Video Article

Isolating Primary Melanocyte-like Cells from the Mouse Heart

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Abstract

We identified a novel population of melanocyte-like cells (also known as cardiac melanocytes) in the hearts of mice and humans that contribute to atrial arrhythmia triggers in mice. To investigate the electrical and biological properties of cardiac melanocytes we developed a procedure to isolate them from mouse hearts that we derived from those designed to isolate neonatal murine cardiomyocytes. In order to obtain healthier cardiac melanocytes suitable for more extensive patch clamp or biochemical studies, we developed a refined procedure for isolating and plating cardiac melanocytes based on those originally designed to isolate cutaneous melanocytes. The refined procedure is demonstrated in this review and produces larger numbers of healthy melanocyte-like cells that can be plated as a pure population or with cardiomyocytes.

Video Link

The video component of this article can be found at http://www.jove.com/video/4357/

Introduction

We recently identified a novel cell population in the heart of mice and humans that express melanin-synthesis enzymes and morphologically resemble cutaneous melanocytes. We called these cells 'cardiac melanocytes' and found they are present in anatomic locations from which atrial arrhythmia triggers often originate in humans. We also found cardiac melanocytes contribute to atrial arrhythmias in mice with germline deletion of Dct. Cardiac melanocytes are sparsely distributed throughout the mouse atrium where they comprise less than 0.1% of all atrial cells. To investigate the electrical and biological properties of cardiac melanocytes we developed a procedure to isolate them from the mouse heart using Cre-inducible melanocyte specific markers. Our initial procedure was developed from those used to isolate neonatal murine cardiomyocytes and yielded very small numbers of cells suitable for patch clamp recordings or PCR analysis. However, to improve the health and viability of cardiac melanocytes, for more in-depth electrophysiological analysis or biochemical studies, we developed a refined procedure from those designed to isolate cutaneous melanocytes. The procedure described and demonstrated in this review has the advantage of producing healthier cardiac melanocytes that can be plated as a pure population or with cardiomyocytes.

Protocol

1. Preparing the Necessary Solutions and Equipment

1. First, sterilize the surgical instruments (straight shape forceps, curved shape forceps, straight shape scissors, and curved shape scissors) by autoclaving them for 15 min. at 121 °C. Prepare 2 sets of instruments, where one set is used for dissecting out the hearts and the other set will be used for mincing up the tissue.
2. Dissolve 1 packet of MCDB153 culture media in 900 ml of sterilized, filtered water and stir slowly until the powder has dissolved. Add 1.2 g sodium bicarbonate, or 15.7 ml of sodium bicarbonate solution [7.5% w/v], for each liter of final volume of medium being prepared and stir until dissolved. Adjust the pH to 7.2 by adding either 1 N HCl or 1 N NaOH, then pour the media through a 0.2 µm bottle top filter under sterile conditions in a tissue culture hood (biosafety cabinet). Then collect the media and store it at 4 °C.
3. Prepare the supplements to add to the culture media (Materials List). The supplements should be added to the media just before the media is needed for plating the cells.
4. Prepare DPBS with 10% FBS plus antibiotics (penicillin and streptomycin).
5. Next, prepare a 0.25% trypsin solution by adding 0.25 g of trypsin (Bovine pancreas, activity > 2,500 USP units/mg, ultrapure; USB) to 100 ml of DPBS plus antibiotics (penicillin and streptomycin).
6. Other required equipment needed to complete this procedure include a stereomicroscope, Petri-dishes (10-cm), 50-ml conical tubes, 40-µm cell strainers, DPBS plus antibiotics, 60-mm culture dishes and 35-mm culture dishes.

2. Isolating Hearts from Neonatal Mouse Pups

1. First, obtain P0 to P2 neonatal mouse pups (n=6 to 8). It is recommended to use pups generated by breeding Dct-Cre homozygous females with males that are heterozygous for either the R26R:EYFP allele or the Z/EG allele to label the melanocyte-like cells. Both R26R:EYFP and Z/EG mice are available from Jackson Laboratories (stock numbers 006148 and 003920, respectively).
2. Next, euthanize the neonatal mouse pups, wipe their chests with an alcohol swab and then place the pups in a 10-cm Petri dish with DPBS.
3. Cut the skin over the chest and sternum with sterilized forceps and scissors and then open the chest carefully under a stereomicroscope. The hearts of neonatal mouse pups are very small so be careful to make sure to remove the entire heart with the atria attached.
4. Wash the hearts in DPBS and transfer them to a new 10-cm Petri dish.

3. Enzymatic Digestion

1. Bring the Petri-dish with the excised hearts to a tissue culture hood (biosafety cabinet) and then follow the steps below using sterile techniques in the hood.
2. First, wash the hearts three times in sterile DPBS plus antibiotics.
3. Place the hearts (n=6-8) in a 60-mm culture dish and add 6-8 ml of 0.5% trypsin solution to the dish.
4. Next, cut the hearts into small pieces (less than 1-mm in size) and incubate them at 37 °C for 30 min in a 5% CO_2 atmosphere.
5. After the incubation period is complete, pour the resulting solution of cardiac tissue and trypsin from the dish into a sterile 50-ml conical tube.
6. Then, using a 10-ml sterile pipette, quickly but gently, triturate the solution in the 50-ml conical tube 30-50 times.
7. Filter the resulting solution through a 40-µm cell strainer on top of a new, clean 50-ml conical tube to collect the filtrate.
8. Next, centrifuge the 50-ml tube at 240 x *g* in a table-top centrifuge for 5 min at room temperature. Then, gently aspirate off the supernatant and resuspend the remaining pellet in 6-8 ml of sterile DPBS. Repeat this step 2 more times. This step pellets the atrial myocytes and cardiac melanocytes but does not bring down the fibroblasts since the fibroblasts are smaller and will remain in the supernatant.

4. Plating Cardiac Melanocyte-like Cells

1. Following the third rinse after centrifugation, carefully drain or aspirate off the wash solution (DPBS) and resuspend the pellet in 6 ml of MCDB culture media to which the supplements have been added.
2. Now, draw the resuspended solution into a sterile transfer pipette and place the cells in a 35-mm dish, or in a well of a 6-well plate, and pool the isolated cardiac cells from 3 neonatal mice in one 35-mm dish or well.
3. Incubate the dishes overnight at 37 °C in a 5% CO_2 atmosphere, aspirate off the culture media along with any unattached cells and then rinse the plates once with DPBS. Then add fresh media to the plates and replace the media every 2 days. Cells isolated from neonatal or embryonic hearts can be maintained in culture for up to 3 weeks, whereas cells isolated from adult hearts can be maintained in culture for up to 10 days.

5. Isolating Cardiac Melanocyte-like Cells from Adult Mouse Heart

1. First, remove the hearts from mature mice (3-4 weeks-old, n=3) through a mid-line sternotomy after administering heparin 100 U intraperitoneal to the mice and then euthanizing them. It is recommended to use pentobarbital to euthanize mice for this procedure, but this drug is not available in all locations.
2. Next, wash the excised hearts in sterile DPBS containing antibiotics in a tissue culture hood (biosafety cabinet). Squeeze the hearts slightly with curved dissecting forceps to remove any remaining blood from them and then rinse the hearts in DPBS one more time.
3. Remove the atria and atrioventricular annulus from the hearts (these structures contain the melanocyte-like cells) and place the excised tissue specimens in a 35-mm dish.
4. Cut the hearts into small pieces using curved scissors and forceps and then add 6-8 ml of 0.5% trypsin solution to the dish. Then incubate the dishes at 37 °C for 45-60 min.
5. Now, follow the steps from the procedure above starting from step 3.6 onwards.

Representative Results

The protocol described in this article is an improved technique compared to the one we previously published for isolating cardiac melanocytes from the murine heart. This procedure employs the use of fluorescent markers driven by melanin synthesis enzyme-specific Cre-recombinase to label cardiac melanocyte-like cells. It is important to use a marker to label cardiac melanocytes when working with freshly isolated embryonic or neonatal cells, since the morphological differences that distinguish cardiac melanocytes from atrial myocytes have not yet developed (**Figure 1A**). As shown in **Figure 1A** when the isolation step has gone well, a fairly large number of atrial myocytes and cardiac melanocytes will adhere to the laminin coated dish with just a few non-adherent cells. In this case the adherent cells will appear flat on the bottom of the dish (yellow arrowheads **Figure 1A**, arrowhead). On the other hand, when the procedure does not work well most of the cells will be non-adherent and often look spherical and rounded up (light blue arrowheads, **Figure 1A**).

When the isolated cells are visualized using green fluorescence imaging, it is clear which cells are cardiac melanocytes and which cells are atrial myocytes (**Figure 1**). As mentioned above in the protocol (step 3.9), the fibroblasts are removed from the pool of isolated atrial cells using low-force centrifugation that pellets the larger atrial myocytes and cardiac melanocytes, but does not bring down the smaller cardiac fibroblasts. The freshly isolated cells may now be used for single cell electrophysiological recordings (**Figure 2**), single cell RNA analysis or biochemical assays.

We have also cultured and maintained isolated neonatal or embryonic atrial cells using this protocol for up to 21 days. Using the culture conditions described in this protocol, the cardiac melanocyte-like cells will further differentiate and proliferate, while the atrial myocytes tend to detach from the dish and die within 2-3 days after they are isolated. Shown in **Figure 3A** are healthy cardiac melanocytes 5 days after they were

isolated from a neonatal mouse heart. Note the lack of any atrial myocytes around these cells (as opposed to the freshly isolated cells shown in **Figure 1**). In addition, these cardiac melanocytes have developed extensive dendritic processes and closely resemble cutaneous melanocytes that have also been in culture for the same number of days (**Figure 3B**).

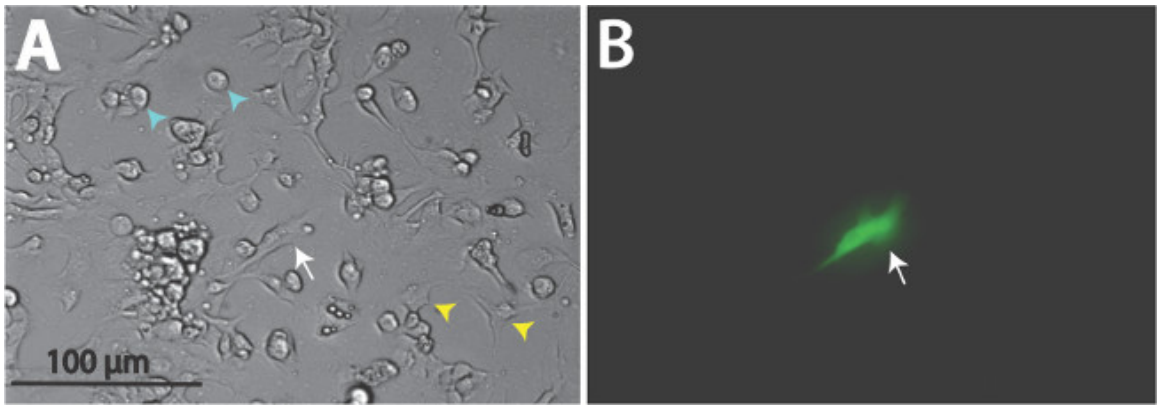

Figure 1. Representative (**A**) DIC and (**B**) GFP images of freshly isolated atrial cells from an embryonic (E19.5) Dct-Z/EG mouse pup. The white arrow identifies the cardiac melanocyte in both panels. Note that without the aid of the fluorescent marker, the cardiac melanocyte is difficult to identify and distinguish from the atrial myocytes (yellow arrowheads) attached to the dish. The light blue arrowheads point to atrial cells that are not well attached to the dish.

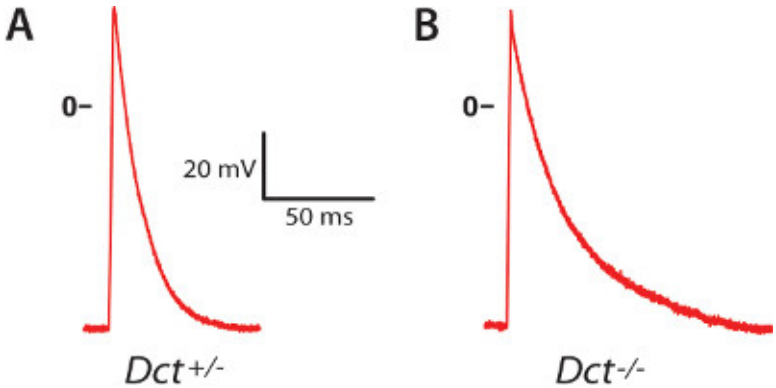

Figure 2. Dct-/- cardiac melanocytes demonstrate prolonged action potential duration. Current clamp recordings from (**A**) cardiac Dct+/- and (**B**) cardiac Dct-/- cells demonstrate increased action potential duration in the absence of Dct. Resting potential of the Dct+/- cell is -62 mV, while the Dct-/- cell has a resting potential of -60 mV. Reproduced, with permission, from Levin *et al. J. Clin. Invest.* **119**, 2920-2936 (2009).

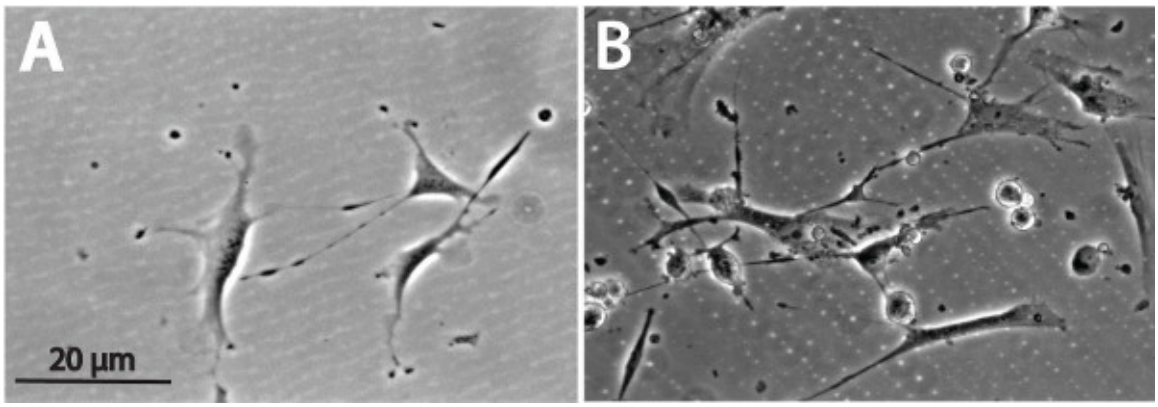

Figure 3. Representative images of isolated (**A**) cardiac melanocyte-like cells and (**B**) cutaneous melanocytes. Melanocytes were isolated from the hearts and skin of neonatal mice (P1), respectively, and then cultured for 5 days.

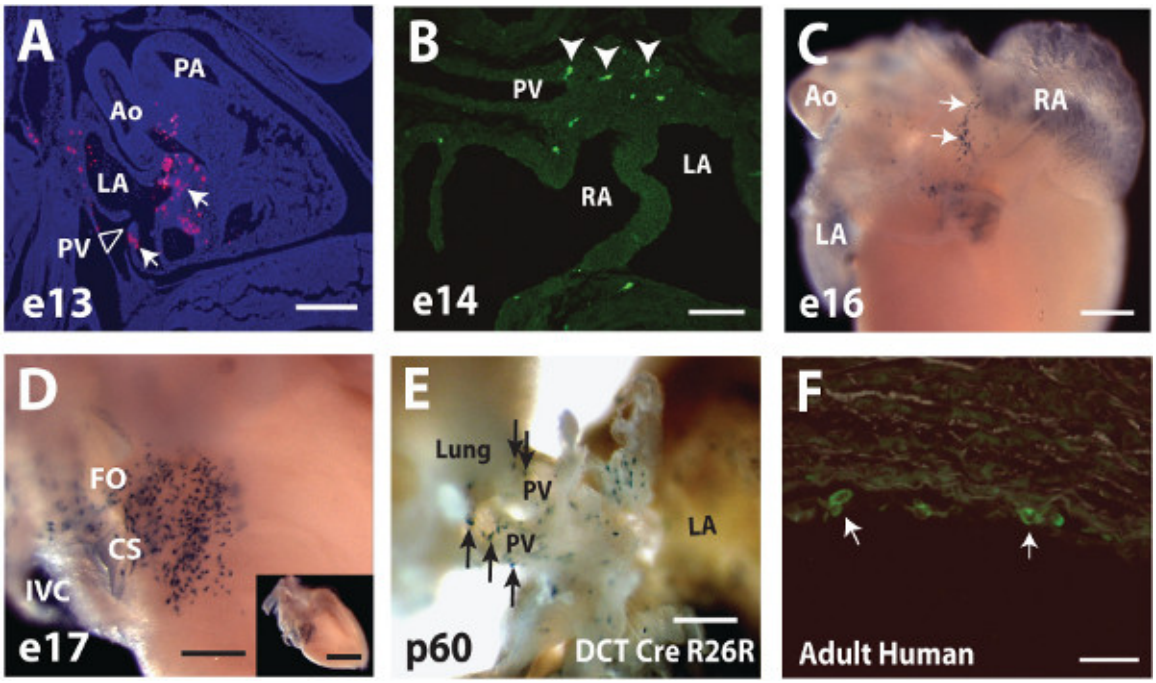

Figure 4. Dct-expressing cells populate the heart. (**A**) *In situ* hybridization of an e13.5 mouse heart shows Dct expression (filled arrows) around the pulmonary vein os (open arrowhead). (**B**) Immunofluorescent staining of Dct-positive cells (arrowheads) within the pulmonary veins. (**C**) X-gal staining of Dct-positive cells in the posterior atrium of an e16.5 mouse Dct-LacZ heart (arrows). (**D**) X-gal staining in a e17.5 Dct-LacZ mouse heart along the RA septum in the regions of the foramen ovale, coronary sinus os and inferior vena cava; inset scale=500 μm. (**E**) Adult mouse (p60) resulting from crossing a DctCre+/- and R26R mouse demonstrates LacZ positive cells (arrows) within the pulmonary veins and left atrium. (**F**) Dct-positive cells detected by immunofluorescent staining (arrows) on the endothelium of a surgically removed human pulmonary vein. Scale=500 μm in all panels except panels (B and D; scale=100 μm) and panel (F; scale=50 μm). Abbreviations: PV, pulmonary vein; LA, left atrium; Ao, aorta; PA, pulmonary artery; RA, right atrium; IVC, inferior vena cava; FO, foramen ovale; CS, coronary sinus. Reproduced, with permission, from Levin *et al. J. Clin. Invest.* **119**, 2920-2936 (2009).

Discussion

Cardiac melanocytes are present in anatomic locations within the heart that commonly give rise to atrial arrhythmia triggers. These regions include the pulmonary veins, the atrioventricular annulus, the posterior left atrium and the foramen ovale (**Figure 4**). To understand the role these cells play in arrhythmogenesis, we develop techniques to isolate them and study their physiology. However, we recognized that improving this technique to yield healthier and larger quantities of cardiac melanocytes would permit further studies to investigate the response of cardiac melanocytes to exogenous stimuli. Furthermore, improved isolation techniques may also permit studies to investigate the interaction of cardiac melanocytes with atrial myocytes during normal physiology and disease.

This paper describes a procedure for isolating viable melanocyte-like cells from the mouse heart that we developed from protocols designed to isolate melanocytes from the skin. The critical step in this protocol is extracting the melanocyte-like cells from the heart. While isolating cardiac melanocytes from neonatal hearts is relatively straight forward, extracting cardiac melanocytes from the mature heart is much more difficult due to the increased amount of interstitial fibrosis. In this protocol we used trypsin digestion to extract melanocyte-like cells from the mature heart. To develop this protocol we tested several different enzymatic combinations that consisted of various concentrations of trypsin, collagenase, and dispase.

We found that treating neonatal atria with a combination of collagenase (for the first 30 min) followed by trypsin (for the next 15 min) initially produced higher yields of melanocyte-like cells, but the isolated cells were less healthy with decreased viability. On the other hand, digestion with trypsin alone produced a moderate number of healthy melanocyte-like cells from mature mouse hearts. Therefore, we recommend just using trypsin to isolate melanocyte-like cells from mature murine hearts. In addition, we found that vigorously triturating the slurry produced after trypsin digestion of mature mouse hearts improved the yield of viable melanocyte-like cells. We should mention that trypsin has the potential to partially digest membrane channels or charge carriers, and this may affect channel density when performing patch clamp studies. While we have not carried out a rigorous analysis to determine if trypsin digestion affects the current density of isolated, mature melanocyte-like cells we have not noticed any significant effects on currents or action potentials in the studies we have performed using this protocol.

Cardiac melanocyte-like cells are a very sparse cell population in the heart (less than 0.1% of all atrial cells) that morphologically resembles cutaneous melanocytes (**Figure 1** and **Figure 3**). Due to the fact there are relatively few cardiac melanocytes present in the heart; until recently we have only been able to use them for single-cell assays (*i.e.* patch clamping recording or single-cell PCR). However, we have obtained larger quantities of cardiac melanocytes by pooling 20-30 neonatal, or 5-8 mature hearts, and found this approach produces a sufficient quantity of tissue for performing immunoblot or biochemical assays. Furthermore, given the low number of melanocyte-like cells in the murine heart, to improve the yield of these cells after they have been isolated we will often place 15-20 mm circular, Teflon inserts on the 35-mm dishes using

sterilized vacuum grease. The cells can then be placed within the inserts to reduce the plating area and increases their density. Using Teflon inserts also improves the viability of melanocyte-like cells following their isolation and during the plating period as well.

Some cardiac melanocyte-like cells have a bipolar morphology following isolation, which we believe represents a less differentiated state of these cells. In addition, we found that culturing cardiac melanocytes for 3 days following isolation seems to induce further differentiation of the melanocyte-like phenotype where the cells start to form dendrites. We have also found this protocol yields melanocyte-like cells from mature hearts that can be maintained for up to 10 days in culture. However, beyond 10 days in culture melanocyte-like cells isolated from mature hearts will stop proliferating and may become senescent. Isolated and plated cardiac melanocytes may be used for cellular electrophysiological analysis or other molecular/cellular biological analysis, such as RNA profiling or studies to assess their physiological responses to exogenous factors.

Disclosures

We have nothing to disclose.

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