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Isolation and Purification of Murine Cardiac Pericytes

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Dear Editor-in-Chief,

I am submitting you the manuscript entitled "Isolation and purification of murine cardiac pericytes".

Pericytes, perivascular cells of microvessels and capillaries, are known to play a part in and endothelial barrier integrity, however, their tissue specific functions in the heart are not well understood. Moreover, there is currently no protocol utilizing readily accessible materials to isolate and purify pericytes of cardiac origin. Our protocol focuses on using the premier mammalian model, the mouse, as our source of cells. Using the enzymatic digestion and dissociation of heart tissue, we obtained a crude cell mixture that was further purified by fluorescence activating cell sorting (FACS) by a plethora of markers.

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Sincerely Yours,
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TITLE:

Isolation and Purification of Murine Cardiac Pericytes

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KEYWORDS:

pericytes, vascular biology, perivascular cells, cardiac, cell isolation, primary cell culture

SUMMARY:

We have optimized a protocol to isolate and purify murine cardiac pericytes for basic research and investigation of their biology and therapeutic potential.

ABSTRACT:

Pericytes, perivascular cells of microvessels and capillaries, are known to play a part in angiogenesis, vessel stabilization, and endothelial barrier integrity. However, their tissue-specific functions in the heart are not well understood. Moreover, there is currently no protocol utilizing readily accessible materials to isolate and purify pericytes of cardiac origin. Our protocol focuses on using the widely used mammalian model, the mouse, as our source of cells. Using the enzymatic digestion and mechanical dissociation of heart tissue, we obtained a crude cell mixture that was further purified by fluorescence activating cell sorting (FACS) by a plethora of markers. Because there is no single unequivocal marker for pericytes, we gated for cells that were $CD31^{-}CD34^{-}CD45^{-}CD140b^{+}NG2^{+}CD146^{+}$. Following purification, these primary cells were cultured and passaged multiple times without any changes in morphology and marker expression. With the ability to regularly obtain primary murine cardiac pericytes using our protocol, we hope to further understand the role of pericytes in cardiovascular physiology and their therapeutic potential.

INTRODUCTION:

Perivascular cells known as pericytes surround the microvessels and capillaries of the vascular tree[1,2]. Physiologically, they are known to promote and play a part in angiogenesis, increase barrier integrity due to their close relationship with endothelial cells as well as stabilize and

mature vessels[1,2]. Moreover, the dysfunction and/or loss of these cells have been implicated in diseases such as Alzheimer's disease[2,3] and various cardiovascular diseases[4]. These cells are found throughout the entire body, but the cell numbers are tissue-dependent. Pericytes have most notably been studied in the brain due to high vascularization of the blood-brain barrier[1,2]. However, in the heart, the biology of pericytes is understudied.

Recently, there are increased interests in the field for cardiac pericytes, but there is currently no streamlined protocol available for their isolation from one of the most used tools in biology – the mouse. There are protocols in the literature on isolating pericytes from the brain[5], retina[6], placenta[7], and skeletal muscle[8,9]; however, few protocols are on isolating pericytes from the heart. There are several groups that have isolated cardiac pericytes. Nees et al. were able to isolate an abundant amount of cardiac pericytes from multiple species including the mouse; however, their methods used specific in-house built equipment which decreases reproducibility[10]. Avolio et al.[11], Chen et al.[12], and Baily et al.[13] also successfully isolated cardiac pericytes from human heart tissue, but human tissues are not always available and hard to obtain for some investigators. Here, we have developed an isolation method to obtain cardiac pericytes from mouse models for investigators to further study their biology with readily available materials.

Using enzymatic digestion and fluorescence activated cell sorting (FACS) with known key pericyte markers[14], our protocol allows us to isolate and purify a population of pericytes that are characterized by $CD31^{-}CD34^{-}CD45^{-}CD140b^{+}NG2^{+}CD146^{+}$. Our panel of markers contain both inclusion and exclusion markers. CD45 is used as a marker to exclude hematopoietic cells. CD31 is used as a marker to exclude endothelial cells. CD34 is used as a marker to exclude both hematopoietic and endothelial progenitor cells. CD146 is a marker for perivascular cells. Lastly, NG2 and CD140b (also known as platelet derived growth factor receptor beta – PDGFRβ) are both accepted markers for pericytes[14]. The primary culture obtained can be cultured and passaged multiple times with no changes in morphology or marker expression. Furthermore, these cells can be co-cultured with endothelial cells to study their interactions and crosstalk with each other. This cell isolation method will allow investigators to study the biology and pathophysiology of cardiac pericytes from wild type, disease, and genetically variant mouse models.

PROTOCOL:

All animals were housed and used in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility and all animal work was conducted under appropriate veterinary oversight and under the Institutional Animal Care and Use Committee (IACUC) approved protocol of Amgen Inc.

1. Preparation of tools and culture media

1.1. Autoclave surgical 9 cm straight tip fine point scissors and 10 cm angled serrated forceps.

1.2. Add 25 mL of 5% fetal bovine serum (FBS) and 5 mL of 1% penicillin streptomycin (P/S) into

a 500 mL bottle of calcium magnesium free Dulbecco's phosphate-buffered saline (CMF-DPBS). Place solution in an ice bath to ensure it will be cold at the time of use. Aliquot 50 mL into a 50 mL conical tube for heart isolation. Add in 250 units/mL of heparin sodium solution into the 50 mL aliquot. This will be referred to as heparinized CMF-DPBS.

1.3. Add 20% FBS (100 mL) and 1% P/S (5 mL) into a 500 mL bottle of high glucose Dulbecco's modified eagle's medium (DMEM). This will be referred to as the enzyme-free culture media. Aliquot 20 mL of DMEM + 20% FBS + 1% P/S and add in 500 µg/mL of collagenase B. This will be referred to as the enzyme solution. Keep both the enzyme-free culture media and enzyme solution warm at 37 °C in an incubator or water bath.

2. Preparation of animal and procurement of cardiac tissue

2.1. Intraperitoneally inject a mouse with 250 units of heparin sodium solution with a 31 G needle syringe. Then wait 10−15 min while the mouse remains active in its home cage.

NOTE: Representative data in this study were obtained from a 4-month-old male C57BL/6 mouse. However, this protocol can be used on any mouse regardless of strain, age, gender, weight, etc.

2.2. Anesthetize the mouse with 5% isoflurane. Once the mouse is no longer responding to the pinch reflex, euthanize the mouse by cervical dislocation.

2.3. Place the anesthetized mouse in supine position and tape down its forelimbs. Carefully open the chest cavity and cannulate the descending aorta using a 25 G butterfly needle.

2.4. Make a nick in the right atrium and perfuse the heart with at least 20 mL of 250 units/mL heparinized CMF-DPBS at 2 mL/min with a variable-flow peristaltic pump. When the PBS comes out of the right atria clean, perfusion is complete.

2.5. Cut the heart out at the aorta and place it into the ice-cold CMF-DPBS.

3. Dissociation of heart tissue

3.1. Transfer the heart into a 15 cm x 15 cm Petri dish. Cut the heart into tiny pieces (1 mm/piece) using spring scissors and fine point forceps with enough enzyme solution to cover the pieces (10−15 mL).

3.2. Transfer the pieces and solution into a 50 mL conical tube, seal with paraffin plastic film, and incubate at 37 °C on an orbital shaker at 120 rpm for 75 min.

3.3. After collagenase digestion with the enzyme solution, decant the liquid through a 100 µm cell strainer into a new 50 mL tube but leave enough solution to make sure the pieces do not dry out.

3.4. Using fine point forceps, take out the tissue from the tube and place a few pieces on a microscope slide. Then grind the tissue between two microscope slides to break up the tissue. Rinse the slides with enzyme free culture media into a new 50 mL conical tube.

3.5. Repeat step 3.4 until all tissue pieces are dissociated.

3.6. Combine the solutions from steps 3.3–3.5 into one tube. Strain the resulting suspension through a 100 µm cell strainer into a new 50 mL conical tube.

3.7. Centrifuge at 220 x *g*, 4 °C, for 5 min. Aspirate off previous solution and gently resuspend cell pellet in fresh enzyme-free culture media.

3.8. Count cells and check viability using a cell counter. Dilute cells to 1 x 10^6/mL with cold FACS staining buffer containing 500 mL of DPBS and 10–25 mL of 2–5% bovine serum albumin (BSA). The cells are ready to be stained and sorted.

4. Purification of pericytes from crude cell mixture using FACS

4.1. Prepare and label 5 mL FACS tubes for all controls and cell samples. Aliquot out cells (1 mL of cells per tube) for an unstained sample, fluorescence minus one (FMO) controls, and isotype-matched controls. Use the remaining cells for the sort. All controls and samples can be prepared and stained at the same time.

NOTE: A total of 13 mL at 0.5 x 10^6 cells/mL were used for the representative sort from one heart. However, the volume depends on how many cells the investigator obtains from their isolation, how many hearts they use, and how well the heart tissue is digested; the size of each heart is also a variable that can alter the volume.

4.1.1. Use compensation beads (**Table of Materials**) to optimize fluorescence compensation controls. Prepare one compensation control for each fluorochrome in the experiment in a labelled 5 mL FACS tube. For this experiment, prepare a total of 9 compensation controls – 2 kinds of unstained beads plus 7 different fluorochromes from the marker panel including NG2-FITC, CD31-APC, CD140b-PE, CD146-BV605, CD34-BV421, CD45-PE-Cy7, and cell viability-APC-Cy7 (**Table of Materials**).

4.1.1.1. Add one drop of compensation beads (~ 50 µL) from the squeeze vial to each tube. Then add 1 µL of antibody to the beads. Repeat for each antibody from the marker panel. Mix vigorously by pulse-vortexing. Incubate for 30 min at 4 °C protected from light except for the cell viability beads which can be left at room temperature protected from light.

4.1.1.2. Next, add 3 mL of FACS staining buffer to each tube and centrifuge at 300 x *g* for 5 min at 4 °C. Aspirate off solution and resuspend each bead pellet in 400 µL of FACS staining buffer. The compensation controls are ready to be used. Keep on ice.

4.1.2. Use FMO controls to optimize background staining due to spectral overlap.

4.1.2.1. Prepare FMO controls by using 1 mL of cells that was aliquoted from section 4.1 in a 5 mL FACS tube and adding in all antibodies from the marker panel described in step 4.1.1 at a 1:100 dilution but excluding one antibody. For example, prepare an NG2-AF488 FMO by including antibodies for CD31-APC, CD140b-PE, CD146-BV605, CD34-BV421, CD45-PE-Cy7, cell viability dye but not the NG2-AF488 antibody. Mix gently by pulse-vortexing. Repeat for each antibody for a total of 7 controls. Incubate for 30 min at 4 °C protected from light.

4.1.2.2. Next, add 3 mL of FACS staining buffer to each tube and centrifuge at 300 x *g* for 5 min at 4 °C. Aspirate off solution and resuspend each cell pellet in 400 µL of FACS staining buffer. The FMO controls are ready to be used. Keep on ice.

4.1.3. Use isotype-matched control antibodies (**Table of Materials**) for nonspecific staining.

4.1.3.1. Prepare isotype controls by adding the isotype-matched control antibody (**Table of Materials**) to 1 mL of cell sample prepared from section 4.1 at a 1:100 dilution each in a 5 mL FACS tube. Mix gently by pulse-vortexing. Incubate for 30 min at 4 °C protected from light.

4.1.3.2. Next, add 3 mL of FACS staining buffer to each tube and centrifuge at 300 x *g* for 5 min at 4 °C. Aspirate off solution and resuspend each cell pellet in 400 µL of FACS staining buffer. The isotype controls are ready to be used. Keep on ice.

4.1.4. Prepare cells to be sorted by adding antibody cocktail to freshly isolated cells.

4.1.4.1. Prepare cell sample from section 4.1 by adding in an antibody cocktail containing anti-mouse NG2-AF488, CD31-APC, CD140b-PE, CD146-BV605, CD34-BV421, CD45-PE-Cy7 at 1:100 dilution each and cell viability dye at 1:1000 dilution. Gently vortex to mix. Incubate samples at 4 °C for 30 min protected from light.

4.1.4.2. After staining, wash cells with FACS staining buffer by centrifugation at 300 x *g* for 5 min 4 °C. Aspirate off solution and resuspend the cell pellet in FACS staining buffer to 0.5 x 10^6 cells/mL.

4.1.4.3. Using new FACS tubes that have 35 µm filter tops, pipette stained cell samples onto the lids and gravity filtrate to obtain single cell suspensions. Keep on ice.

4.2. Use a cell sorter to purify cells.

4.2.1. Run the unstained cells on the cell sorter to set voltages and correct for the background signal (for example, set voltages for forward scatter to 490−560 and for side scatter to 180−250).

4.2.2. Run each isotype control one at a time and this data can be used to adjust gates for nonspecific binding if there are any.

4.2.3. Run each FMO sample one at time and adjust voltages for each channel to correct for spectral bleed through due to a multi-color panel.

4.2.4. Run each single-color compensation bead sample one at a time to adjust voltages for each channel and adjust gates for the positive signal. Collect data. Use the software to calculate for spectral overlap by calculating the compensation matrix. All voltages are ready and set.

4.2.5. Run the stained cell samples in the cell sorter and collect cells in 10 mL enzyme-free culture media (DMEM + 20% FBS + 1% P/S) in a 15 mL conical collection tube. Use the following gating strategy: gate for single cells, gate for live cells, gate for CD45 negative cells, gate for CD34 and CD31 negative cells, gate for NG2 positive cells, and finally gate for CD146 and CD140b positive cells.

5. Culturing of pericytes

5.1. Coat a 24-well plate with 0.2% gelatin for 5 min and aspirate off gelatin solution. Seed freshly obtained cells from step 4.2.5 in DMEM + 20% FBS + 1% P/S up to 2 x 10^4 cells/cm^2. Culture cells in a cell incubator set at 37 °C, 5% CO_2 and 95% O_2.

5.2. Passaging of pericytes

5.2.1. Once the cells are 95% confluent, wash cells with warm 1x DPBS, and lift cells with 200 µL of 0.1% trypsin in each well at room temperature for 3−5 min.

5.2.2. Gently tap the plate to loosen the cells.

5.2.3. Neutralize the trypsin with 3.5x the amount of culture media (700 µL DMEM + 20% FBS + 1% P/S) and seed passage two (P2) cells onto an uncoated 6-well plate at to 2 x 10^4 cells/cm^2.

5.2.4. Each well, when confluent, can be moved into a single T-75 flask as P3 cells which then can be split at a 1:6 ratio.

6. Characterization of pericytes

6.1. Flow cytometry analysis

6.1.1. Use the same FACS staining protocol and gating strategy as previously described in section 4.

6.1.2. Run controls and stained samples on the flow cytometer. Collect data and analyze data using the analysis software (**Table of Materials**).

6.2. To collect brightfield images, grow cells in a flask in a cell incubator set at 37 °C, 5% CO_2 and 95% O_2. Capture images on a microscope after cells attach to the surface.

6.3. Immunocytochemistry

6.3.1. Grow cells in a 96-well plate until 90% confluent. Wash cells with warm 1x DPBS and fix with 4% paraformaldehyde for 30 min at room temperature.

6.3.2. Wash cells 3x with 1x DPBS and permeabilize with 0.1% detergent for 10 min at room temperature.

6.3.3. Incubate cells with blocking buffer for 1 h at room temperature. After blocking, add primary antibodies (one antibody per well) diluted 1:100 in blocking buffer and incubate at 4 °C overnight. Primary antibodies are: anti-NG2, anti-CD140b, anti-CD31, anti-vimentin, anti-desmin, and anti-alpha smooth muscle actin.

6.3.4. Next day, wash cells 3x with wash buffer (**Table of Materials**). Add secondary antibody diluted 1:1000 in blocking buffer and incubate for 2 h at room temperature in the dark. Secondary antibody is an anti-rabbit conjugated to FITC.

6.3.5. Wash cells 3x with wash buffer. Add 300 µM nuclear stain diluted at 1:1000 for 5 min at room temperature.

6.3.6. Wash cells 3x with 1x DPBS and mount with mounting media.

6.3.7. Image cells with a confocal microscope.

REPRESENTATIVE RESULTS:

After enzymatic digestion and dissociation of the whole heart and before FACS purification of the cells, cells are a crude mixture that contains many different cell types from the heart (**Figure 1A**). After FACS purification and culturing, cells are homogenous. They are single nucleated, quite flat, and have the typical pericyte rhomboid morphology (**Figure 1B**).

Using FACS, cells are purified to homogeny. The unstained control cell sample is used to show the gating strategy (**Figure 2**). First, debris and doublets were gated out based on forward and side scatter distributions. Then dead cells were gated out due to their amine reaction with the dye which produces a signal greater and more intense than live cells. Of the live cells, hematopoietic cells were gated out by being $CD45^+$. To further remove hematopoietic and endothelial cells, $CD34^+$ and $CD31^+$ cells were gated out. Finally, $NG2^+$ and $CD140b^+/CD146^+$ cells were selected for being perivascular cells with expression of typical pericyte markers (**Figure 3**). The marker panel was also tested on mouse coronary endothelial cells as a control (**Supplemental Figure 1**). Only about 1% of crude cell mixture consisted of pericytes after sorting.

To validate that the cells were indeed pericytes, we passaged the cells for further characterization. Cells grew rapidly once they reached P3 in the T-75 flasks without changes in viability as they became older (**Supplemental Figure 2**). When compared with human brain pericytes, the cells had a similar morphology (**Figure 4A**). When compared with mouse and human smooth muscle cells, the cells had a different morphology (**Figure 4A**). There were also no observed changes in morphology or marker expression at P7 when immunostained or by flow cytometry analysis after passaging (**Figure 4B,C**).

FIGURE LEGENDS:

Figure 1: Crude cells versus purified cells. (**A**) The brightfield image of crude cell mixture post whole heart enzymatic digestion and dissociation which has been cultured in a T25 flask for 14 days. (**B**) The brightfield image of a homogenous population of cardiac pericytes post-sorting and culturing after 14 days. Scale bar = 100 µm.

Figure 2: Representative images of FACS analysis of unstained cells. Schematic representation of the gating strategy used to purify crude cell mixture. Gate for cells that are single, live, $CD45^-$, $CD31^-$, $CD34^-$, $NG2^+$, $CD146^+$, and $CD140b^+$.

Figure 3: Representative images of FACS analysis of crude cells. Schematic representation of the sorting used to obtain a homogenous population of cardiac pericytes. Roughly 1% of crude cells are $CD31^-CD34^-CD45^-CD140b^+NG2^+CD146^+$.

Figure 4: Characterization of primary isolated cardiac pericytes (**A**) Brightfield images of cultured cells from human brain (hPC) and mouse hearts (mPC) show similar pericyte cell morphology but different morphology from human smooth muscle cells hSMC) and mouse smooth muscle cells (mSMC). Scale bar = 100 µm. (**B**) Phenotypic characterization of cells at P7 by immunocytochemistry for pericyte markers. Scale bar = 100 µm. (**C**) Analysis by flow cytometry of the pericytes at P7 where they were gated for negative markers CD31, CD34, CD45 and positive markers NG2, CD140b, and CD146. Population remains homogenous.

Supplemental Figure 1: Representative images of flow cytometry analysis of endothelial cells using marker panel. A mouse coronary endothelial cell line was used as control for the binding specificity for the markers. Using the same gating strategy that was used in the sort except for a positive gate for CD31 instead of a negative gate, the endothelial cells were negative for CD45, CD34, NG2, CD140b, and CD146 but positive for CD31 as expected.

Supplemental Figure 2: Representative images of flow cytometry analysis of different passages of mPC. Primary isolated cardiac pericytes were cultured and passaged up to passage 12. Cells were stained with propidium iodide and analyzed on a flow cytometer. Control population is a mixture of dead cells and live cells. There were no significant differences in number of viable cells between passages.

DISCUSSION:

As studies on cardiac pericytes are relatively new, the role of pericytes in cardiovascular physiology and pathophysiology have yet to be defined. In other organs, they have been shown to play key roles in vessel homeostasis and perfusion[1,2]. Compared to the literature of pericytes from other organs such as the brain, there are significantly fewer publications on cardiac pericytes. The isolation of cardiac pericytes is critical to the understanding of their functional characteristics and signaling mechanisms. Therefore, this protocol will provide investigators with an easier way to access cardiac pericytes from a more readily available tissue source and promote studies on their biology. It will help answer questions on how cardiac pericytes contribute to cardiac homeostasis and pathophysiology as well as investigate their therapeutic potential.

The pericyte population isolated from murine heart and characterized by $CD31^{-}CD34^{-}CD45^{-}CD140b^{+}NG2^{+}CD146^{+}$ has been passaged multiple times (up to P12 and was still going strong), which does not decrease in viability and propagates quickly (**Supplementary Figure 2**). The cells have also been cryofrozen and recovered with at least 95% viability. However, we prefer to use cells P7 or younger for our experiments. Comparing brightfield images of our pericytes with human brain pericytes, the two cell lines have comparable cell morphology (**Figure 4A**) while they differ in morphology from smooth muscle cells (**Figure 4A**). Our P7 cells were characterized by immunocytochemistry for pericyte markers, some from our FACS panel (NG2 and CD140b), and a few not in the panel (vimentin, desmin, αSMA) and we found that the cells expressed pericyte markers homogenously (**Figure 4B**). Additionally, our P7 cells were analyzed by flow cytometry again with the same marker panel to assess for changes in marker expression due to passaging and we found that there were no changes (**Figure 4C**). Therefore, both phenotypically and morphologically, our cells are pericytes.

The studies by Nees et al.[10], Avolio et al.[11], Chen et al.[12], and Baily et al.[13] have shown successful cardiac pericyte isolations. However, the use of an in-house custom built equipment to detach the pericytes from the microvessels by Nees et al.[10] involved two chambers with pumps that perfused protease solution back and forth through a mesh net stack, which was hard to replicate as they did not provide a schematic and/or picture of the apparatus and how it was built. Although Nees et al.[10] successfully isolated cardiac pericytes from many species, we were never able to reproduce their method. Our pericyte detachment step in our protocol simply uses an orbital shaker (to dissociate all cells) which is available in most, if not all laboratories, with the tissue and enzyme solution in a conical tube followed by a mechanical dissociation step. There is no custom apparatus required. Secondly, the remaining protocols involve the use of human tissues and thus the procurement of human tissue is limiting to investigators. Our protocol is a modification and optimization of current protocols[9,12] using mouse models (wild type, genetically modified, diseased) and materials that are readily available to all investigators.

Because perivascular cells in general are sensitive, viability of the cells is critical to obtain a good yield. During procurement of cardiac tissue and staining of cells, the tissue/cells need to be kept ice cold. Secondly, the enzymatic digestion of the tissue may require optimization on an individual basis. Depending on the units of activity on one's vials of enzymes, concentration and

digestion time may need to be optimized. Make sure that the enzymatic solution is prepared fresh each time otherwise yield will decrease. Thirdly, the crude mixture contains a lot of cells, some dead and/or dying, it is best to lower the concentration of FBS in the staining buffer from 5% to 2%. If you are having trouble with cells clogging the nozzle during sort, enrich the cells first by using a dead cell removal kit. You can also add EDTA/HEPES buffer or DNase treatment to the cell pre-sort to prevent cell clumping. Lastly, because our panel of antibodies is rather large and uses many fluorophores, be sure your FMO controls and compensation controls are done correctly.

One limitation to this method is the amount of cardiac pericytes that can be obtained per heart. In our case, only 1.1% of our crude mixture from one mouse heart were pericytes which is comparable to the percent in the human heart isolations, but the number of cells is significantly less due to the amount of heart tissue a mouse provides. Because the starting number of cells is so low after FACS, it would be better to isolate from multiple hearts at once. However, the problem with that is the sheer number of cells that you need to sort through in one day. If you have more than 30 million cells, it will be difficult to get through the sort without affecting the viability of the cells. If the investigator had multiple cell sorters, isolating from multiple hearts in a day would be doable. Another limitation is that because we do not know if there are subpopulations of pericytes in the heart like there is skeletal muscle[15,16], we do not know if we are eliminating a subtype in our gating strategy. We are in the process of characterizing our cardiac pericytes and thus far in our unpublished data, they are functionally like other pericytes in the literature.

Our protocol will enable investigators to answer questions on cardiac pericyte properties, characteristics, functionality, and other aspects that will help define their contribution to cardiac homeostasis and hemodynamics. These cells could have therapeutic potential to cardiovascular disease once their biology is better understood.

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DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Armulik, A., Abramsson, A., Betsholtz, C. Endothelial/pericyte interactions. *Circulation Research.* **97** (6), 512-523 (2005).
2. Armulik, A., Genove, G., Betsholtz, C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Developmental Cell.* **21** (2), 193-215 (2011).
3. Sengillo, J. D. et al. Deficiency in mural vascular cells coincides with blood-brain barrier disruption in Alzheimer's disease. *Brain Pathology.* **23** (3), 303-310 (2013).
4. Avolio, E., Madeddu, P. Discovering cardiac pericyte biology: From physiopathological mechanisms to potential therapeutic applications in ischemic heart disease. *Vascular*

Pharmacology. **86**, 53-63 (2016).
5. Dore-Duffy, P. Isolation and characterization of cerebral microvascular pericytes. *Methods in Molecular Medicine.* **89**, 375-382 (2003).
6. Bryan, B. A., D'Amore, P. A. Pericyte isolation and use in endothelial/pericyte coculture models. *Methods in Enzymology.* **443**, 315-331 (2008).
7. Maier, C. L., Shepherd, B. R., Yi, T., Pober, J. S. Explant outgrowth, propagation and characterization of human pericytes. *Microcirculation.* **17** (5), 367-380 (2010).
8. Crisan, M., Corselli, M., Chen, W. C., Peault, B. Perivascular cells for regenerative medicine. *Journal of Cellular Molecular Medicine.* **16** (12), 2851-2860 (2012).
9. Crisan, M. et al. Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. *Methods in Cellular Biology.* **86**, 295-309 (2008).
10. Nees, S. et al. Isolation, bulk cultivation, and characterization of coronary microvascular pericytes: the second most frequent myocardial cell type in vitro. *American Journal of Physiology Heart Circulatory Physiology.* **302** (1), H69-84 (2012).
11. Avolio, E. et al. Expansion and characterization of neonatal cardiac pericytes provides a novel cellular option for tissue engineering in congenital heart disease. *Journal of the American Heart Association.* **4** (6), e002043 (2015).
12. Chen, W. C. et al. Human myocardial pericytes: multipotent mesodermal precursors exhibiting cardiac specificity. *Stem Cells.* **33** (2), 557-573 (2015).
13. Baily, J. E. et al. Isolation of Perivascular Multipotent Precursor Cell Populations from Human Cardiac Tissue. *Journal of Visualized Experiments.* (116), e54252 (2016).
14. Murray, I. R. et al. Skeletal and cardiac muscle pericytes: Functions and therapeutic potential. *Pharmacology & Therapeutics.* **171**, 65-74 (2017).
15. Birbrair, A. et al. Role of pericytes in skeletal muscle regeneration and fat accumulation. *Stem Cells Development.* **22** (16), 2298-2314 (2013).
16. Birbrair, A. et al. Skeletal muscle pericyte subtypes differ in their differentiation potential. *Stem Cell Research.* **10** (1), 67-84 (2013).

Figure 1

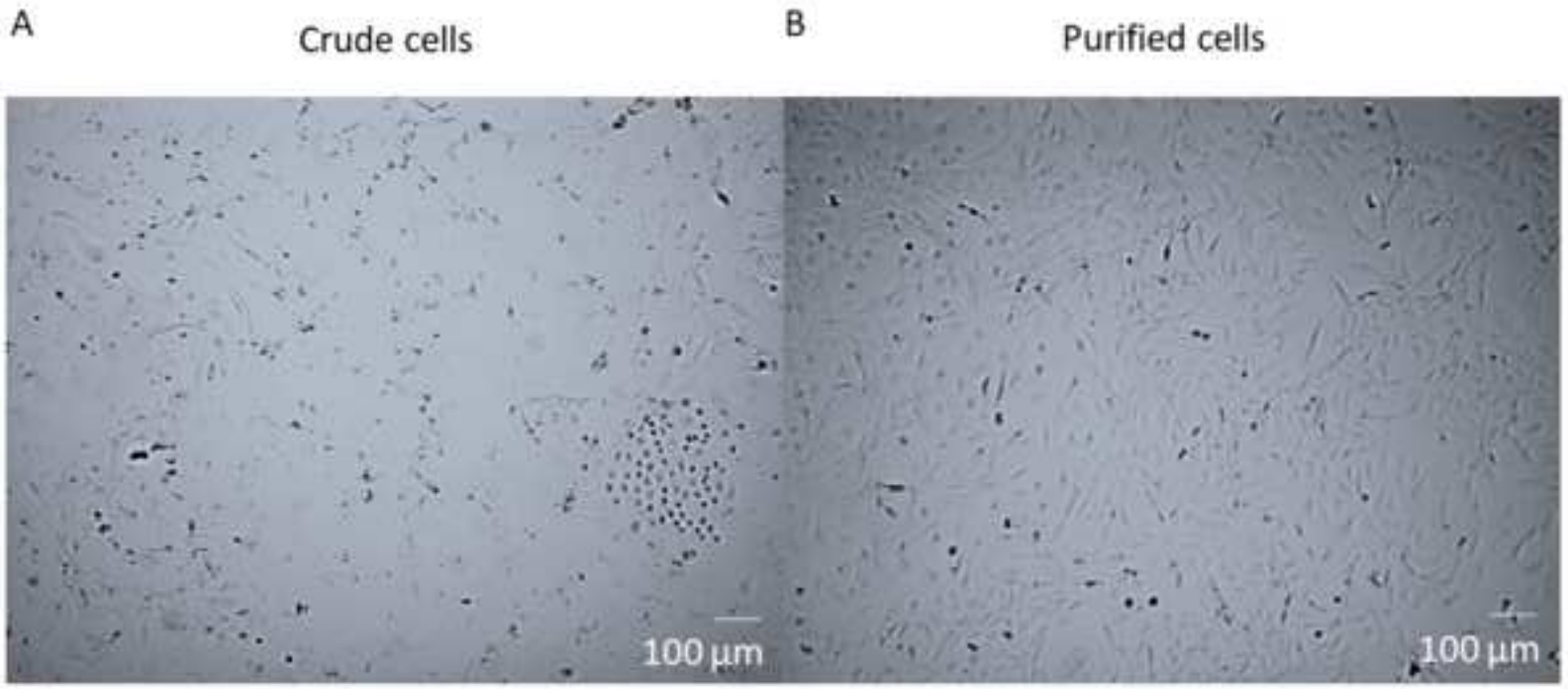

Figure 2

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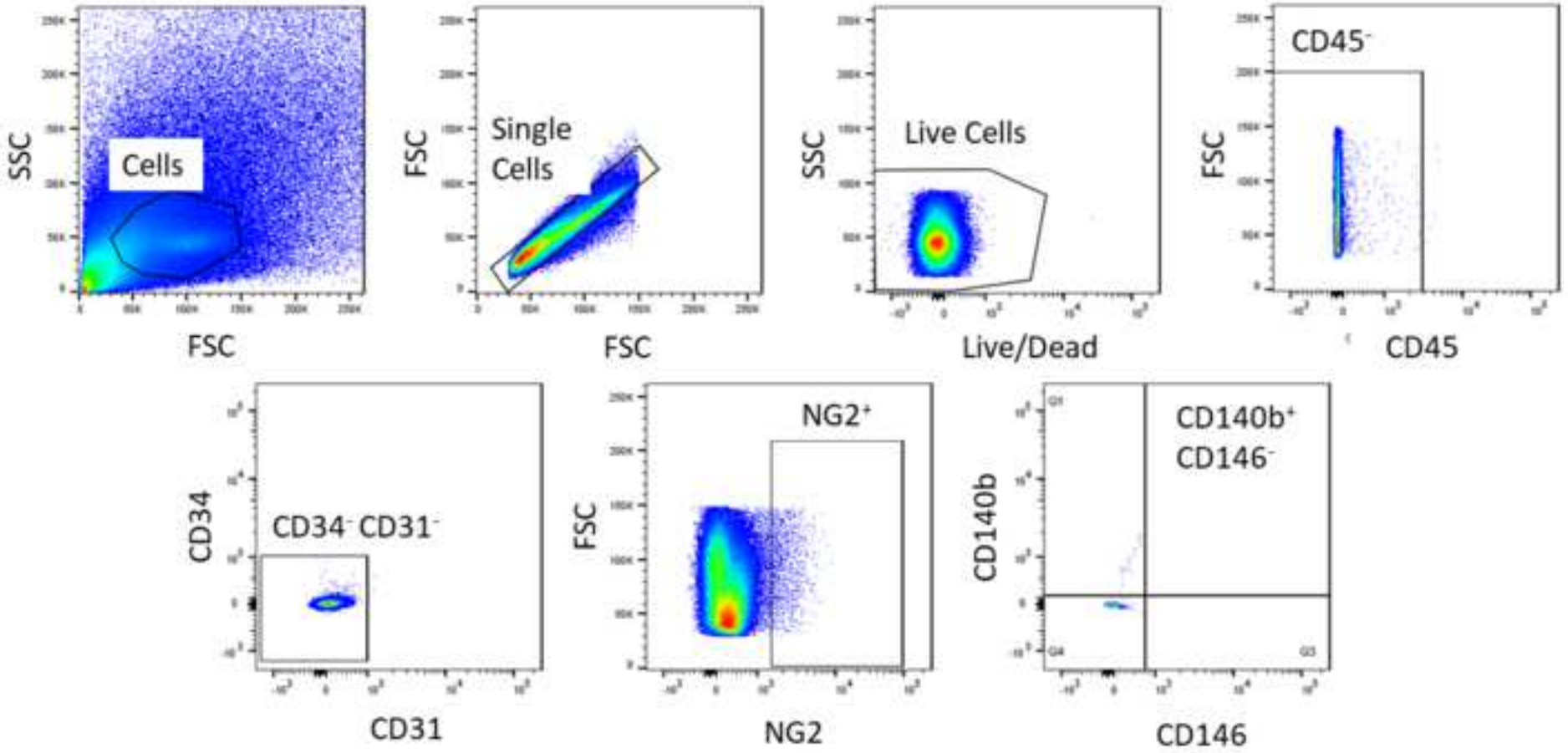


Figure 3

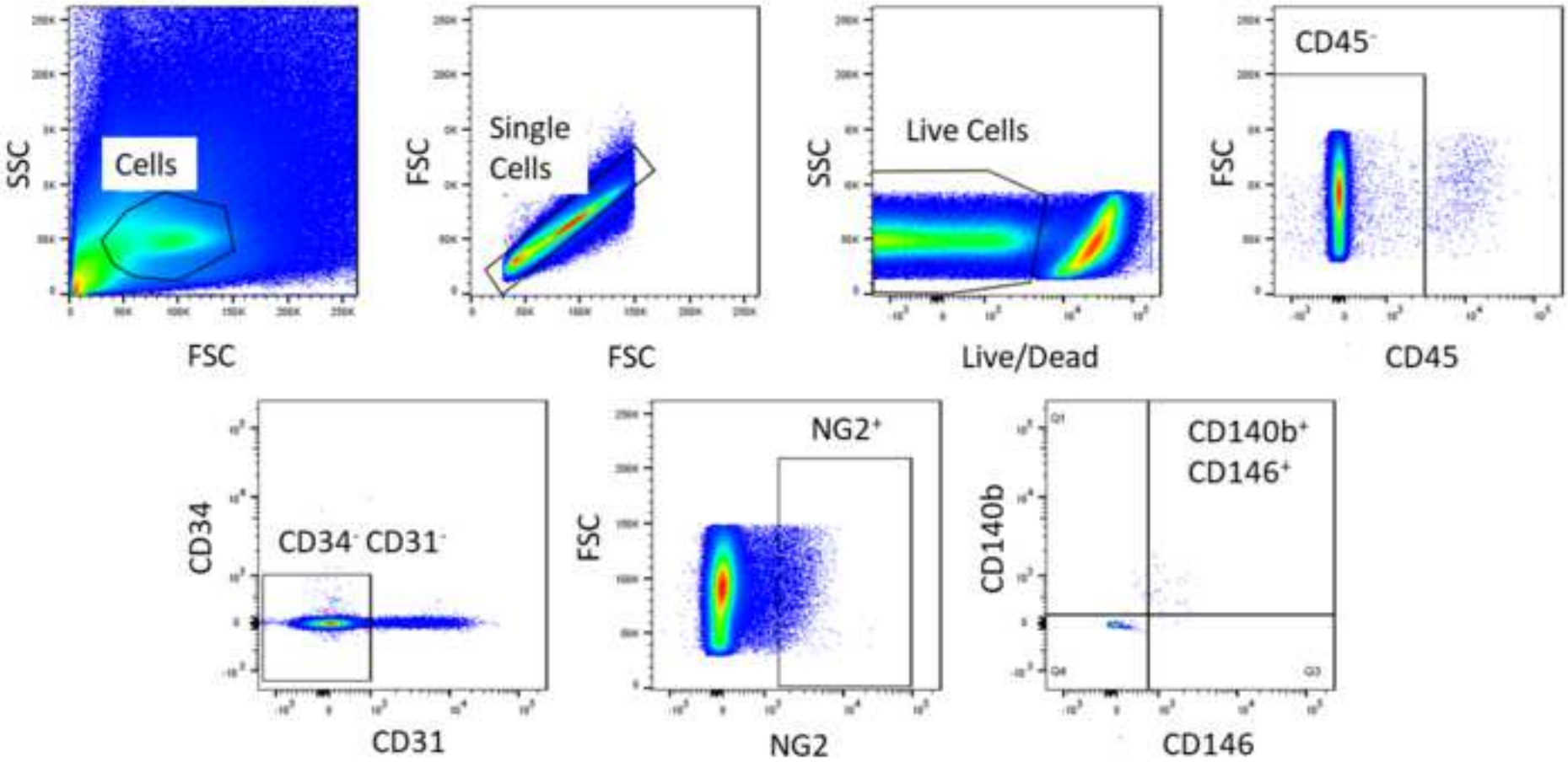
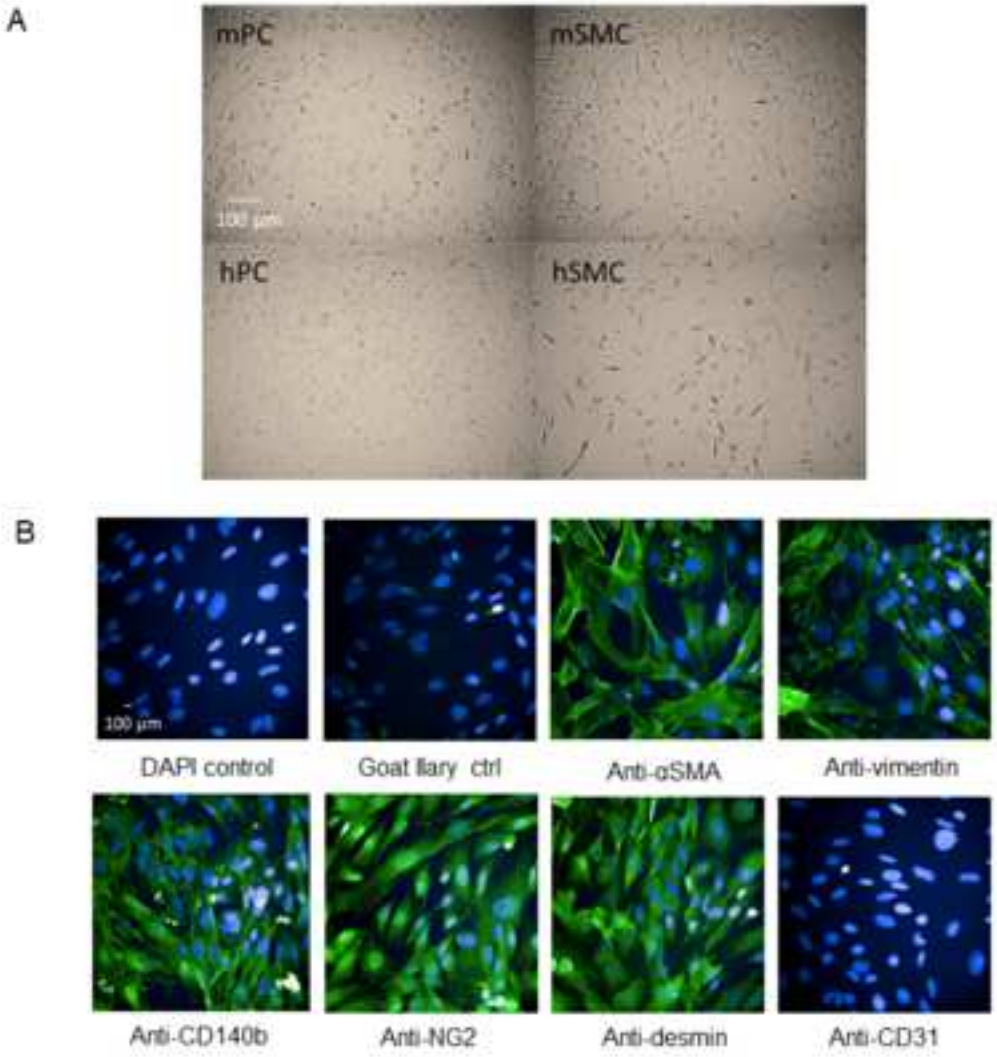


Figure 4



Name of Reagent/ Equipment	Company	Catalog Number
0.25% Trypsin-EDTA	Corning	25-053-Cl
100 µM Cell strainer	FisherSci	22363549
15 mL Falcon conical tubes	BD	352096
24-well plate	Corning	CLS3527
25 gauge butterfly needle	FisherSci	22-253-146
31 gauge needle syringe	FisherSci	B328446
50 mL Falcon conical tubes	BD	352098
6-well plate	Corning	CLS3516
anti-alpha smooth muscle actin rabbit mAb	abcam	ab32575
anti-CD140b rabbit mAb	Cell Signaling	28E1
anti-CD31 rabbit pAb	abcam	ab28364
anti-desmin rabbit pAb	abcam	ab8592
anti-NG2 conjugated to AF488	Millipore	MAB5384A4
anti-vimentin rabbit mAb	abcam	ab92547
ArC Amine Reactive Compensation bead kit	Invitrogen	A10346
Brightfield Microscope		
CD140b-PE (clone APB5)	eBioscience	12-1402-81
CD146-BV605 (clone ME-9F1)	BD	740434
CD31-APC (clone MEC 13.3)	BD	551262
CD34-BV421 (clone RAM 34)	BD	56268
CD45-PE-Cy7 (clone 30-F11)	BD	552848
Centrifuge	eppendorf	
Collagenase B	Roche	11088815001
Confocal Microscope		
DAPI	ThermoFisher	D1306
DMEM with 4.5 g/L glucose, L-glutamine & sodium pyruvate	Corning	10-013-CV
Dowell scissors	FST	15040-11
Dulbecco's Phosphate-Buffered Saline (DPBS)	Corning	21-030-CV

Dulbecco's Phosphate-Buffered Saline without Ca and Mg (CMF-DPBS)	Corning	21-031-CV
Dumont #5 Fine Forceps	FST	11254-20
FACSAria cell sorter	BD	
FACSAria software	BD	
Falcon tube round-bottom polypropylene, 5 mL	BD	38057
Falcon tube with cell strainer cap, 5 mL	BD	08-771-23
Fetal Bovine Serum	Corning	35-015-CV
Fine scissors	FST	14060-09
FlowJo software	FlowJo LLC	
Fortessa LSR flow cytometer	BD	
Gelatin-based coating	Cell Biologics	6950
Goat anti-rabbit IgG (H+L) Cross-Absorbed Secondary antibody, Alexa Fluor 488	Invitrogen	A-11008
Graefe Forceps	FST	11049-10
Heparin sodium solution	Hospira	NDC 0409-2720-02
Incubator		
Live/Dead-Near IR	Life Technologies	L10119
Microscope slides	FisherSci	12-550-343
NG2-FITC	Millipore	AB5320A4
Oribital shaker	VWR	
Paraformaldehyde	FisherSci	50-980-487
Penicillin-Streptomycin	Corning	30-002-CI
Petri dish	FisherSci	FB0875714
Pipette and tips		
ProLong Diamond	ThermoFisher	P36965
Propidum Iodide	ThermoFisher	
Rabbit IgG FITC	eBiosciences	11-4614-80

Rat IgG2a APC	Biolegend	400512
Rat IgG2a BV421	Biolegend	400536
Rat IgG2a BV605	BD	563144
Rat IgG2a PE	Biolegend	400308
Rat IgG2b PE-Cy7	Biolegend	400617
SuperBlock	ThermoFisher	37515
T75	ThermoFisher	156499
Triton X-100	Sigma	X100
UltraComp beads	Invitrogen	01-2222-42
Variable-Flow Peristaltic Pump	FisherSci	13-876-1
ViCell Cell counter	Beckman	
Wash buffer		

Comments/Description
dilute with 1x DPBS to get 0.1%
Antibody used in ICC 1:100 dilution
Antibody used in ICC 1:100 dilution
Antibody used in ICC 1:100 dilution
Antibody used in ICC 1:100 dilution
Antibody used in ICC 1:100 dilution
Antibody used in ICC 1:100 dilution
compensation beads for Live/Dead Near IR dye
camera attached
Antibody used in FACS 1:100 dilution
Antibody used in FACS 1:100 dilution
Antibody used in FACS 1:100 dilution
Antibody used in FACS 1:100 dilution
Antibody used in FACS 1:100 dilution
0.226 U/mg lyo.
nuclear stain
500 mL
500 mL

500 mL
Lasers: 405 nm 50 mW, 488 nm 100 mW, 561 nm 50mW, 633 nm 11 mW
500 mL
Lasers: 405 nm 50 mW, 488 nm 100 mW, 561 nm 50mW, 633 nm 11 mW
Antibody used in ICC 1:1000 dilution
10,000 USP units/10 mL; from porcine intestines
set at 37 °C, 5% CO2, 95% O2
Antibody used in FACS 1:100 dilution
Inside 37 °C incubator or room
dilute with 1x DPBS to get 4%
mounting media
cell viability dye for supplemental figure 2
Isotype control antibody - FITC

Isotype control antibody - APC
Isotype control antibody - BV421
Isotype control antibody - BV605
Isotype control antibody - PE
Isotype control antibody - PE-Cy7
blocking buffer
detergent, dilute with x DPBS to get 0.1%
compensation beads
1:10 dilution of Superblock in 1x DPBS

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Author(s): Linda L. Lee, Aarif Khakoo, Vishnu Chintalgattu

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CORRESPONDING AUTHOR

Name:	Vishnu Chintalgattu
Department:	Cardio Metabolic Disorders
Institution:	Amgen Inc.
Title:	Sr. Scientist
Signature:	Vishnu
Date:	12/14/2018

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JOVE manuscript (JoVE59571) reviewers comments

All references to "Lines" are based on the track changes version of the manuscript.

Editorial comments:

Changes to be made by the Author(s):

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.

Thank you for this opportunity. We have proofread the manuscript and made minor changes throughout.

2. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u. Please abbreviate liters to L to avoid confusion.

We have used SI abbreviations for all units.

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

Spaces between all numerical values and units have been included.

4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Added in Line 77-79: All animals were housed and used in an AAALAC accredited facility and all animal work was conducted under appropriate veterinary oversight and under the IACUC approved protocol of Amgen Inc.

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Manuscript has been updated to remove commercial sounding language.

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. 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. See examples below.

7. 1.2, 1.3: What volume of FBS and P/S is added? Please spell out CMF-DPBS.

For 5% FBS, 25 mL of FBS was added to 500 mL media. For 20% FBS, 100 mL of FBS was added to 500 mL media. For 1% P/S, 5 mL of P/S was added to 500 mL media. Volumes have been added in Line 86 and Line 92.

CMF-DPBS is Calcium-Magnesium free – Dulbecco's phosphate-buffered saline. See Line 87.

8. 2.1: Please specify the type, gender and age of mouse used.

Male C57BL/6 mice, 4 months old has been added in Line 103.

9. 2.3, 2.4: Please specify all surgical tools used. What is heparnized CMF-DPBS?

Surgical tools information has been added in Line 83-84.

Heparinized CMF-DPBS is CMF-DPBS with 250 units/mL of heparin sodium solution in it. This information has been clarified and added in Line 89-90.

10. 3.1: Does the enzyme solution refer to the ice-cold CMF-DPBS?

Enzyme solution is the 20 mL of media + 500 ug/mL of collagenase B, see section 1.3.

11. 3.3: The unit for the mesh size should be µm, instead of µM. Please correct.

Corrected in Line 128.

12. 3.4: Please specify the composition of enzyme-free culture media.

Enzyme-free culture media is the 500 mL of DMEM + 20% FBS + 1% P/S, see 1.3 Line 95.

13. 4.1: Please spell out FMO. What volume of cells are used for the sort?

Fluorescence minus one has been added in Line 150.

A total of 13 mL at 0.5 x 10 ^-6 cells/mL were used for the representative sort from one heart, however, volume depends on how many cells the investigator gets from their isolation and how many hearts they used. How well the heart tissue is digested and the size of each heart are also variables that can alter the volume. Information has been added in section 4.1, Lines 155-158.

14. 4.1.1: Please describe how the controls samples are prepared.

One compensation control is prepared for each fluorochrome in the experiment in a labelled 5 mL FACS tube. For this experiment, we have a total of 9 compensation controls – 2 kinds of unstained beads plus 7 different fluorochromes. Add one drop of beads to each tube. Then add 1 µL of antibody to the beads. Mix vigorously by pulse-vortexing. Incubate for 30 min at 4 °C protected from light except for the Live/Dead Near-IR beads which can be left at room temperature protected from light. Next, add 3 mL of staining buffer to each tube and centrifuge at 300 x g for 5 min at 4 °C. Aspirate off solution and resuspend each bead pellet in 400 µL of staining buffer.

FMO controls are prepared by using 1 mL of cells in a 5 mL FACS tube and adding in all antibodies in the marker panel but one. For example, a NG2-AF488 FMO will include antibodies for CD31-APC, CD140b-PE, CD146-BV605, CD34-BV421, CD45-PE-Cy7, Live/Dead-Near IR but not the NG2-AF488 antibody. Next, add 3 mL of staining buffer to each tube and centrifuge at 300 x g for 5 min at 4 °C. Aspirate off solution and resuspend each cell pellet in 400 µL of staining buffer.

Isotype controls are prepared by adding the isotype-matched control antibody to 1 mL of cell sample and incubate for 30 min at 4 °C in the dark to stain. Next, add 3 mL of staining buffer to each tube and centrifuge at 300 x g for 5 min at 4 °C. Aspirate off solution and resuspend each cell pellet in 400 µL of staining buffer.

Details have been heavily modified and more details have been added. Section 4.1 Lines 148-189.

15. 4.1.2: Please describe how to perform staining.

Section 4.1 have been heavily modified for clarification. See Lines 149-190.

16. 4.3: Please describe FACS analysis and gating strategies.

The gating strategies should be as follows – gate for single cells, gate for live cells, gate for CD45 negative cells, gate for CD34 and CD31 negative cells, gate for NG2 positive cells, and finally gate for CD146 and CD140b positive cells. This information has been added in Lines 204-206.

17. Lines 195-196: Please cite the relevant references here.

Citations have been updated via EndNote8.

18. Please revise the Acknowledgements to be a complete sentence.

We would like to thank the Amgen Flow Cytometry Core for their help with fluorophore panel design, troubleshooting, and cell sorting. The revised complete sentence has been added in Lines 349-350.

19. References: Please do not abbreviate journal titles.

References have been modified to not have abbreviated journal titles.

20. Table of Materials: Please use SI abbreviations for all units (L, mL, µL, µm) and include a space between all numerical values and their corresponding units (15 mL, 37 °C, etc.). Please sort the items in alphabetical order according to the name of material/equipment. Please provide lot numbers and RRIDs of antibodies, if available.

Changes have been made and tracked as requested in the JoVE_materials excel file.

Reviewers' comments:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

Using enzymatic digestion and fluorescence activated cell sorting (FACS) with inclusion/exclusion of known key pericyte markers CD31-CD34-CD45-CD140b+NG2+CD146+, the protocol allows users to isolate and purify a population of murine cardiac pericytes.

As known, there are different approaches about pericyte isolation, characterization and culture but there has been no common protocol yet because of the lack of defined pericyte-specific marker. For human cardiac tissue, one protocol published in the JOVE used antibody labeling and FACS to isolate perivascular multipotent precursor cell populations (also called pericytes).1 One other study obtained pericytes by outgrowth from microvessel fragments recovered after enzymatic digestion of human placental tissue by a plethora of markers and FACS.2 There are many literatures available using similar methods and mainly FACS. Therefore, my major concerns are: 1) lack of novelty 2) lack of critical data

which should validate the isolated cell population, 3) What is isolation efficacy? In line 181 also line 214, authors claim the approximately 1% of crude cells are targeted cells, but it should be an important estimate for readers to reproduce the result. How many hearts to start with? This limitation is critical to perform the experiment successfully. Authors need to discuss further. 4). How long can the isolated cells survive and propagate?

1.) Our protocol is optimized for the mouse model and uses materials that are readily available to all investigators. There is currently no step-by-step protocol available specifically for murine cardiac pericytes and we believe this is the novelty of our manuscript. As mentioned in Lines 49-60 of the introduction and brought up by the reviewer, there are publications about pericyte isolations from tissues such as the brain, skeletal muscle, and retina. There are a few publications from the heart (Baily *et al.*; Avolio *et al*.; Nees *et al*.). The protocols that are available for the heart are from human (Baily *et al.*) and human neonatal tissues (Avolio *et al*.) which not all investigators have access to. Nees *et al*. isolated pericytes from multiple species, including the mouse, but their method included in-house equipment which we have not been able to reproduce. Because the mouse model is a widely used biological tool, pericytes from healthy or disease mouse models such as myocardial infarction or atherosclerosis can be isolated using our protocol. This is discussed in Lines 301-314.
2.) We have included immunofluorescence staining of our cells for phenotypic characterization and brightfield images of our cells for morphological characterization as a supplemental figure (Figure 4). In our immunofluorescence images, we stained our cells with a plethora of known pericyte markers (Armulik *et al.*, Murray *et al.*). Secondly, pericyte morphology is distinguishable from other vascular cells (Armulik *et al*.). Our brightfield images show comparable morphology with a commercial pericyte cell line as well as the difference in morphology from commercial smooth muscle cell lines (Figure 4). Detailed functional characterization of our cell population will be in a follow-up manuscript and we believe that it is beyond the scope of this method focused manuscript.
3.) In every isolation that we've done, cells expressing the desired markers have been isolated (each time has been 1 % of crude cells). Each isolation prep was done with one heart, just like the representative results. However, if the investigator chooses to bulk sort multiple hearts, it is possible provided they have multiple sorters. Investigators should use one heart to start with. This has been noted in Lines 154-157. This limitation was discussed in Lines 329-341.
4.) Cells can survive for multiple passages. They propagate quickly and have been passaged up to passage 12 with no changes in morphology. Cells can also be frozen down and recovered to at least 95% viability. This is discussed in Lines 286-289 and added as supplemental Figure 2, Lines 269-272.

1. Baily, J. E. et al. Isolation of Perivascular Multipotent Precursor Cell Populations from Human Cardiac Tissue. J. Vis. Exp. (116), e54252, doi:10.3791/54252 (2016).

This citation was already cited.

2. Maier C et al. Explant Outgrowth, Propagation and Characterization of Human Pericytes. Microcirculation. 2010 Jul; 17(5): 367-380. doi: 10.1111/j.1549-8719.2010.00038.x

This citation has been added in Line 52.

Reviewer #2:

Manuscript Summary:

This manuscript reports a method for isolation and purification of murine cardiac pericytes. The authors generate a protocol using the enzymatic digestion and dissociation of heart tissue to obtain the crude cells and purify the pericytes by fluorescence activating cell sorting by the combination of the markers CD31-CD34-CD45-SD140b+NG2+CD146+. This manuscript is well organized and the method is critical for this field development. Nevertheless, there are a number of critical questions that need to be addressed.

Major Concerns:

1. The JOVE if featured by the visualization of the experiment procedures. However, the video is not found in this manuscript.

The video is made and produced by JoVE once the manuscript is peer reviewed and accepted.

2. Please make each step more detailed, so that the readers could follow. For example, at the step 4.3 starting line 143, please add what voltages the authors used, or suggested voltage range, etc.

We have heavily revised and added more details to section: Purification of pericytes from crude cell mixture using FACS, Lines 147-206.

3. Finally and most importantly, please provide the evidence of these purified cells are pericytes.

We have included immunofluorescence staining of our cells for phenotypic characterization and brightfield images of our cells for morphological characterization as a supplemental figure (Figure 4). In our immunofluorescence images, we stained our cells with a plethora of known pericyte markers (Armulik *et al.*, Murray *et al.*). Secondly, pericyte morphology is distinguishable from other vascular cells (Armulik *et al*.). Our brightfield images show comparable morphology with a commercial pericyte cell line as well as the difference in morphology from commercial smooth muscle cell lines (Figure 4). Detailed functional characterization of our cell population will be in a follow-up manuscript and we believe that it is beyond the scope of this method focused manuscript.

Minor Concerns:
1. On step 3.1, line 100, "15*15mm petri dish" was used. Is this too small to operation? The heart was cut into "5mm/piece". Is 5mm too big for digestion? Please confirm these.

Should be 15x15 cm petri dish. Should be 1mm/piece. Corrected in Line 120.

2. On the discussion part, from line 200 to 210, these tricks could put in the according protocol part as notes.

Per JoVE manuscript instructions, troubleshooting and limitations should be noted in the discussion section.

3. In figure 3, the last panel, should the "CD146-" be "CD146+"?

Yes, it should be CD146+. Figure updated.

Reviewer #3:

Manuscript Summary:

In the manuscript, Lee et al provide a protocol for isolation and purification of murine cardiac pericytes. It is certainly worthy of publication. The method is generally easy to perform. It has also been well-described. However, the following, important controls (largely to establish the purity and viability of the isolated cells) and information, must be provided.

Major Concerns:

1. The authors agree that contamination of cardiac pericytes with skeletal muscle cells is a possibility. The authors must provide evidence (FACS etc) to determine the extent of this contamination.

The mention of skeletal muscle cells are only in two places: Line 52 and Line 338. In Line 52, we are referring the protocols in the literature that are available for pericyte isolation. Skeletal muscle is one of the tissues beds where pericytes have been isolated from and published on. In Line 338, we are referring to possible subpopulations of cardiac pericytes because in skeletal muscle subpopulations have been identified. There is no possibility for skeletal muscle cell contamination as our markers are not specific to skeletal muscle cells. Secondly, after multiple passages from our primary isolation, the cells were analyzed by flow cytometry again to check for changes in marker expression and we found that there were no changes. Thirdly, we have immunostained our culture cells for pericyte markers, some from our FACS panel (NG2 and CD140b), and a few not in the panel (vimentin, desmin, alpha-SMA) and we found that the cells expressed pericyte markers homogenously.

2. In addition, some evidence of the isolated cardiac pericytes being functional is essential.

We have included immunofluorescence staining of our cells for phenotypic characterization and brightfield images of our cells for morphological characterization as a supplemental figure (Figure 4). In our immunofluorescence images, we stained our cells with a plethora of known pericyte markers (Armulik *et al.*, Murray *et al.*). Secondly, pericyte morphology is distinguishable from other vascular cells (Armulik *et al*.). Our brightfield images show comparable morphology with a commercial pericyte cell line as well as the difference in morphology from commercial smooth muscle cell lines (Figure 4). Detailed functional characterization of our cell population will be in a follow-up manuscript and we believe that it is beyond the scope of this method focused manuscript.

3. The authors must include a brief explanation of why the tested markers were used. It will be a useful reference for the readers and users of this protocol.

Our panel of markers contained both inclusion and exclusion markers. CD45 is used as a marker for hematopoietic cells. CD31 and CD34 are both markers for endothelial cells. CD146 is a marker for perivascular cells. Lastly, NG2 and CD140b are both accepted markers for pericytes. Brief explanation has been added to the introduction Lines 64-69.

4. Please provide FACS results of the tested markers from an unrelated cell suspension, as a control.

We have used mouse coronary endothelial cells in FCM experiment as a control for tested markers. Please see supplemental Figure 1.

5. Please include information about the number of passages to which the pericyte culture can grow. Also, does the viability of these cells reduce with each passage (please provide FACS data for live/dead staining to determine this)?

Information has been added to discussion section along with further details about their characterization in Lines 286-298 and added in Figure 4. We have performed flow cytometry analysis with our cells at P6, P8, P10, and P12 using L/D staining and there were no differences in viability between the passages. Please see supplemental Figure 2.

6. The authors generally mention that the other protocols (Nees et al etc) require the use of in-house equipment. Please provide specific instances of these equipments and how the current manuscript overcomes this limitation.

Nees *et al.* use of in-house custom built equipment to detach the pericytes from the microvessels involved two chambers with pumps that perfused protease solution back and forth through a mesh net stack was hard to replicate as they did not provide a schematic and/or picture of the apparatus and how it was built. Although Nees *et al.* successfully isolated cardiac pericytes from many species, we were never able to reproduce their method. Our pericyte detachment step in our protocol simply uses an orbital shaker (to dissociate all cells) which is available in most, if not all laboratories, with the tissue and enzyme solution in a conical tube. There is no custom apparatus required. Secondly, the remaining protocols involves the use of human tissues and thus the procurement of human tissue is limiting to investigators. This have been added to the discussion section Lines 301-314.

Reviewer #4:

Manuscript Summary:

The submission by Lee, Khakoo and Chintalgattu describes a protocol to isolate and purify pericytes from murine hearts.

Major Concerns:

None.

Minor Concerns:

Mouse "arms" should be described as forelimbs.

Corrected Line 110.

For a single heart, the term atrium is appropriate (not atria).

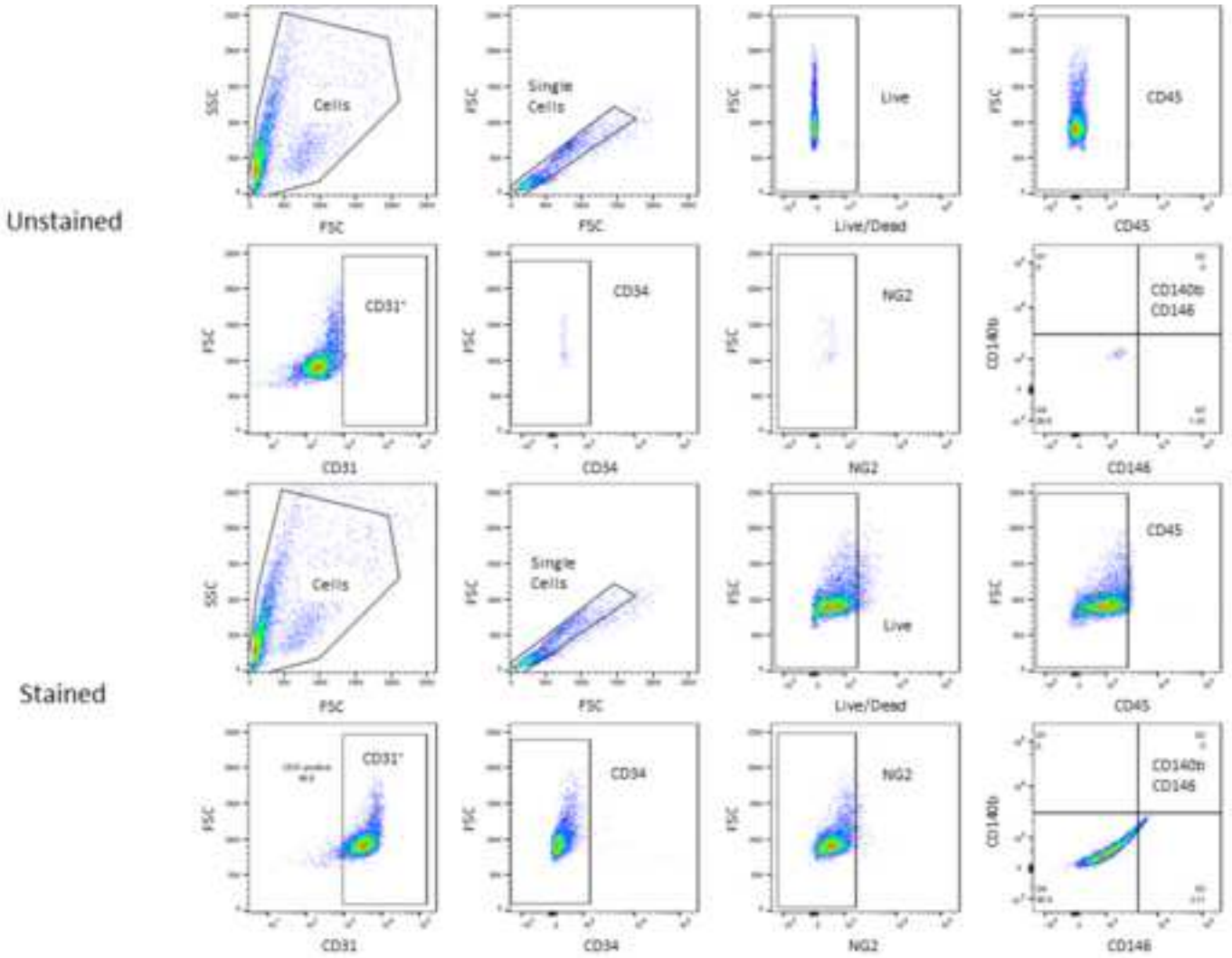
Corrected Line 112.

Please clarify the control statement in Section 4.1.1. I think isotype-matched controls should always be performed. This is particularly important in cells from tissue with high levels of auto-fluorescence (like the heart).

Auto-fluorescence can be corrected by looking at the unstained sample (negative control for the system). Isotype controls are for non-specific binding or staining issues. We feel that the gating boundaries identified using the FMO controls are sufficient enough to eliminate non-specific staining. We do agree that it is best practice to always use all controls and we have eliminated the option of using isotype controls and have included the use of isotype controls.

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);JOVE Suppl Figure 1 300dpi.tif



Supplemental Figure 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);JOVE Suppl Figure 2 300dpi.tif