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Epithelial-to-mesenchymal transformation alters electrical conductivity of human epicardial cells

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Abstract

The myocardium of the developing heart tube is covered by epicardium. These epicardial cells undergo a process of epithelial-to-mesenchymal transformation (EMT) and develop into epicardium-derived cells (EPDCs). The ingrowing EPDCs differentiate into several celltypes of which the cardiac fibroblasts form the main group. Disturbance of EMT of the epicardium leads to serious hypoplasia of the myocardium, abnormal coronary artery differentiation and Purkinje fibre paucity. Interestingly, the electrophysiological properties of epicardial cells and whether EMT influences electrical conductivity of epicardial cells is not yet known. We studied the electrophysiological aspects of epicardial cells before and after EMT in a dedicated *in vitro* model, using micro-electrode arrays to investigate electrical conduction across epicardial cells. Therefore, human adult epicardial cells were placed between two neonatal rat cardiomyocyte populations. Before EMT the epicardial cells have a cobblestone (epithelium-like) phenotype that was confirmed by staining for the cell-adhesion molecule β-catenin. After spontaneous EMT *in vitro* the EPDCs acquired a spindle-shaped morphology confirmed by vimentin staining. When comparing both types we observed that the electrical conduction is influenced by EMT, resulting in significantly reduced conductivity of spindle-shaped EPDCs, associated with a conduction block. Furthermore, the expression of both gap junction (connexins 40, Cx43 and Cx45) and ion channel proteins (SCN5a, CACNA1C and Kir2.1) was down-regulated after EMT. This study shows for the first time the conduction differences between epicardial cells before and after EMT. These differences may be of relevance for the role of EPDCs in cardiac development, and in EMT-related cardiac dysfunction.

Keywords: epicardium • epithelial-to-mesenchymal transformation • EPDCs • electrical conduction

Introduction

Cardiogenesis is partly dependent on a proper development of the epicardium. The epicardium originates from the pro-epicardial organ (PEO), from which epicardial cells migrate and cover the primitive heart tube during embryogenesis. Part of these epicardial cells undergo epithelial-to-mesenchymal transformation (EMT), thereby forming epicardium-derived cells (EPDCs). As a result of EMT, cell–cell and cell–matrix interactions change, allowing EPDCs to migrate into the subepicardium and subsequently into the myocardium [1, 2]. In the myocardium, EPDCs initially differentiate into interstitial fibroblasts [3] and a later population forms smooth muscle cells and adventitial fibroblasts of the coronary vasculature [4, 5]. Besides this cellular contribution to heart development, EPDCs also have a regulatory role in cardiogenesis by interacting with surrounding structures. Although the mechanisms underlying this regulatory process are largely unknown, it is likely that cell–cell communication is of great importance. Moreover, several studies demonstrated a crucial role for EPDCs in growth of the compact myocardium and the organization of the myocardial architecture [1, 6, 7]. Loss of proper EPDC-function results in a thin hypoplastic myocardium [8, 9]. Also, an inductive role for EPDCs in the development of the avian Purkinje fibre

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network of the ventricular conduction system has been reported [3, 10]. However, the aforementioned roles of EPDCs do not seem to be restricted to embryonic development, as adult rat epicardial cells delayed the dedifferentiation of rat ventricular cardiomyocytes (CMCs) *in vitro* [11]. In addition, Van Tuyn *et al.* demonstrated that human adult EPDCs can undergo EMT spontaneously and obtain characteristics of smooth muscle cells *in vitro* [12]. Interestingly, recent *in vivo* studies suggest that adult EPDCs can reactivate their embryonic program [13–15]. In more detail, induced hyperplastic cardiac growth in adult zebrafish was associated with epicardial expression of embryonic markers such as *raldh2* and *tbx18*. Also, epicardial cells proliferated to expand the epithelial covering of the ventricles, suggesting that adult epicardium is a dynamic tissue still able to contribute EPDCs to the adult ventricular wall [14–16]. Furthermore, a recent study demonstrated that human adult EPDCs injected into the infarcted myocardium, preserved cardiac function and reduced remodeling both early and late after the onset of infarction [13, 17]. These findings suggest that EPDCs could be suitable for cell-based cardiac repair.

For cardiac cell therapy to be successful and of therapeutic value, the transplanted cells should be able to couple and functionally integrate with native, excitable cardiac tissue. Cell–cell coupling was shown to be of importance, as absence of functional gap junctions between transplanted cells and native cardiomyocytes was associated with electrical disturbances [18]. However, the working myocardium also contains a large number of fibroblasts that, in a more passive manner, are involved in electrical conduction. Cardiac fibroblasts (cFBs) are not excitable, and may therefore contribute to conduction by gap junction-mediated electrotonic interaction [19].

Despite the many effects of epicardial cells during cardiac development and their potency to preserve cardiac function after myocardial infarction, knowledge about their electrical properties is still lacking. Illustrated by their wide spectrum of constructive, regulatory and therapeutic properties, it is of importance to know whether and to which extent EMT of epicardial cells influences their capacity to conduct electrical impulses. We therefore developed a controlled *in vitro* model [20] to study the conductivity of human adult epicardial cells, before and after EMT, by culturing them in-between two fields of CMCs.

Materials and methods

Animal experiments and human specimens

Animal experiments were approved by the Animal Experiments Committee of the Leiden University Medical Center and conformed to the Guide for the Care and Use of Laboratory Animals as stated by the US National Institute of Health. In addition, all experiments with human tissue specimens were carried out according to the official guidelines of the Leiden University Medical Center and with the approval of the institutional ethical committee.

Isolation and culturing of cardiomyocytes

CMCs were dissociated from hearts of 2-day-old male Wistar rats of which the ventricles were minced and dissociated with collagenase and DNase, as described previously [20].

Harvesting and preparation of human epicardium-derived cells

Cultures of human epicardial cells were prepared as described previously [12]. When outgrowth of epicardial cells was confluent, the cells were detached from the bottom of the culture dish with trypsin/EDTA (Invitrogen, Paisly, UK) solution and were divided into two subcultures. The first subculture was seeded in a high density and cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) and medium 199 (M199) (Invitrogen) containing 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen) and 10% inactivated foetal calf serum (FCSi) (Invitrogen), to maintain the epithelium-like morphology. The cells in this subculture of epicardial cells will be referred to as cobblestone-like EPDCs (cEPDCs). The second subculture was seeded in a low density and cultured in aforementioned medium supplemented with 20 ng/ml basic fibroblast growth factor (bFGF; Sigma-Aldrich, St. Louis, USA). This subculture will be referred to as spindle-shaped EPDCs (sEPDCs). The purity of the human EPDC cultures was certified with immunohistochemical staining for Wilm's Tumor-1 protein (WT1) (Calbiochem, San Diego, USA) at a dilution of 1:50 (Fig. S1A and B).

Immunofluorescence microscopy

cEPDCs and sEPDCs were cultured on glass chamber-slides and subjected to immunofluorescent staining as described previously [20] (Table 1). The details of the antibodies used for immunofluorescence microscopy are listed in Table 2. To investigate their morphology, the cells were stained for β-catenin for epithelium-like morphology and vimentin for spindle-shaped morphology. Next, the cells were labelled with antibodies against connexins (Cx40, Cx43 and Cx45) and ion channels. For the ion channels, we used antibodies for a voltage-gated sodium channel (SCN5a), inward rectifier potassium channel (Kir2.1) and voltage-gated L-type calcium channel (CACNA1C). Incubation with primary and appropriate secondary antibodies (Table 2) was carried out overnight and for 2 hrs at room temperature, respectively. Nuclei were stained with Hoechst 33342 (diluted 1:1000 in PBS, Invitrogen). Finally, the slides were mounted with Vectashield (Vector, Burlingame, USA). Examination of the slides was performed using a fluorescence microscope equipped with a digital camera (Eclipse, Nikon Europe, Badhoevedorp, the Netherlands).

Micro-electrode arrays

To study the functional effect of EMT on electrical conduction across epicardial cells we used a standardized *in vitro* model, described in our previous studies [21, 22]. Isolated CMCs were cultured in micro-electrode array culture dishes (MEA, Multichannel Systems, Reutlingen, Germany; number of titanium nitride electrodes: 60; inter-electrode distance: 200 μm; electrode diameter: 30 μm). To improve attachment of the cells to the glass surface, MEAs were glow-discharged and coated with collagen. After 3 days of culture, the confluent synchronously beating monolayer was divided into

Table 1 Summarizing table of IHC, PCR and MEA experiments

	cEPDCs		sEPDCs	
	24 hrs	48 hrs	24 hrs	48 hrs
Immunofluorescent staining				
WT1	X	X	X	X
β-catenin		X		X
Connexin40		X		X
Connexin43		X		X
Connexin45		X		X
Vimentin		X		X
Kir2.1		X		X
SCN5a		X		X
CACNA1C		X		X
PCR				
GAPDH		X		X
Connexin40		X		X
Connexin43		X		X
Connexin45		X		X
Kir2.1		X		X
SCN5a		X		X
CACNA1C		X		X
MEA				
250–270 μm	X	X	X	X
350–370 μm	X	X	X	X

Table 2 Antibodies used for immunofluorescence microscopy

Antigen	Source	Clone	Isotype	Label	Species
β-catenin	BD	14	IgG1	–	Mouse
CACNA1C (A-20)	SC	–	–	–	Goat
Cx40 (C-20)	SC	–	–	–	Goat
Cx43 (C-363–382)	SC	–	–	–	Rabbit
Cx45 (C-19)	SC	–	–	–	Goat
Kir2.1 (N-18)	SC	–	–	–	Goat
SCN5a (C-20)	SC	–	–	–	Goat
Vimentin	SA	V9	IgG1	Cy3	Mouse
Goat IgG	MP	–	–	Alexa Fluor 568	Rabbit
Mouse Ig	Dako	–	–	FITC	Rabbit
Mouse Ig	BD	–	–	FITC	Goat
Rabbit IgG	MP	–	–	Alexa Fluor 568	Goat

BD: BD Biosciences; Bio: Biocarta; CLB, Sanquin; Dako: Dako Cytomation; MP: Molecular Probes; SA: Sigma-Aldrich; SC: Santa Cruz; FITC: fluorescein isothiocyanate; PE: phycoerythrin. All antibody preparations were used at the concentrations recommended by the suppliers. For an explanation of the abbreviations of the antigens, see the main text of the paper.

two fields of CMCs by an a-cellular channel of either (A) 250–270 μm or (B) 350–370 μm wide, using a laser dissection microscope (P.A.L.M. microlaser system, including PALM robosoftware 4.0, Microlaser Technologies GmbH, Bernried, Germany). This a-cellular channel electrically separated the two CMC fields, and served as the site for cell transplantation. After ensuring that no cells or cell debris were present in the channel and after confirming the presence of a conduction block between the two CMC fields, cells could be transplanted into the a-cellular channel. Either 50×10^3 (250–270 μm wide channel) or 75×10^3 (350–370 μm wide channel) eGFP-labelled cEPDCs or sEPDCs were applied in-between the two CMC fields. The technique of labelling of cells with eGFP by adenoviral transduction has been described in an earlier study [12]. All cultures were thoroughly screened for inhomogeneities as assessed by light-microscopy and extracellular recordings. Both types of EPDCs were applied into the channel with a pipette mounted in a micro-manipulator and a light microscope (20× magnification), which resulted in a confluent strip of cells connecting both fields, within 24 hrs after application of the cells (Fig. S2). The average size of a single EPDC alters with EMT as the overall morphology of the cell will change, from a cobblestone-like morphology to a spindle-shape, but ranges between 30 and 70 μm. The inter-electrode distance is 200 μm, and therefore three to six EPDCs will be present in-between two electrodes (Fig. S2). Simultaneous high-density mapping of these cultures was performed 24 and 48 hrs after cell seeding (Table 1), using a dedicated data acquisition system (sampling rate 5 kHz/channel, Multi Channel Systems, Reutlingen, Germany). Electrograms were analysed off-line using MC-Rack software (version 3.6.8, Multi Channel Systems). Cell cultures were electrically stimulated *via* an external pipette electrode producing bipolar rectangular pulses (1.5× threshold, pulse width: 10 ms), placed in close contact to the cell culture and at least 5 mm apart from the measurement sites. Cultures were stimulated for at least 30 sec, before recordings were started. Conduction velocities were calculated from local activation times recorded at eight fixed measuring points, distributed equally over the two lines of electrodes next to the channel [20, 21].

Semiquantitative RT-PCR

Epicardial cells before and after EMT were lysed in TriPure (Roche, Indianapolis, IN, USA). Total RNA was isolated by using Nucleospin RNAII (Macherey-Nagel, Düren, Germany) as described by the manufacturer. cDNA was synthesized with 750 ng RNA per sample, using iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands). cDNA samples were subjected to semiquantitative RT-PCR (Table 1). Primer sequences and annealing temperatures are available on request. Results were analysed on 1.5% agarose gel stained with ethidium bromide and quantitative expression of genes was normalized for expression of *β-actin*.

Statistics

Statistical analysis of the electrical conductivity data was performed using SPSS14.0 for Windows (SPSS Inc., Chicago, USA). *P*-values <0.05 were

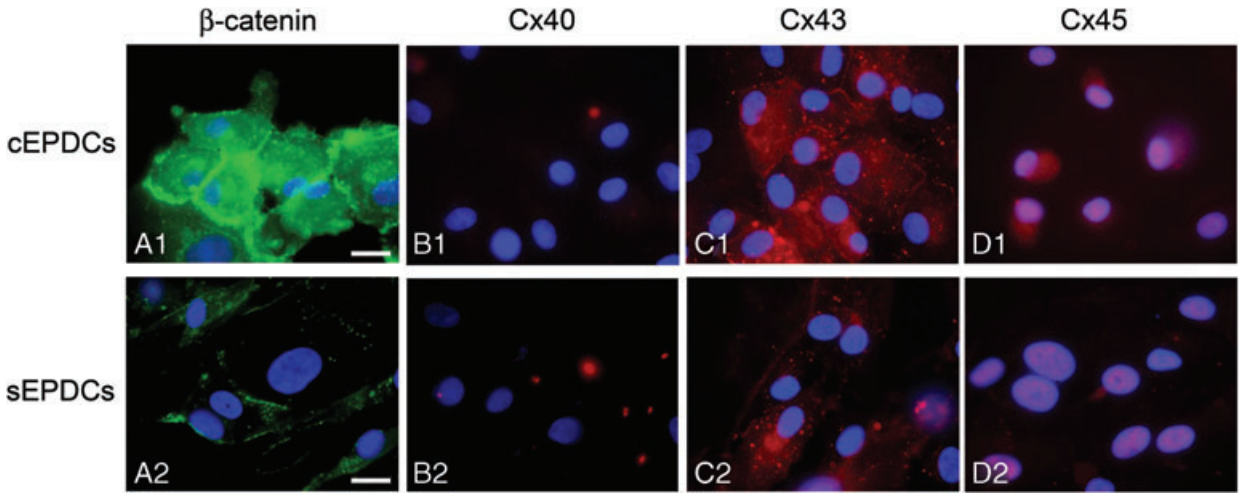

Fig. 1 Epithelium-to-mesenchymal transformation is accompanied by a decrease in β-catenin and connexins expression levels. Immunofluorescence microscopy of cEPDCs (before EMT) and sEPDCs (after EMT) labelled with antibodies directed against β-catenin (**A**), Cx40 (**B**), Cx43 (**C**) and Cx45 (**D**). The expression of β-catenin is strongly expressed at the cell borders of cEPDCs. Expression of β-catenin is downregulated in the cytoplasm of sEPDCs. Scale bar, 20 μm.

considered statistically significant. Electrical conductivity data were compared with the one-way or two-factor mixed ANOVA test with Bonferroni correction for multiple comparisons, and expressed as mean ± S.D. Statistical analysis of the semiquantitative RT-PCR data was performed with an independent sample t-test, relative to an internal control, *β-actin*. Significance was assumed when $P < 0.05$ using SPSS 16.0 software program (SPSS Inc.). Graphics of statistical analysis were composed by Graphpad software.

Results

Immunofluoresence microscopy

To determine the differentiation state of the EPDCs we investigated the presence of WT1, normally only present in EPDCs if they are in an undifferentiated state. Immunohistochemistry showed that all cEPDCs and sEPDCs 48 hrs after seeding expressed WT1 (Fig. S1C and D).

To evaluate whether EMT may have an effect on their conductivity of epicardial cells, we first analysed their capacity to couple. Therefore, the two types of EPDCs were analysed by immunofluorescence microscopy using antibodies recognizing specific connexins (Fig. 1) and ion channels (Fig. 2) antibodies. EMT induced changes in cellular morphology were confirmed using immunofluorescent staining for β-catenin (Fig. 1A1 and A2) and vimentin (Fig. 2A1 and A2). cEPDCs displayed a marked membrane expression of β-catenin, especially at sites of cell–cell contact, confirming their epithelial nature (Fig. 1A1). After EMT, the level of β-catenin was reduced and in the sEPDCs the protein was down-regulated in the cytoplasm in sEPDCs (Fig. 1A2). Cx40 (Fig. 1B1 and B2) and Cx45 (Fig. 1D1 and D2) were weakly expressed in the cytoplasm of both cEPDCs and sEPDCs, whereas some of some Cx45 staining was also present in the nucleus (Fig. 1D1 and D2). Analysis of mRNA expression confirmed that both *Cx40* and *Cx45* were present before and after EMT (Fig. 3). The quantification of *Cx40* mRNA expression showed a 3.1 times higher expression in sEPDCs as in cEPDCs ($P < 0.05$) (Fig. 3A). The mRNA expression of *Cx45* was not significantly affected by EMT as the *Cx45/β-actin* ratio was almost equal in cEPDCs and sEPDCs (Fig. 3C and F). Cx43 was present in the cytoplasm of cEPDCs and between adjacent cEPDCs. The punctuated pattern of the staining reflected the presence of gap junctions (Fig. 1C1). Cx43 levels were reduced in the cytoplasm of sEPDCs and between adjacent sEPDCs (Fig. 1C2) compared to cEPDCs. Quantification of PCR analysis revealed that the expression of *Cx43* mRNA was 53% higher in cEPDC ($P < 0.05$) (Fig. 3B).

The organization of the intermediate cytoskeleton filaments, stained with vimentin, was a reference to delineate the morphology of spindle-shaped cells. In cEPDCs, the intermediate filaments are tightly packed reflecting their epithelial nature (Fig. 2A1). After EMT, in the sEPDCs the intermediate filaments are more visible (Fig. 2A2) compared to cEPDCs, as was confirmed by a more marked vimentin expression.

Staining for the inward rectifier potassium channel (Kir2.1) revealed presence of this channel in the cytoplasm of cEPDCs, although the expression intensity was rather heterogeneous among cells (Fig. 2B1). Protein expression levels of Kir2.1 was almost absent in sEPDCs compared to cEPDCs (Fig. 2B1 and B2). Analysis of *Kir2.1* mRNA expression showed no expression in both cEPDCs and sEPDCs after 35 cycles (Fig. 3F). Expression of SCN5a, encoding the voltage-gated fast sodium channel, was present in both cEPDCs and sEPDCs, however, in cEPDCs the expression of SCN5a was higher and the distribution pattern was more distinct

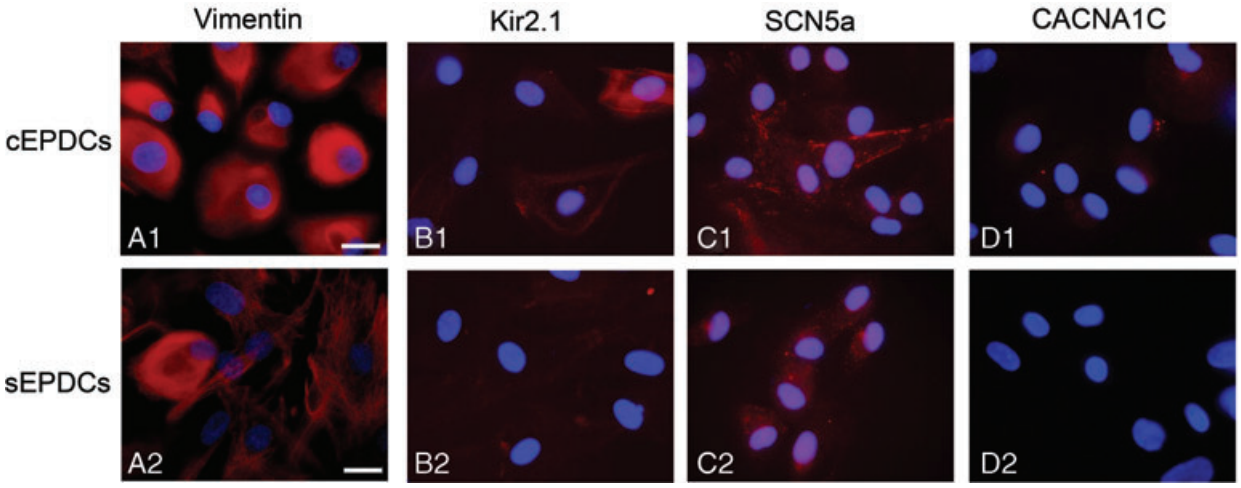

Fig. 2 Immunofluorescent staining of ion channels in epicardial cells before EMT (cEPDCs) and after EMT (sEPDCs). Immunofluorescence analysis of Kir2.1 (**B**), SCN5a (**C**) and CACNA1C (**D**) in EPDCs before and after EMT. Expression of ion channels was reduced by EMT. Vimentin was used to determine cell morphology of sEPDCs (**A**). Scale bar, 20 μm.

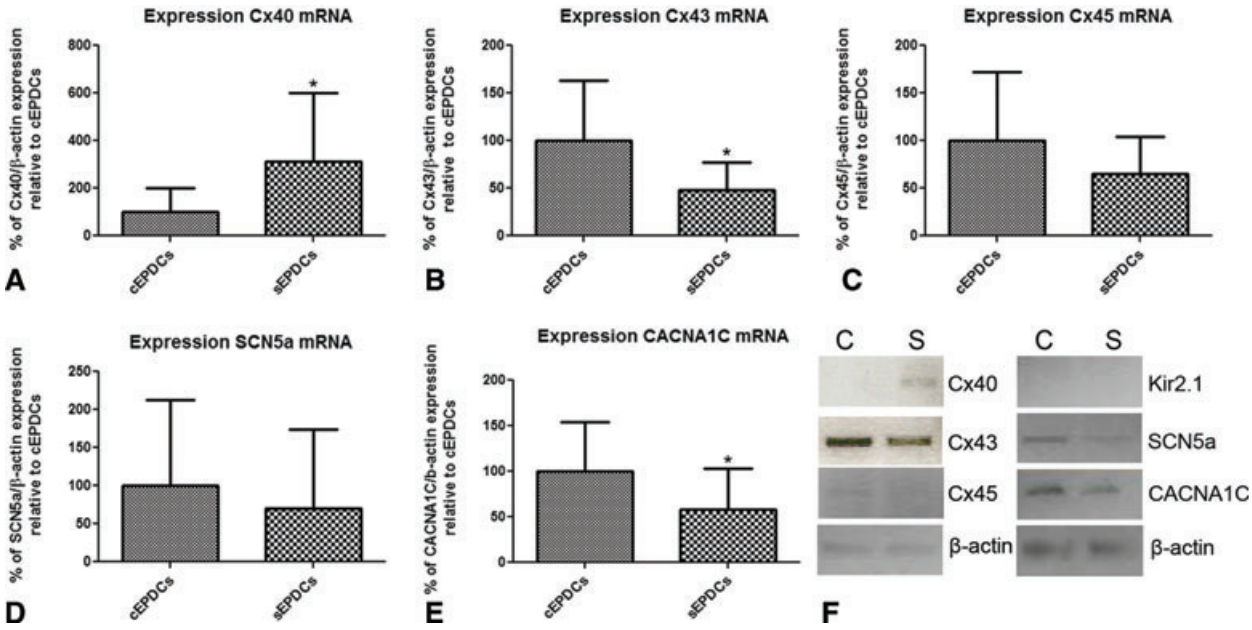

Fig. 3 Semiquantitiative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of connexins and ion channels in EPDCs before and after EMT. mRNA levels of Cx40 (**A**, **F**), Cx43 (**B**, **F**), Cx45 (**C**, **F**) and ionchannels Kir2.1 (**F**), SCN5a (**D**, **F**) and CACNA1C (**E**, **F**) were quantified. Equal amounts of input cDNA were used as indicated by β-actin (**F**). C: cEPDCs; S: sEPDCs.

compared to that in sEPDCs (Fig. 2C2). PCR analysis showed that the expression of *SCN5a* was reduced in sEPDCs by 30.2% compared to cEPDCs (Fig. 3D and F), although not significantly.

Low amounts of voltage-dependent L-type calcium channels (CACNA1C) were present in the cytoplasm of cEPDCs and sEPDCs, although the expression was higher in cEPDCs, which was confirmed on the mRNA level (42.3%, $P < 0.05$) (Fig. 3E and F).

Electrical conduction across EPDCs before and after EMT

To study the effect of EMT on electrical properties of epicardial cells, we examined their conduction properties, both before and after EMT, using multi-electrode arrays.

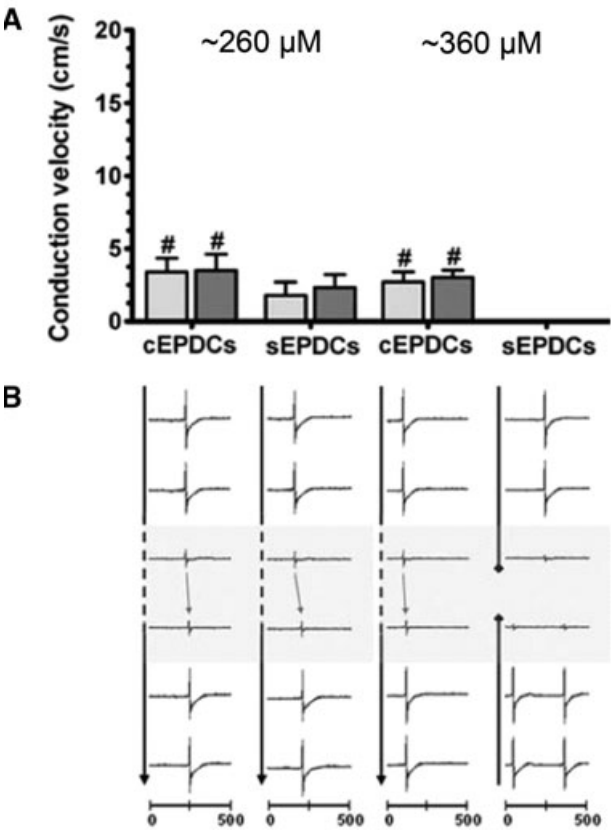

Fig. 4 Overview of conduction velocities, and corresponding conduction delays, measured in EPDCs in-between two adjacent fields of CMCs. Conduction velocity was significantly decreased in sEPDCs. In fact, with increasing distance (360 μm) sEPDCs were no longer able to conduct the electrical impulse across the channel, resulting in asynchronized beating of the two CMC fields. No significant differences were found during follow-up at 48 hrs (**A**). Extracellular electrograms derived from cocultures of labelled EPDCs and neonatal rat cardiomyocytes, 24 hrs (light grey) and 48 hrs (dark grey) after plating (**B**). These electrograms clearly show the decremental nature of conduction across EPDCs, regardless of EMT. However, as conduction across these EPDCs depends on electrotonic interaction, the decrease in connexin levels that occurs in these cells during EMT is expected to result in conduction block over a certain distance (**B**). $^{\#}P <$ 0.05 *versus* sEPDCs.

Multi-electrode recordings of electrical conduction across cEPDCs and adjacent CMC fields showed persistent electrical interaction between the two CMC fields. Electrograms recorded from the site of cEPDCs showed their ability to conduct electrical impulses over a 270-μm-wide channel ($n = 8$), resulting in electrical activation of the distal CMC field. Further analysis of electrogram characteristics confirmed a substantial conduction delay between the two CMC fields, caused by relatively slow conduction across these cEPDCs (4.2 ± 0.9 cm/sec) (Fig. 4A and B). To study the functional effects of EMT, the separated CMC fields were now connected by seeding sEPDCs in the channel ($n = 8$). Similar to cEPDCs, these sEPDCs also functioned as a conductive cellular bridge in-between the two CMC fields within 24 hrs after application. However, EPDC-related conduction delays were significantly increased, and consequently, conduction velocity across sEPDCs was significantly decreased as compared to cEPDCs, now reaching values of 1.8 ± 1 cm/sec.

Importantly, follow-up till 48 hrs after transplantation did not show any significant differences concerning conduction velocity across transplanted cEPDCs ($n = 8$) and sEPDCs ($n = 8$) (respectively, 4.3 ± 1.4 and 2.0 ± 0.9 cm/s) (Fig. 4A and B).

Both cell types showed decremental conduction, defined here as decreased electrogram amplitudes combined with increased conduction times. To further define this depressed conduction across EPDCs, we increased the channel width (360 μm), to assess the magnitude of decremental conduction in EPDCs. Seeding the a-cellular channel with a monolayer of cEPDCs ($n = 7$) resulted in electrical restoration between the two CMC fields, although with extensive conduction delay and slow conduction (2.2 ± 0.7 cm/sec) (Fig. 4A and B). Interestingly, transplantation of sEPDCs did not result in electrical interaction between the two adjacent CMC fields. In fact, these sEPDCs ($n = 7$) now imposed a cellular conduction block, thereby prohibiting electrical impulse conduction from one CMC field to the other. Follow-up at 48 hrs did not show any significant differences as compared to conduction velocities across cells measured 24 hrs after transplantation (Fig. 4A and B).

Discussion

To the best of our knowledge, this is the first study describing the electrical behavior of human adult epicardial cells before and after EMT. The key findings of this study are: (1) Adult epicardial cells are able to connect to functionally active CMCs and to conduct electrical impulses over significant distances, although this is characterized by slow and decremental conduction. (2) EMT in adult epicardial cells is associated with a decrease in conduction velocity, consistent with a decrease in connexin and ion channels expression levels.

Role of EPDCs in conductivity

During embryogenesis, in the splanchnopleuric mesoderm two crescent-shaped heartforming fields develop [1, 23, 24]. The second heart field, which is also positioned in this splanchnopleuric mesoderm, can be divided into two fields related to the cranio-caudal axis of the primary heart tube. From this second heart field, cells are added to both the outflow [anterior heart field (AHF)] and inflow [posterior heart field (PHF)] of the heart [25]. The PHF contributes to the development of the cardiac conduction system [26], and the epicardium that covers the heart develops also from the PHF [1, 9]. Because of this shared PHF-origin in early development, these cells

can have several characteristics in common. They will distinguish themselves from each other through differentiation. After the heart is covered by epicardium, these cells undergo EMT and migrate into the sub-epicardial space and thereafter into the myocardium, where they will differentiate into interstitial fibroblasts, smooth muscle cells and fibroblasts of the coronary vasculature. These EPDCs are also involved in the induction of Purkinje fibre differentiation of the ventricular conduction system [27, 28].

Recent literature suggests that the epicardium is also a source of cardiac progenitors based on the fact that WT1-positive pro-epicardial cells are likewise descendants of $Nkx2.5^+/Isl^+$ precursors, as most cardiomyocytes are [29]. In these studies it has been overlooked that Van Tuyn *et al.* described that adult EPDCs and CMCs share the expression of GATA4 and cardiac troponinT [12]. These findings support the common origin of CMC and epicardial cells from the PHF but do not unambiguously support the origin of CMCs from EPDCs. Future studies need to resolve this issue. The present *in vitro* electrophysiological study demonstrates that cultured adult cEPDCs and sEPDCs can connect to functionally active CMCs and are able to conduct a cardiac action potential. EMT is probably the onset for the differentiation of EPDCs, although the EPDCs in this study were confirmed to be in a undifferentiated state. Differentiation of EPDCs into electrically active smooth muscle cells of the coronary vasculature is dependent on signals from the endothelium of the coronary vessels [1]. EPDCs can also differentiate into interstitial fibroblasts, which are unexcitable cells that form supportive layers within the myocardium, and modulating cardiac action potential propagation [19, 30]. The present data show that conduction velocity of sEPDCs is decreased compared to that in cEPDCs, and that this velocity is comparable to that found in cFBs. Therefore, EMT of epicardial cells might represent the onset of differentiation into cFB-like cells.

Role of epithelial-to-mesenchymal transformation in conductivity

EMT is a critical process in the development of the heart. Not only for the development of cardiac structures, like the cardiac valves [31], but it is also needed for the development of gap junctions [32, 33]. Previous studies have shown that classical cadherins support gap junctional stabilization. Cadherins are cell surface molecules anchored *via* catenins to the cytoskeleton [34]. Cadherins and catenins are localized at cell–cell adherent junctions, especially in cells with an epithelial-like morphology. In the heart, Cx43 colocalizes with β-catenin in the junctional membrane [35] and the association between β-catenin and Cx43 is required for the development of gap junctions [33]. The present data show that the downregulation of β-catenin and Cx43 and consequent decreased expression levels of connexins and ion channels in human adult epicardial cells during EMT is associated with less cell–cell coupling and a decrease in conduction velocity. Previous studies have shown that N-cadherin and β-catenin control targeting of Cx43 to adherens junctions and that the stabilization of Cx40 and Cx43 can be regulated by the N-cadherin/β-catenin complex [36].

It was shown that in embryonic carcinoma cells, EMT is followed by repression of Cx43 [37].

The low expression of Cx40 and Cx45 is explained to the fact that Cx40 is mainly found in atrial tissue and the conduction system. The upregulation of mRNA expression of Cx40 after EMT could be related to the differentiation of the cells initiated by EMT. EPDCs can differentiate into vascular smooth muscle cells and previous studies showed that these cells express Cx40 [38]. The weak expression of Cx45, which is not affected by EMT, is because of the fact that Cx45 is predominantly detected during early cardiac development [39], and in the His bundle and peripheral Purkinje fibres [40].

Little is known about how EMT effects ion channel formation or stabilization. Ion channel levels are decreased in epicardial cells when undergoing EMT. Previously it has been shown that metanephrogenic mesenchyme-to-epithelium transition (MET) induced profound expression changes of ion channels [41]. This effect is mediated by E-cadherin and β-catenin, factors that play a crucial role in early epithelial polarization by mediating cell–cell adherens junctions. E-cadherin is also important for the integration and retention of Na^+-K^+-ATPase in membrane–cytoskeleton complexes [41]. In atrial myocyte cultures intracellular measurements show that transforming growth factor beta1 (TGF-β_1), which is also an EMT stimulator of epicardial cells [42], decreases cardiac muscle L-type Ca^{2+} channels [43]. TGF-β_1 also decreases epithelial sodium channel functionality and thereby decreases the electrical current in renal collecting ducts [44]. The downregulation of ion channel protein expression levels after EMT suggests that these decreases in protein expression may contribute to the EMT-related changes in conduction velocity.

Immunofluoresecence microscopy showed the presence of a variety of ion channels in EPDCs, which could be involved in the generation of action potentials. However, the relatively low velocity by which the action potential is conducted across EPDCs and the decremental nature of conduction indicates that EPDCs, like the cFB [20] they will differentiate into, passively conduct action potentials from the CMCs instead of actively contributing to the conduction process by excitation.

Limitations

In this study we compared adult human EPDCs from multiple donors were compared with neonatal rat CMCs and rat cFBs. Given possible species- and individual-specific differences, ideally EPDCs, CMCs and cFBs from the same human patient(s) should have been used for our experiment. However, there are considerable technical and ethical objections to their use. Furthermore, this model is a simplified representation of what occurs *in vivo*.

Conclusions

Electrical impulse transmission across human adult epicardial cells is characterized by slow and decremental conduction, and

EMT is associated with a decrease in conduction velocity in these cells. This is of importance as epicardial cells are crucial for proper cardiac development and might be applicable for cell-based therapy to repair the injured heart.

Future research regarding the potential of adult epicardial cells in models of cardiac injury and disturbed EMT may further emphasize their importance.

Conflicts of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Expression of Wilm's Tumor 1.

Fig. S2 Model of the Multi Electrode Array data acquisition system.

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