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Enhancing epicardial EMT to repair the heart

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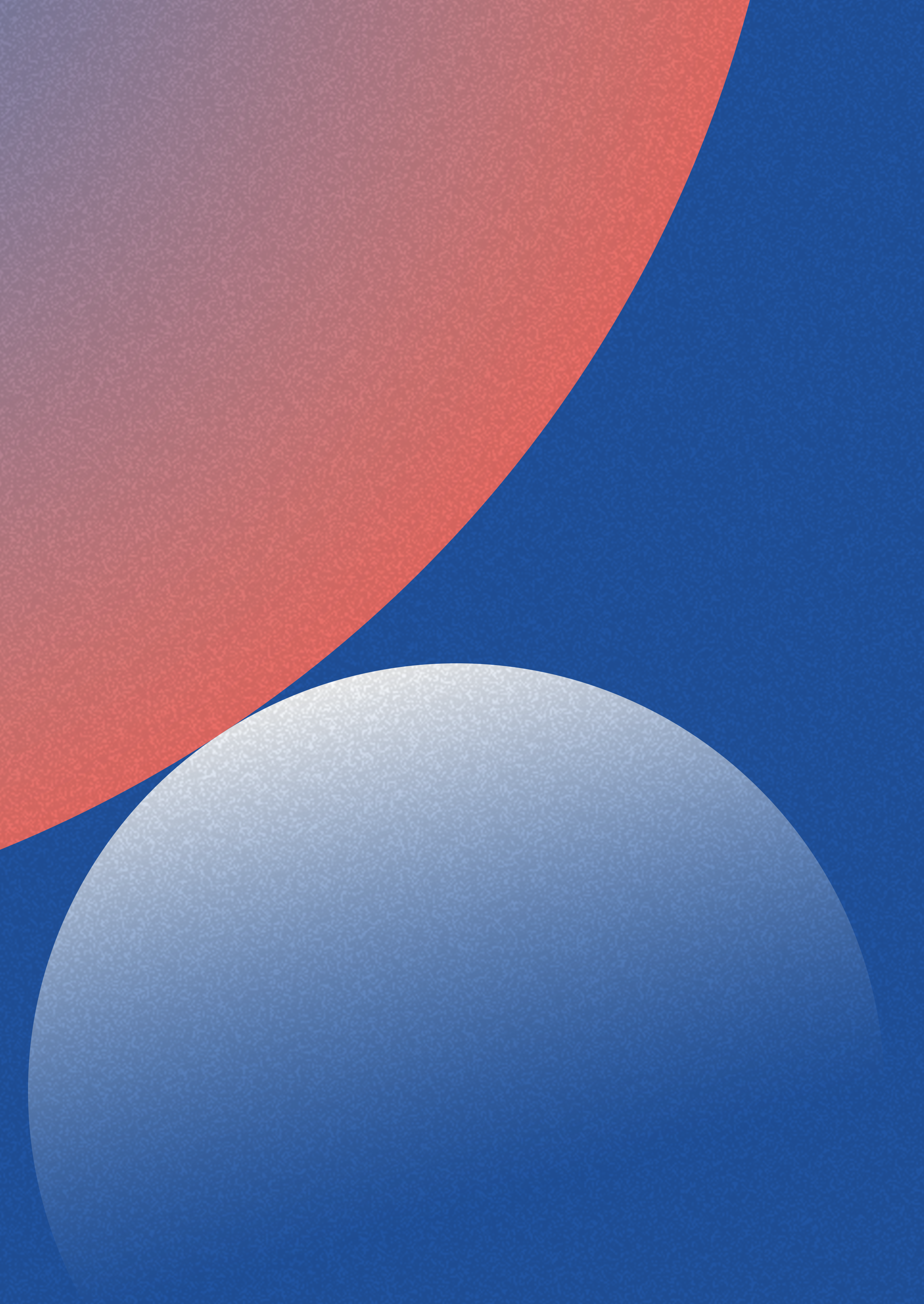

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The Isolation and Culture of Primary Epicardial Cells Derived from Human Adult and Fetal Heart Specimens

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ABSTRACT

The epicardium, an epithelial cell layer covering the myocardium, has an essential role during cardiac development, as well as in the repair response of the heart after ischemic injury. When activated, epicardial cells undergo a process known as epithelial to mesenchymal transition (EMT) to provide cells to the regenerating myocardium. Furthermore, the epicardium contributes via secretion of essential paracrine factors. To fully appreciate the regenerative potential of the epicardium, a human cell model is required. Here we outline a novel cell culture model to derive primary epicardial derived cells (EPDCs) from human adult and fetal cardiac tissue. To isolate EPDCs, the epicardium is dissected from the outside of the heart specimen and processed into a single cell suspension. Next, EPDCs are plated and cultured in EPDC medium containing the ALK 5-kinase inhibitor SB431542 to maintain their epithelial phenotype. EMT is induced by stimulation with TGFβ. This method enables, for the first time, the study of the process of human epicardial EMT in a controlled setting, and facilitates gaining more insight in the secretome of EPDCs that may aid heart regeneration. Furthermore, this uniform approach allows for direct comparison of human adult and fetal epicardial behavior.

Keywords

Developmental Biology, Human, Epicardium, EPDC, Primary Cell Culture, Cardiac Development, Cardiac Regeneration, Epithelial to Mesenchymal Transition, EMT

INTRODUCTION

The epicardium, a single-cell epithelial layer that envelopes the heart, is of vital importance for cardiac development and repair (reviewed in Smits *et al.*[1]). Developmentally, the epicardium arises from the proepicardial organ, a small structure located at the base of the developing heart. Around developmental day E9.5 in mouse, and 4 weeks post-conception in human, cells start to migrate from this cauliflower structure and cover the developing myocardium[2]. Once a single epithelial cell layer is formed, a portion of the epicardial cells undergoes epithelial to mesenchymal transition (EMT). During EMT, cells lose their epithelial characteristics, such as cell-cell adhesions, and obtain a mesenchymal phenotype which gives them the capacity to migrate into the developing myocardium. The formed epicardial derived cells (EPDCs) can differentiate into several cardiac cell types including fibroblasts, smooth muscle cells, and potentially cardiomyocytes and endothelial cells[3], although differentiation of the latter two cell populations remains subject to debate (reviewed in Smits *et al.*[4]). Furthermore, the epicardium provides instructive paracrine signals to the myocardium to regulate its growth and vascularization[5,6,7,8]. Multiple studies have demonstrated that impaired epicardial formation leads to developmental defects in cardiac muscle[9,10], vasculature[11], and conduction system[12], emphasizing the essential contribution of the epicardium to the formation of the heart.

Although in the adult heart the epicardium is present as a dormant layer, it becomes reactivated upon ischemia[13]. Epicardial reactivation post-injury recapitulates several of the processes described for cardiac development, including proliferation and EMT[14], albeit less efficiently. Interestingly, although the exact mechanism is not fully understood, the epicardial contribution to repair can be improved by treatment with, *e.g.*, Thymosin β4[15] or modified VEGF-A mRNA[16], resulting in ameliorated cardiac function after myocardial infarction. The epicardium is therefore considered an interesting cell source to enhance endogenous repair of the injured heart.

Mechanisms of cardiac development are often recapitulated during injury, although in a less efficient manner. In search of epicardial activators, it is paramount that we can determine and compare the full capacity of the fetal and adult epicardium. Moreover, from a therapeutic point of view, it is important that, in addition to animal experiments, we extend knowledge regarding the response of the human epicardium. Here, we describe a method to isolate and culture human adult and fetal epicardial derived cells (EPDCs) in an epithelial-cell-like morphology and to induce EMT. With this model, we aim to explore and compare adult and fetal epicardial cell behavior.

The main advantage of this protocol is the use of human epicardial material, which has not been thoroughly studied. Importantly, the described isolation and cell culture protocol provides a single uniform method to derive both fetal and adult cobble EPDCs, enabling a direct comparison between these two cell sources. Additionally, since the epicardium is isolated based on its location, it is ensured that the cells are actually epicardially derived[17].

While human EPDC isolation methods have been established previously, these mostly rely on outgrowth protocols where pieces of cardiac or epicardial tissue are plated onto a cell culture dish[18,19]. This approach thereby selects specifically for cells that partially lose their epithelial phenotype in order to migrate, and that are more prone to undergo spontaneous EMT. In the current protocol, the epicardium is first processed into a single cell solution which allows the isolated EPDCs to maintain their epithelial state. This method therefore provides a solid *in vitro* model to study epicardial EMT.

PROTOCOL

All experiments with human tissue specimens were approved by the ethics committee of the Leiden University Medical Center and conforms to the Declaration of Helsinki. All steps are performed with sterile equipment in a cell culture flow cabinet.

1. Preparations

1. Prepare EPDC medium by mixing Dulbecco's modified Eagle's medium (DMEM low- glucose) and Medium 199 (M199) in a 1:1 ratio. Add 10% heat inactivated fetal bovine serum (FBS, heat inactivated for 25 min at 56 °C) and supplement with 100 U/mL penicillin and 100 mg/mL streptomycin. Pre-warm the EPDC medium in a 37 °C water bath.
2. Pre-warm Trypsin 0.25%/EDTA (1:1) in a 37 °C water bath.
3. Coat wells with gelatin. Add 0.1% gelatin/PBS to each well and incubate the plates for at least 15 min at 37 °C. Guidelines for the required cell culture plate are summarized in **Table 1**. Carefully remove all fluid before plating cells.
4. Prepare a stock solution of 10 mM SB431542 (SB), diluted in DMSO (CAUTION). Make 50 μL aliquots in conical bottom polypropylene centrifuge tubes, like Eppendorf tubes, and store them at -20 °C. Note that SB aliquots can be thawed only once.

Table 1: Guideline for selecting the appropriate cell culture plate. The required well size depends on the amount of epicardial cells isolated. These guidelines can be used as a rule of thumb to select the appropriate cell culture plate.

Tissue	Well type	Cell Growth Area (cm^2)	Volume of medium (mL)	Addition of SB
Fetal	24	1.9	0.5	Directly
Adult small (<4 cm)	12	3.8	1	After 1st passage
Adult large (>4 cm)	6	9.6	2	After 1st passage

2. Retrieval and Storage of Adult and Fetal Heart Specimens

1. Store human adult auricles directly upon removal during surgery in a 50-mL tube with 15 mL high-glucose DMEM containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 4 °C. Samples are generally 3 - 5 cm in size and can be stored up to 48 h after dissection.
2. Store fetal hearts obtained from elective abortion material in EPDC medium at 4 °C up to 24 h after isolation. For this protocol, use samples with a gestational age between 12 - 22 weeks. Note that the whole heart can be used for isolation of the epicardium.

3. Isolation of the Epicardial Layer

1. In the laminar flow cabinet, prepare a 100-mm cell-culture dish with PBS and place a separate droplet (~200 µL) of PBS in the lid. Fill a 15-mL tube with 5 mL pre-warmed EPDC medium.
2. Place the tissue in the cell-culture dish filled with PBS. Make sure that the tissue is moistened frequently during the procedure.
3. Using a stereomicroscope, remove as much of the epicardial layer from the tissue sample as possible by peeling it off using forceps. Note: The epicardium can be recognized as a very thin, transparent layer tightly adhered to the outside of the heart (**Figure 1A**). Try to avoid contamination with epicardial adipose tissue and blood vessels since this will hamper the isolation.
4. Collect pieces of epicardial tissue in the droplet of PBS on the lid.
5. Cut the epicardial tissue into small pieces (0.5 mm3) using a scalpel (**Figure 1B**), or using the sharp tips of small forceps.Note that the pieces should be able to pass a P1,000 pipet tip (step 3.6).
6. Add 1 mL of trypsin to the epicardial tissue and collect the pieces and trypsin with a P1,000 pipet tip. Transfer the tissue into a 1.5-mL conical centrifuge tube (**Figure 1C**).

7. Incubate the tube in a 37 °C water bath for 10 min, while shaking at ~60 rpm.
8. Remove the tube from the water bath, and clean the outside of the tube with 70% ethanol.
9. Allow the tissue to sink to the bottom of the tube (**Figure 1D**) and carefully transfer the supernatant containing epicardial cells to the 15-mL tube containing 5 mL EPDC medium to inactivate the trypsin.
10. Replenish the remaining tissue in the conical centrifuge tube with 1 mL trypsin, and mix by gently pipetting up and down.
11. Repeat step 3.7 to 3.10 two times. At the final step, after a total of 30 min of trypsin incubation, transfer both the supernatant and the remaining epicardial tissue into the tube containing EPDC medium.
12. When all cells are collected in EPDC medium, gently pass the suspension through a 10-mL syringe with a 19-gauge needle into a new 15-mL tube to mechanically dissociate the cells (**Figure 1E**). Note: Make sure to homogenize the suspension by pipetting up and down before passing the solution through the needle to prevent the needle getting obstructed.
13. To further dissociate the cells, repeat step 3.12 with a 21-gauge needle.
14. Place a 100-µm cell strainer on top of a 50-mL tube and transfer the medium containing the epicardial cells onto the strainer using a 10-mL pipette to remove all remaining clumps (**Figure 1F**).
15. Wash the strainer to collect residual cells by pipetting 5 mL EPDC medium onto the strainer.
16. Pipet the cell suspension from the 50-mL tube into a 15-mL tube. Since cells sink to the bottom of the tube, shake the solution gently before pipetting. Note: While this step is not necessary, a smaller tube aids visualization of the pellet after centrifugation.
17. Centrifuge at 200 x g for 5 min at room temperature (**Figure 1G**).
18. Remove the supernatant and resuspend the cell pellet (**Figure 1H**) in the required volume of EPDC medium (**Table 1**). Note: For fetal EPDCs, directly use EPDC medium containing 10 µM of the ALK5 kinase inhibitor (EPDC+SB). Adult cells can be plated without SB during the first passage.
19. Plate the cell suspension on the gelatin coated culture plates (**Figure 1I**). The size of the well depends on the size of the epicardial tissue sample (**Table 1**). Note that low confluency will induce the occurrence of EMT.
20. Place the cells in the incubator for at least 48 h at 37 °C, 5% CO2 to allow the cells to attach to the culture plate.

4. Culture of EPDCs

1. Replenish the EPDC+SB medium at least every 3 days.
2. Inspect the cells at least every 3 days using the microscope. When the cells reach confluency, *i.e.*, when the culture plate is fully covered with cells, passage the cells in a 1:2 surface ratio. Note that reaching confluency can take ~5 - 10 days.

1. Aspirate the medium using a pipet or an aspiration system with, *e.g.*, a glass pipet.
2. Wash the cells carefully by adding PBS to the cells. Gently swirl the plate and aspirate the PBS.
3. Add trypsin to the plate. Gently rotate the plate to cover all cells with trypsin and incubate the plate for 1 min at 37 °C. NOTE: Use as little trypsin as possible (indication: 200 µL per well of a 6 well plate)
4. Tap the plate to mechanically detach the cells from the bottom of the plate. Use the microscope to visually check if cells have detached. If not, incubate the cell culture plate for an extra minute.
5. Add the required volume of EPDC+SB medium to the cells, resuspend by pipetting up and down, and transfer the cell suspension to a new gelatin coated culture plate. NOTE: In general, cells can be kept in a cobblestone morphology up to passage 8.

5. Induction of EMT in EPDCs

1. To induce EMT, dissociate the cells with trypsin and transfer the cells to new gelatin coated wells in a 1:2 surface ratio in EPDC+SB medium, as described in 4, and incubate for at least 24 h at 37 °C, 5% CO2.
2. Check if confluency is 50 - 70% and if EPDCs have a cobblestone morphology. NOTE: Confluency affects the ability of EPDCs to undergo EMT.
3. Aspirate the medium from the cells and wash the cells carefully with PBS (see step 4.2.1 - 4.2.2).
4. Stimulate cells with 1 ng/mL TGFβ3 in EPDC medium and place them in an incubator at 37 °C, 5% CO2 for 5 days. Note that during stimulation, the EPDC medium containing TGFβ3 does not have to be replenished.
5. Monitor the cells daily. After 5 days, cells that underwent EMT are recognized by a spindle-shaped morphology (**Figure 2A**).
6. Culture spindle-shaped EPDCs according to the method described in 4 without addition of SB or TGFβ to the EPDC medium. Note that in general, spindle-shaped EPDCs can be cultured up to passage 20.
7. Validate the occurrence of EMT with immunofluorescent staining using antibodies against the mesenchymal markers αSMA or Vimentin or by phalloidin

to detect the formation of F-actin stress fibers (**Figure 2B**), or using qRT-PCR for EMT-related genes (*e.g.*, WT1, Periostin, Col1A1, αSMA, N-Cadherin, MMP3, Snail, Slug) (**Figure 2C** and **Table 2**).

Table 2: Primer sequences used for validation of EMT in EPDCs. Sequences of forward and reverse primers used for qRT-PCR to determine the expression of several EMT related genes in adult EPDCs.

Gene	Sequence
WT1 Forward	CAG CTT GAA TGC ATG ACC TG
WT1 Reverse	TAT TCT GTA TTG GGC TCC GC
N-Cadherin Forward	CAG ACC GAC CCA AAC AGC AAC
N-Cadherin Reverse	GCA GCA ACA GTA AGG ACA AAC ATC
POSTN Forward	GGA GGC AAA CAG CTC AGA GT
POSTN Reverse	GGC TGA GGA AGG TGC TAA AG
SMA Forward	CCG GGA GAA AAT GAC TCA AA
SMA Reverse	GAA GGA ATA GCC ACG CTC AG
MMP3 Forward	TGG ATG CCG CAT ATG AAG
MMP3 Reverse	CAG AAA TGG CTG CAT CGA
COL1A1 Forward	CCA GAA GAA CTG GTA CAT CAG CA
COL1A1 Reverse	CGC CAT ACT CGA AAT GGG AAT
GAPDH Forward	AGC CAC ATC GCT CAG ACA C
GAPDH Reverse	GCC CAA TAC GAC CAA ATC C
B2M Forward	ACA CTG AAT TCA CCC CCA CT
B2M Reverse	GCT TAC ATG TCT CGA TCC CAC T

REPRESENTATIVE RESULTS

Here, we outline a straightforward protocol to isolate EPDCs from human adult and fetal cardiac tissue (**Figure 1**). This protocol takes advantage of the easily accessible location of the epicardium on the outside of the heart (**Figure 1A**). Staining of the heart auricle after dissection demonstrates that the WT1+ epicardium is removed while the underlying subepicardial extracellular matrix and myocardial tissue remain intact (**Figure 1J**). Extensive characterization has been performed before, demonstrating that EPDCs express epicardial markers, *e.g.*, ALDH1A2, TBX18, KRT8, KRT19 and lack expression of other heart-resident cell types, *e.g.*, PECAM1, ISL1, CD34, and TNNT2.[17]

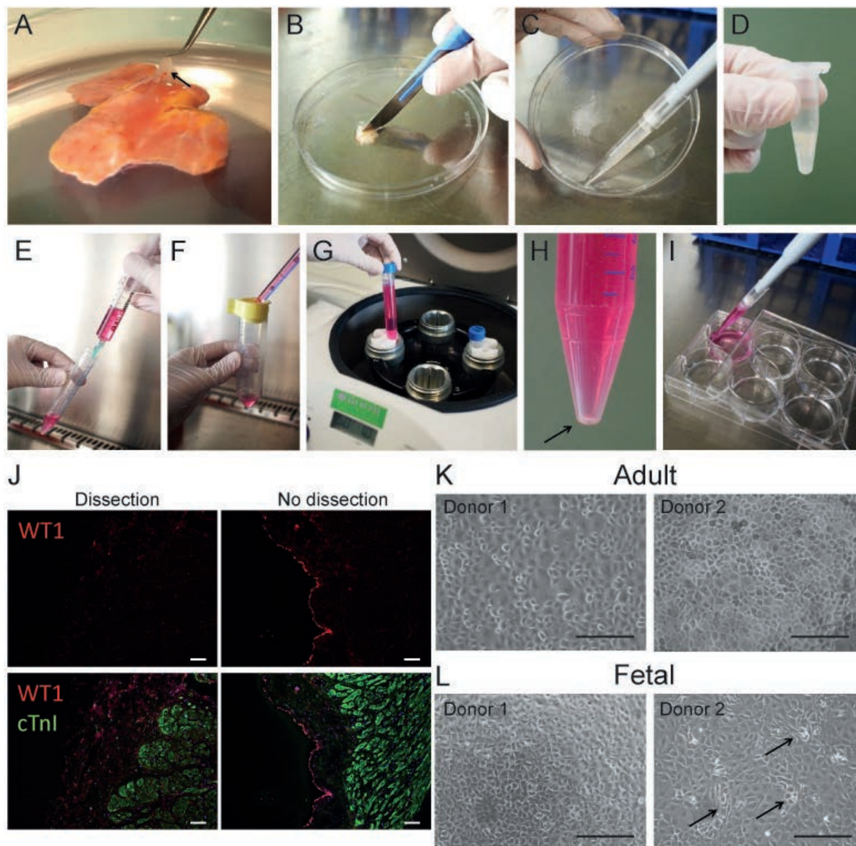

Figure 1 | Isolation of Epicardial Derived Cells (EPDCs). (**A**) Depicted is an adult auricle removed from the human heart with a thin outer membranous layer, the epicardium, which is removed. The black arrow points to the epicardium. (**B-I**) Visual representation of the isolation method of EPDCs. The black arrow points to the cell pellet. (**J**) Immunofluorescent staining of a heart auricle with and without dissection of the epicardium. Scale bar: 50 µm. (**K**) Representative pictures of two different adult EPDC isolations cultured with SB. (**L**) Representative pictures of two different fetal EPDC isolations cultured with SB. Black arrows indicate mesenchymal-like cells in fetal EPDC culture. Scale bar: 200 µm.

Both adult (**Figure 1K**) and fetal (**Figure 1L**) EPDCs cultured in the presence of ALK5 kinase inhibitor SB431542 show a cobblestone morphology. However, depending on the donor and culture conditions, fetal EPDCs can undergo EMT despite the presence of SB. This can result in spindle-shaped cells within the population of cobblestone cells (see arrows in **Figure 1L**). Please be aware that the isolation of cobblestone-shaped cells from fetal tissue is not always feasible, and may result in the derivation of mostly

spindle-shaped cells. Since these cells grow faster, they will rapidly overgrow other cell types.

EMT can be induced by incubating with TGFβ3[20] for 5 days in both adult and fetal cells, as demonstrated by a clear morphological transition to spindle-shaped cells (**Figure 2A**). As demonstrated with the untreated control ("Empty"), fetal EPDCs will undergo spontaneous EMT upon removal of SB, while adult EPDCs will only undergo EMT upon stimulation with TGFβ3. To validate EMT, EPDCs were immunostained with alpha-smooth muscle actin (αSMA), a mesenchymal marker (**Figure 2B**). Furthermore, EMT was confirmed in adult EPDCs using qRT-PCR showing downregulation of the epicardial marker WT1 and upregulation of the mesenchymal markers POSTN, αSMA, Collagen 1A1, MMP3, and N-Cadherin (**Figure 2C**). Comprehensive experiments regarding adult and fetal epicardial EMT are published before[17].

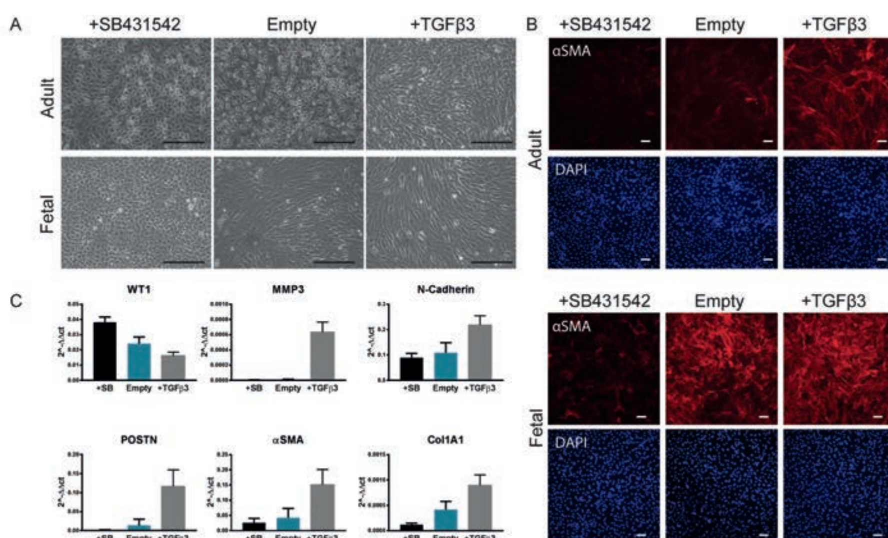

Figure 2 | Validation of EMT in human adult and fetal Epicardial Derived Cells (EPDCs). Adult and fetal EPDCs were cultured with SB, not treated (Empty) or stimulated with TGFβ3 for 5 days. (**A**) Representative bright field pictures. Scale bar: 200 μm. (**B**) Immunostaining for DAPI and αSMA. Scale bar: 100 μm. (**C**) mRNA levels of EMT-related genes, determined in adult EPDCs using qRT-PCR. Measured values were normalized to GAPDH and B2M expression. Values are depicted as mean + SD $2^{-\Delta\Delta ct}$ (n = 2). Abbreviations: WT1:Wilms' tumor 1, MMP3:Matrix Metalloproteinase 3, POSTN:Periostin, αSMA: alpha Smooth Muscle Actin, Col1A1:Collagen 1A1.

DISCUSSION

Here we describe a detailed protocol to isolate and culture primary epicardial cells derived from human adult and fetal hearts. Extensive characterization of these cells has been previously published[17]. We have shown that both cell types can be maintained as epithelial cobblestone-like cells when cultured with the ALK5 kinase inhibitor SB431542. EMT is an integral part of epicardial activation *in vivo* during both development and the post-injury response. EMT can be studied using this method by addition of TGFβ. Importantly, we previously observed that fetal EPDCs rapidly undergo spontaneous EMT when SB is removed, while adult EPDCs only undergo EMT upon stimulation[17]. Studying these processes in fetal EPDCs may aid in understanding how to optimally activate the adult epicardium after damage.

The presented method relies on patient material, which is obtained during surgery. Therefore, one can expect several variations in the state of the material isolated, which are either due to patient variability or the speed at which the material is collected in the operating theater. This variation may explain differences in the procedure: 1) how easily the epicardium can be dissected from the myocardium, 2) adherence of isolated cells to the plate, 3) the ability to prevent or undergo EMT, and 4) proliferation speed. In general, a quick isolation is preferable for cell survival. The main critical point is peeling the epicardium from the myocardium. If the epicardium is strongly adhered to the underlying tissue, a 15-min pre-treatment of the cardiac tissue with trypsin can help to remove the epicardium more easily. Furthermore, the efficacy of the trypsin treatment depends on several factors, including the trypsin activity and the composition of the tissue. Therefore, if a low yield is observed, the incubation time with trypsin can be adjusted. Additionally, if no cell pellet is visible after spinning down, the solution might not have been completely dissociated before running through the filter. Mixing the solution before passing the solution through the syringe or using an extra, smaller syringe could be useful to dissociate the cells. The well size for seeding EPDCs should be considered carefully to enable cells to maintain their epithelial state. **Table 1** gives an indication, but one can deviate from this guideline when, for example, only a small part of fetal heart can be used and plating in a smaller well is more convenient.

Since fetal cells will undergo EMT immediately without SB[17], fetal EPDCs should always be plated with SB. However, adult EPDCs tend to maintain their epithelial morphology and therefore can be plated without SB during the first 48 h of the first passage, to promote cell adherence. After the first passage, both adult and fetal EPDCs are

continuously cultured with SB to prevent spontaneous EMT. In addition, low cell density is an important trigger for EMT. EPDCs should therefore never be cultured below 50% confluency. On the other hand, if EMT is desired, make sure that EPDCs are not seeded too densely, and stay below 70% confluency. Though this protocol utilizes TGFβ3, stimulation with TGFβ1 and 2 can induce EMT in EPDCs as well (observations unpublished).

In this protocol, cells are split directly without spinning down, since we observe a lower cell survival when using the centrifuge. Therefore, it is vital to use low volumes of trypsin to ensure its deactivation when serum containing medium is added.

After ~6 - 8 passages, EPDCs with an epithelial morphology will either stop growing or will undergo EMT spontaneously. Therefore, for experiments, we use cobble EPDCs between passage 3 and passage 6. In contrast, EPDCs with a mesenchymal morphology are less vulnerable and can be cultured up to passage 20. In experiments, however, we have never used spindle EPDCs after the 10th passage.

Since the epicardium is located at the outside of the heart and is easy to separate from underlying tissue, the authenticity of the cells is evident, and the chance of significant contamination is low. Although adipose tissue or blood vessels sometimes stick to the isolated epicardial layer, the majority of those cells will not pass the strainer during the isolation, and otherwise cannot survive in EPDC cell culture. Furthermore, as mentioned before, using human material provides a unique model to investigate human epicardial cell behavior. It is known that, for instance, epicardial adipose tissue is different between species, emphasizing the necessity of human epicardial cell models[21].

It should be noted that the heart auricles were obtained during surgery on diseased hearts, and therefore the differences in disease, used medication, age, and gender of the donor may influence the reproducibility of the experiments. Experiments should therefore be performed on several isolations to obtain valid results and allow solid conclusions to be drawn. Furthermore, depending on the research question, one could choose specifically to only use epicardium from patients with ischemic disease or patients with non-ischemic valvular disease, which are expected to behave differently.

It has been suggested that atrial epicardium may have distinct characteristics from ventricle epicardium[2], thereby questioning if epicardium derived from the atrial heart

auricle provides a valid model for epicardial behavior. In this context, it is important to point out that we could not find differences between fetal atrial and ventricular derived EPDCs (unpublished data). However, using epicardium from the human adult ventricle would be the ultimate cell source to verify this. Yet, collecting large specimens of human adult ventricles for EPDC isolation is highly invasive for the patient, and is therefore currently not feasible in this hospital.

We observed that SB is not always sufficient to prevent EMT, mainly in fetal EPDCs. When fetal EPDCs undergo EMT in the presence of SB, we exclude them from experiments. As a consequence, cells used for experiments are selected for their ability to maintain an epithelial phenotype in response to SB. We hypothesize that fetal cells can already be beyond a certain threshold in the process of EMT, and that inhibition with SB is not able to stop this.

This epicardial cell model has several applications, since both the developing and the adult epicardium can be investigated. In our lab, we focus on improvement of the epicardial regenerative response after cardiac damage. Adult EPDCs can be used to test compounds which induce EMT, aiming to find a potential therapeutic drug for an ameliorated regenerative response of the epicardium. In addition, it is possible to measure factors secreted by EPDCs to comprehend the paracrine signaling to the (re)generating myocardium. Furthermore, since we observed that fetal EPDCs are more prone to undergo EMT spontaneously compared to adult EPDCs, we investigate the differences between the fetal and adult EPDCs. Determining the underlying mechanism of increased fetal activation could provide a cue to improve epicardial activation in the adult heart.

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MATERIALS

Name of Material	Company	Catalog Number	Comments/Description
Dulbecco's modified Eagle's medium + GlutaMAX™	Gibco™	21885-025	
Medium 199	Gibco™	31150-022	
Fetal Bovine Serum	Gibco™	10270-106	
Penicillin G sodium salt	Roth	HP48	
Streptomycin sulphate	Roth	HP66	
Trypsin 1:250 from bovine pancreas	Serva	37289	
EDTA	Sigma	E4884	
Gelatin	Sigma-Aldrich	G1393	
Culture plates 6 well	Greiner bio-one	657160	
Culture plates 12 well	Corning	3512	
Culture plates 24 well	Greiner bio-one	662160	
SB 431542	Tocris	1614	
Dimethyl Sulfoxide (DMSO)	Merck	102931	
100-1000μL Filtered Pipet Tips	Corning	4809	
10-ml pipet	Greiner bio-one	607180	
5-ml pipet	Greiner bio-one	606180	
Cell culture dish 100/20 mm	Greiner bio-one	664160	
PBS	Gibco™	10010056	Or home-made and sterilized
Eppendorf tubes 1.5 mL	Eppendorf	0030120086	
15-ml centrifuge tubes	Greiner bio-one	188271	
50-ml centrifuge tubes	Greiner bio-one	227261	
10 mL Syringe	Becton Dickinson	305959	

Needles 19 Gauge	Becton Dickinson	301700
Needles 21 Gauge	Becton Dickinson	304432
EASYstrainer™ Cell Sieves, 100 µm	Greiner bio-one	542000
TGFβ3	R&D systems	243-B3
Monoclonal Anti-Actin, α-Smooth Muscle	Sigma	A2547
Anti-Mouse Alexa Fluor 555	Invitrogen	A31570
Alexa Fluor™ 488 Phalloidin	Invitrogen	A12379

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