficiency of endothelial differentiation, whereby LN proved to be a more suitable substrate than FN under these conditions. We used two distinct types of CPCs: rat CPCs, which were used in a cell line-like manner, and mouse SP-CPCs, which were isolated and expanded. Notably, the protocol was applicable to both types of CPCs. Current techniques allow scientists to isolate and expand primary CPCs from genetically modified mice and from preclinical disease models, as well as from humans[28,36]. Using this protocol on isolated CPCs could be helpful to the investigation of not only disease mechanisms but also to the exploration of novel therapeutic targets.

CPCs are currently in clinical testing for cell therapy[4,5], whereby cells are isolated from patients and transplanted back after *in vitro* amplification. In this regard, the protocol presented here could help identify strategies to enhance the differentiation potential of such CPCs before transplantation. However, cell therapy still suffers from limited efficacy due to low retention, survival, and engraftment of transplanted cells. Mechanistic *in vitro* studies based on this protocol or on adaptations thereof have the potential to contribute to a better understanding of the molecular mechanisms driving the differentiation process—in particular, mechanisms related to cell density, substrate, and growth factors. New knowledge retrieved from such studies may ultimately be used for cell reprogramming and applied to directly influence resident CPCs to differentiate after injury.

Limitations of this Protocol:

Isolated primary CPCs are heterogeneous populations that show different phenotypes with respect to cell size and cell growth rate depending on the isolation, even when using the same markers and identical isolation technique. Therefore, the following three points need to be

defined: (1) the cell seeding numbers according to cell size, (2) the ideal serum concentration (<0.5%), and (3) the incubation time for the first step (induction of lineage) with a very low serum concentration. Here, we show differences in differentiation efficiency depending on cell density. However, although we compared low serum (0.1% FBS) *versus* culture serum concentrations (3.5% FBS), we did not examine other concentrations within the <0.5% FBS range. In addition, depending on the isolation markers used and the species, the efficiency of the induction of lineage commitment may vary. Therefore, the protocol should be optimized for each cell type.

Pharmacological and genetic inhibition/induction techniques could be used for examining disease mechanisms with this protocol, instead of genetically modified or disease animal models. In this case, the protocol should be redesigned: before the treatment with low-serum-containing medium, the cells will be treated with specific reagents or genetic tools. As a consequence, the cells may be more vulnerable than nontreated cells. Using low serum in the first step is the key point in this protocol. Therefore, a careful validation of the serum concentration and incubation time for the first step to allow for slowing the cell proliferation while maintaining viability under serum deprivation is critical to whether this protocol can be a success or not.

Summary:

In summary, isolated CPCs from animal models provide a valuable tool for cardiac disease and regeneration studies. Upon expansion, human CPCs may also be directly used for cell therapy. We, here, provide a protocol for the efficient endothelial lineage induction and differentiation of CPCs based on careful adaptations of cell density and serum concentrations. The advantage of this protocol is its applicability to different types of CPCs and its potential to contribute a basis for the study of and/or the establishment of other novel cardiac regeneration tools.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

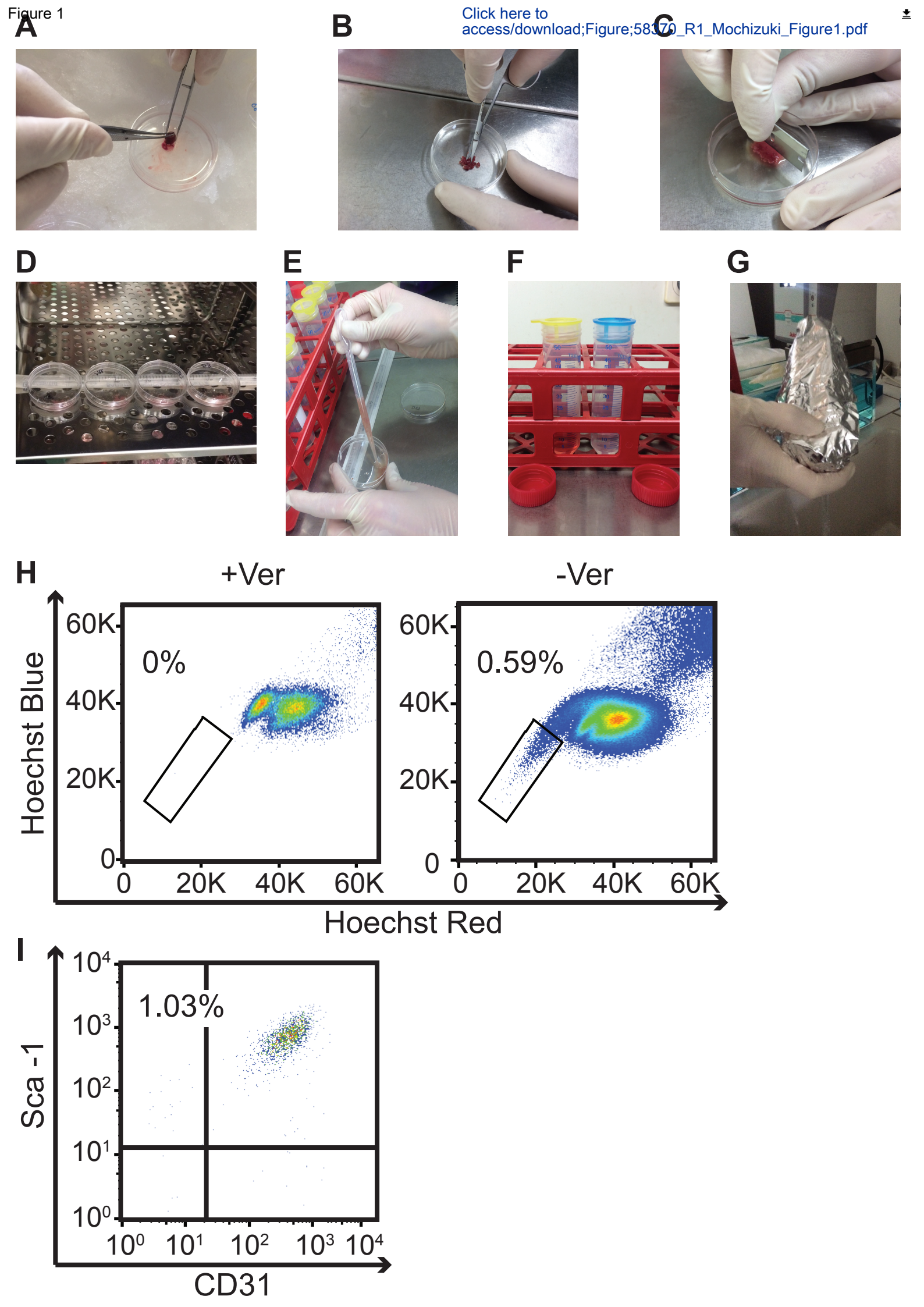

Figure 2

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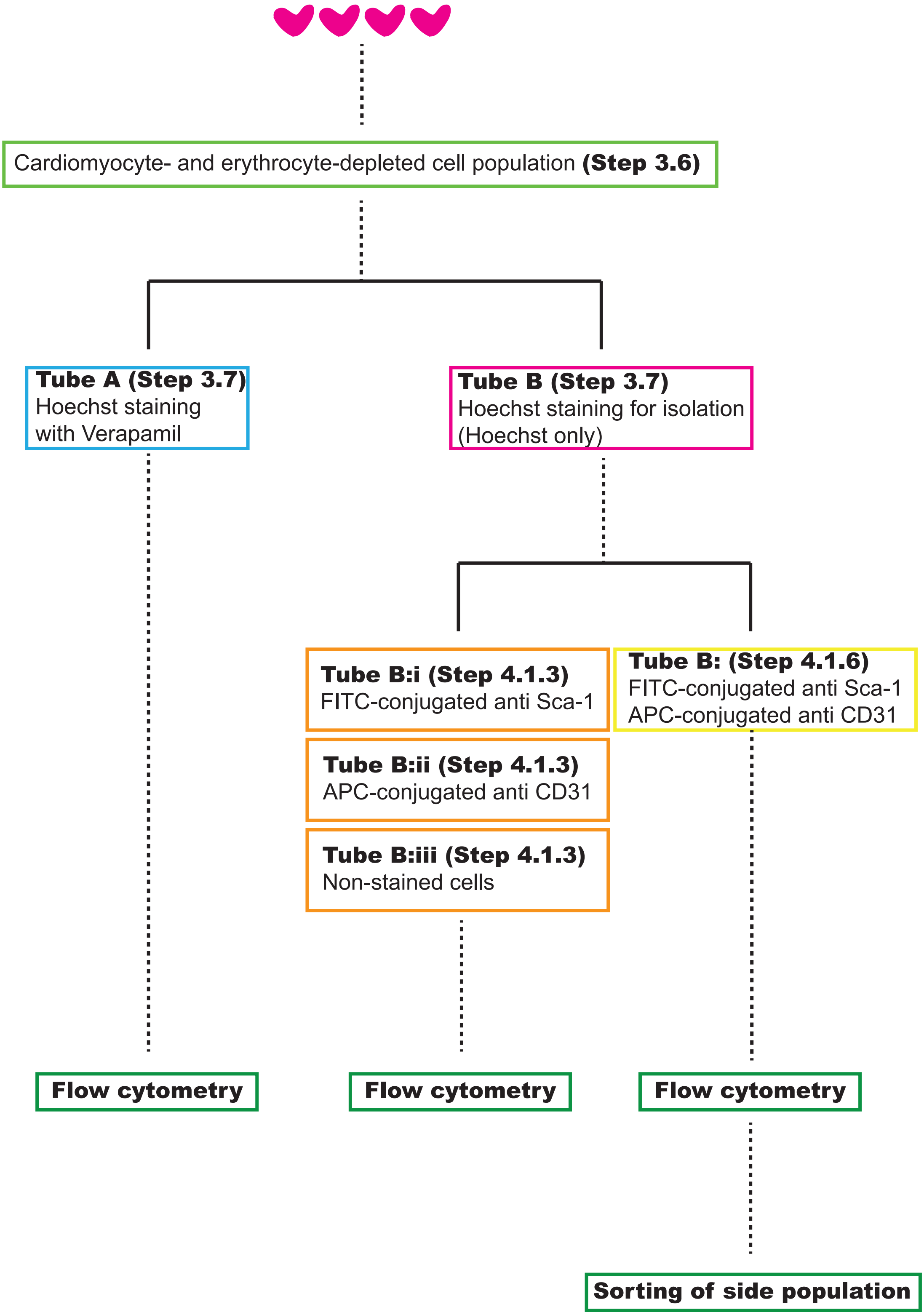

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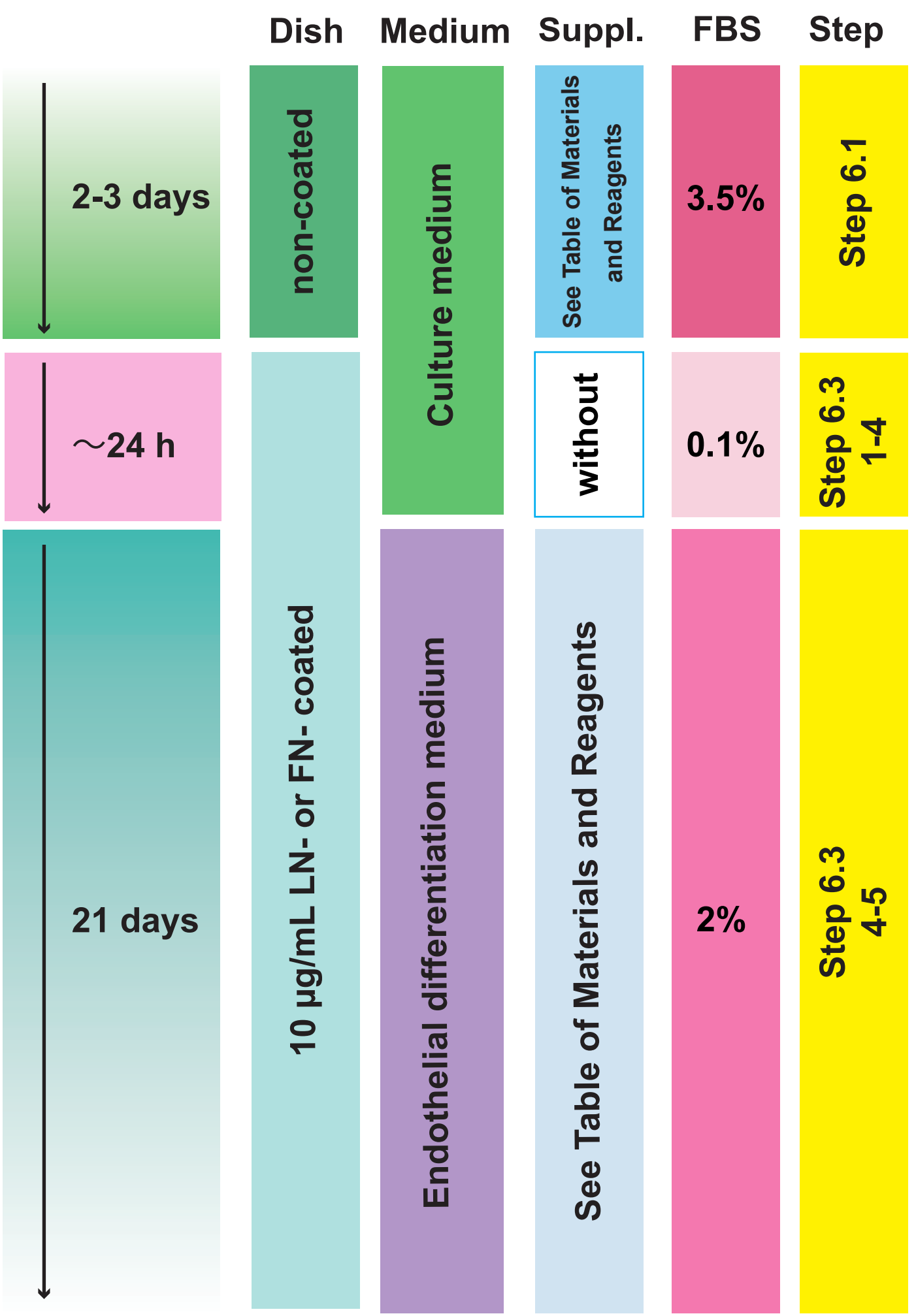

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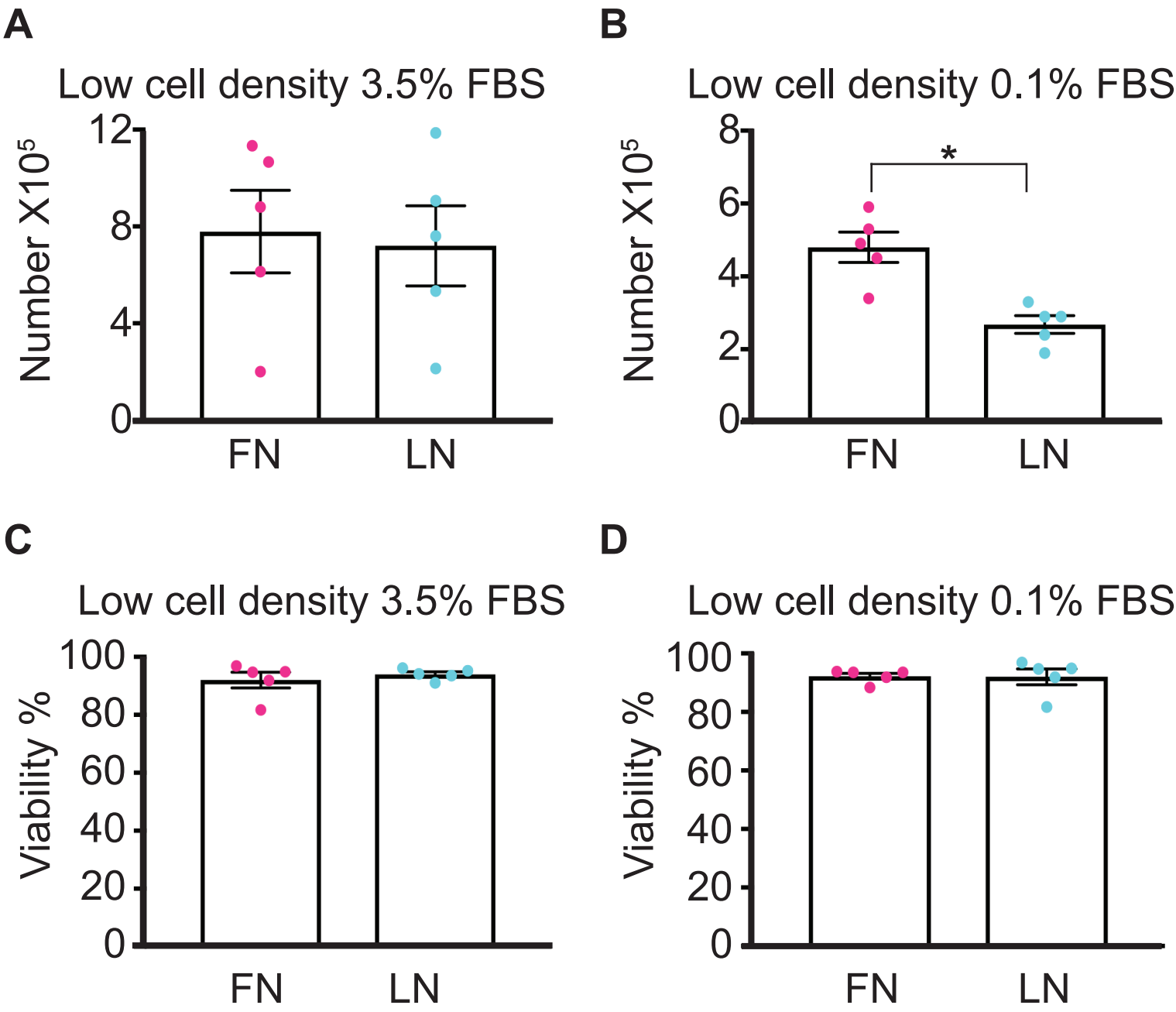

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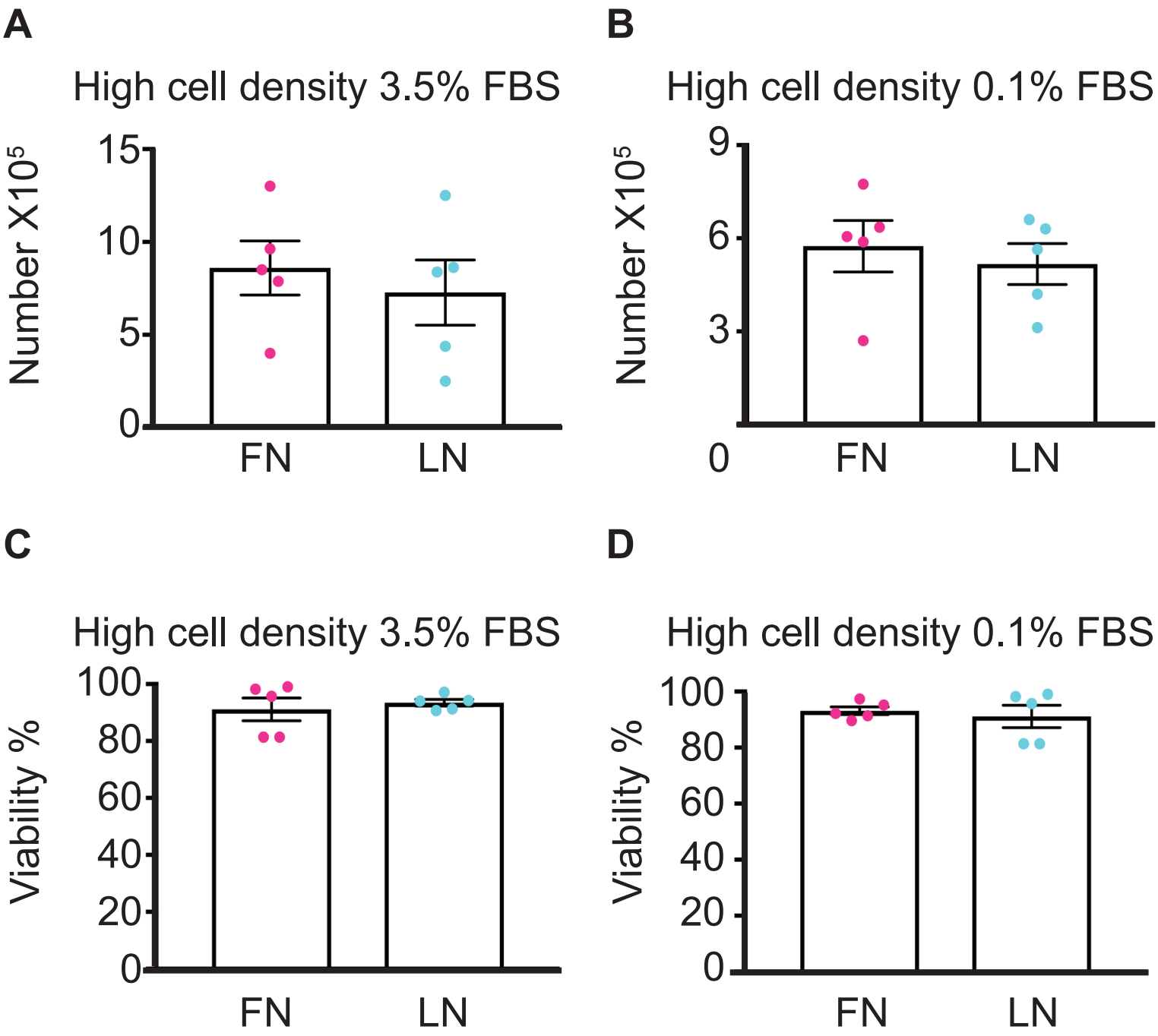

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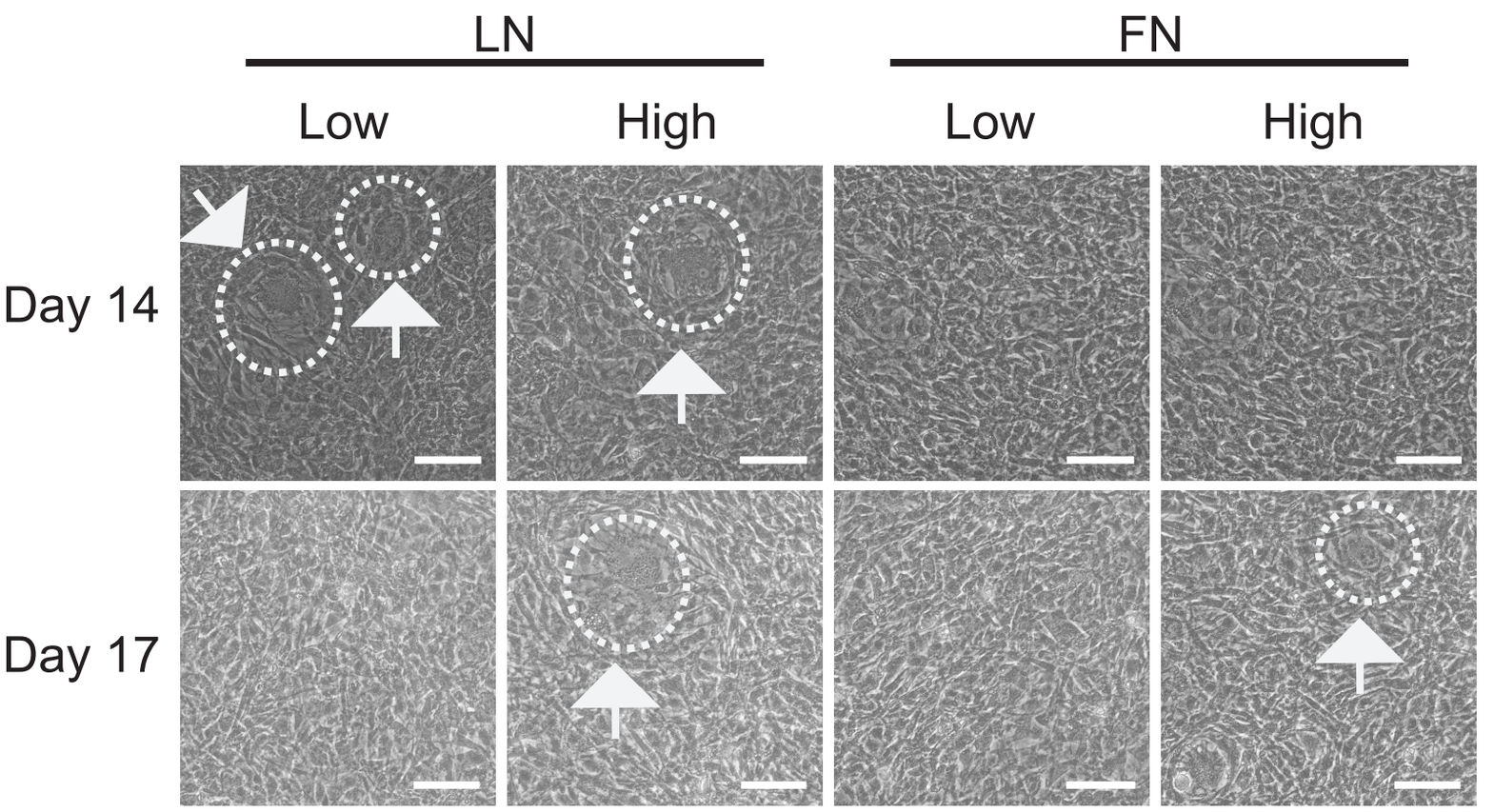


Figure 7

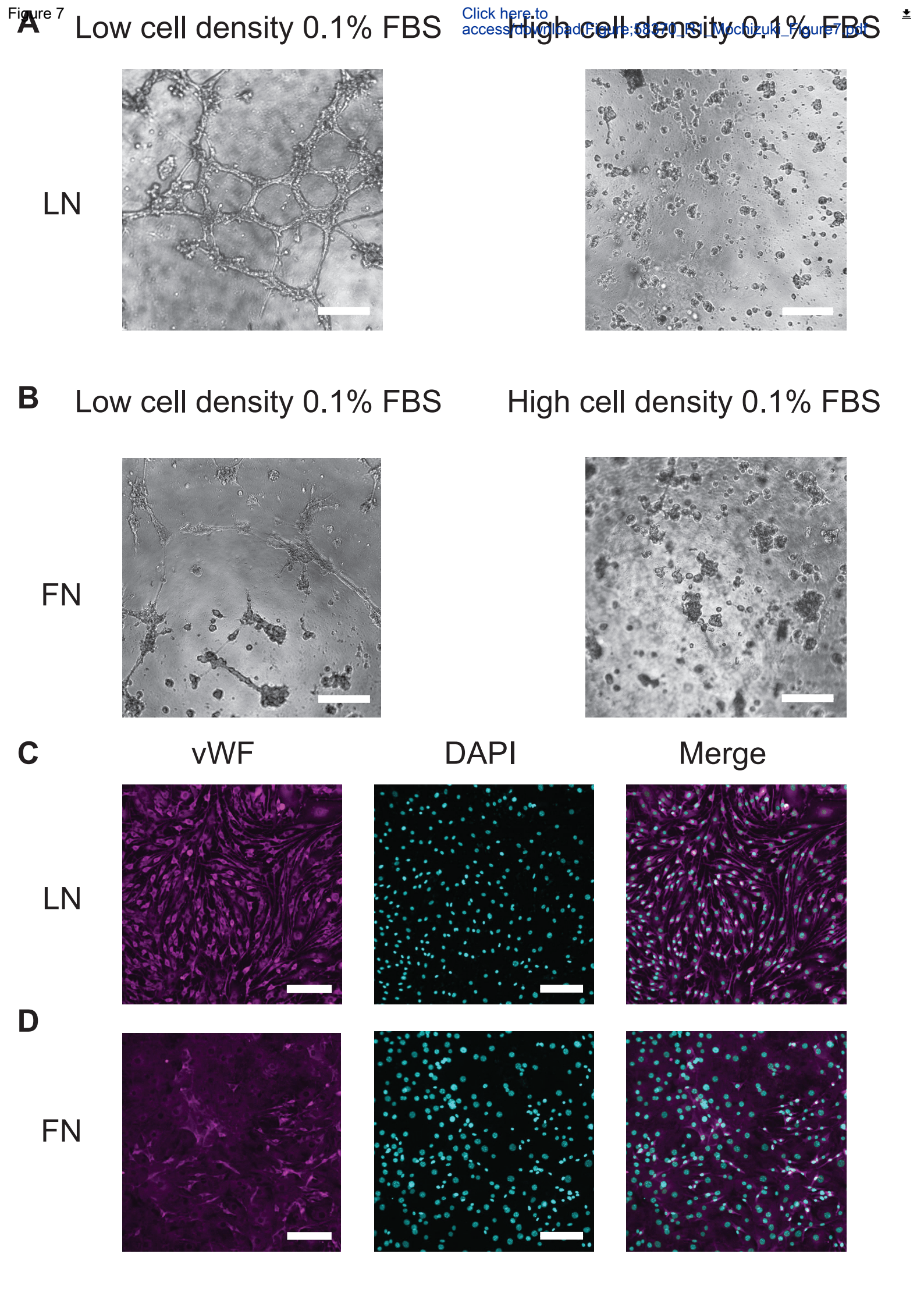
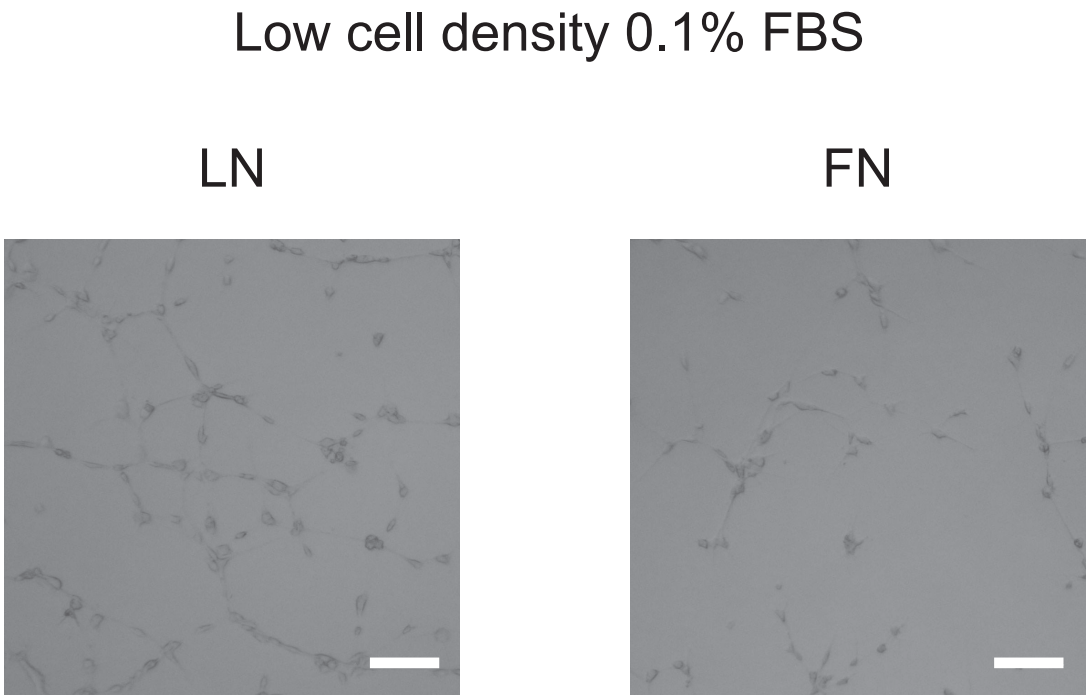


Figure 8



Table

Species
Human, rat, mouse
Mongrel dogs
Mouse
Mouse
Mouse
Mouse, rat, human

* Isl-1 is found in the embry

Isolation, detection marker
c- kit $^{+}$ /Lin^{-}, c-kit^{+}/CD45^{-}, c-kit^{+}/CD45^{-}/CD31^{-}
Lin $^{-}$, plus: c-kit^{+}, MDR1^{+} or Sca1^{+}
Sca-1 $^{+}$
Side population , plus: Abcg2^{+}, Sca1^{+}/CD31^{-}, Sca1^{+}/PDGFRα^{+}
Colony forming unit-fibroblast , plus: CD45^{-}, CD31^{-}, Sca1^{+}, PDGFRα^{+}
Isl-1 $^{+}$ *** , plus: CD31^{-}, c-kit^{-}, Sca1^{-}, Gata4^{+}, Nkx2.5^{+}

ɣonic or fetal myocardium, not in the adult heart.

Reference
Beltrami, Cell 2003; Bolli, Lancet 2011; Ellison, Cell 2013; Smith, Nat Protoc 2014; Vicinanza, Cell Death Differ 2017
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Oh, PNAS 2003
Hierlihy, FEBS Lett 2002; Martin, Dev Biol 2004; Pfister, Circ Res 2005; Noseda, Nat Commun 2015
Pelekanos, Stem Cell Res 2012
Laugwitz, Nature 2005; Bu, Nature 2009

Table of Materials

Product name	Company	Catalogue No.	Note
Culture medium			
Iscove's Modified Dulbecco's Medium (IMDM)	ThermoFisher	#12440	
Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F12 Ham	Merck	#D8437	
Penicillin-Streptomycin (P/S)	ThermoFisher	#15140122	
Fetal Bovine Serum (FBS)	Hyclone	#SH30071	3.5% (0.1% for lineage induction)
L-Glutamine	ThermoFisher	#25030	Final concentration 2 mM
Glutathione	Merck	#G6013	
Recombinant Human Epidermal Growth Factor (EGF)	Peprotech	#AF-100-15	
Recombinant Basic Fibroblast Growth Factor (FGF)	Peprotech	#AF-100-18B	
B27 Supplement	ThermoFisher	#17504044	
Cardiotrophin 1	Peprotech	#250-25	
Thrombin	Diagontech AG, Switzerland	#100-125	
Hanks' Balanced Salt Solution (HBSS) CaCl2(-), MgCl2(-)	ThermoFisher	#14170	
0.05 % Trypsin-EDTA	ThermoFisher	#25300	
T75 Flask	Sarstedt	#83.3911	
Endothelial differentiation			
Endothelial Cell Growth Medium (EGM)-2 BulletKit	Lonza	#CC-3162	
Ham's F-12K (Kaighn's) Medium	ThermoFisher	#21127	
Laminin	Merck	#L2020	
Fibronectin	Merck	#F4759	Dilute in ddH2O
6 Well Plate	Falcon	#353046	
Formaldehyde Solution	Merck	#F1635	Diluite 1:10 in PBS (3.7% final concentration)
Triton X-100	Merck	#93420	0.1% in ddH2O
Normal Goat Serum (10%)	ThermoFisher	#50062Z	
Anti-von Willebrand Factor antibody	Abcam	#ab6994	1:100 in 10% goat serum
Goat anti-Rabbit IgG, Alexa Fluor 546	ThermoFisher	#A11010	1:500 in 10% goat serum
4',6-diamidino-2-phenylindole, dihydrochloride (DAPI)	ThermoFisher	#62247	1:500 in ddH2O
SlowFade Antifade Kit	ThermoFisher	#S2828	
BX63 widefield microscope	Olympus		
Tube formation			
96 Well Plate	Falcon	#353072	
5 ml Round Bottom Tube with Strainer Cap	Falcon	#352235	
Matrigel Growth Factor Reduced	Corning	#354230	
IX50 widefield microscope	Olympus		

Sca-1+/CD31- cardiac side population isolation[24]			
Reagents			
Pentobarbital Natrium 50 mg/ml ad usum vet.	in house hospital pharmacy	#9077862	Working solution: 200 mg/kg
Phosphate Buffered Saline (PBS) CaCl2(-), MgCl2(-)	ThermoFisher	#20012	
Hanks' Balanced Salt Solution (HBSS) CaCl2(-), MgCl2(-), phenol red (-)	ThermoFisher	#14175	Prepare HBSS 500 mL + 2% FBS for quenching Collagenase B activity
Dulbecco's Modified Eagle's Medium (DMEM) 1g/L of D-Glucose, L-Glutamine, Pyruvate	ThermoFisher	#331885	Prepare DMEM + 10% FBS + 25 mM HEPES+ P/S for Hoechst stanining
Penicillin-Streptomycin (P/S)	ThermoFisher	#15140122	
HEPES 1 M	ThermoFisher	#15630080	Final concentration 25 mM
Fetal Bovine Serum (FBS)	Hyclone	#SH30071	
RBC LysisBuffer (10X)	BioLegend	#420301/100mL	Dilute to 1X in ddH2O and filter through a 0.2 µm filter
Collagenase B	Merck	#11088807001	Final concentration 1 mg/mL in HBSS, filtered through a 0.2 µm filter
bisBenzimide H33342 Trihydrochloride (Hoechst)	Merck	#B2261	Prepare 1 mg/mL in ddH2O
Verapamil-hydrochloride	Merck	#V4629	Final concentration 83.3 µM
APC Rat Anti-Mouse CD31	BD Biosciences	#551262	0.25 µg/10^7cells
FITC Rat Anti-Mouse Ly-6A/E (Sca-1)	BD Biosciences	#557405	0.6 µg/10^7cells
7-Aminoactinomycin D (7-ADD)	ThermoFisher	#A1310	0.15 µg/10^6cells
APC rat IgG2a k Isotype Control	BD Biosciences	#553932	0.25 µg/10^7cells
FITC Rat IgG2a k Isotype Control	BD Biosciences	#554688	0.6 µg/10^7cells
Material			
Needles 27G	Terumo	#NN-2719R	
Needles 18G	Terumo	#NN-1838S	
Single Use Syringes 1 mL sterile	CODAN	#62.1640	
Transferpipette 3.5 mL	Sarstedt	#86.1171.001	
Cell Strainer 40 µm blue	BD Biosciences	#352340	
Cell Strainer 100 µm yellow	BD Biosciences	#352360	
Lumox Dish 50	Sarstedt	#94.6077.305	
Culture Dishes P100	Corning	#353003	
Culture Dishes P60	Corning	#353004	

Mouse		
Line	**Age**	**Breeding**
C57BL/6NRj / male	12 weeks	in house

Overview of medium compositions. Some of this infomation is identical with the one provided above, but sorted according to the composition of Media 1-3.

Product Name	Company	Catalogue No.	Medium 1[8]	Medium 2	Medium 3
Reagents			**Culture**	**Lineage induction**	**Endothelial diff.**
Iscove's Modified Dulbecco's Medium (IMDM)	ThermoFisher	#12440	35%	35%	
Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F12 Ham	Merck	#D8437	65%	65%	
Penicillin-Streptomycin (P/S)	ThermoFisher	#15140122	1%	1%	
Fetal Bovine Serum (FBS)	Hyclone	#SH30071	3.5%	≤0.1%	
L-Glutamine	ThermoFisher	#25030	2 mM	2 mM	
Glutathione	Merck	#G6013	0.2 nM	0.2 nM	
B27 Supplement	ThermoFisher	#17504044	1.3%		
Recombinant Human Epidermal Growth Factor (EGF)	Peprotech	#AF-100-15	6.5 ng/mL		
Recombinant Basic Fibroblast Growth Factor (FGF)	Peprotech	#AF-100-18B	13 ng/mL		
Cardiotrophin 1	Peprotech	#250-25	0.65 ng/mL		
Thrombin	Diagontech AG, Switzerland	#100-125	0.0005 U/mL		All necessary components included in the kit
Endothelial Cell Growth Medium (EGM)-2 BulletKit	Lonza	#CC-3162			

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Author(s): Michika Mochizuki, Giacomo Della Verde, Habiba Soliman, Otmar Pfister, Gabriela M. Kuster

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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.

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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, µL, 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.

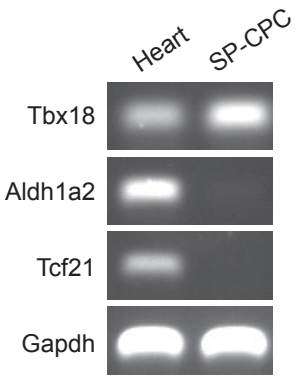


Figure: Gene expression of epicardial markers. mRNA was isolated from SP-CPC and mouse whole heart, then cDNA was generated and PCR was performed PCR. Aldh1a2: aldehyde dehydrogenase 1 family member a2; Tcf21: transcription factor 21; Heart: RNA from adult mouse heart serving as positive control.

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.

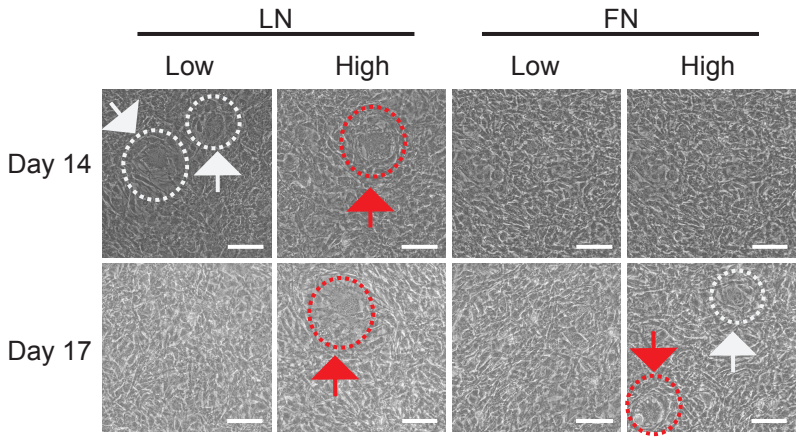
Cell fate decisions are executed by dynamic shifts in the transcriptome that occur during specific phases of the cell cycle. Cell cycle lengthening allows for a fate-switch from proliferation to lineage commitment and differentiation during the prolonged gap phases and this has been shown to involve 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 Sca 1+ 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 heterogenous 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.

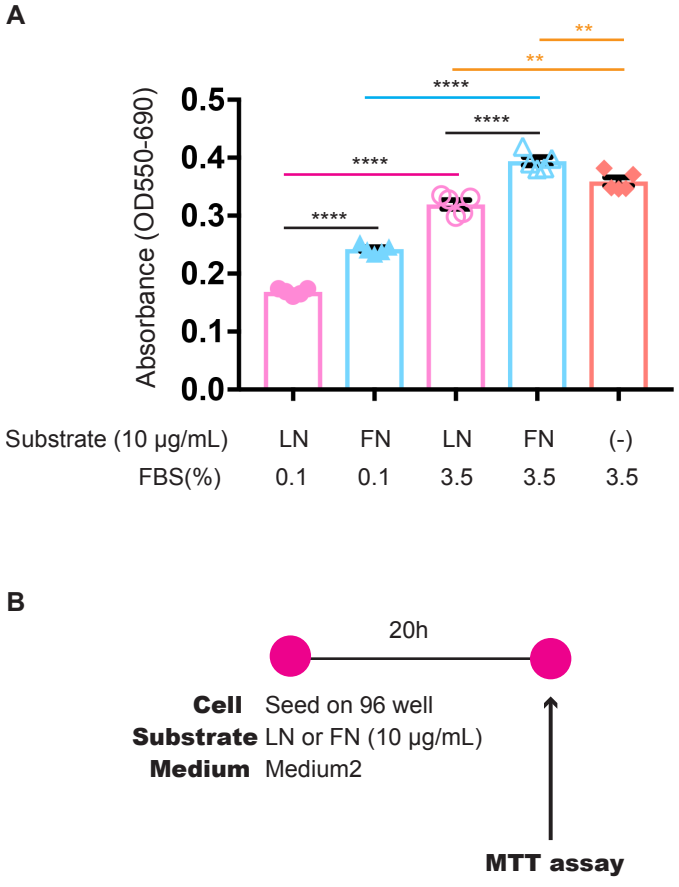


Figure: Viability of SP-CPCs under culture settings that are considered suitable to assess LDL uptake: (A) MTT assay with SP-CPCs. (B) Protocol. Cells were seeded at low density on a 96 well plate precoated with LN or FN with Medium 2 (including 0.1% FBS) for 20 h, then the MTT assay was performed. No substrate (-) and 3.5% FBS-containing medium were used as positive controls. Data are given as mean ±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 cm^2/well), which is now indicated in the manuscript.

JoVE58370 Response to Editorial Comments

We thank the Editors for their time and consideration given to our manuscript.

Editorial Comments:

1. **Comments 1-8, 10-11.**
New references have now been added.

2. **Comment 9.**
The sentence has been revised.

3. **Comment 13.**
PBS has been defined as phosphate buffer saline.

4. **Comment 14.**
The sentence now reads: "Cut the pieces into smaller pieces"

5. **Comment 15.**
"Mince strongly" has been replaced by "mince thoroughly".

6. **Comment 16.**
"Add to the dish from step 1.6" has been added.

7. **Comment 17.**
Concentration has been added.

8. **Comment 18.**
Information has been added.

9. **Comment 19-21, 24, 26-27, 33, 36-37,**
Step numbers have been updated in the manuscript and in Figures 2 and 3 according to the new edited protocol.

10. **Comment 22.**
"The cells" has been added.

11. **Comment 23.**
Information has been added.

12. **Comment 25.**
Culture conditions are now mentioned.

13. **Comment 28.**
Information has been added.

14. **Comment 29.**
Concentration has been added.

15. **Comment 30.**

Step 6.2.7 has been added.

16. **Comment 31.**
“Centrifuge speed should be adjusted depending on the machine rotor” has been removed.

17. **Comment 32.**
The concentrations of Laminin and Fibronectin given as % have been added.

18. **Comment 34.**
Details on the vWF staining as verification step of endothelial differentiation have been added in the manuscript and in the Table of Materials and Reagents.

19. **Comment 35.**
Each note has been moved under the relevant step.

20. **Comment 38.**
Information has been added.

21. **Comment 39.**
Microscopy settings have been added.

22. **Comment 40.**
Laminin (LN) and Fibronectin (FN) have been defined.

23. **Comment 41.**
“A” has been removed.

24. **Comment 42.**
The Table of Materials and Reagents has been referenced in Figure 3.

25. **Comment 43-45.**
Microscopes have been added to the Table of Materials and Reagents.

26. **Comment 46.**
References have been added to the Table of Materials and Reagents.

In addition, more detail has been added to the trypsinization steps in 6.2.2, 6.3.2 and 6.4.2.

Re: Request regarding Copyright

JAHA Dept Account <jaha@journalaha.org>

Mo 23.04.2018 15:59

An:Kuster Pfister Gabriela <Gabriela.Kuster@usb.ch>;

Cc:Michika Mochizuki <m.mochizuki@unibas.ch>;

Dear Dr. Kuster,

This is fine, **as long as** the modified figure is not being used for commercial purposes. If this were the case, JAHA would not be able to allow it.

Sincerely,

JAHA Editorial Office
jaha@journalaha.org

On Mon, Apr 23, 2018 at 9:29 AM, Kuster Pfister Gabriela <Gabriela.Kuster@usb.ch> wrote:
> Dear Madam/Sir,
>
>
> Thank you for your fast response.
>
>
> As to my understanding, we can reproduce in case no adaptations or
> modifications are made. However, we would not like to reproduce, but
> actually to provide a modification of a Figure published in the previous
> paper.
>
>
> May I kindly ask you how we have to proceed and whom we have to contact to
> receive permission to do that? Our previous request to Wiley unfortunately
> remained unanswered.
>
>
> Thank you for your support!
>
>
> Kind regards,
>
>
> Gabriela Kuster
>
>
>
>
>
> _____

> Von: JAHA Dept Account <jaha@journalaha.org>
> Gesendet: Montag, 23. April 2018 15:03
> An: Kuster Pfister Gabriela
> Betreff: Re: Request regarding Copyright
>
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>
> On Mon, Apr 23, 2018 at 8:57 AM, Kuster Pfister Gabriela
> <Gabriela.Kuster@usb.ch> wrote:
>> Dear Madam/Sir,
>>
>>
>> Based on our previously published paper in J Am Heart Assoc (Mochizuki et
>> al., J Am Heart Assoc 2017), we were asked by JoVE to write a
>> methods/protocol article based on the differentiation technique for
>> cardiac
>> progenitor cells used in the JAHA paper. We now drafted such an article
>> conducting independent experiments and technical adaptations that will be
>> used to illustrate the basics of this technique using in principle a
>> different type of cells than the one the respective data included in the
>> JAHA paper are retrieved from.
>>
>>
>> To demonstrate that this technique is applicable to different types of
>> cells, though, we would like to provide one representative picture from
>> our
>> study published in JAHA. This picture is newly generated, but contains
>> data
>> that are shown in the original Figure 2C-D. This newly generated Figure
>> would be labelled as "modified from Mochizuki et al., JAHA 2017" followed
>> by
>> a reference to the original paper.
>>
>>
>> May I kindly ask you to please let us know whether this is in agreement
>> with
>> the copyright licensing or how we have to proceed to include this Figure
>> in
>> the planned manuscript?
>>
>>
>> Thank you very much for your support.
>>
>>
>> Kind regards,

>>
>>
>> Gabriela Kuster
>>
>>
>>
>> PD Dr. med. Gabriela M. Kuster Pfister
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