Video Article

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

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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/61126/

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[1,2,3,4,5,6,7]. 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. Prepare the digestion buffer using the reagents listed in **Table 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.
   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.

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.

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. 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.
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.
4. Wash the heart with 50 mL of cold 1x Hanks' balanced salt solution (HBSS) buffer 3x to remove excessive blood.
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**).
   1. Lay the heart on its flatter, posterior face to identify the left and right side.
   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**).
   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**).
6. Under the microdissection microscope, remove the left ventricular free wall into a 5 mL tube containing DMEM (**Figure 1C**,**D**).
   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

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

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

3. Incubate the tube in a 37 ˚C water bath for about 15–20 min. Vortex every 2–3 min.
4. Pipette 4 mL of EC medium into the 5 mL tube to terminate the digestion.
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

1. Centrifuge the tubes at 300 x g for 10 min at room temperature (RT) and then aspirate the supernatant.
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.
3. Incubate the tubes in a 37 ˚C water bath for 5 min.
4. Pipette 10 mL of PBS into the 50 mL tubes to stop the lysis.
5. Centrifuge the tubes at 300 x g for 10 min at RT, and then aspirate the supernatant.

6. EC sorting

1. Add 90 µL of sorting buffer and 10 µL of anti-CD31 PE antibody into the 50 mL tubes.
2. Vortex the tubes and then incubate them for 10 min in a 4 ˚C fridge.
3. Add 10 mL of sorting buffer into the tubes, mix thoroughly, and then centrifuge at 300 x g for 10 min at RT.
4. Aspirate the supernatant and resuspend the pellet in 80 µL of sorting buffer and 20 µL of anti-PE microbeads.
5. Vortex the tubes and incubate at 4 ˚C for 15 min.
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.
7. Aspirate the supernatant and resuspend the pellet in 500 µL of sorting buffer.
8. Place a column containing superparamagnetic spheres into a magnetic separator and rinse the column with 3 mL of sorting buffer.
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**).
10. Remove the columns from the separator and place them on top of a new 15 mL collection tube.
11. With a pipette, add 5 mL of EC medium to the columns, and propel the solution through using a column plunger.
12. Centrifuge the collection tubes at 300 x g for 10 min at RT and then aspirate the supernatant.
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.
14. Reverse transcribe 500 ng of RNA to get cDNA using a random primer and a kit, following the manufacturer's instructions.
15. Perform quantitative PCR for the validation of endocardial and endothelial populations (**Figure 2H**). Primer sequences are listed in **Table 2**.
    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.
    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.
    3. Seal the plate with plastic film and centrifuge for 1 min at 300 x g at RT.
    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**).
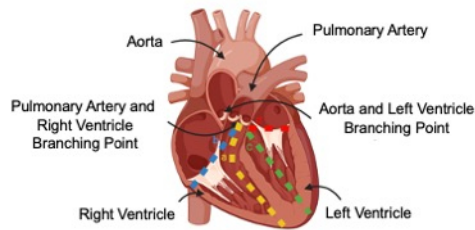
Figure 1: Diagram of heart dissection. (**A**) First cut made to separate the right ventricle free wall from the septum. (**B**) Second cut made to liberate the right ventricle completely. (**C**) First cut made to separate the left ventricle free wall from the septum. (**D**) Second cut made to liberate the left ventricle completely. Please click here to view a larger version of this figure.
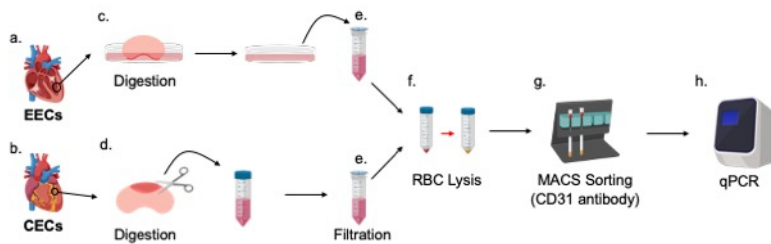
Figure 2: Diagram of digestion set-up of the CECs and EECs and the following arrangement for cell sorting. (**A**) Innermost free ventricular wall and (**B**) outermost ventricular free wall were (**C**) immersed in digestion buffer or (**D**) digested in digestion buffer respectively. (**E**) Collection 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. Please click here to view a larger version of this figure.
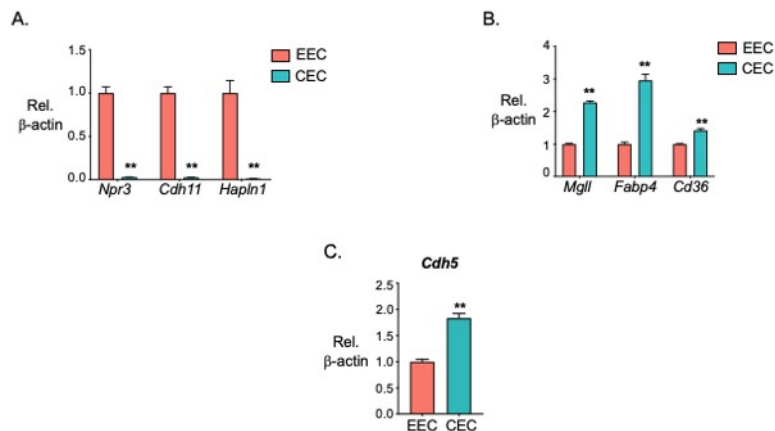
Figure 3: qPCR data verifying isolation of CECs and EECs. (**A**) Gene expression levels of the EEC markers *Npr3*, *Cdh11*, and *Hapln1* in EECs and CECs were quantified by real-time PCR. (**B**) Gene expression levels of the CEC markers *Mgll*, *Fabp4*, and *Cd36* in EECs and CECs were quantified by 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. Please click here to view a larger version of this figure.

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 1: Recipe for the digestion buffer.

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 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.

Disclosures

The authors have nothing to disclose.

Acknowledgments

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