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 fiber 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 downregulated 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.
INTRODUCTION

Cardiogenesis is partly dependent on a proper development of the epicardium. The epicardium originates from the proepicardial 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 fiber 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) \textit{in vitro} \(^11\). In addition, van Tuyn \textit{et al.} demonstrated that human adult EPDCs can undergo EMT spontaneously and obtain characteristics of smooth muscle cells \textit{in vitro} \(^12\). Interestingly, recent \textit{in vivo} studies suggest that adult EPDCs can reactivate their embryonic program \(^2,13,14\). In more detail, induced hyperplastic cardiac growth in adult zebrafish was associated with epicardial expression of embryonic markers such as \textit{raldh2} and \textit{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 \(^13,15\). Furthermore, a recent study demonstrated that human adult EPDCs injected into the infarcted myocardium, preserved cardiac function and reduced remodelling both early and late after the onset of infarction \(^2,16\). 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 \(^17\). 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. 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 to study the conductivity of human adult epicardial cells, before and after EMT, by culturing them in-between 2 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.

Harvesting and preparation of human epicardium-derived cells
Cultures of human epicardial cells were prepared as described previously. 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 fetal 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), to stimulate EMT. 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 (Appendix Figure 1a,b).

**Immunofluorescence microscopy**
cEPDCs and sEPDCs were cultured on glass chamber-slides and subjected to immunofluorescent staining as described previously 19 (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 labeled 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 2hrs 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 20,21. 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). In order 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 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 $50 \times 10^4$ (250-270 μm wide channel) or $75 \times 10^5$ (350-370 μm wide channel) eGFP-labeled cEPDCs or sEPDCs were applied in-between the two CMC fields. The technique of labeling 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 (20x magnification), which resulted in a confluent strip of cells connecting both fields, within 24 hrs after application of the cells (Appendix Figure 2). 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 3 to 6 EPDCs will be present in-between 2 electrodes (Appendix Figure 2). Simultaneous high density mapping of these cultures was performed 24 hrs 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 analyzed 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 (1.5x 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 s, 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.

Semiquantitative RT-PCR
Epicardial cells before and after EMT were lysed in TriPure (Roche). Total RNA was isolated by using Nucleospin RNAII (Macherey-Nagel) as described by the manufacturer. cDNA was synthesized with 750 ng RNA per sample, using iScript cDNA synthesis kit (Bio-Rad). cDNA samples were subjected to semiquantitative RT-PCR (Table 1). Primer sequences and annealing temperatures are available on request. Results were analyzed 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 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±SD. 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. Chicago, USA). Graphics of statistical analysis were composed by Graphpad software.
RESULTS

Immunofluorescence 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 (Appendix Figure 1c,d).

To evaluate whether EMT may have an effect on their conductivity of epicardial cells, we first analyzed their capacity to couple. Therefore, the two types of EPDCs were analyzed by immunofluorescence microscopy using antibodies recognizing specific connexins (Figure 1) and ion channels (Figure 2) antibodies. EMT induced changes in cellular morphology were confirmed using immunofluorescent staining for β-catenin.

| Table 1. Summarizing table of IHC, PCR and MEA experiments |
|-------------------------------------------------------------|
|                                                            |
| **Immunofluorescent staining**                              |
|                                                            |
| WT1  X  X  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  X                                  |
| 350-370 μm  X  X  X  X  X                                  |
cEPDCs displayed a marked membrane expression of \( \beta \)-catenin, especially at sites of cell-cell contact, confirming their epithelial nature (Figure 1a1). After EMT, the level of \( \beta \)-catenin was reduced and in the sEPDCs the protein was downregulated in the cytoplasm in sEPDCs (Figure 1a2). Cx40 (Figure 1b1-b2) and Cx45 (Figure 1d1-d2) were weakly expressed in the cytoplasm of both cEPDCs and sEPDCs, while some of some Cx45 staining was also present in the nucleus (Figure 1d1-d2). Analysis of mRNA expression confirmed that both Cx40 and Cx45 were present before and after EMT (Figure 3). The quantification of Cx40 mRNA expression, showed a 3.1 times higher expression in sEPDCs as in cEPDCs (P<0.05) (Figure 3a). The mRNA expression of Cx45 was not significantly affected by EMT as the Cx45/\( \beta \)-actin ration was almost equal in cEPDCs and sEPDCs (Figure 3c,f). Cx43 was present in the cytoplasm of cEPDCs and between adjacent cEPDCs. The punctated pattern of the staining reflected the presence of gap junctions (Figure 1c1). Cx43 levels were reduced in the cytoplasm of sEPDCs and between adjacent sEPDCs (Figure 1c2) compared to cEPDCs. Quantification of PCR analysis revealed that the expression of Cx43 mRNA was 53% higher in cEPDC (P<0.05) (Figure 3b).

### Table 2. Antibodies used for Immunofluorescence microscopy

| Antigen         | Source | Clone | Isotype | Label          | Species |
|-----------------|--------|-------|---------|----------------|---------|
| \( \beta \)-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    |

Abbreviations used in this table: 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.
The organization of the intermediate cytoskeleton filaments, stained with vimentin, was a reference for morphology spindle-shaped cells. In cEPDCs the intermediate filaments are tightly packed reflecting their epithelial nature (Figure 2a1). After EMT, in the sEPDCs the intermediate filaments are more visible (Figure 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 (Figure 2b1). Protein expression levels of Kir2.1 was almost absent in sEPDCs compared to cEPDCs (Figure 2b1-b2). Analysis of Kir2.1 mRNA expression showed no expression in both cEPDCs and sEPDCs after 35 cycles (Figure 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 compared to that in sEPDCs (Figure 2c2). PCR analysis showed that the expression of SCN5a was reduced in sEPDCs by 30.2% compared to cEPDCs (Figure 3d,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) (Figure 3e,f).

Figure 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) labeled 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, 20μm.
Figure 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, 20μm.

Figure 3. Semi-quantitative 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 ion channels 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). *P<0.05, vs sEPDCs. C, cEPDCs; S, sEPDCs.
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. 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/s) (Figure 4a,b). In order 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/s. 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 cm/s and 2.0±0.9 cm/s) (Figure 4a,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/s) (Figure 4a,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 (Figure 4a,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 the present 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\textsuperscript{1,22,23}. 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\textsuperscript{24}. The PHF contributes to the development of the cardiac conduction system\textsuperscript{25}, and the epicardium that covers the
heart develops also from the PHF. Due to 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 subepicardial 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 fiber differentiation of the ventricular conduction system. Recent literature suggests that the epicardium is also a source of cardiac progenitors based on the fact that WT1-positive proepicardial cells are likewise descendants of Nkx2.5+/Isl+ precursors, as most cardiomyocytes are. 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. 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 an 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. EPDCs can also differentiate into interstitial fibroblasts, which are unexcitable cells that form supportive layers within the myocardium, and modulating cardiac action potential propagation. 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 cardiac fibroblast-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, but it is also needed for the development of gap junctions. Previous studies have shown that classical cadherins support gap junctional stabilization. Cadherins are cell surface molecules anchored via catenins to the cytoskeleton. 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 and the association between β-catenin and Cx43 is required for the development of gap junctions. 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. It was shown that in embryonic carcinoma cells, EMT is followed by repression of Cx43.

The low expression of Cx40 and Cx45 is explained by 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. The weak expression of Cx45, which is not affected by EMT, is due to the fact that Cx45 is predominantly detected during early cardiac development, and in the His bundle and peripheral Purkinje fibers.

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. 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. In atrial myocyte cultures intracellular measurements show that transforming growth factor beta1 (TGF-β1), which is also an EMT stimulator of epicardial cells, decreases cardiac muscle L-type Ca²⁺ channels. TGF-β1 also decreases epithelial sodium channel functionality and thereby decreases the electrical current in renal collecting ducts. 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.

Immunofluorescence 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 cardiac fibroblast, will differentiate into, passively conduct action potentials from the CMCs in stead 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.
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APPENDIX

RESULTS

Epicardial cells isolated from the human atrial appendages, mainly with an epithelium-like or cobblestone-like shape, demonstrated expression of WT1 (Appendix Figure 1). Epicardial cells that transformed into spindle-shaped cells or sEPDCs still expressed WT1.

To study the functional effect of EMT on electrical conduction across epicardial cells we used the micro-electrode array culture dishes (MEA). A synchronously beating monolayer of neonatal rat CMCs was divided into two fields CMCs by an a-cellular channel. EPDCs before and after EMT, which were labelled by eGFP, were transplanted into the a-cellular channel.
Appendix Figure 2. Model of the Multi Electrode Array data acquisition system
Light microscopy (a) and fluorescence microscopy (b) of micro-electrode array (MEA) cultures after application of eGFP-labelled human sEPDCs in a 260 μm channel. The position of the electrodes is marked by the white circles. Scale bar, 50μm.
Epicardium-derived cells and electrical conduction