Chapter 15

Isolation and Characterization of Embryonic and Adult Epicardium and Epicardium-Derived Cells

Bin Zhou and William T. Pu

Abstract

Epicardium is the outer cell layer of the heart. Its integrity and function are essential for normal heart development. To study the role of epicardium in both fetal and adult hearts, it is desirable to isolate and culture pure populations of these cells. Here we describe methods with Cre-loxP technology to lineage tag epicardial cells (EpiCs) and epicardium-derived cells (EPDCs), dissociate and isolate them by flow-activated cytometry sorting (FACS), and characterize them by quantitative PCR and immunostaining. This platform allows further characterization and manipulation of EpiCs and EPDCs for expression studies and functional assays.

Key words: Epicardium, Epicardium-derived cells, Flow-activated cytometry sorting, Quantitative PCR, Immunostaining

1. Introduction

The epicardium is an epithelial sheet of cells covering the heart. It is gaining recognition as important cells and source of signals that modulate heart development and postnatal heart function (1). Epicardial cells (EpiCs) orginate from the proepicardium (PE), an outgrowth of the embryonic septum transversum. Between E9.5 and ~E11.0, cells from the PE migrate over the surface of the heart to form an epithelial sheet, the epicardium. EpiCs are critical for normal cardiac development, as impaired formation of epicardium leads to midgestation lethality and aberrant heart development, including abnormal thin compact myocardium and deficient

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coronary vessel formation (2–4). Disruption of epicardial genes such as Wilm's tumor (Wt1), retinoid X receptor α (RXRα), β-catenin, erythropoietin (EPO), and integrin α4 likewise cause embryonic lethality and abnormal heart formation (5–9). Epicardium participates in heart development through reciprocal signaling with myocardium, and by undergoing epithelial to mesenchymal transition (EMT) to form epicardium-derived mesenchymal cells (EPDCs). These mesenchymal cells are a major source of coronary vascular smooth muscle and cardiac fibroblasts (4, 10, 11). Recently, we and others found that a subset of epicardial cells differentiated into cardiomyocytes during normal heart development (12, 13).

As a vasculogenic and myogenic signaling center and source of mesenchymal cells and cardiomyocytes in the developing heart, epicardium has great potentials to be reactivated in the mature heart after myocardial infarction (12–14), which yield great interest in the epicardium and its potential roles in regenerative approaches to myocardial injury.

One of the major challenges in characterizing EpiCs and EPDCs in both embryo and adult hearts is to isolate the specific population without contamination of other lineages. We have generated genetic reagents to facilitate studies of EpiCs and their fate. These reagents are based on regulatory elements of Wt1, a transcription factor highly expressed in the developing kidney and in the mesothelial covering of most visceral organs (6, 15). In the fetal heart, Wt1 expression is confined to the PE and epicardium, not within myocardium (6, 10). As EpiCs undergo EMT and migrate into the myocardium, Wt1 is rapidly down-regulated, so that it is not detected in most migrating mesenchymal cells (10, 16). We knocked either GFP or tamoxifen-inducible Cre cDNA into the Wt1 locus, so that Wt1-driven GFP fluorescence marks EpiCs, or Cre-activated reporter gene expression marks EpiCs plus their descendants.

Here we describe the way of using of these genetic reagents to isolate and characterize EpiCs and EPDCs from both embryo and adult hearts. In outline, steps consist of induction of genetic labeling of EpiCs, collagenase-based dissociation of heart cells, FACS isolation of the labeled population, and downstream analysis of the isolated population by quantitative RT-PCR (qRT-PCR), and immunostaining (see Fig. 1a). This system provides a platform to study important biological aspects of EpiCs and EPDCs, such as their differentiation potential, proangiogenic activity, and cardiomyotrophic activity. These methods will allow further genome-wide screening to discover genes and pathways responsible for the beneficial properties of epicardium.

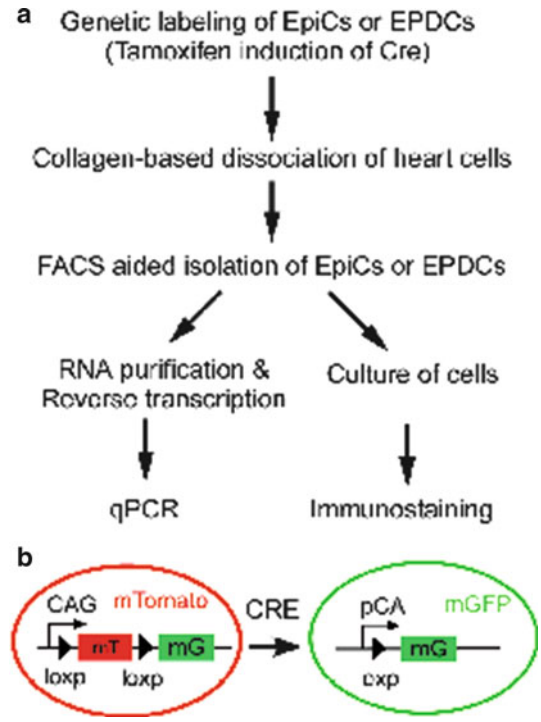

Fig. 1. (**a**) Schematic figure showing the major procedures of isolation and characterization of epicardium cells (EpiCs) and epicardium-derived cells (EPDCs). (**b**) Rosa26$^{mTmG/+}$ reporter shows switching mGFP from mTomato expression after Cre recombination.

2. Materials

2.1. Induction of Labeling

1. Wt1^{GFPCre}, Wt1^{CreERT2}, and Rosa26^{mTmG} mice (Jackson Laboratories, stock numbers 010911, 010912, 007676).
2. Tamoxifen stock solution: Dissolve 25 mg of tamoxifen in 1 mL of 100% ethanol. Store at −20°C.
3. Tamoxifen working solution: Mix tamoxifen stock sol`ution with sesame oil at a 2:1 (v/v). Sonicate for half an hour to mix them at 37°C.
4. Plastic feeding tubes for gavage.

2.2. Dissociation of Heart Cells

1. Digestion stock solution:
   (a) 44.5 mL Hanks' Balanced salt solution.
   (b) 4 mL 1% Collagenase IV. Dissolve collagenase IV in sterile water at 1% (w/v). Stir for half an hour to 1 h at 4°C, filtrate through a 0.45- m filter, and store at −20°C (see Note 1).
   (c) 1 mL 2.5% trypsin.
   (d) 0.5 mL Chicken serum.

2. Heat inactivated horse serum.
3. Deoxyribonuclease I in PBS buffer at 10 mg/mL. Store at –20°C. Add 1:100 when used in the digestion solution.
4. Surgical tools (Fine scientific tools), ice bucket, 50-mL falcon tubes and shaker in 37°C incubator.

2.3. FACS Analysis and Isolation

1. Collecting medium: mesenchymal stem cell growth medium (MSCGM) with 50% FBS for collection of isolated GFP^+ or GFP^- populations for subsequent cell culture.
2. For immunophenotyping by FACS analysis, we used APC-conjugated rat anti-mouse CD45, CD29, CD90 et al. antibodies (e-Biosciences) together with isotype negative control IgG.
3. FACS buffer is made fresh by adding 0.5 g of BSA (weight/volume 0.5% in final working solution) and 400 L of 0.5 M EDTA (2 mM final) into 100 mL PBS.
4. 16% Paraformaldehyde solution and 0.5 M EDTA for antibody staining before FACS.
5. Cell strainers (70 m) for filtrate digested cells to remove clumps.

2.4. qRT-PCR

1. Trizol reagent in 5-mL tube for subsequent RNA isolation.
2. RNase-free or DEPC-treated water for RNA experiments. Dilute 1 mL DEPC in 1 L water and mix overnight at room temperature in the hood. Autoclave it to inactivate DEPC.
3. Superscript III first strand synthesis system.
4. Sybr Green master mix and oligonucleotide primers for qRT-PCR.
5. Some primers that are useful for initial validation of sorting and qRT-PCR are as follows:

   Gapdh, foward acaactttggcattgtgg, reverse gatgcagggatgatgttc.

   Wt1, foward gccttcaccttgcacttctc, reverse gaccgtgctgtatccttggt.

   Raldh2, forward atatgggagccctcatcaag, reverse tctatgccgatgtgagaag.

   Msln, forward tggacaagacctacccacaa, reverse tggtgaggtcacattccact.

2.5. Culture and Immunostaining of EpiC or EPDC

1. 2% Gelatin solution for coating culture dishes to improve cell adhesion.
2. 8-well chamber coated with 2% gelatin.
3. Antibiotic-antimycotic (100×).
4. Mesenchymal stem cell growth medium (MSCGM).
5. 4% fresh PFA for fixation.

6. Triton X-100 for membrane permeabilization.
7. 5% normal donkey serum in PBS for blocking.
8. Antibodies:
   (a) Epicardial markers: WT1 and Epicardin.
   (b) Mesenchymal markers: alpha smooth muscle actin.
   (c) Genetic labeled reporter marker GFP.
   (d) Secondary antibodies. Donkey anti-rabbit or anti-mouse Alexa 555 or 647.
9. VectaShield mounting medium with DAPI to cover slides and stain nucleus. Seal coverslip edges by nail polish.

3. Methods

In heart, Wt1 expression is confined in the epicardium. We used $Wt1^{GFPCre}$ or $Wt1^{CreERT2}$ to enable FACS purification of EpiC or EPDC cells respectively. The $Wt1^{GFPCre}$ allele expresses GFP in $Wt1^{+}$ cells, and therefore GFP^{+} cells represent EpiC. The $Wt1^{CreERT2}$ allele drives CreERT2 expression in the Wt1 expression domain (17). CreERT2 is a fusion protein of Cre recombinase and a portion of the estrogen receptor engineered to be selectively activated by tamoxifen (18). In the absence of tamoxifen, the fusion protein is retained in the cytoplasm (and hence not active as a DNA recombinase). In the presence of tamoxifen, CreERT2 translocates into nucleus, where it catalyzes recombination of loxP sequences. The reporter mouse line we used is $Rosa26^{mTmG/+}$ (19), where a CAG-loxP-mTomato-loxP-mGFP cassette is knocked into the Rosa26 locus (see Fig. 1b). The strong CAG promoter in this permissive locus is widely expressed in most adult tissues including mesothelium. In contrast, other Cre reporters we have tested (e.g., $Rosa26^{fsLz}$ (20) and Z/Red (21)) show less activity in adult mesothelium (BZ and WTP, unpublished). Without Cre recombinase, the cell expresses membrane localized RFP. After excision of the floxed mTomato cassette by Cre, the cell ceases to express mTomato and instead expresses membrane localized GFP, allowing genetic lineage identification. The labeling is irrevocable and heritable, and thus the EpiC and all of its descendants (EPDCs) will express mGFP.

In normal adult mouse epicardium, Wt1 is expressed in a small fraction of cells, making it challenging to isolate enough cells for further characterization. Recently it was reported that after injury, the epicardium reactivates fetal gene organ size-wide (22). Consistent with this observation, we have also found that myocardial injury strongly up-regulated Wt1 expression in large areas of epicardium (Zhou et al., unpublished data). Thus myocardial injury makes it possible to isolate Wt1-expressing cells and their

derivatives. Here we surgically ligate the left descending coronary artery to generate an experimental myocardial infarction (MI).

To isolate EpiC or EPDC from embryonic or adult heart, we use collagenase IV and trypsin to dissociate the heart into single cells for subsequent FACS isolation of the GFP^+ population. The purified cells can be directly analyzed by immunostaining or by qRT-PCR, or can be grown in culture medium. When the purified cells need to be cultured for downstream analysis, strict sterile technique should be followed.

3.1. Mouse Breeding and Models

All mice experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Children's Hospital Boston. To generate E14.5–E15.5 $Wt1^{CreERT2/+}$;$Rosa26^{mTmG/+}$ embryos for EPDC isolation, we crossed $Wt1^{CreERT2/+}$ male mice with $Rosa26^{mTmG/mTmG}$ female mice. For EpiC isolation, we cross a $Wt1^{GFPCre/+}$ male with wild-type females. Mice were checked each morning for vaginal plugs. Record the age of its embryos as E 0.5 at noon of the day when the vaginal plug is seen. At E10.5, female mice were treated with tamoxifen (0.1 mg/g body weight) via gavage to induce CreERT2 activity (see Note 2). Timed pregnant mice were sacrificed for retrieval of embryos on E14.5 (see Note 3).

For study of adult epicardium, we first inject tamoxifen at 0.2 mg/g body weight twice each week for 3 weeks, and then ligate the left anterior descending artery to generate a model of MI and to induce reactivation of epicardium, thus allowing isolation of enough EpiC or EPDCs for further analysis and culture. The method to make a MI model was described before (23). At 1 week after MI, we sacrificed the mice and obtained the infarcted heart for cell isolation.

3.2. Dissociation of Embryonic EpiCs and EPDCs

1. If cells will be grown in culture medium after sorting, cover the tissue culture dishes with sterile 2% gelatin and incubate at 37°C for 1 h. Remove gelatin and wash with PBS once before adding cells.
2. Prepare 50 mL digestion solution for one-time isolation from a pair of positive and negative samples (see step 3 below). Keep the digestion solution on ice.
3. Remove hearts from embryos or adult mice. Keep the heart intact (i.e., do not mince into pieces as commonly done for cardiomyocyte preparations) as this selectively exposes the epicardial surface for digestion. Wash the isolated hearts in cold HBSS several times to remove excess blood cells. Put hearts with the same genotype (up to four embryo hearts or two adult hearts per pool) into a sterile 5-mL polypropylene tube. For best FACS analysis, it is important to process in parallel equivalent samples known to be GFP^-.

The genotype of adult mice should be known, but for embryos it is useful to identify embryos without the proper genotype (approximately 50% of embryos from the described matings will have the correct genotype). For $Wt1^{GFPCre/+}$ embryos, the green fluorescence of $Wt1^{GFPCre/+}$ hearts is too weak to distinguish from background under a dissecting microscope. However, GFP⁺ kidneys could be observed in $Wt1^{GFPCre/+}$ embryos. Using this way, we pool $Wt1^{GFPCre/+}$ hearts and $Wt1^{+/+}$ littermate control hearts for further isolation (see Fig. 2a). Unlike $Wt1^{GFPCre/+}$ hearts, $Wt1^{CreERT2/+}$;$Rosa26^{mTmG/+}$ hearts are easily identified by their green and red fluorescence (see Fig. 2b, c), while $Wt1^{+/+}$;$Rosa^{mTmG/+}$ littermate controls exhibit only red

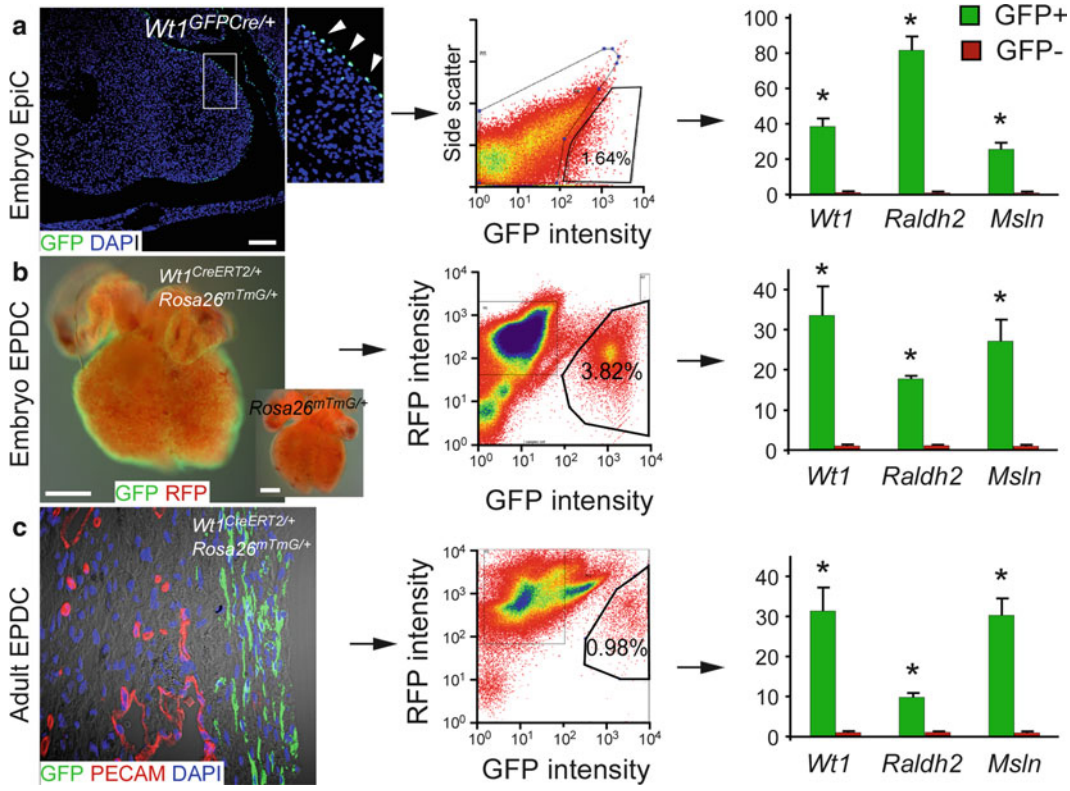

Fig. 2. Isolation of embryonic and adult EpiC and EPDC. (**a**) GFP endogenous expression in E14.5 *Wt1*$^{GFPCre/+}$ heart. Bar = 200 m. *White arrowheads* indicate the epicardium expression of GFP. GFP⁺ cells were sorted by FACS and analyzed by qPCR. Three epicardium markers *Wt1*, *Raldh2*, and *Msln* were found highly enriched in sorted GFP⁺ cells compared with GFP⁻ cells suggesting the high purity of epicardium cells in GFP⁺ population. (**b**) After tamoxifen injection, E14.5 *Wt1*$^{CreERT2/+}$;*Rosa26*$^{mTmG/+}$ embryo heart shows green fluorescence compared with littermate control *Wt1*$^{+/+}$;*Rosa26*$^{mTmG/+}$. Bar = 500 m. Quantification of *Wt1*, *Raldh2*, and *Msln* suggests the enrichment of these markers in isolated GFP⁺ population. (**c**) Adult *Wt1*$^{CreERT2/+}$;*Rosa26*$^{mTmG/+}$ mice were treated with tamoxifen and myocardium infarction (MI). The injured heart (7 days post-MI) was stained with GFP and PECAM. GFP⁺ and GFP⁻ cells were isolated by FACS and subsequently analyzed by qPCR. All three epicardium genes were highly up-regulated in GFP⁺ population. $n = 3$, $^*P < 0.05$.

fluorescence and no detectable green signal. Pool the double fluorescent hearts ($Wt1^{CreERT2/+}$;$Rosa26^{mTmG/+}$) into one group and red-only embryo hearts ($Wt1^{+/+}$;$Rosa^{mTmG/+}$) as negative control into a second group.

4. Add 4 mL digestion solution to each tube.
5. Rock gently in 37°C shaker (~60 times/min) for 6–7 min. Pipet up and down three times and let the tube stand for 15 s. Remove the supernatant containing dissociated cells to a 50-mL falcon tube and add 0.5 mL horse serum to neutralize the digestion solution. Keep the collecting tube containing dissociated cells on ice throughout all steps.

   If material traps tissues are sticky during digestion, add DNase I (1:100) to cells just after adding digestion solution.
6. Add 4 mL digestion solution again. Pipet the hearts up and down. Repeat step 5.
7. Repeat steps 5 and 6 for seven to eight times (usually the outer layer of the heart will be first digested and come off after 7–8 rounds). To avoid photobleaching, turn off the light in the hood.
8. After the final digestion, filtrate the cells through 70- m filter and pellet cells by centrifuging at 200 × *g* for 5 min at 4°C. If another marker when co-expressed in EpiC or EPDCs will be analyzed, resuspend the cells in 5 mL FACS buffer and follow the proceed in step 1, Subheading 3.3. If downstream signaling is to be studied on EpiC or EPDCs, resuspend the cells of around $2–5 \times 10^6$ cells/mL in 0.5 mL HBSS and see step 2 in Subheading 3.3.
9. If the cells will be used for RNA isolation, prepare two FACS collection tubes containing 1 mL Trizol each. If the cells will be used for culture, prepare two FACS collection tubes containing 1 mL 50% FBS-MSCGM for collection of isolated EPDCs.

3.3. FACS Analysis or Isolation of EpiC or EPDCs

For analyzing co-expression of another marker in EpiC or EPDCs, follow Subheading 3.3.1. For isolating purified populations of EpiC or EPDCs for downstream studies, proceed directly to Subheading 3.3.2.

3.3.1. Immunostaining Prior to FACS Analysis

Time estimate: 1 h

1. FACS analysis requires antibody staining for the marker of interest. Since the hearts already have GFP and RFP fluorescence, we use APC or Alexa647 conjugated antibodies to label the cells. Keep antibodies on ice.
2. Wash cell pellets with 5 mL FACS buffer. Spin down cells at 200 × *g* on bench centrifuge for 5 min and then wash again

with 1 mL FACS buffer. Transfer to 1.5-mL Eppendorf tubes. For some intracellular epitopes, a fixation and permeabilization step is necessary. 1% FPA is used for fixation on ice for 10 min and 0.1% Triton X100 to permeabilize the membrane for 5 min.

3. Spin down at 200×*g* for 5 min, and resuspend the cells with 100 L FACS buffer containing 1 L APC-conjugated antibody. Incubate at 4°C for 20 min and mix gently on shaker in dark room or with protection from light in tinfoil paper (see Note 4).
4. Spin down cells at 300×*g* on bench centrifuge for 3 min and then resuspend the cells with 1 mL FACS buffer. Repeat it for two times.
5. After final centrifugation, suspend the cells with 250 L 1% PFA fixation on ice.
6. Transfer the samples from Eppendorf tubes to FACS tubes and proceed for FACS analysis. Alternatively, the samples could be stored in the dark at 4°C until analysis.

3.3.2. FACS Isolation

1. Prepare the FACS machine for sterile isolation of cells according to the FACS instrument protocol.
2. Gate on forward and side scatter to focus on single cells, and then gate on GFP and RFP signal intensity to isolate the GFP^+/RFP^- subset (see Fig. 2b, c, middle panels). When using the $Rosa26^{mTmG}$ reporter, two fluorescence colors could be seen: green (GFP^+) and red (RFP^+). Proper control group should be used due to crosstalk between fluorescence channels. The GFP^- control sample is essential to properly set compensation and gating for cells from GFP^+ samples. For APC-conjugated antibodies analysis, a new gate should be set to identify the subset of GFP^+ cells that are also positive for APC.
3. The GFP^+ cells are isolated and put into prepared tubes by FACS. Collection of the GFP^- cells is also necessary. For RNA analysis, collect samples in Trizol at room temperature. For cell culture, collect cells in sterile polypropylene tubes containing culture medium with fetal bovine serum (see Note 5). High speed (around 1,000–2,000 cells/s) is used in the sorted cells to minimize introduction of excess volume into the collection tubes (see Note 6).

3.4. Characterization of EpiC or EPDC by qRTPCR

Prepare everything by using RNase-free or DEPC-treated water. In general, we proceed with Trizol purification immediately, then store RNA until enough samples are collected to proceed to qRT-PCR. It is desirable to have at least three independent samples per group for quantitative comparisons between groups.

1. Purify RNA from cells following the Invitrogen Trizol protocol (see Note 7).

2. At the end of the procedure, dissolve the RNA pellet with 30 L RNase-free water and incubate for 10 min at room temperature. Measure RNA concentration and quality using a spectrophotometer. Store RNA at −80°C until enough RNA is available to proceed.
3. Reverse transcribe RNA to cDNA. ~0.3–0.5 g total RNA is needed and the Invitrogen Superscript III protocol using oligo (dT) primers is followed. A control sample lacking RT is highly recommended.
4. Perform qRT-PCR. Sybr Green detection chemistry is used and qRT-PCR is performed with technical triplicates. Expression values are relative to an internal control that is assumed not to vary substantially between conditions. Gapdh is used as an internal control (see Note 8).

3.5. Culture of EpiCs or EPDCs and Characterization by Immunostaining

For immunostaining, it is important not to allow samples to dry between steps. Perform incubations in a humidified slide staining chamber. Examples of immunostained EPDCs are shown in Fig. 3.

1. GFP$^+$ cells were plated onto gelatin-coated wells in MSCGM supplemented with 20% FBS and antibiotic-antimycotic.

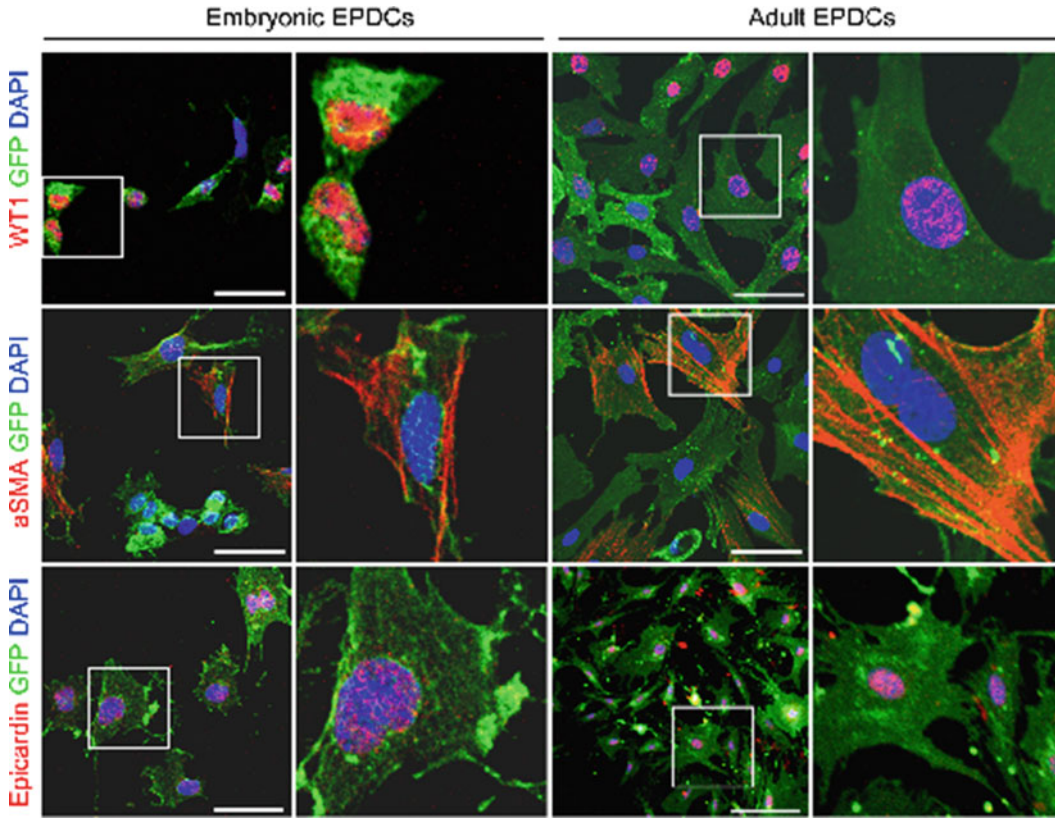

Fig. 3. Representative immunostaining figure of attached primary epicardium-derived cells from embryo or adult hearts. Bar = 20 m.

Before staining, remove the medium and wash cells twice with PBS. Cells are then fixed in 4% fresh PFA for 10 min, followed by two-time washes in PBS.

2. Antigen block and permeabilization: serum with the same species as the secondary antibody should be used to block nonspecific binding sites. In our case, cells were treated with 5% normal donkey serum and 0.1% Triton X-100 in PBS for 30 min at room temperature. Remove the block solution gently.
3. Primary antibody: incubate the slides overnight at 4°C with primary antibody diluted in block solution. Some antibodies are useful for initial characterization of EpiC and EPDC cultures such as Wt1 1:100, Epicardin 1:100, smooth muscle actin 1:200. After incubation, aspirate primary antibody and wash with PBS three times each for 5 min (see Note 9).
4. Secondary antibody: add donkey anti-mouse or anti-rabbit Alexa 555 or 647 antibodies at 1:1,000. Incubate at room temperature for half an hour in dark room or covered with tinfoil paper (see Note 10).
5. Wash with PBS twice each for 5 min, counter stain with DAPI, and wash with PBS twice again each for 5 min.
6. Add vectashield and mount coverslips. Use nail polish to seal coverslip edges.
7. Acquire images using an epifluorescent or confocal microscope using blue, green, and red or far red channels.

4. Notes

1. Unless stated otherwise, all solutions should be prepared in water that has a resistivity of at least 18.2 MΩ-cm and has been filtered through a 0.22- m filter. This standard is referred to as "water" in this text.
2. For gavage of tamoxifen, be cautious when inserting the tube, or it will injure the pharynx or esophagus. Make sure the insertion of the tube occurs with the mouse swallowing and align the angle of the tube to that of the esophagus. Insert the tube about 3 cm into the stomach and inject the tamoxifen-sesame oil mixture slowly.
3. Autoclave the surgical tools prior to use and spray the dissecting platform with 70% ethanol. Retrieve embryos under a dissecting microscope in a dissecting hood. It is critical to keep everything sterile during all procedures for culturing the isolated cells. Including 1% antibiotic-antimycotic in culture media helps to avoid contamination by bacteria and fungi.

4. The dilution of each antibody needs to be optimized. For conjugated antibodies we have had good experience starting at a dilution of 1:100.
5. The amount of serum in the collection media influences cell viability. The combination of 50% serum and 50% medium is the best.
6. One E14.5 embryo heart typically yields ~1×10^4 EpiCs or 2–3×10^4 EPDCs. One adult post-MI heart typically yields ~5×10^4 EPDCs.
7. Usually we obtain 1×10^5 cells or less, making the RNA pellet difficult to visualize. Adding 2 L glycoblue to 1 mL Trizol reaction at this step enhances recovery and visualization of the pellet.
8. When performing qPCR, primer pairs should be validated in pilot experiments to be highly efficient and to generate a single specific product without significant primer dimers. To avoid issues related to genomic DNA contamination, amplicons should be optimally designed to span an intron.
9. Optimal antibody dilution varies with different antibodies and different lots. The initial testing of dilutions could be 1:100, 1:300, and 1:1,000. The incubation time and temperature also need to be empirically optimized. 2 h at room temperature and overnight at 4°C are both reasonable.
10. The secondary antibody is directed against the species of origin of the primary antibody. If the red channel is available, use Alexa 555. However, if the red channel is occupied, e.g., by the mTomato reporter from $Rosa26^{mTmG}$, then use Alexa 647. $Rosa26^{mTmG}$ expresses either mGFP or mTomato. Therefore, if pilot experiments show that sorting was effective and nearly all cells are GFP^+, then the red channel can be considered unoccupied.

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