Journal of Visualized Experiments

Isolation of Endocardial and Coronary Endothelial Cells from the Ventricular Free Wall of the Rat Heart

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61126R1
Full Title:	Isolation of Endocardial and Coronary Endothelial Cells from the Ventricular Free Wall of the Rat Heart
Section/Category:	JoVE Developmental Biology
Keywords:	Endocardium, Coronary Endothelium, Isolation, Heart, Rat, CD31
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Additional Information:	
Question	**Response**
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Cover Letter

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December 15, 2019

Dear Dr. Jewhurst,

We would like to submit our new manuscript entitled "**Isolation of Endocardial and Coronary Endothelial Cells from the Ventricular Free Wall of the Rat Heart**" for consideration for publication in JoVE. We believe that the novel method for isolating endocardial cell and coronary endothelial cell presented in our manuscript would be of great interest and value to the diverse readership of JoVE.

Significance
It has been shown that endocardial endothelial cells (EECs) and coronary endothelial cells (CECs) differ in origin, development, markers, and functions. Consequently, these two cell populations play unique roles in cardiac diseases. Current studies involving isolated endothelial cells investigate cell populations consisting of both EECs and CECs, making it infeasible for researchers to determine cell-type specific mechanisms for heart development and diseases. Thus, the ability to isolate EECs and CECs independently from the heart is crucial for cell specific characterization.

Novelty and Strengths
1) We are the first group reporting the protocol for isolating EECs and CECs independently from the rat heart.
2) We used newly reported EECs and CECs specific markers for verification of successful isolation through gene expression analyses.
3) This method maintains cell phenotype characteristics upon isolation, allowing subsequent cell culturing and downstream functional analysis.
4) This method could be further modified for isolating different subtypes of ECs from the human heart tissue.

Graphical Abstract

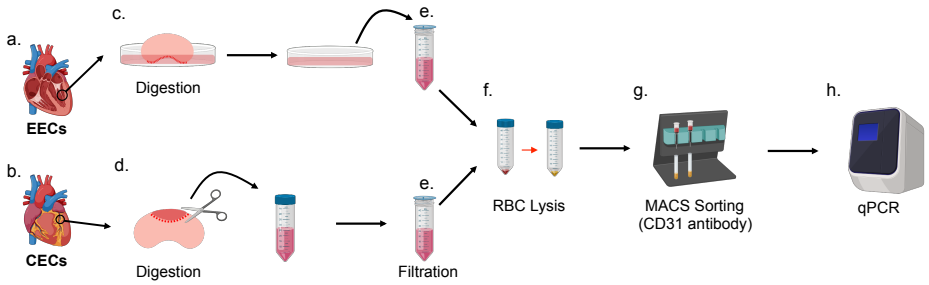

Figure Caption: Diagram of digestion set up of the CECs and EECs, and following arrangement of cell sorting. (a) Innermost free ventricular wall and (b) outermost ventricular free wall were (c) immersed in digestion buffer or (d) chopped into small pieces and digested in digestion buffer respectively. (e) Collection and filtration of cell solutions following with (f) RBC lysis and (g) MACS sorting using CD31 antibody. (h) Purified ECs were processed for qPCR verification. RBC: red blood cells; MACS: Magnetic-activated cell sorting.

Summary
Successful isolation of EECs and CECs is necessary to achieve comprehensive knowledge of these two cell populations, which can be utilized in both the research and clinical setting. Determining growth and differentiation factors of these cell populations would provide a reference for the differentiation of endothelial subtypes from induced pluripotent stem cells. Further, complete identification of the variances in the development, regulation, and function of EECs and CECs is vital for understanding the genomic and epigenomic factors responsible for numerous heart diseases in a cell-type specific manner. Because of the importance and broad opportunity for utilization of our protocol, we believe that it would be of value to JoVE readers.

Submission Invitation
9/30/2019: Received publication inquiry from Dr. Kyle Jewhurst.
10/01/2019: Received invitation to submit a manuscript to JoVE from the editorial.

Suggest Reviewers
We would like to suggest the following investigators with strong expertise in the fields of endothelial biology and heart development, as potential reviewers for our study.

Zhen Chen, Assistant Professor, Department of Diabetes Complications and Metabolism, City of Hope National Medical Center, zhenchen@coh.org;

Sang Ging Ong, Assistant Professor, Pharmacology and Medicine, University of Illinois Chicago, sangging@uic.edu

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John Shyy, Professor, Institute of Engineering in Medicine, University of California San Diego, jshyy@ucsd.edu

You have our assurance that all co-authors have read and are in agreement with the final submitted version of the manuscript. This manuscript is not under consideration for publication elsewhere.

We are grateful for your time and consideration of this manuscript for potential publication in JoVE, and we look forward to your feedback.

Sincerely,

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TITLE:

Isolation of Endocardial and Coronary Endothelial Cells from the Ventricular Free Wall of the Rat Heart

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KEYWORDS:

endocardium, coronary endothelium, isolation, heart, rat, CD31

SUMMARY:

We present a protocol for the isolation of endocardial and coronary endothelial cells from rat hearts through sequential tissue digestion in a digestion buffer, cell collection from recurrent centrifuge cycles, and cell purification using anti-rat CD31 microbeads.

ABSTRACT:

It has been shown that endocardial endothelial cells (EECs) and coronary endothelial cells (CECs) differ in origin, development, markers, and functions. Consequently, these two cell populations play unique roles in cardiac diseases. Current studies involving isolated endothelial cells investigate cell populations consisting of both EECs and CECs. This protocol outlines a method to independently isolate these two cell populations for cell-specific characterization. Following the collection of the left and right ventricular free wall, endothelial cells from the outer surface and inner surface are separately liberated using a digestion buffer solution. The sequential digestion of the outer surface and the inner endocardial layer retained separation of the two endothelial cell populations. The separate isolation of EECs and CECs is further verified through the identification of markers specific to each population. Based on previously published single cell RNA profiling in the mouse heart, the *Npr3*, *Hapln1*, and *Cdh11* gene expression is unique to EECs; while *Fabp4*, *Mgll*, and *Cd36* gene expression is unique to CECs. qPCR data revealed enriched expression of these characteristic markers in their respective samples, indicating successful EEC

and CEC isolation, as well as maintenance of cell phenotype, enabling further cell-specific functional analysis.

INTRODUCTION:

This article provides a detailed protocol (modified from Gladka et al.[1]) for the dissection and subsequent isolation of endocardial endothelial cells (EECs) and coronary endothelial cells (CECs) from rat hearts. The ability to investigate these cell populations independently would enable the exploration of cell type-specific mechanisms underlying a variety of heart diseases that could serve as potential therapeutic targets. A successful method for the collection of these cell populations independently has yet to be published, however.

CECs differ from EECs in regards to their origin, markers, and functions during heart development and disease[1-7]. EECs stem from the ventral surface of the cardiac mesoderm[3]. They arise from $Flk1^{+}$ progenitor cells in response to VEGF and HIF signaling and form the innermost layer of the three discrete regions of the developing heart: the atrium, ventricle, and sinus venosus[3,6]. Genetic lineage tracing suggests that the pluripotent endocardial cells of the sinus venosus derive venous cells, which migrate to form the subepicardial layer[3]. Subsequently, the subepicardial layer differentiates into coronary arteries and veins, including CECs, which remain across the peripheral ventricular free wall[3,4]. This endocardial to endothelial pathway is regulated by VEGFC, ELA/APJ, and SOX17 signaling[3,4,6,8,9]. The ventricular endocardium derives the fewer CECs of the interventricular septum by an unknown mechanism[3]. Subsequently, localized differentiation between EECs and CECs is suggested by markers specific to these two cell populations, including *Mgll*, *Fabp4,* and *Cd36* expression in CECs, or *Npr3, Cdh11,* and *Hapln1* expression in EECs[3,5,10].

EECs and CECs play different roles in cardiac function. Endocardial to mesenchymal transition, valve formation, chamber maturation, outflow tract regulation, and atrioventricular canal development are contingent on EECs[6]. Alternatively, CECs contribute to vasomotor tone and inflammation of coronary arteries[11]. These variances in function result in individualized roles in disease development[4,12]. For instance, evidence suggests that malfunctioning EECs may lead to congenital valve disease[6], noncompaction myocardium[6], atrioventricular septal effect[6], endocardial fibroelastosis[13], hypoplastic left heart syndrome[13], ventricular hypoplasia[13], and cardiac hypertrophy[12]. Similarly, studies have found that abnormal CECs contribute to coronary artery disease[14] and thrombosis[11].

Successful isolation of EECs and CECs is necessary to achieve comprehensive knowledge of these two cell populations, which could be utilized in both the research and clinical settings. Determining the growth and differentiation factors of these cell populations would provide a reference for the differentiation of endothelial subtypes from induced pluripotent stem cells (iPSCs). Further, complete identification of the variances in the development, regulation, and function of EECs and CECs is vital for understanding the genomic and epigenomic factors responsible for numerous heart diseases in a cell type-specific manner. This article outlines steps for the successful collection of EECs and CECs independently, and provides evidence of separation by assessing the gene expression levels of cell type-specific markers.

PROTOCOL:

All animal procedures were approved by the Administrative Panel on Laboratory Animal Care (APLAC31608) at Stanford University.

1. Preparation of buffers

1.1. Prepare the digestion buffer using the reagents listed in **Table 1**.

1.1.1. Prepare the DNase I stock solution: Dissolve DNase I in RNase-free water for a stock solution of 2,000 units/mL (1 mg/mL). Aliquot and store the stock solution at -20 ˚C.

1.1.2. Prepare the liberase solution: Dissolve liberase with RNase-free water for a stock solution of 5 mg/mL. Rotate it on a roller bank at 4 ˚C for 30 min. Aliquot and store the stock solution at -20 ˚C.

1.2. Prepare the sorting buffer by diluting 0.5 M ethylenediaminetetraacetic acid (EDTA) and 10% bovine serum albumin (BSA) stock solution with 1x phosphate buffered saline (PBS) for a final concentration of 2 mM of EDTA and 0.5% of BSA.

2. Heart collection

NOTE: Six Sprague Dawley rats weighing 50–100 g were used in this protocol. No gender difference was observed.

2.1. Euthanize the rat with CO_2 and place it in a supine position on a dissecting platform. Pin the extremities down and sterilize both the abdomen and chest with 70% ethanol.

2.2. Open the abdomen with scissors and insert a 21 G needle into the portion of the posterior vena cava located behind the intestines. Pull the syringe plunger back, withdrawing the blood from the vein until the liver appears lighter in color and no more blood can be withdrawn, indicating sufficient blood removal.

2.3. Using scissors, open the chest carefully to avoid damaging the lung and the heart. Lift the aorta arch/atrium with the forceps and cut the aorta, pulmonary artery, pulmonary veins, and vena cava to liberate and remove the heart.

2.4. Wash the heart with 50 mL of cold 1x Hanks' balanced salt solution (HBSS) buffer 3x to remove excessive blood.

2.5. Under a microdissection microscope, remove the right ventricular free wall into a 5 mL tube containing Dulbecco's modified Eagle medium (DMEM) (**Figure 1A**,**B**).

2.5.1. Lay the heart on its flatter, posterior face to identify the left and right side.

2.5.2. Locate the pulmonary artery, the most anterior of the veins and arteries branching from the top of the heart and cut through the pulmonary artery down to the right ventricular chamber. On the anterior face of the heart, cut along the septum until reaching the apex. Continue cutting from the apex up the posterior side of the heart along the septum until the pulmonary artery and right ventricular chamber junction point (**Figure 1A**).

2.5.3. Starting from the pulmonary artery and right ventricular junction point, cut both the anterior and posterior side of the heart perpendicularly to the previous dissection and away from the septum, until the right ventricular free wall is liberated from the rest of the heart (**Figure 1B**).

2.6. Under the microdissection microscope, remove the left ventricular free wall into a 5 mL tube containing DMEM (**Figure 1C**,**D**).

2.6.1. Locate the aorta, branching from the top of the heart behind the pulmonary artery, and repeat the method described in step 2.5 to cut off the left ventricular free wall.

3. EEC digestion

3.1. Place both right and left ventricular free wall tissues in a 60 cm culture dish with the inner surface lying flat, facing down (**Figure 2**).

NOTE: The inner surface is the interior face of the slightly concave shaped ventricle as shown in **Figure 2A**,**C**.

3.2. Add 0.5–1 mL of digestion buffer to the dish, placing the tip of the pipette directly under the tissue. Continue until only the inner surface is immersed.

3.3. Incubate the dish in a 5% CO_2, 37 ˚C incubator for 5 min.

3.4. Add EC medium to the culture dish to stop the digestion.

NOTE: Keep the amount of digestion buffer to EC medium as a 1:5 ratio.

3.5. Use a 1 mL pipette to flush the inner surface of the ventricles with EC medium and transfer the runoff into a 50 mL collection tube through a 40 mm strainer (**Figure 2E**). Keep the collections on ice for downstream purification.

4. CEC digestion

4.1. Cut along the outer surface of the left ventricle without contamination from the inner layer (**Figure 2B**,**D**) and place each in a separate 5 mL tube containing 1 mL of the digestion buffer.

NOTE: The left ventricle can be identified as the thicker ventricle, and the outer surface can be determined as the exterior face of the slightly concave ventricle.

4.2. Using dissection scissors, mince the ventricular wall into small, 1 mm^3 pieces.

4.3. Incubate the tube in a 37 ˚C water bath for about 15–20 min. Vortex every 2–3 min.

4.4. Pipette 4 mL of EC medium into the 5 mL tube to terminate the digestion.

4.5. Transfer the solution with a 5 mL serological pipette into a 50 mL tube through a 40 mm strainer (**Figure 2E**). Keep the collections on ice for downstream purification.

5. Cell collection

5.1. Centrifuge the tubes at 300 x *g* for 10 min at room temperature (RT) and then aspirate the supernatant.

5.2. If the pellet appears red, resuspend the pellet in 1–2 mL of 1x red blood cell (RBC) lysing buffer (**Figure 2F**).

NOTE: If there are no RBCs, proceed to step 6.

5.3. Incubate the tubes in a 37 ˚C water bath for 5 min.

5.4. Pipette 10 mL of PBS into the 50 mL tubes to stop the lysis.

5.5. Centrifuge the tubes at 300 x *g* for 10 min at RT, and then aspirate the supernatant.

6. EC sorting

6.1. Add 90 µL of sorting buffer and 10 µL of anti-CD31 PE antibody into the 50 mL tubes.

6.2. Vortex the tubes and then incubate them for 10 min in a 4 ˚C fridge.

6.3. Add 10 mL of sorting buffer into the tubes, mix thoroughly, and then centrifuge at 300 x *g* for 10 min at RT.

6.4. Aspirate the supernatant and resuspend the pellet in 80 µL of sorting buffer and 20 µL of anti-PE microbeads.

6.5. Vortex the tubes and incubate at 4 ˚C for 15 min.

6.6. Wash the cells by adding 10 mL of sorting buffer into the tubes, mix thoroughly, and then centrifuge them at 300 *x g* for 10 min at RT.

6.7. Aspirate the supernatant and resuspend the pellet in 500 μL of sorting buffer.

6.8. Place a column containing superparamagnetic spheres into a magnetic separator and rinse the column with 3 mL of sorting buffer.

6.9. After the column are "flow stop", pipette 500 μL of the cell suspension into the columns and then wash the columns with sorting buffer. Repeat the wash 3x, each with 3 mL of sorting buffer (**Figure 2G**).

6.10. Remove the columns from the separator and place them on top of a new 15 mL collection tube.

6.11. With a pipette, add 5 mL of EC medium to the columns, and propel the solution through using a column plunger.

6.12. Centrifuge the collection tubes at 300 x *g* for 10 min at RT and then aspirate the supernatant.

6.13. Extract the RNA from the cell pellet of the EEC and CEC samples using a kit, following the manufacturer's instructions. A minimum of 500 ng of RNA can be obtained.

NOTE: After RNA extraction, the sample can be stored at -80 ˚C.

6.14. Reverse transcribe 500 ng of RNA to get cDNA using a random primer and a kit, following the manufacturer's instructions.

6.15. Perform quantitative PCR for the validation of endocardial and endothelial populations (**Figure 2H**). Primer sequences are listed in **Table 2**.

6.15.1. Add 5 μL of EEC or CEC cDNA (1 ng/μL) into the wells of a 384 qPCR plate. Add the EEC or CEC cDNA into enough wells so that there are three wells per number of primers.

6.15.2. Mix 6 μL of 2x qPCR SYBR green buffer with 0.5 μL of each 10 μM primer, including both forward and reverse primers, and add them into a single well corresponding to each candidate gene.

6.15.3. Seal the plate with plastic film and centrifuge for 1 min at 300 x *g* at RT.

6.15.4 Place the plate into a qPCR machine and then start the program at 95 ˚C for 3 min, followed by 95 ˚C for 15 s, and then 55 ˚C for 60 s. Repeat the subsequent two steps for 45 cycles.

REPRESENTATIVE RESULTS:

The process of EEC and CEC isolation is described in **Figure 2**. The successful isolation of EECs and CECs was determined by assessing the presence of pan-endothelial cell markers, as well as those

distinct to the two subtype populations. As predicted, qPCR revealed that relative to *β-actin*, EECs expressed higher levels of endocardial markers *Npr3*, *Hapln1,* and *Cdh11* compared to CECs (**Figure 3A**). Likewise, CECs expressed higher levels of coronary markers *Fabp4*, *Mgll*, and *Cd36* compared to EECs (**Figure 3B**). Additionally, both EECs and CECs expressed the pan-EC marker gene *Cdh5*, with slightly higher levels in CEC (**Figure 3C**).

FIGURE LEGENDS:

Figure 1: Diagram of heart dissection. (**A**) First cut made to separate the right ventricle free wall from the septum. (**B**) Second cut made to liberate the right ventricle completely. (**C**) First cut made to separate the left ventricle free wall from the septum. (**D**) Second cut made to liberate the left ventricle completely.

Figure 2: Diagram of digestion set-up of the CECs and EECs and the following arrangement for cell sorting. (**A**) Innermost free ventricular wall and (**B**) outermost ventricular free wall were (**C**) immersed in digestion buffer or (**D**) digested in digestion buffer respectively. (**E**) Collection and filtration of cell solutions followed by (**F**) red blood cell (RBC) lysis and (**G**) magnetic-activated cell sorting (MACS) using the CD31 antibody. (**h**) Purified ECs were processed for gene expression verification using qPCR.

Figure 3: qPCR data verifying isolation of CECs and EECs. (**A**) Gene expression levels of the EEC markers *Npr3*, *Cdh11*, and *Hapln1* in EECs and CECs were quantified by real-time PCR. (**B**) Gene expression levels of the CEC markers *Mgll*, *Fabp4*, and *Cd36* in EECs and CECs were quantified by real-time PCR. (**C**) The pan-EC marker *Cdh5* was quantified in EECs and CECs by real-time PCR. (n = 3 in each group). Bars represent mean ± SEM. *$p < 0.05$, **$p < 0.01$ vs. EEC, unpaired t-test.

Table 1: Recipe for the digestion buffer.

Table 2: Primer Sequences

DISCUSSION:

CECs and EECs differ in origin, markers, and functions, and thus could play unique roles in development and disease. Existing protocols for endothelial cell isolation are limited to macrovascular tissues, neglecting the collection of EECs, thus restricting the study of CEC- and EEC-specific functions. It is essential to isolate and study these two populations independently, as this knowledge would provide a reference for the differentiation of iPSCs into CECs and EECs for future studies and facilitate the examination of these cell populations for potential therapeutic targets for various cardiac diseases. This novel protocol outlines a method for the isolation of EECs from the inner surface and CECs from the outer surface of the ventricular free wall of adult rats.

It is critical to control the timing for each step very precisely in this protocol. Because the number of EECs is very limited in rat heart tissue, we minimized the digestion time of the inner layer of the heart to prevent cellular damage and more importantly, CEC contamination. Immediate addition of the EC medium solution to terminate the enzymatic reaction is also very important

for maintaining high cell viability. A red pellet following cell collection suggests the presence of a large number of RBCs. Depending on the amount of RBC contamination, 1–2 mL of RBC lysis buffer can be added to degrade the RBCs. Incubation must be carefully timed to avoid significant damage to the cells, and PBS is promptly added to terminate the reaction. Using the current protocol, we were able to isolate 10^5 EECs from six rat hearts. These cells could be seeded onto a cell culture dish for further expansion and characterization.

When preparing the tissue for free wall collection, the heart was washed with HBSS to remove the majority of the remaining RBCs. HBSS is the recommended medium due to its clear appearance, which enables the visualization of blood cells, in contrast to DMED containing phenol red. The composition of the digestion buffer ensures sufficient liberation of endothelial cells, where liberase digestion enzyme strips the tissue of the exposed cells, DNase I eliminates DNA from the dead cells to promote cell detachment that may be inhibited by DNA's adhesive quality, HEPES buffer balances the pH, and DMEM is a modified basal medium with a higher concentration of amino acids and vitamins for better cell maintenance and contains the calcium necessary to activate the liberase.

According to the single cell RNA sequencing (scRNA-seq) obtained from both human[15] and mouse heart[10], several markers attributed to specific EC populations were reported. We selected some of the most enriched genes to examine the purity of isolated EECs (i.e., *Npr3*, *Cdh11*, and *Hapln1*) and EECs (i.e., *Mgll*, *Fabp4*, and *Cd36*). Relative to *β-Actin*, *Npr3*, *Cdh11*, and *Hapln1* markers demonstrated increased expression in EECs compared to CECs. Similarly, the expression of *Mgll*, *Fabp4*, and *Cd36* markers was greater in CECs compared to EECs. The uniquely expressed markers for each sample is in accord with markers characteristic to EECs and CECs respectively, indicating successful isolation.

However, the current protocol cannot rule out cross contamination between the two EC populations during the isolation, even with carefully controlled sequential digestions. Therefore, some cell surface markers can be applied for further purification. For example, NPR3 generally labels the endocardium[10], whereas APJ can trace a majority of the CECs[16]. Because these two markers are expressed on the cell surface, they could be used for fluorescence activated cell sorting (FACS), and antibodies used to further purify distinct EC populations. In addition, human[15] and mouse[10] heart scRNA-seq can confirm enrichment of *Npr3* in EECs, and *Cd36* can be potentially used for CEC purification.

In conclusion, the presented protocol outlines the independent isolation of EECs and CECs from the rat heart. The comprehensive identification of cellular properties, enabled by cell isolation, can be utilized for significant downstream applications.

ACKNOWLEDGEMENTS:
The authors greatly appreciate Dr. Lingli Wang for her help with the animal protocol, and the Department of Pediatrics at Stanford for infrastructure support. This work was supported by NIH/NHLBI K99 HL135258 (to M.G.).

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Gladka, M. M. et al. Single-Cell Sequencing of the Healthy and Diseased Heart Reveals Cytoskeleton-Associated Protein 4 as a New Modulator of Fibroblasts Activation. *Circulation.* **138** (2), 166-180 (2018).
2. Koren, C. W., Sveinbjornsson, B., Smedsrod, B. Isolation and culture of endocardial endothelial cells from Atlantic salmon (Salmo salar) and Atlantic cod (Gadus morhua). *Cell and Tissue Research.* **290** (1), 89-99 (1997).
3. Tian, X. et al. Subepicardial endothelial cells invade the embryonic ventricle wall to form coronary arteries. *Cell Research.* **23** (9), 1075-1090 (2013).
4. Wu, B. et al. Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF signaling. *Cell.* **151** (5), 1083-1096 (2012).
5. Li, Y., Lui, K. O., Zhou, B. Reassessing endothelial-to-mesenchymal transition in cardiovascular diseases. *Nature Reviews in Cardiology.* **15** (8), 445-456 (2018).
6. Zhang, H., Lui, K. O., Zhou, B. Endocardial Cell Plasticity in Cardiac Development, Diseases and Regeneration. *Circulation Research.* **122** (5), 774-789 (2018).
7. Yu, S. Y. et al. Isolation and characterization of human coronary artery-derived endothelial cells in vivo from patients undergoing percutaneous coronary interventions. *Journal of Vascular Research.* **46** (5), 487-494 (2009).
8. Red-Horse, K., Ueno, H., Weissman, I. L., Krasnow, M. A. Coronary arteries form by developmental reprogramming of venous cells. *Nature.* **464** (7288), 549-553 (2010).
9. Kamimura, T., Yamagishi, T., Nakajima, Y. Avian coronary endothelium is a mosaic of sinus venosus- and ventricle-derived endothelial cells in a region-specific manner. *Development, Growth and Differentiation.* **60** (2), 97-111 (2018).
10. Zhang, H. et al. Endocardium Minimally Contributes to Coronary Endothelium in the Embryonic Ventricular Free Walls. *Circulation Research.* **118** (12), 1880-1893 (2016).
11. Kinlay, S., Libby, P., Ganz, P. Endothelial function and coronary artery disease. *Current Opinion in Lipidology.* **12** (4), 383-389 (2001).
12. Jacques, D., Bkaily, G. Endocardial endothelial cell hypertrophy takes place during the development of hereditary cardiomyopathy. *Molecular and Cell Biochemistry.* **453** (1-2), 157-161 (2019).
13. Grossfeld, P., Nie, S., Lin, L., Wang, L., Anderson, R. H. Hypoplastic Left Heart Syndrome: A New Paradigm for an Old Disease? *Journal of Cardiovascular Development and Disease.* **6** (1), (2019).
14. Li, Y. et al. Down-regulated RGS5 by genetic variants impairs endothelial cell function and contributes to coronary artery disease. *Cardiovascular Research.* 10.1093/cvr/cvz268, (2019).
15. Miao, Y. et al. Single-Cell RNA-Seq Reveals Endocardial Defect in Hypoplastic Left Heart Syndrome. *bioRxiv.* 10.1101/809731, (2019).
16. Chen, H. I. et al. The sinus venosus contributes to coronary vasculature through VEGFC-stimulated angiogenesis. *Development.* **141** (23), 4500-4512 (2014).

Figure 1.

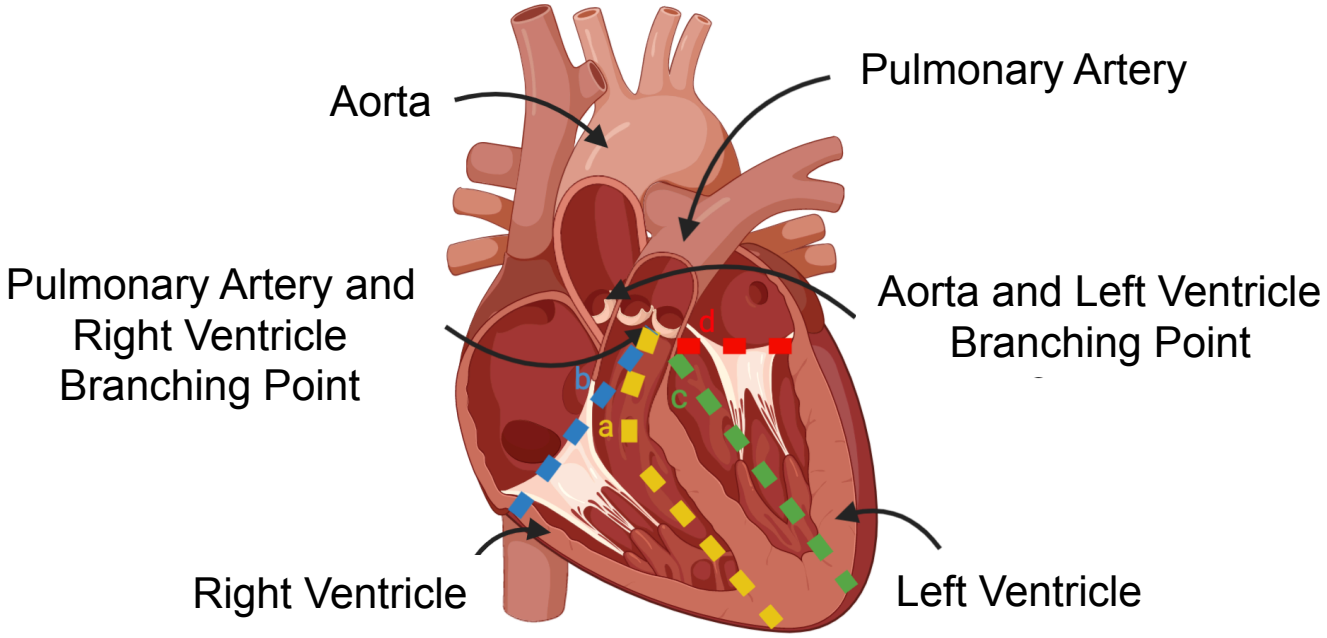
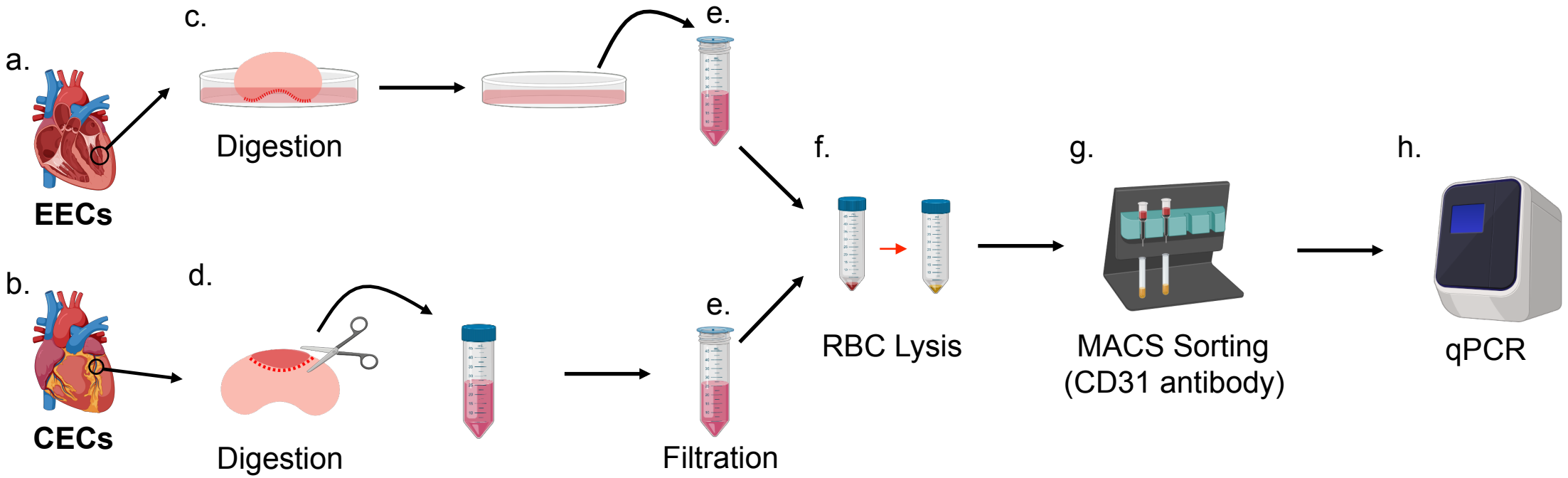


Figure 2.


a.
EECs
b.
CECs
c.
Digestion
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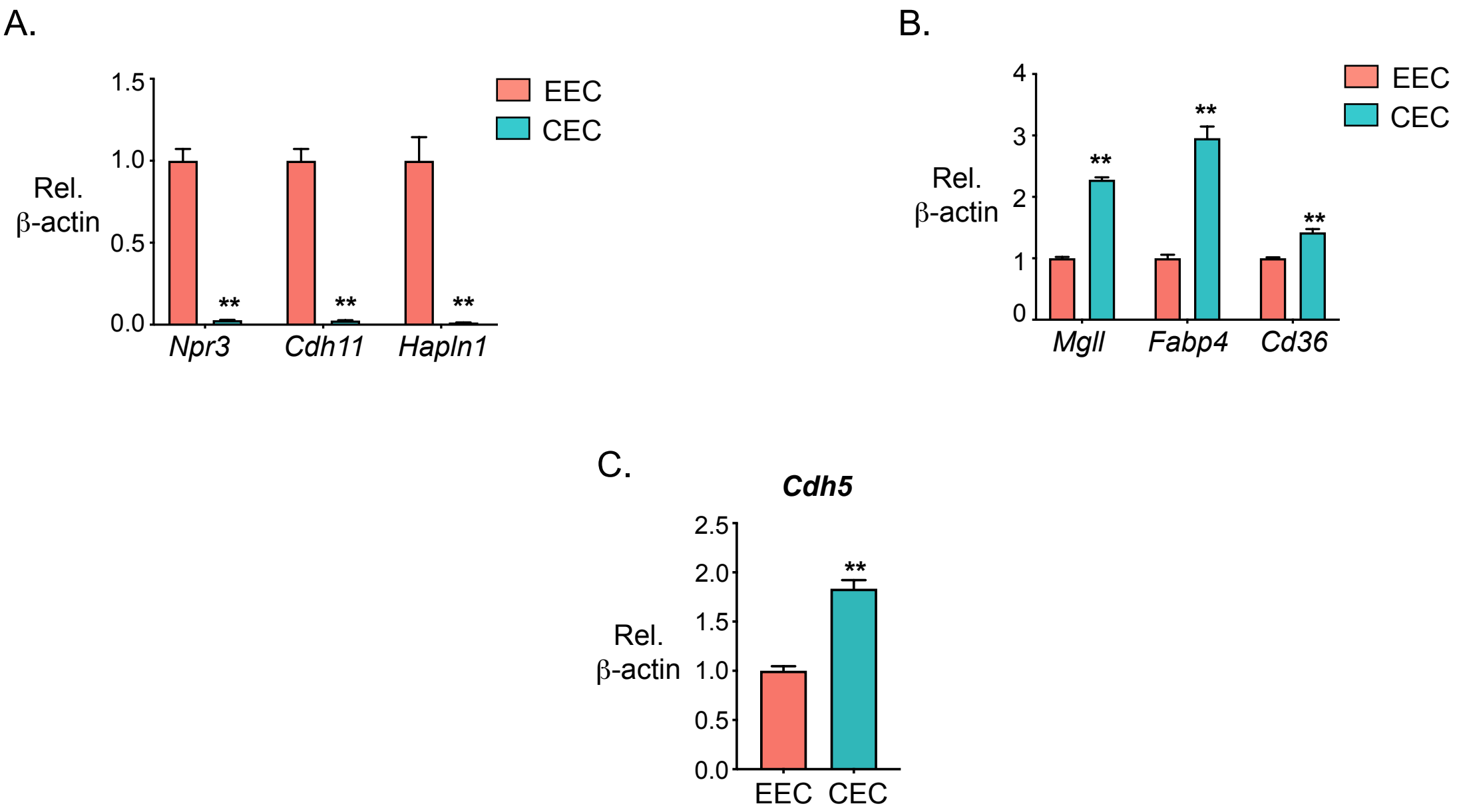
Filtration
f.
RBC Lysis
g.
MACS Sorting
(CD31 antibody)
h.
qPCR

Figure 3.


A.
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EEC
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Npr3
Cdh11
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EEC
CEC
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Mgll
Fabp4
Cd36
C.
Cdh5
Rel.
β-actin
2.5
2.0
1.5
1.0
0.5
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**
EEC
CEC

Table

Click here to access/download;Table;Table 1. Digestion Buffer.xlsx

Table 1. Digestion Buffer (1.5ml)			
	Stock Con.	Final Con.	Volume
Liberase TM	5 mg/ml	0.5 mg/ml	150 ul
Dnase I	1 mg/ml	20 ug/ml	30 ul
HEPES	1 M	10 mM	15 ul
DMEM	-	-	1305 ul

Table 2. Primer Sequences

Gene Name	Forward	Reverse
Npr3	TCCTTGCAAATCATGTGGCCTA	GGAATCTTCCCGCAGCTCTC
Cdh11	GTGAATGGGACTGGGACTGG	GTAATTTCTGGGGCCCTTGC
Hapln1	CCAGCTAAGTGGGACTCGAAG	GGGCCATTTTCAGCTTGGATG
Mgll	CCCGGGGCCCAAAGAC	GAAGATGAGGGCCTTGGGTG
Fabp4	AGAAGTGGGAGTTGGCTTCG	ACTCTCTGACCGGATGACGA
Cd36	GCAAAACGACTGCAGGTCAA	CCCGGTCACTTGGTTTCTGA
Cdh5	CCATTGAGACAGACCCCGAC	TGTGGAACGTGTACTGCTGG
B-actin	TCTGTGTGGATTGGTGGCTC	CGGACTCATCGTACTCCTGC

Table of Materials

Click here to access/download;Table of Materials;20200115_Materials and Reagents.xlsx

Name	Company
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Gibco
15ml Tubes	Eppendorf
50ml Tubes	Nunc
5ml Tubes	Eppendorf
ACK Lysing Buffer	Gibco
Anti-CD31 PE	Miltenyi Biotec
Anti-PE microbeads	Miltenyi Biotec
Bovine serum albumin (BSA)	Miltenyi Biotec
CFX384 Touch Real-Time PCR Detection System	Bio-rad
DNAse I	Worthington
Dulbecco's Modified Eagle Medium (DMEM)	Gibco
Endothelial Cell Growth Medium-2 (EGM-2)	Lonza
Ethylendiaminetetraacetic Acid (EDTA)	Invitrogen
Hank's Balanced Salt Solution (HBSS) X1	Gibco
Hard-Shell 384-Well PCR Plates, thin wall, skirted, clear/white	Bio-rad
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems
Liberase TM	Sigma Aldrich
MACS LS Column	Miltenyi Biotec
MACS MultiStand	Miltenyi Biotec
Microseal 'B' PCR Plate Sealing Film, adhesive, optical	Bio-rad
Narrow Pattern Forceps	F.S.T
Nylon Sterile Strainer	Falcon
Phosphate Buffered Saline (PBS) X1	Gibco
PowerUp SYBR Green Master Mix	Applied Biosystems
Quick-RNA Microprep Kit	Zymo Research
S&T Forceps - SuperGrip Tips	F.S.T
Strabismus Scissors - Tungsten Carbide	F.S.T
Vannas Spring Scissors - Microserrated	F.S.T

Catalog Number	Comments
15630080	1M
0030122151	
339652	
30119401	
A1049201	
130-115-505	
130-048-801	
130-091-376	10%
1855485	For qPCR
LK 003172	1 mg/mL in water
10313021	
CC-3162	EC Medium
15575020	0.5 M
14025092	
HSP3805	For qPCR
4368813	
5401119001	5 mg/mL
130-042-401	For EC isolation
130-042-302	For EC isolation
MSB1001	For qPCR
11002-12	For heart harvest
352340	40 mm
1001023	
A25780	For qPCR
R1050	For RNA extraction
00632-11	For heart harvest
14574-11	For heart harvest
15007-08	For heart harvest

Dear Editor and reviewers:

Thank you for the thorough review and constructive suggestions. We have made corrections and modification according to each comment in blue, and our revision in the manuscript was highlighted in red.

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.

- All authors have proofread the manuscript.

2. JoVE cannot publish manuscripts containing commercial language. 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: Liberase TM buffer, Worthington Biochemical, Cat# LK003172, Sigma-Aldrich, Cat# 5401119001P, EGM-2 Medium (Lonza, Cat# CC-3162), Gibco, Cat# 10010023, Gibco, Cat# 14025092, Gibco, Cat# A1049201, Miltenyi Biotec, Cat# 130-116-505, Miltenyi Biotec, Cat# 130-048-801, Quick-RNA Microprep Kit (Zymo Research, Cat# R1050), MACS, Eppendorf, etc.

- All commercial language has been removed from the manuscript, excluding the Materials and Reagents excel document.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

- The protocol has been revised to contain only the imperative tense, in complete sentences, and without aforementioned phrases.

4. The Protocol should contain only action items that direct the reader to do something.

- The protocol has been revised to contain only action items.

5. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

- The protocol has been revised to include more detail, specifically those addressing the "how" question. For instance, all instruments used are addressed in each step, further instruction on dissection has been added, and qPCR conditions are outlined.

6. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

- The embedded tables have been removed and are submitted separately as .xlsx files.

7. Please move all the materials to the table of materials instead.
- All materials have been removed from the protocol.

8. 2.2: Where is the needle inserted? How much blood is collected and how?
- The needle is inserted into the portion of the posterior vena cava located behind the intestines. The blood is removed by withdrawing the plunger of the syringe, and this is done until the tissue of the liver appears lighter in color and no blood can be further withdrew. These specifics have been added to the protocol.

9. 2.4: Please include volume and concentrations of all the solutions and reagents used.
- The volume and concentration of all the solutions and reagents has been added to the protocol.

10. 2.5: How is this done? How do you visually identify each of them?
- You can visually determine the ventricles by laying the heart on the flatter posterior face, enabling the identification of the left and right ventricles. The pulmonary artery is identified as the most anterior of the veins and arteries that branch from the top of the heart, and the aorta is directly behind it. The right ventricle is removed by starting from the branching point of the pulmonary artery from the right ventricle, and cutting down the anterior face of the heart along the septum and back up the posterior face. Starting from the same branching point, one cuts perpendicular to the previous dissection, away from the septum, until the ventricle is liberated from the remaining heart. This same procedure is repeated to remove the left ventricle, but utilizing the branching point of the aorta from the left ventricle. This detail has been added to the protocol. Please see image below for clarity. Both the image and this description have been added to the protocol on page 7, Figure 1.

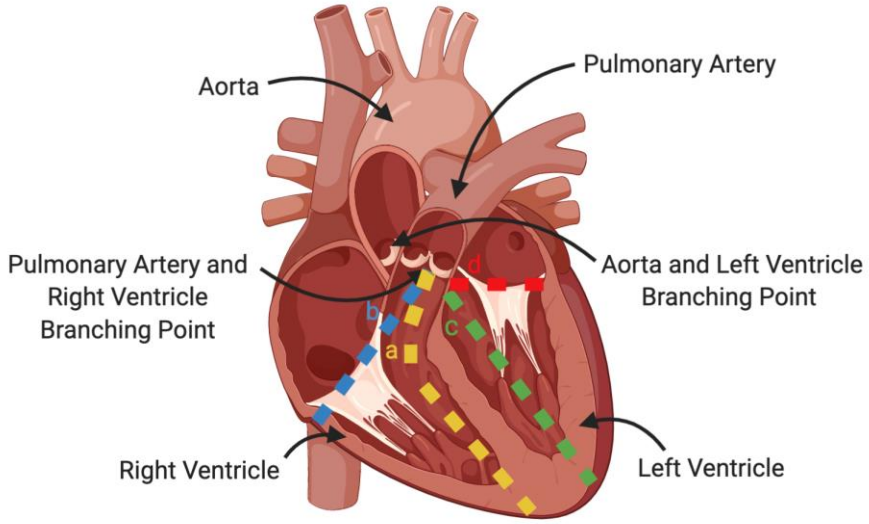

Figure legend: Diagram of heart dissection. a) First cut made to separate the right ventricle free wall from the septum; b) Second cut made to liberate the right ventricle completely; c) First cut made to separate the left ventricle free wall from the septum; d) Second cut made to liberate the left ventricle completely.

11. 3: How do you visually differentiate between inner and outer surface of left ventricle.
- The ventricles maintain a slight concave shape, enabling the differentiation between the inner and outer surface. This specification has been added to the protocol.

12. 6.14: How do you perform qPCR. Please detail the conditions.
- The detail conditions have been added to the protocol on page 6, steps 6.15.1-6.15.4. These details include the addition of 5uL of EEC or CEC cDNA into wells of a 384 qPCR plate, the mixing of 6ul of 2X qPCR SYBR Green buffer with 1 ul of each primer, the addition of the buffer and primer mixture into the wells, the final preparation of the plate with a plastic seal and 1 min 300 g at room temperature centrifuge, and the qPCR protocol. This consists of starting with 95 ^{o}C for 3 minutes, followed by 95 ^{o}C for 15 seconds, and then 55 ^{o}C for 60 seconds, and repeating the subsequent two steps for 45 cycles. The detailed information has been added to the protocol (6.15).

13. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
- Approximately 2.5 pages of the protocol have been highlighted.

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
- The manuscript does not contain any figures form a previous publication.

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
- The critical steps highlighted in the discussion include digestion time, digestion termination, RBC lysis, lysis termination, HBSS wash, and digestion buffer preparation. Digestion time was discussed on page 7, paragraph 5, lines 1-3; digestion termination was noted on page 7, paragraph 5, lines 4 and 5; RBC lysis was highlighted on page 7, paragraph 5, lines 5-7; lysis termination was mentioned on page 7, paragraph 5, lines 7 and 8; HBSS wash was discussed on page 7 and 8, paragraph 6, lines 1-4; and digestion buffer was noted on page 8, paragraph 1, lines 4-9.

b) Any modifications and troubleshooting of the technique
- The discussion notes a modification in the digestion time of the endocardium, on page 7, paragraph 5, lines 1-3, which we reduced to prevent cellular damage. Meanwhile, the potential EC populations cross-contamination was also discussed on page 8, paragraph 3, lines 1-8, with feasible solutions.

c) Any limitations of the technique

- The greatest limitations of the technique are the small number of EECs in rat tissue, noted on page 7, paragraph 5, lines 1-3, and potential cross-contamination of EC populations, discussed on page 8, paragraph 3, lines 1-8. Additionally, the delicate timing of digestion and incubation with lysis buffer creates opportunity for error, mentioned on page 7, paragraph 5, lines 4 and 5. Under digestion could lead to insufficient collection of cells, but over digestion results in cell damage. Similarly, as highlighted on page 7, paragraph 5, lines 5-8, if incubation with lysis buffer is terminated too quickly, RBCs may still be present. However, if left for too long, the lysis buffer will damage the CECs and EECs.

d) The significance with respect to existing methods

- Existing methods of endothelial cell isolation, added on page 7, paragraph 4, lines 2-4, are limited to macrovascular tissues, and thus do not provide a mechanism for collecting endocardial endothelial cells. The inability to collect EECs and CECs independently prevents the examination and identification of cell specific functions and unique roles in diseases. This significance has been added to the discussion.

e) Any future applications of the technique

- In the discussion, it is mentioned on page 7, paragraph 4, lines that this technique can be used to understand cell specific roles in development and disease; which could be used as a reference for iPSC differentiation, or the examination of these cell populations as therapeutic targets.

Reviewer #1:

1. The only concern I have is how to avoid contamination of coronary ECs when authors use digestion buffer to the immersed inner surface. It is very likely that some coronary ECs on the flanks or in the subendocardial myocardium could be digested and collected. It would be more valuable if authors could find a surface marker that could distinguish endocardial cells from coronary ECs, which would permit FACS isolation and purification. Since there are published data (RNA-Seq or scRNA-Seq) on these two distinct cell populations, exploration of one surface marker for endocardial cells that distinguish from coronary EC would be future direction. Authors may add some discussion on this point.

- We completely agreed with reviewer's concern. As we have revealed recently (Miao et al., Single-Cell RNA-Seq Reveals Endocardial Defect in Hypoplastic Left Heart Syndrome. bioRxiv, 2019), EEC and CEC represented distinguished sets of genes. In combination with previous mouse heart single cell gene expression analysis (Zhang et al., Endocardium Minimally Contributes to Coronary Endothelium in the Embryonic Ventricular Free Walls. Circulation Research, 2016), several genes expressed on the cell surface shared high expression specificity to distinct EC populations, e.g. *Npr3* to EEC and *Cd36/Apj* to CECs. As mentioned by reviewer, it will be worthwhile further purifying the cells using FACS or MACS with suitable antibodies specific to these surface markers. In fact, recently we have successfully isolated EECs from a heterogeneous cell population using FACS sorting with Npr3 antibody. These points were well-taken, and added to the 5th paragraph of the Discussion.

2. why Nfatc1 is also high in CECs? Is coronary vessel expressing Nfatc1? could authors discuss on this?

- According to Zhang et al's research, noted in the paper "Endocardium Minimally Contributes to Coronary Endothelium in the Embryonic Ventricular Free Walls", Nfatc1 was found to be expressed in both EECs and a subset of CECs, albeit sporadically. This potentially explains its expression pattern in both cell populations from our qPCR data. Plus, our current protocol can not completely exclude minor cross-contamination between the two EC populations, which might also cause Nfatc1 expression in both. Since Nfatc1 is not a clean endocardial marker, we have removed Nfatc1 qPCR data from our results to avoid misunderstanding.

Reviewer #2:

1. Please indicate the strain of rats.
- The rats' strain is Sprague Dawley, and this information was added to the protocol on page 3, as a note under step 2.

2. Step 3.3: 5%CO2 incubator?
- Yes it is in 5%CO_2 incubator. This has been noted in the protocol.

3. Step 3.4: why is there a range of 1-5ml of volume to add? Why not an exact volume?
- This is due to the variability of digestion buffer used. The protocol specifies that digestion buffer is added until just the inner surface is submerged. Because the size of the ventricles or the number of ventricles digested may vary, the amount of digestion buffer added is inconstant. The amount of EC medium added should be a consistent ratio to the amount of digestion buffer present in the dish. This clarification has been noted.

4. Step 4.2: mince with which instrument?
- The tissue is minced with dissection scissors. This specification has been added.

5. Step 5.3: in water bad?
- Yes, incubation takes place in a water bath. This specification has been added.