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Posterior heart field and epicardium in cardiac development : PDGFRα and EMT

Bax, N.A.M.

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CHAPTER 6

In vitro epithelial-to-mesenchymal transformation in human adult epicardial cells is regulated by TGFβ-signalling and WT1

Noortje A.M. Bax[1], Angelique A.M. van Oorschot[2], Saskia Maas[1], Jerry Braun[3], John van Tuyn[4], Antoine A.F. de Vries[4], Adriana C. Gittenberger-de Groot[1], Marie-José Goumans[2].

1 Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands
2 Department of Molecular Cell Biology and Centre for Biomedical Genetics, Leiden University Medical Center, Leiden, The Netherlands
3 Department of Cardiothoracic Surgery, Leiden University Medical Center, Leiden, The Netherlands
4 Virus and Stem Cell Biology Laboratory, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands

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ABSTRACT

Adult epicardial cells are required for endogenous cardiac repair. After myocardial injury, they are reactivated, undergo epithelial-to-mesenchymal transformation (EMT) and migrate into the injured myocardium where they generate various celltypes, including coronary smooth muscle cells and cardiac interstitial fibroblasts, which contribute to cardiac repair. To understand what drives epicardial EMT, we used an *in vitro* model for human adult epicardial cells. These cells have an epithelium-like morphology and markedly express the cell surface marker vascular cell adhesion marker (VCAM-1). In culture, epicardial cells spontaneously undergo EMT after which the spindle-shaped cells now express endoglin. Both epicardial cells before and after EMT express the epicardial marker Wilm's tumor 1 (WT1). Adding transforming growth factor β (TGFβ) induces loss of epithelial character and initiates the onset of mesenchymal differentiation in human adult epicardial cells. In this study, we show that TGFβ-induced EMT is depending on type-1 TGFβ receptor activity and can be inhibited by soluble VCAM-1. We also show that epicardial-specific knockdown of Wilm's-tumor-1 (WT1) induces the process of EMT in human adult epicardial cells, through transcriptional regulation of platelet-derived growth factor alpha (*Pdgfrα*), *Snai1* and *VCAM-1*. These data provide new insights in the process of EMT in human adult epicardial cells which might provide opportunities to develop new strategies for endogenous cell-based cardiac repair.

INTRODUCTION

The transformation of epithelial cells to a mesenchymal (EMT) phenotype is an important process for proper heart development. The development of the cardiac valves and septa of the heart is depending on EMT of endocardial cells underlying the atrioventricular canal and endocardial cells underlying the outflow tract forming the endocardial cushions [1,2]. A distinct population of cells derived from the epicardium also undergoes EMT during cardiogenesis [3]. During embryogenesis, the epicardium originates from the proepicardial organ (PEO) recruited from the coelomic wall mesothelium at the venous pole of the heart. Cells derived from the PEO protrude to and cover the primitive heart tube. Part of these epicardial cells undergo EMT, thereby forming epicardium-derived cells (EPDCs). As a result of EMT, EPDCs migrate into the subepicardium and subsequently into the myocardium [3,4]. In the myocardium, EPDCs initially differentiate into interstitial fibroblasts [5] and later on in smooth muscle cells and adventitial fibroblasts of the coronary vasculature [6,7]. The contribution of EPDCs to heart development is not only structural, they also have a regulatory role in cardiogenesis. The mechanisms underlying this regulatory process are largely unknown, although it is likely that cell-cell communication is of importance [3].

Previous *in vitro* studies have identified transforming growth factor beta (TGFβ) as a key regulator of EMT in the embryonic PEO and embryonic epicardium in both mouse and chicken [2,8,9]. The TGFβ regulated EMT of chicken embryonic epicardial cells is dependent on the kinase activity of the TGFβ type I receptor or activin receptor-like kinase (ALK) 5 [9]. Furthermore, soluble vascular cell adhesion molecule-1 (VCAM-1) is able to inhibit TGFβ induced EMT [2]. Examination of the expression patterns of other growth factors present during embryonic development implies a role for fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) in epicardial EMT [10,11]. Interestingly, the aforementioned roles of epicardial cells and EPDCs do not seem to be restricted to embryonic development. In the adult heart the epicardium is a simple squamous epithelium which functions as a smooth surface on which the heart slides in the pericardial cavity during contraction [3]. Over the last years, several studies have focused on the role of adult epicardium and EPDCs in heart repair. Recently, we demonstrated that human adult spindle-shaped EPDCs injected into the infarcted myocardium, preserved cardiac function and reduced remodelling both early and late after the onset of infarction [12]. These findings suggest that EPDCs could be a suitable cell source for cardiac cell therapy. Interestingly, recent *in vivo* studies showed that endogenous epicardium is reactivated after a myocardial infarction, thereby re-expressing embryonic markers such as *raldh2*, *Tbx18* and *WT1* [12-16]. Furthermore, re-activated epicardial cells proliferate and are able to undergo EMT where after they migrate into the injured myocardium were they contribute

to cardiac regeneration [12-16]. These studies suggest that adult epicardium is a dynamic tissue that is still able to generate EPDCs that can contribute to the adult ventricular wall. More insight in the signals that direct the process of EMT in adult epicardial cells would be beneficial for endogenous regulated cell-based cardiac repair, thereby providing the opportunity to investigate the possibilities to bypass the need for cell transplantation by stimulating repair by endogenous cells.

Therefore, the aim of this study was to gain more insight into the EMT process of human adult epicardial cells and to determine which factors are involved in EMT of human adult epicardium. We characterized human adult epicardial cells *in vitro* before and after EMT. Here we describe that the cobblestone-like VCAM-1 expressing EPDCs (cEPDCs) upon TGFβ stimulation undergo specific morphological changes including the loss of epithelial characteristics. Spindle-shaped EPDCs (sEPDCs) have the appearance of a smooth muscle-like morphology and express endoglin, while VCAM-1 expression is inhibited in an ALK5 dependent manner. Furthermore, we show that WT1 is a repressor of the EMT process in human adult epicardial cells. Taken together with the expression patterns of TGFβ and WT1 after myocardial infarction, these new insights in the process of EMT in human adult epicardial cells may help to the development of therapies for cardiac repair.

MATERIALS AND METHODS

Human specimens

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.

Harvesting and preparation of human epicardial cells

Cultures of human epicardial cells with an epithelium-like morphology also referred to as cobblestone-like EPDCs (cEPDCs) were prepared as described previously [17]. Briefly, 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. Cells were 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), to maintain the epithelium-like morphology. Medium was refreshed every 3 days. Epicardial cells from passage 2-4 were used for stimulation experiments. The purity of the human epicardial cell cultures was certified with immunohistochemical staining

for Wilm's Tumor-1 protein (WT1) (Calbiochem, San Diego, USA) at a dilution of 1:50. For the *in vitro* stimulation experiments cells were plated at a density of 20.000 cells/cm^2 and were allowed to attach for 24hrs prior to growth factor or inhibitor addition.

Growth factor or inhibitor addition

Medium was supplemented with the growth factors and inhibitors 24hrs after seeding of the human adult epicardial cells. Growth factors TGFβ3 (1 ng/ml) and sVCAM-1 (100 ng/ml) or small molecule inhibitors SB431542 (iALK5, 10 μM), α-Endoglin antibody (0.5 μg/ml) and Y27632 (iROCK 2.33 μg/ml) were used After 48hrs of addition of stimulators, cells were us for further analysis by immunohistochemical analsyis, qPCR, Western blot analsyis and for a MTT assay.

Immunophenotyping

The surface antigen expression profiles of epicardial cells and epicardium-derived cells (EPDCs) were determined by flow cytometry as described previously [17]. The antibodies used for flow cytometric analysis are listed in Table 1.

Immunohistochemical analysis

cEPDCs were cultured on glass chamber-slides and 48hrs after stimulation slides were subjected to immunohistochemical analysis [17]. Briefly, after fixation and permibilization with 0.1% Triton slides were incubated overnight with primary antibodies. The details of the antibodies are listed in Table 1. To investigate their morphology, the cells were immunofluorescent stained for β-catenin and phalloidin. Staining for β-catenin was performed overnight to detect cells with epithelial morphology, using an anti mouse FITC labelled secondary antibody. Prior to incubation (1hr) with phalloidin directly labelled with Rhodamine, cells were blocked with 3% BSA/PBST for 45 min. For immunofluorescence nuclei were stained with 4',6-diamidino-2-phenylinodole (DAPI) (diluted 1:1000 in PBS). Finally, the slides were mounted with Vectashield (Vector, Burlingame, USA).

Furthermore, cells were immunohistochemical labelled with an antibody against α-smooth muscle actin (αSMA) and an appropriate secondary antibody (See Table 1), carried out overnight and for 2hrs at room temperature, respectively. Cells were counterstained with heamatoxylin (Merck, Darmstadt, Germany), followed by rinsing with tapwater for 10 min. Finally, all slides were dehydrated and mounted with Entallan (Merck). Examination of the slides was performed using a fluorescence microscope and light microscope equipped with a digital camera (Diagnostics, RT3 slider, Sterling Heights, Michigan, USA).

Table 1. Antibodies Used for Flow Cytometry and Immunohistochemistry

Antigen	Source	Clone	Isotype	Label	Species
β-catenin	BD	14	IgG1	-	Mouse
α/β-tubulin	CellSig	-	-	-	Rabbit
αSMA	SA	-	IgG2a	-	Mouse
CD31	CLB	HEC/75	IgG1	FITC	Mouse
CD34	BD	8G12	IgG1	PE	Mouse
CD44	BD	G44-26 C26	IgG2b	PE	Mouse
CD46	BD	E4.3	IgG2a	FITC	Mouse
CD90	BD	5E10	IgG1	FITC	Mouse
CD105	Bio	SN6	IgG1	PE	Mouse
CD106	BD	51-10C9	IgG1	PE	Mouse
Endoglin	HM	-	-	-	Rabbit
GAPDH	MP	6C5	IgG1		Mouse
Id1	SC				Rabbit
Pai-1	SC				Rabbit
pSmad1	HM	-	-	-	Rabbit
pSmad2	HM	-	-	-	Rabbit
VE-cadherin	SC	F8	IgG1	-	Mouse
Vimentin	SA	V9	IgG1	Cy3	Mouse
WT1	CA	-	-	-	Rabbit
Goat IgG	MP	-	-	Alexa Fluor 568	Rabbit
Mouse Ig	Dako	-	-	FITC	Rabbit
Mouse Ig	BD	-	-	FITC	Goat
Mouse IgG1	BD	X56	IgG1	PE	Rat
Rabbit IgG	MP	-	-	Alexa Fluor 568	Goat
Rabbit IgG	VL	-	-	Biotin	Goat
Mouse IgG	VL		-	Biotin	Horse

Abbreviations used in this table: BD, BD Biosciences; Bio, Biocarta; CA, Calbiochem; CLB, Sanquin; CellSig, Cell Signalling; Dako, Dako Cytomation; HM, homemade; MP, Molecular Probes; SA, Sigma Aldrich; SC, Santa Cruz; VL, Vector Labs; 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.

Western blotting

For total protein extraction of cEPDCs under the described culture conditions, cells were trypsinized and cell pellet was lysated in sample buffer (SB) containing 10% SDS, 20% Glycerol, 0,1% broomphenol blue, 5% β-mercapto-ethanol, Tris-HCl pH 6.8. Homogenates were size-fractionated on 10% PAGE gels and transferred to Hybond PVDF membranes. After blocking non-specific binding sites, membranes were incubated overnight with the anti-pSmad1 (previously described by Goumans *et al*.[18]), pSmad2 (previously described by Goumans *et al*.[18]), inhibitor of differentiation 1 (Id1), plasminogen activator inhibitor-1 (Pai-1), endoglin (previously described by Lebrin *et al*.[19]) and αSMA antibodies (Table 1) followed by incubation with a horseradish peroxidase (HRP)-labelled secondary antibody (goat anti-rabbit or rabbit anti-goat) for 1hr. A competitive Western blot for GAPDH and α/β-tubulin was performed to correct differences in protein concentration. Chemiluminescence was induced by ECL Advanced Detection reagent and detected by exposure to Hyperfilm ECL.

MTT assay

The total number of living cells as measure for proliferation and viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma Aldrich) assay (n=3). Cells were seeded in quatro in 95 wells plates with 20.000 cells/ cm^2 and stimulated as described previously. In addition, the different cultures were incubated for 3hrs with MTT (50µg per well) in fresh culture medium. Medium was removed and crystallized formazan dye in the cells was solubilised by adding dimethylsulfoxide (DMSO). Absorbance was measured at 540nm using 690nm as reference. Quantification was carried out by comparison absorbance after 48hrs of stimulation with absorbance before stimulation.

RNA Interference

Endoglin was knocked down by using shRNA pTER technology[20] targeting the following 19-nucleotide sequence: 5'-GAAAGAGCTTGTTGCGCA-3'[21]. Oligonucleotides were cloned into the pTER vector under the control of the H1-RNA promoter. H1-RNA promoter and targeting sequence were subsequently transferred to the pRRL-CMV-GFP vector using *PstI* and *XhoI* sites (opposite orientation of CMV-GFP cassette). The plasmid was then used for lentivirus production. cEPDCs were transduced with lentivirus expressing shRNAs that specifically target human Endoglin, as described above, and control GFP virus. After 6hrs culturing in the presence of 8 µg/ml polybrene the medium was refreshed. The day after the cells we plated in 6-well plates and two days after transduction, cells were stimulated with TGFβ3 (1 ng/ml) comparable to the other stimulation experiments.

mRNA isolation and Quantitative RT-PCR analysis

Total RNA from cEPDCs stimulated with described conditions was isolated using TriPure (Roche, Almere, The Netherlands) as described by the manufacturer. cDNA was synthesized of 750 ng RNA per sample, using iScript cDNA synthesis kit (Fermentas). cDNA samples were subjected to quantitative RT-PCR (qRT-PCR) by using SYBRgreen (Roche) and a primer concentration of 10μM. Primers were designed with Primer3 and qPrimerDepot (htp://primerdepot.nci.nih.gov/). Primer sequences and annealing temperatures are available on request. qRT-PCR was performed for the following factors: *WT1* isoform A, *WT1* isoform D, *TGFβ1*, *2*, and *3*, *ALK1*, *ALK5*, *VCAM-1*, *E-cadherin*, *endoglin* and *Snai1*. qRT-PCR reactions were run on a 7900HT Applied Biosystems. PCR conditions were: 10min. at 95°C followed by 40 cycles of 30 seconds at 95°C, 30 seconds annealing temperature (60°C) and 30 seconds at 72°C. All samples were corrected for input based on housekeeping gene *β-actin*, which was not influenced by the different culture conditions (data not shown) and normalized to unstimulated conditions.

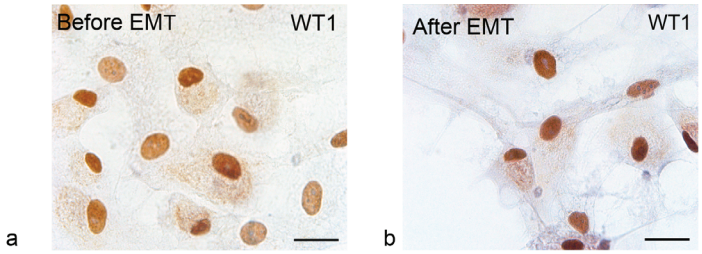
Statistics

All data of the volume measurements are presented as average ± SEM. The PCR data were quantified using the $2^{-\Delta\Delta Ct}$ method, by comparing signal of the sh-RNA treated groups with that of the control group, both relative to an internal control, *β-actin*. Analysis of the qRT-PCR data was performed with T-test. 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. For simplification of the interpretation of the data, in all graphs representing the expression of mRNA of epithelial and EMT markers the fold inductions of the control and TGFβ3-stimulation were displayed.

RESULTS

Analysis of cell surface marker profile

We expanded human epicardial cells from several atrial appendages and certified purity of the cultures by WT1 staining (Figure 1a,b). In both cEPDCs (Figure 1a) and sEPDCs (Figure 1b) WT1 was predominantly localized in the nuclei. The surface antigen profile of cEPDCs and sEPDCs was analyzed by flow cytometry (Table 1). As previously reported [17], sEPDCs abundantly expressed the TGFβ co-receptor endoglin (CD105) at their plasma membrane (Figure 1c,d). The surface of cEPDCs was not decorated with endoglin but contained substantial amounts of VCAM-1 (CD106) (Figure 1c-e). For the other surface markers that were tested, cEPDCs and sEPDCs yielded similar results, i.e. there were low surface levels of hyaluronate receptor (CD44), membrane



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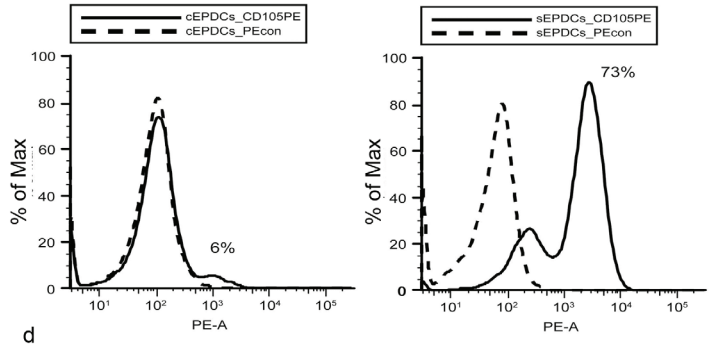
Antigen	*cEPDCs*	*sEPDCs*
CD31 (PECAM-1)	-	-
CD34 (sialomucin)	-	-
CD44 (HCAM)	++	++
CD46 (MCP)	+	+
CD90 (Thy-1)	+	+
CD105 (endoglin)	-	++
CD106 (VCAM-1)	+	-
VE-cadherin	-	-



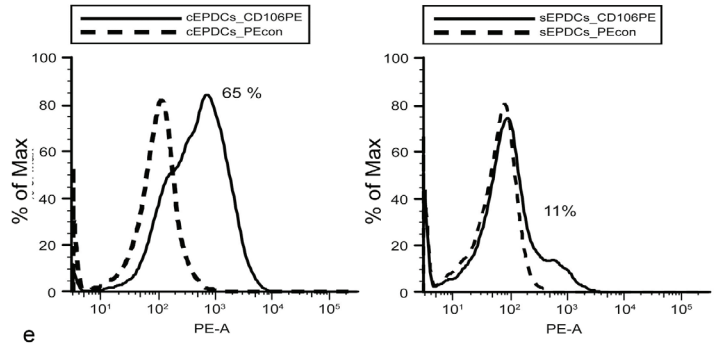


Figure 1. Surface marker profile of human adult epicardial cells

EPDC cultures were stained using WT1 to certify the purity of the culture (a,b). Flow cytometric analysis of cultured adult human EPDCs before (cEPDCs) and after EMT (sEPDCs) was performed (c). Histograms of endoglin (CD105) (d) and VCAM-1 (CD106) (e) are shown with isotype control (dashed line) and the specific signal (solid line). Scale bar, 20µm.

cofactor protein of the complement system (MCP; CD46) and major T-cell antigen (Thy1; CD90). Neither cEPDCs nor sEPDCs expressed the hematopoietic marker CD34 or endothelial markers such as platelet-endothelial cell adhesion molecule-1 (PECAM-1; CD31) and vascular endothelial (VE)-cadherin at their cell surface (Figure 1c).

TGFβ-signalling in epicardial cells

Among the many molecules that regulate the EMT process in many different organs, there is one common element among them involving the TGFβ superfamily. Therefore we examined the expression of the TGFβ type I receptors, ALK1 and ALK5 and found both ALK1 and ALK5 expressed in cEPDCs (Appendix Figure 1 and Figure 2a) confirming that cEPDCs are able to respond to TGFβ. Interestingly, addition of TGFβ increased the expression of *ALK5* mRNA by 1.6 fold ($P<0.05$) (Figure 2a) while the expression of *ALK1* decreased by 30% ($P<0.05$) (Appendix Figure 1). Furthermore, qRT-PCR confirmed the presence of *TGFβ1*, *TGFβ2* and *TGFβ3* transcripts in the human adult cEPDCs (Appendix Figure 2).

To test whether the TGFβ signalling pathway is functional in epicardial cells, we analyzed the epression of two defined downstream targets using Western blot analysis, Id1 and Pai-1 as downstream targets of pSmad1/5 and pSmad2/3, respectively. We were not able to detect any Id1 protein two days after TGFβ stimulation (data not shown), but Pai-1 was present in all samples (Figure 2b). The expression of Pai-1 was increased after TGFβ stimulation and markedly reduced if the ALK5 kinase inhibitor was added to the medium (Figure 2b).

TGFβ causes loss of epithelial character

To determine whether the TGFβ signalling pathway is involved in EMT of human adult epicardial cells, we examined the effect TGFβ stimulation had on the epithelial character of these cells (Figure 3a,b). Incubating cEPDCs with TGFβ for 48hrs resulted in elongation of the cells (Figure 3b), which is indicative for EMT. To further confirm EMT, we stained with the epithelial marker β-catenin to mark cell-cell junctions and phalloidin to visualize filamentous actin. In untreated cells, β-catenin was localized in cell junctions (adherens junctions) and actin filaments were associated with these junctions (cortical actin filaments) (Figure 3a2,3). When stimulated with TGFβ, the cEPDCs showed decreased expression and cytoplasmic redistribution of β-catenin (Figure 3b2), while the actin filaments were clearly visible across the cells (stress fibers) (Figure 3b3). These changes were consistent with the cellular events know to occur during EMT in which dissolution of organized cell junctions and reorganization of actin into stress fibers is needed to allow actin-myosin based contraction. TGFβ stimulation also caused increased staining of αSMA (Figure 3b4). The increase in αSMA was confirmed by Western blot

analysis (Figure 2c). To further explore this apparent loss of epithelial character, we examined the mRNA expression of *E-cadherin*, *VCAM-1* and *Snai1* by qRT-PCR (Figure 2d) and the expression of endoglin by Western blot (Figure 2c). Quantitative analysis of the epithelial markers E-cadherin and VCAM-1 after incubation with TGFβ showed significant decrease in *VCAM-1* expression by 97% (P<0.05) while the expression of *E-cadherin* did not change significantly (Figure 2d). The expression of *Snai1*, which is best known for its induction of phenotypic changes, increased by 1.5 fold (Figure 2d).

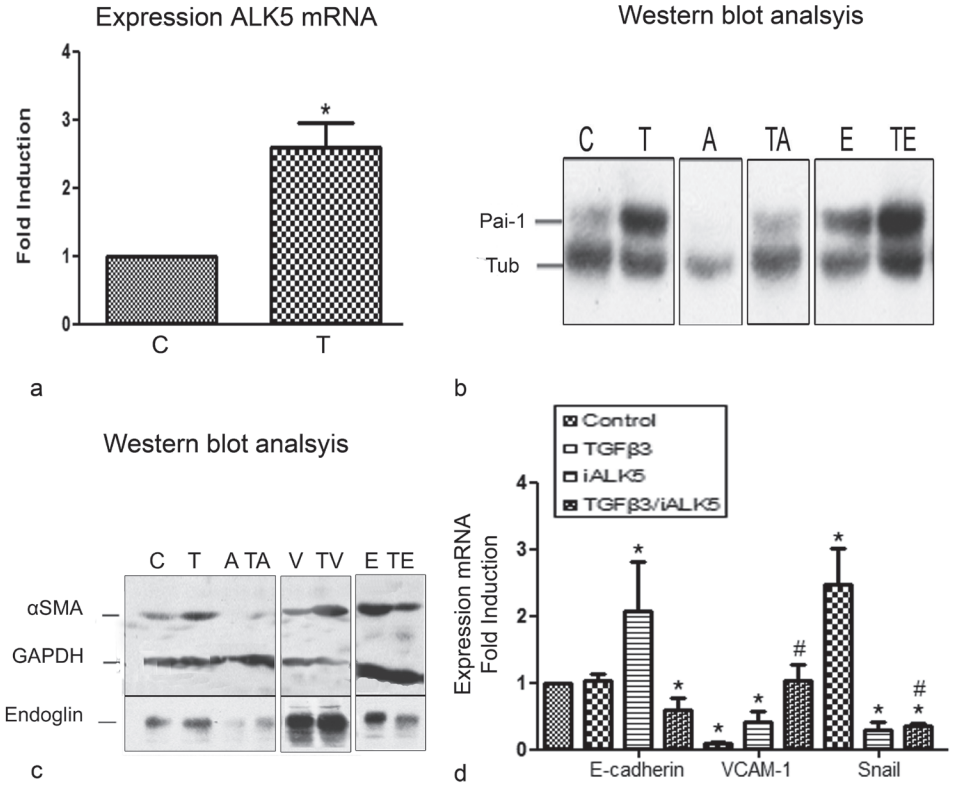


Figure 2. Western blot and qRT-PCR analysis

Gene expression of cEPDCs (control; C) and cEPDCs treated with 1 ng/ml TGFβ3 (T) showed that the expression of *ALK5* was increased by TGFβ (a). Western blot analysis of protein samples isolated from cEPDCs stimulated in presence or absence of 1 ng/ml TGFβ3 and α-Endoglin probed for Pai-1 (b). As a loading control, α/β-tubulin was used (b). Western blot analysis of αSMA and endoglin in epicardial cells stimulated by sVCAM-1 (100 ng/ml) and α-Endoglin (0.5 μg/ml) independent or simultaneously with TGFβ3 (1 ng/ml) (c). GAPDH was used as a loading control in this experiment (c). Gene expression of cEPDCs and cEPDCs treated with TGFβ3, iALK5 and both simultaneously. Treatment with TGFβ showed significant decrease in epithelial markers and increase in EMT marker *Snai1* which is dependent on ALK5 kinase activity (d). *P<0.05, vs control, #P<0.05 vs TGFβ3 stimulation. Abbreviations: A, iALK5; C, control; E, α-Endoglin T, TGFβ3; TA, TGFβ3/iALK5; TE, TGFβ3/α-Endoglin; TV, TGFβ3/sVCAM-1; V, sVCAM-1.

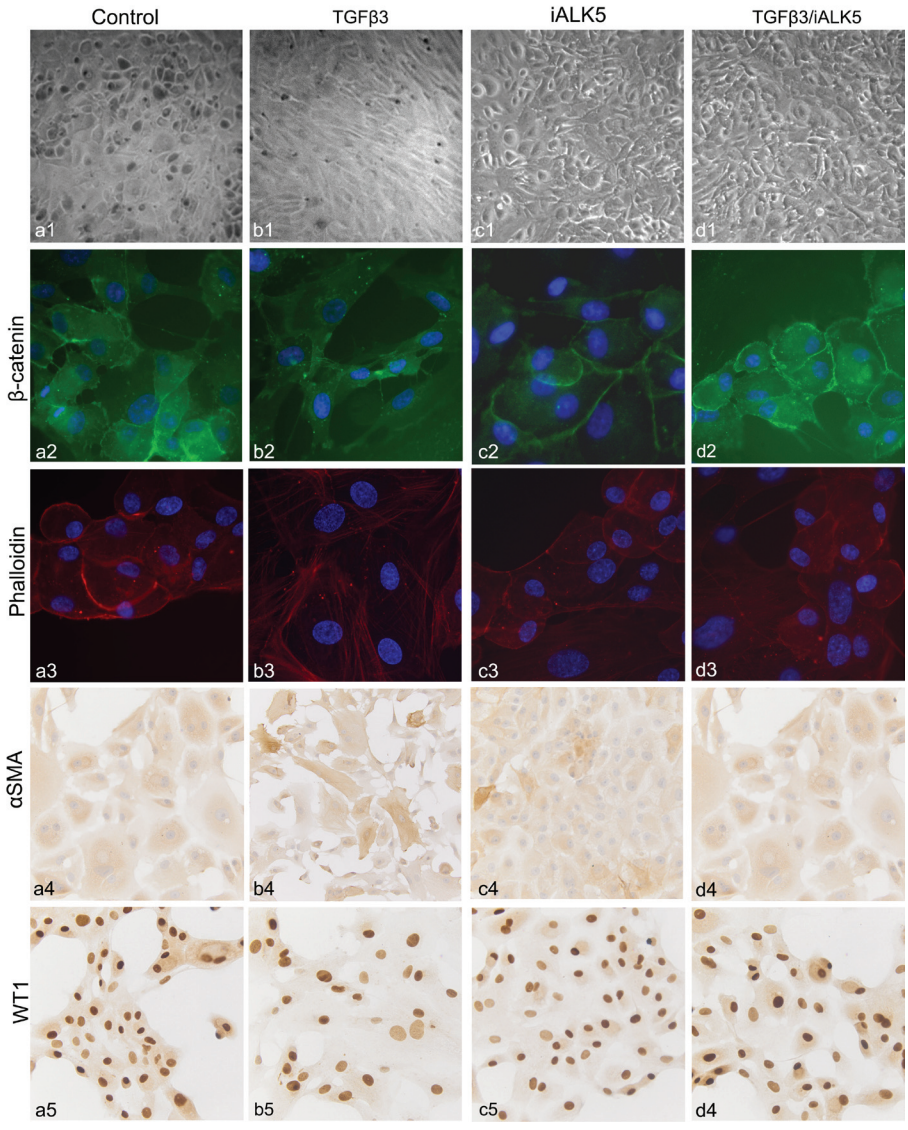


Figure 3. TGFβstimulated EMT is dependent on ALK5 kinase activity

Transforming growth factor (TGFβ) induces epithelial-mesenchymal transformation (EMT) in human adult epicardial cells and ALK5 is required for the effects of TGFβ. Epicardial cells were treated with 1ng/ml TGFβ3, 10 μM SB431542 (iALK5) an ALK5 kinase inhibitor for 48hrs before fixation. Untreated epicardial cells display an epithelial phenotype (a) accompanied with expression of β-catenin (a2) and phalloidin (a3) at the cell-cell borders. Cells incubated with TGFβ are elongated and have lost β-catenin expression and phalloidin was visualized across the cells in stress fibers (b1-3). Cells treated with TGFβ express αSMA in organized consistent with a smooth muscle phenotype (b4). In the presence of iALK5 cells display an epithelial phenotype (c) consistent with the untreated cells (a). Cells treated simultaneously with TGFβ and iALK5 retain expression of β-catenin at the cell border and no phalloidin staining across the cells is present (d). All untreated and treated cells express WT1 (a5-e5). X100 in a1-e1, X400 in a4-e5, X650 in a2-e3.

Western blot analysis revealed that the expression of endoglin was not altered by addition of TGFβ. These data show that in EPDCs TGFβ causes loss of epithelial characteristics and induces the onset of differentiation into a smooth muscle cell phenotype.

ALK5 is required and sufficient for loss of epithelial character

To determine whether the TGFβ induced morphological changes which we observed in human adult epicardial cells is ALK5-dependent, we incubated epicardial cells with TGFβ in the presence or absence of the ALK5 kinase inhibitor (iALK5), SB431542, and we observed that cEPDCs stimulated in the presence of iALK5 preserve their epithelial appearance. qRT-PCR analysis confirmed this epithelial phenotype, as the expression of *E-cadherin* mRNA was increased by 107% ($P<0.05$) in the presence of iALK5 although the expression of *VCAM-1* decreased by 70% ($P<0.05$) (Figure 2d). Western blot analysis showed that addition of iALK5 markedly reduced endoglin levels. Cells incubated simultaneously with TGFβ and iALK5 did not elongate (Figure 3d), and β-catenin and phalloidin were persistent at the cell-junctions (Figure 3d2,3). The decrease in expression of *VCAM-1* and the increase of *Snai1* mRNA by TGFβ was completely neutralized when iALK5 was added (Figure 2d). Simultaneous addition of TGFβ and iALK5 markedly decreased the protein level of endoglin, comparable to the addition of iALK5 alone.

RhoA in epicardial EMT

TGFβ is known to activate several downstream pathways independent of Smad signalling, e.g. via RhoA mediated activation [8]. To address the potential role of RhoA in TGFβ-stimulated loss of epithelial character in human adult epicardial cells, we inhibited the p160 rho kinase, a downstream effector of RhoA. cEPDCs were treated with Y27632, a specific p160 rho kinase inhibitor (iROCK) in the absence or presence of TGFβ3. The addition of iROCK caused elongation of the cells which was accompanied with the decrease of β-catenin and phalloidin at the cell-junctions (Appendix Figure 3). Stress fibers were not detected by phalloidin (Appendix Figure 3) and also staining for αSMA was below detection levels. Simultaneous addition of TGFβ and iROCK resulted in similar morphological changes as addition of iROCK alone, although stress fibers became visible across the cells. These data show that p160 rho kinase activity is not mediating the expression of smooth muscle cell markers in response to TGFβ.

VCAM-1 and endoglin alter the cytoskeleton of human adult EPDCs

Analysis of the cell surface marker profile showed spatiotemporal differences in VCAM-1 and endoglin expression. As the soluble form of the α4β1-integrin ligand VCAM (sVCAM) stimulates change in epicardial cells which restrict EMT and the fact that endoglin is part of the TGFβ-receptor complex [2,22], the question raised if VCAM-1 and

endoglin are involved in the EMT process in human adult epicardial cells. To determine the cellular mechanism through which VCAM-1 inhibits epicardial EMT we treated human adult cEPDCs with sVCAM-1 for 48hrs and did not observe morphologic changes of the cells (Figure 4b). The expression of β-catenin was increased in cells stimulated with sVCAM-1 (Figure 4b2) while there was a decrease in cortical actin filaments (Figure 4b3). qRT-PCR for the epithelial markers *E-cadherin* and *VCAM-1* and the EMT marker *Snai1* showed that treatment with sVCAM-1 did not significantly change their mRNA expression (Figure 4d).

Since sVCAM-1 might stimulate the preservation of intercellular adhesion, we simultaneously treated cEPDCs with TGFβ and sVCAM-1. Remarkably, the presence of

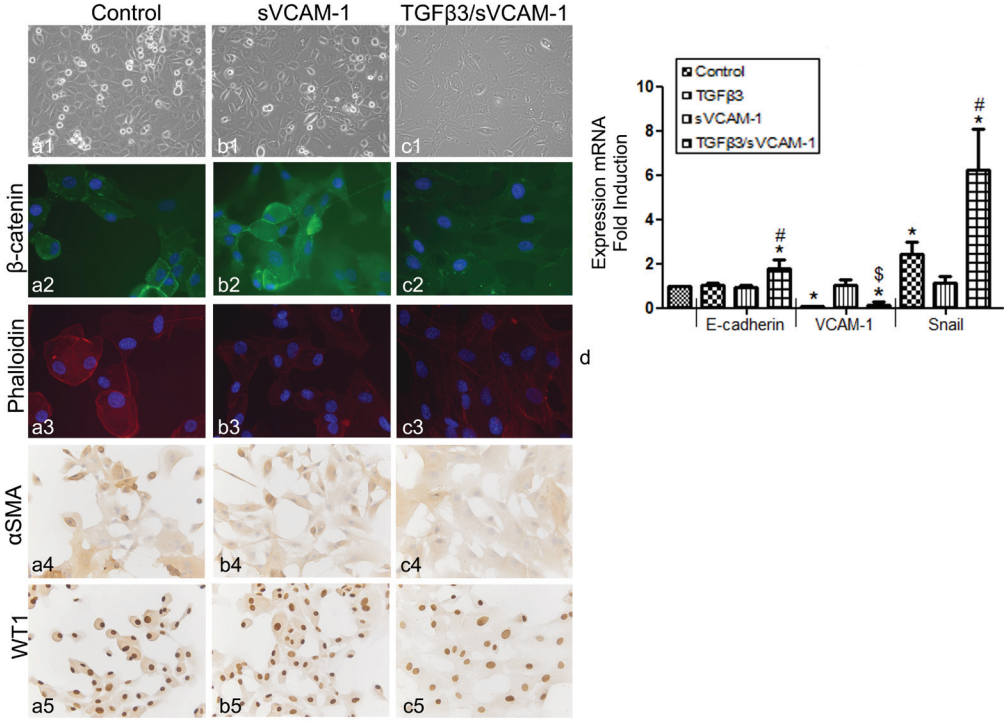


Figure 4. sVCAM-1 inhibits cell shape changes in human adult epicardial cells treated with TGFβ3
Unstimulated cells (a) and stimulated with sVCAM-1 (100ng/ml) (b) and simultaneously with TGFβ3 (1ng/ml) and sVCAM-1 (100ng/ml) (c) are stained for β-catenin (a2-c2) and with phalloidin to visualize filamentous actin (a3-c3). Onset of differentiation into smooth muscle cells was visualized by staining for αSMA (a4-c4) and the state of differentiation was visualized by WT1 (a5-c5). The process of EMT was confirmed by qRT-PCR (d) analysis for epithelial and EMT markers. Magnification 100x in a1-c1, 400x in a4-c5, 650x in a2-c3. *P<0.05, vs control, #P<0.05 vs TGFβ3 stimulation, $P<0.05 stimuli vs simultaneous TGFβ3/stimuli

sVCAM-1 not only prevented the morphologic changes of the cells (Figure 4c) but it also prevented the formation of filamentous actin (Figure 4c3), although there was alteration of β-catenin in cell junctions (Figure 4c2). Strikingly, qRT-PCR data showed that the expression of *E-cadherin* and *Snai1* both increased significantly by 1.8 and 6.2 times, respectively, while the expression of *VCAM-1* mRNA decreased significantly (83%) in the presence of TGFβ and sVCAM-1 (Figure 4d). These data show that sVCAM-1 is able to restrict TGFβ stimulated EMT.

Endoglin is a co-receptor modulating the TGFβ signaltransduction pathway [23,24] and is markedly present at the surface of epicardial cells after EMT. Endoglin has been postulated to be involved in the cytoskeletal organization affecting cell morphology and migration [1].

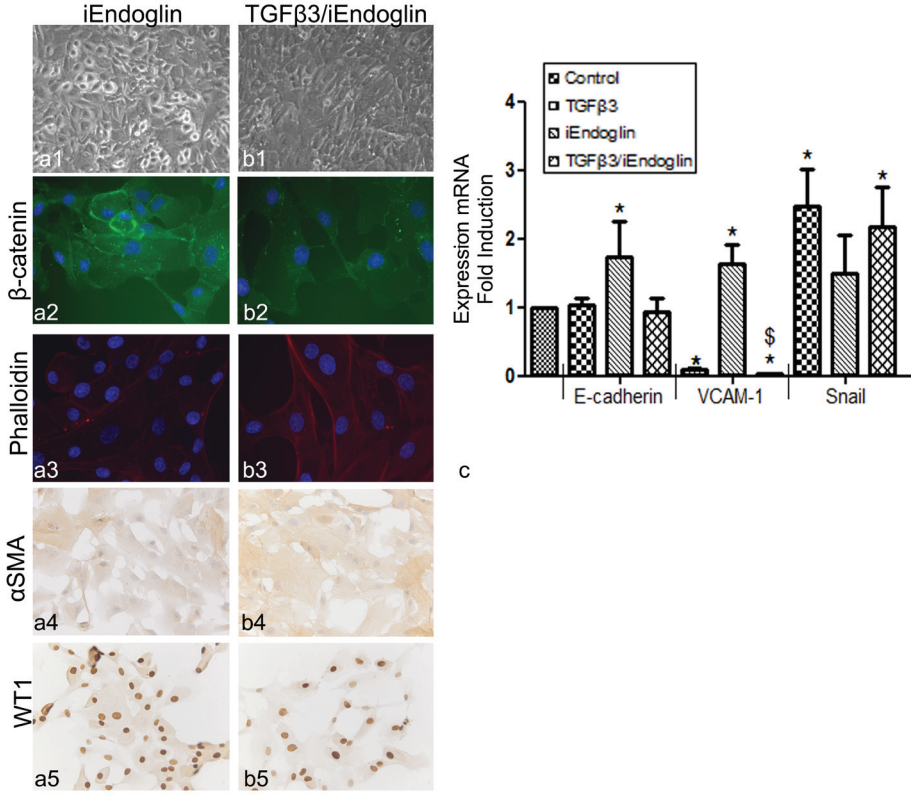


Figure 5. Inhibition of endoglin expression can not block the process of TGFβ-stimulated EMT

Blocking of endoglin increased the expression of epithelial markers (a,c). In simultaneous treatment of epicardial cells with TGFβ and α-Endoglin, the decrease in epithelial and increase in mesenchymal and EMT markers stimulated by TGFβ could not be prevented (a-c). *P<0.05, vs control, $P<0.05 stimuli vs simultaneous TGFβ3/stimuli. Magnification: 100x in a1-c1, 400x in a4-c5, 650x in a2-c3.

To understand if and how endoglin functions in the EMT process, cEPDCs were incubated with an α-Endoglin antibody (Figure 5a). α-Endoglin did not induce morphologic changes of cEPDCs. In cells treated with α-Endoglin the expression of β-catenin increased, which was accompanied with increased expression of *E-cadherin* and *VCAM-1* by 74% and 64% ($P<0.05$), respectively (Figure 5b,c). Interestingly, Western blot analysis showed that addition of α-Endoglin increased the protein levels of endoglin in human EPDCs (Figure 2). The α-Endoglin antibody was not able to prevent the morphologic changes induced by TGFβ, as evident by decreased β-catenin expression and an increased

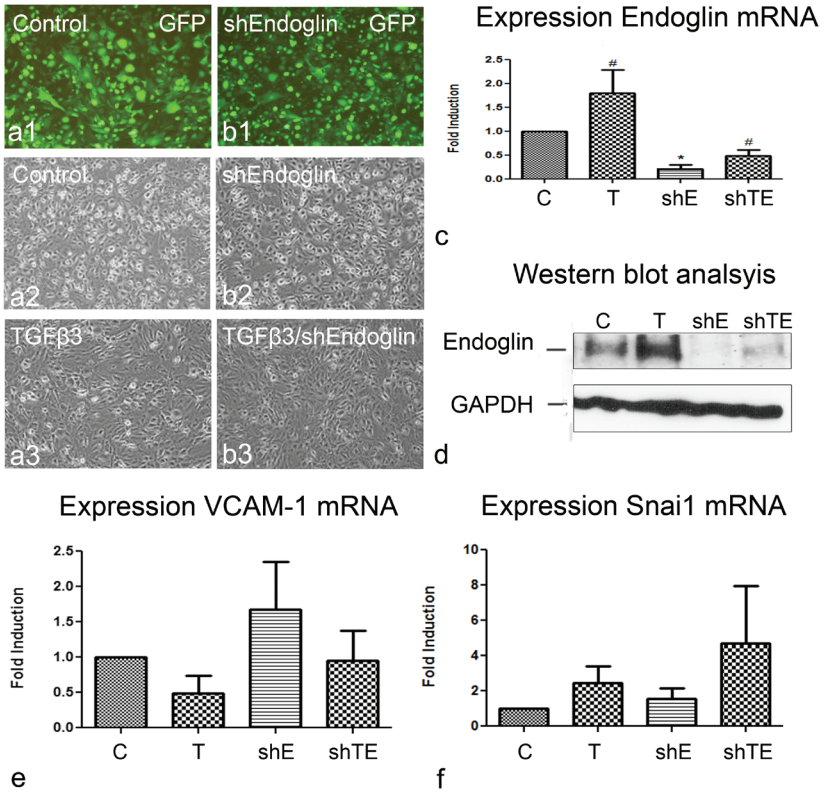


Figure 6. Knockdown of endoglin expression can not block the process of TGFβ-stimulated EMT
Transduction of epicardial cells with lentivirus expression shRNAs for control GFP virus (Control) and human Endoglin (shEndoglin) was visualized by the expression of GFP (a1,b1) and did not effect the cell morphology (a2,b2). Addition of TGFβ caused morphological changes in both shEndoglin transduced cEPDCs and control (a3,b3). qRT-PCR (c) and Western blot (d) analysis showed reduction of endogenous endoglin expression after transduction (c,d). Addition of TGFβ caused increase in endoglin expression in both transduced and control epicardial cells. Analysis of epithelial marker *VCAM-1* (e) and EMT marker *Snai1* (f) showed that knockdown of endoglin could not prevent the effects of TGFβ on these markers. X100 in a1-c2. *$P<0.05$, vs control, #$P<0.05$ TGFβ3 stimulation vs nonstimulated. Abbreviations: C, control; T, TGFβ3; shE, shEndoglin; shTE, TGFβ3/shEndoglin.

expression of phalloidin (Figure 5b). qRT-PCR data showed a decrease in mRNA levels of *VCAM-1* by 96% and increase of *Snai1* by 118% (Figure 5c). These data show that incubating human EPDCs with an endoglin antibody did not affect TGFβ induced EMT. EMT in EPDCs is accompanied by a strong induction of endoglin. To ensure that the inability of the antibody to block TGFβ induced EMT is not caused by the high levels of endoglin, we analyzed the effect of endoglin knockdown on TGFβ induced EMT. Human adult cEPDCs were transduced with a lentivirus expressing a shRNA for human *endoglin* (*shEndoglin*) or eGFP as a control (Figure 6a1-b1). Transduction of cEPDCs with shRNAs did not affect the epithelial morphology (Figure 6a2,b2), nor did it affect TGFβ induced morphological changes (Figure 6a3,b3). EMT was confirmed by qRT-PCR showing a decrease in *VCAM-1* (Figure 6e) and increase of *Snai1* mRNA (Figure 6f). Although endogenous endoglin levels were reduced on both mRNA (78%, $P<0.05$) and protein levels (Figure 6c,d) addition of TGFβ3 was able to induce endoglin at both mRNA (2.2 times, $P<0.05$) and protein level in *shEndoglin* transduced cells (Figure 6c,d). These data show that knockdown of *endoglin* is not able to restrict TGFβ stimulated EMT and increase in endoglin expression.

Role of WT1 in the process of EMT

EMT is the onset for migration and/or differentiation of the epicardium. Therefore, we determined the differentiation state of the treated epicardial cells by analyzing the presence of WT1. WT1 is only expressed in epicardial cells if they are in an undifferentiated state. Immunohistochemistry showed that all treated epicardial cells express WT1 (Figure 2-5). To determine if the level of WT1 expression is affected by the treatment of the cells with several stimulators and inhibitors, we performed qRT-PCR analysis on *WT1* mRNA expression. We observed decrease *WT1* expression in the presence of TGFβ by 68% ($P<0.05$) (Figure 7a). Simultaneous addition of TGFβ with either iALK5, sVCAM-1 or α-Endoglin caused a decrease in *WT1* expression by 45%, 35% and 78%, respectively (Figure 7a). Addition of iALK5, sVCAM-1 or α-Endoglin alone did not change the expression of *WT1* significantly (Figure 7a).

As WT1 is high in the presence of factors preventing EMT and low in the presence of EMT stimulating factors, we explored the role of WT1 in the process of EMT. Therefore, we transduced human adult cEPDCs with shRNAs for *WT1* (*shWT1*). Morphological analysis showed elongation of the WT1 knockdown cells (Figure 7d) when compared to the control and noncoding shRNA transduced cells (Figure 7b,c). qRT-PCR analysis for *WT1* after transduction showed that *shWT1* transduction decreased the mRNA expression of the *WT1* isoform A and isoform D by 79% ($P<0.05$) (Figure 7e) and 83% ($P<0.05$) (Appendix Figure 4), respectively. The observed elongation of the epicardial cells was accompanied with a decrease in the epithelial marker *E-cadherin* by 65% (Figure 7f). The expression

of α*4-integrin* and its ligand *VCAM-1* (Figure 7g,h) decreased by 14% and 35% (P<0.05) respectively, and the EMT marker *Snai1* was increased by 98% after transduction of shWT1 (P<0.05) (Figure 7i).

Role of Platelet-derived growth factors in the process of EMT

The platelet-derived growth factor (PDGF) signalling pathways were found to have a crucial role in EMT during cancer progression, since PDGF-A and its receptor PDGFR-α were highly elevated and secretion of PDGF-A was induced upon TGFβ-induced EMT [25,26]. We previously showed that there is a relation between WT1 and Pdgfrα which may affect the process of EMT [27]. Interestingly, mRNA expression of *WT1* in human adult EPDCs is increased after *shPdgfr*α transduction [27]. Furthermore, *WT1* knockdown increased the expression of *Pdgfr*α mRNA by 85% (P<0.05) (Figure 7j). To address the potential role of PDGFR-α and its ligand PDGF-A in TGFβ-stimulated loss of epithelial character and the onset of smooth muscle differentiation in human epicardial cells, we analyzed the expression of *Pdgfa* (Figure 8a) and *Pdgfr*α (Figure 8b) mRNA after addition of the different stimulators and inhibitors. In response to TGFβ3, the expression of *Pdgfa* increased by 1.5 fold while the expression of *Pdgfr*α decreased by 70% (P<0.05) (Figure 8). iALK5 inhibited the effect of TGFβ on the expression of *Pdgfa* and *Pdgfr*α (Figure 8). Addition of sVCAM-1 and α-Endoglin were not able to influence the effect TGFβ had on the expression of the *Pdgf*α and *Pdgfr*α.

Regulation of proliferation/viability

Several studies showed that the TGFβ pathway modulates cell growth and viability. Using an MTT assay the total number of living cells can be used as a measure for proliferation and viability. We observed that addition of TGFβ reduced the number of living epicardial cells by 31% compared to untreated cells (P<0.05) (Figure 9). Addition of iALK5 and sVCAM-1 increased the number of living epicardial cells by 37% and 130% (P<0.05), respectively (Figure 9). Simultaneously addition of TGFβ with iALK5 did not affect the number of epicardial cells compared to untreated cells, but there was a significant increase compared to cells treated with TGFβ alone (Figure 9). Stimulation with sVCAM-1 could block the negative effect of TGFβ on the number of living cells completely and the cell number increased by 58% (P<0.05) after simultaneous addition (Figure 9). Inhibition of endoglin (α-Endoglin) significantly increased the number of living epicardial cells by 39% (Figure 9). Blocking of endoglin was not able to inhibit the effect of TGFβ, as cells treated simultaneously with TGFβ and α-Endoglin showed significant decrease in cell number by 38.4% (P<0.05) (Figure 9).

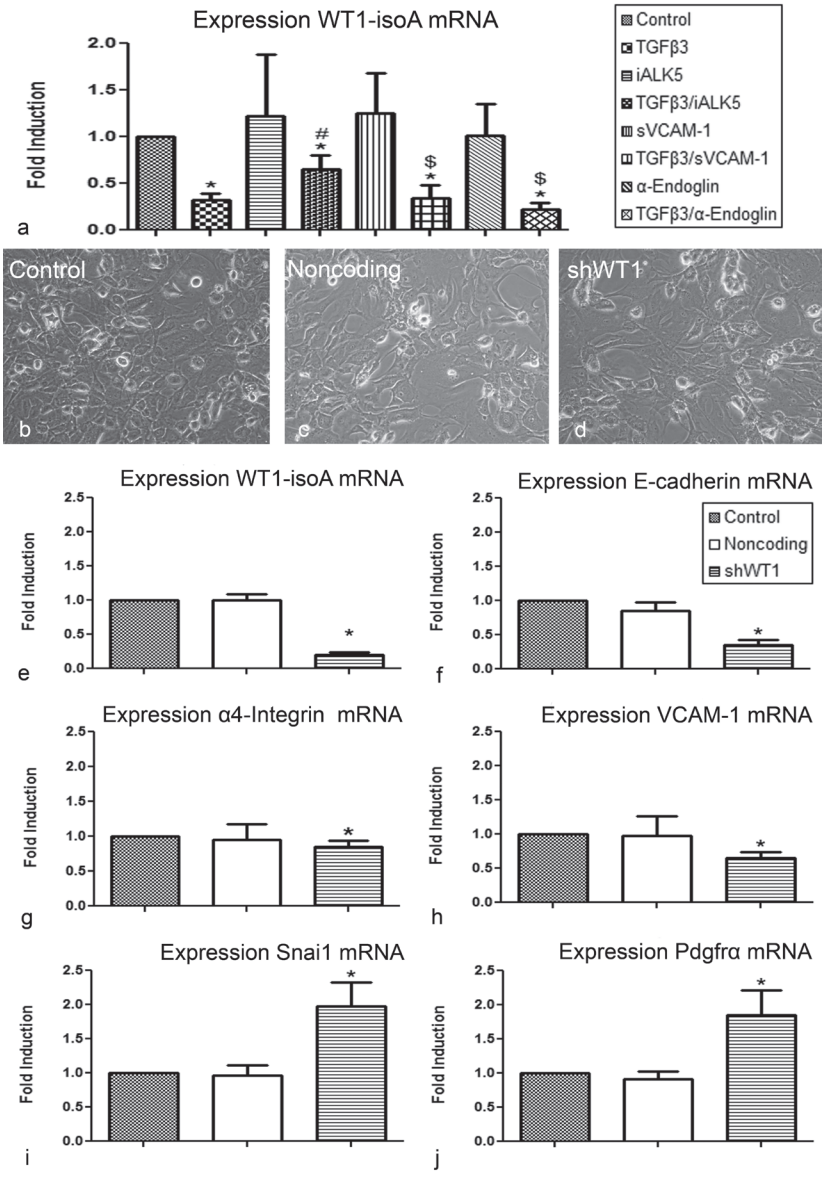


Figure 7. Role of WT1 in the EMT process of human adult epicardial cells

Quantification of *WT1* isoform A mRNA expression in human adult epicardial cells decreased after treatment with TGFβ3 (a). Knockdown of *WT1* by shRNA results in loss of epithelial character of epicardial cells (b-d). Quantification of *WT1* mRNA expression was significantly reduced in epicardial cells after knockdown of *WT1* ($P<0.05$) (e and Appendix Figure 3). The expression of epithelial markers *E-cadherin (f)*, *α4-Integrin (g)* and *VCAM-1* (h) mRNA was decreased in epicardial cells after knockdown of WT1 ($P<0.05$). The expression of EMT-marker *Snai1* increased significantly after knockdown of WT1 ($P<0.05$) (i). WT1 knockdown caused a significant decrease in *Pdgfrα* mRNA expression ($P<0.05$) (j). *$P<0.05$, vs control, #$P<0.05$ vs TGFβ3 stimulation, $$P<0.05$ stimuli vs simultaneous TGFβ3/ stimuli.

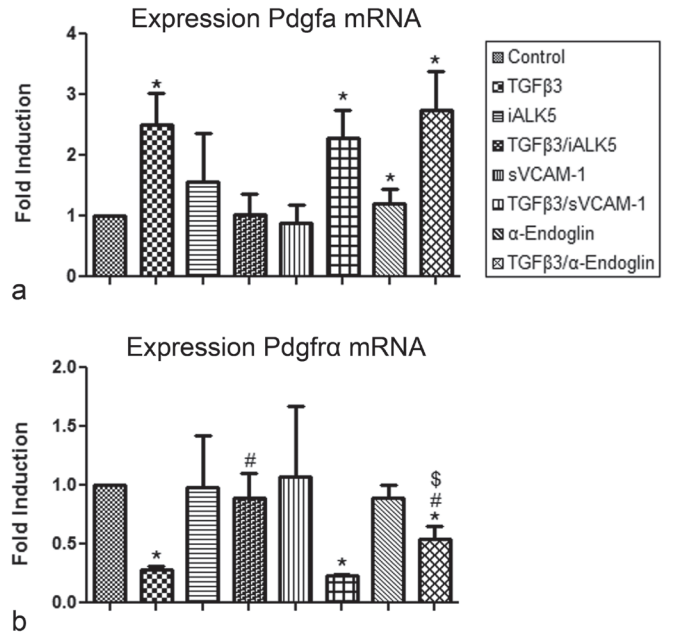


Figure 8. PDGF-signalling in EMT

Quantification of *Pdgfa* (a) and *Pdgfrα* (b) mRNA expression in human adult epicardial cells. Expression of *Pdgfa* increased (a) significantly (P<0.05) after treatment with TGFβ3 in contrast to decrease of *Pdgfrα* expression (b) (P<0.05). Addition of sVCAM-1 or α-Endoglin were both not able block the effect of TGFβ3 on *Pdgfa* (a) and *Pdgfrα* (b) mRNA expression (P<0.05). *P<0.05, vs control, #P<0.05 vs TGFβ3 stimulation, $P<0.05 stimuli vs simultaneous TGFβ3/stimuli.

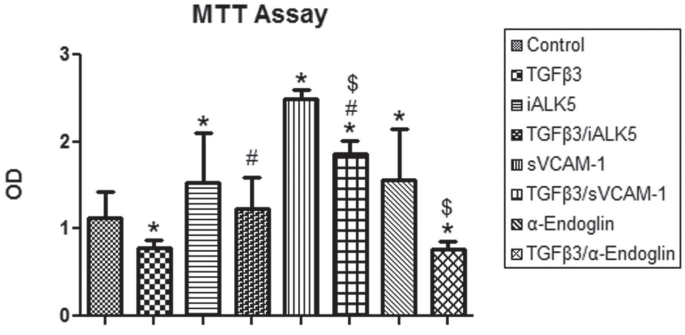


Figure 9. Quantification of the number of epicardial cells by a MTT assay

TGFβ3 reduced the number of epicardial cells. Stimulation by inhibition of ALK5 (iALK5) and Endoglin (α-Endoglin) induced the number of cells as also did addition of sVCAM-1. Simultaneous addition of TGFβ3 and iALK5 did not alter cell numbers compared to control and was able to block the effect of TGFβ3. Addition of sVCAM-1 was also able to block the effect of TGFβ3 and significantly increased the total cells number. Inhibition of α-Endoglin was not able to block the effect of TGFβ3 and number of epicardial cells reduced. *P<0.05, vs control, #P<0.05 vs TGFβ3 stimulation, $P<0.05 stimuli vs simultaneous TGFβ3/stimuli.

DISCUSSION

In this study we show that human adult epicardial cells loose their epithelial character and gain αSMA expression when stimulated by TGFβ. These effects are dependent on ALK5 kinase activity. TGFβ-stimulated loss of epithelial character in adult epicardial cells seem to be independent of endoglin levels, but can be inhibited by induction of VCAM-1. Furthermore we show that WT1 in relation to *Pdgfrα* both have a role in epicardial EMT. Our data support a role for TGFβ in the regulation of EMT of human adult epicardial cells, which is dependent on ALK5 kinase activity, and is the onset of differentiation. The switch from VCAM-1 positive cEPDCs to endoglin sEPDCs is accompanied with downregulation of VCAM-1 which triggers EMT. These new insights in the process of EMT in human adult epicardial cells may help to develop new therapies for cardiac repair which is focussed on modulation of VCAM-1 and WT1.

Adult EPDCs express VCAM-1 before, but endoglin after EMT

We analyzed epicardial cells isolated from adult atrial appendages before and after EMT, and observed that morphological changes during EMT are accompanied by a reduction of β-catenin levels. More interestingly, flow cytometric analysis of cEPDCs and sEPDCs demonstrated that while cEPDCs could be characterized by expression of VCAM-1 on their cell surface and an absence of endoglin, sEPDCs did express endoglin and were negative for VCAM-1. Previous studies revealed that VCAM-1 restricts TGFβ-stimulated EMT by inhibiting TGFβ-mediated loss of β-catenin from intercellular junctions and enhances cell-cell adhesion, concomitant with increased association of β-catenin with intercellular junctions [2]. Therefore, the surface expression of VCAM-1 and β-catenin are supportive for the epithelial nature of cEPDCs. Epicardial cells loose their epithelial characteristics under influence of TGFβ [8,9]. Endoglin is an ancillary TGFβ co-receptor expressed on mesenchymal stem cells [28], smooth muscle cells [21] and is involved in EMT [23,24]. Together with the decreased expression of β-catenin at the plasma membrane of sEPDCs, endoglin expression in sEPDCs confirms EMT.

Human adult epicardial EMT *in vitro*

Our data demonstrate that TGFβ signalling induces loss of epithelial character of human adult cEPDCs and is the onset of differentiation towards fibroblasts and/or smooth muscle cells. These data are consistent with the well-described actions of TGFβ in mediating EMT in embryonic mouse and chicken epicardial cells [2,8,9]. As TGFβ is expressed in the injured myocardium [29], reactivated epicardial cells probably undergo EMT and invade the myocardium due to the availability of active TGFβ in the myocardium.

All human TGFβ isoforms (TGFβ1-3) signal via binding to a heterotetrameric complex of

transmembrane serine/threonine kinase receptors [30]. Upon ligand binding to the TGFβ type II receptor (TGFβRII), the type I receptor (TGFβRI or ALK5) is recruited into the complex and phosphorylated. The activated TGFβRI kinase transduces the signal into the nucleus by phophorylating Smad proteins. There is a third class of TGFβ receptors, the co-receptors (type III receptors (TGFβRIII)) namely β-glycan and endoglin. Our data demonstrates that EMT of human adult epicardial cells requires ALK5 kinase activity. Furthermore, addition of TGFβ increased ALK5 expression and decreased the expression of ALK1. Also the levels of Pai-1 were elevated, a known downstream target of the ALK5/Smad2/3 signalling cascade, and inhibition of the ALK5 kinase decreased Pai-1 expression, also confirming that EMT in epicardial cells is regulated via the TGFβ/ALK5 pathway. Increased expression of ALK5 mRNA after TGFβ stimulation suggests a feed-forward loop. We were unable to show phosphorylated Smad1 and Smad2 in our study, which might be due to time of sampling, 48hrs after TGFβ stimulation.
Addition of TGFβ resulted in decreased number of human adult epicardial cells, suggesting less proliferation or cell viability. Previous studies using endothelial cells (ECs) showed that TGFβ inhibits the proliferation of ECs via the ALK5 pathway, while the ALK1 signalling cascade is responsible for the activation of proliferation [18,19]. Although both type 1 receptors are expressed in epicardial cells, only the ALK5 pathway seems to be necessary for TGFβ induced EMT. Therefore epicardial cells regulate their growth via the ALK5 pathway to the contrary of ECs which are able to respond to TGFβ stimulation via both ALK1 and ALK5 pathways.

VCAM-1 antagonizes TGFβ induced EMT in adult epicardial cells

Soluble VCAM-1 treatment inhibited TGFβ-dependent loss of epithelial character and E-cadherin expression. sVCAM-1 treatment leads to events that increase cortical actin association with the adherens junction and stabilizes adherens junction components at the membrane comparable with the observation of Dokic *et al.* [2] in embryonic chicken epicardial cells and rat epicardial mesothelial cells (EMCs) [2]. Interestingly, although sVCAM-1 treatment inhibited TGFβ-dependent loss of epithelial character, the effect of TGFβ on the expression of VCAM-1 and Snai1 was not blocked by sVCAM-1. Although the decrease in VCAM-1 mRNA expression could not by blocked by sVCAM-1, the total decrease in VCAM-1 mRNA was less compared to addition of TGFβ and simulataneous addition of TGFβ and α-Endoglin. This suggests that the modulation of VCAM-1 is important for EMT. The discrepancy could be caused by the differences in VCAM-1 mRNA and VCAM-1 protein at the cell surface. Another cause of this discrepancy coud be the activation of the Smad pathways. VCAM-1 is associated with Smad1, as $Smad1^{-/-}$ embryos lack the expression of VCAM-1 in the placenta [31]. Furthermore, expression of VCAM-1 is increased in human umbilical vein endothelial cells infected with constitutively active form

of ALK1 [32]. These observations indicate that the ability of VCAM-1 to alter TGFβ stimulated EMT is probably depending on the TGFβ/ALK5 pathway and suggests that VCAM-1 is also involved in the TGFβ/ALK1 pathway in other processes. To date we have no evidence that ALK1 is activated upon TGFβ and it is important to know if sVCAM-1 is able to activate ALK1. The upregulation of Snai1 seen in simultaneous stimulation of epicardial cells by TGFβ3 and sVCAM-1 is probably regulated by the ALK1 pathway and leads to decrease in the epithelial markers, although this pathway in the regulation of EMT markers is not responsible for the actual process of EMT as cells treated with TGFβ3/sVCAM-1 have a epithelial morphology. Further studies of the signalling pathway altered by VCAM-1 and the modulation of VCAM-1 in human adult epicardial cells should allow us to elucidate the important role of VCAM-1 in regulating EMT.

The role of endoglin in EMT of adult epicardial cells

The presence of endoglin on the surface of epicardial cells that underwent EMT, suggested a role for endoglin in the process of EMT. This was supported by Mercado-Pimental *et al*. who showed that loss of endoglin expression resulted in a direct perturbation of EMT during cardiac valve formation and reduced expression of EMT markers including slug and Runx2 [1]. However, in endothelial cells, endoglin, preferentially binds TGFβ1 and TGFβ3, when associated with TGFβRII [22] and stimulates Alk1 signalling and antagonizes ALK5 signalling [19,33]. In our study, blocking of endoglin function by addition of α-Endoglin antibody was not able to inhibit TGFβ-induced loss of epithelial character of the EPDCs. Although, previous studies in cardiac cushions [1] showed that TGFβ-induced EMT was dependent on endoglin. One explanation for this discrepancy is the availability of endoglin for signalling. Using the antibody, we might just not be able to occupy all receptors on the cell surface. Therefore, we performed knockdown of endoglin by shRNAs in human adult epicardial cells. Comparable to the α-Endoglin antibody, knockdown of endoglin also did not restrict TGFβ-induced mophologic changes in epicardial cells. So both interference with endoglin function or absence of the endoglin is not able to prevent TGFβ-induced EMT in EPDCs. qRT-PCR and Western blot analysis revealed that expression of endoglin is still increased after addition of TGFβ even when cells are transduced by *shEndoglin*. These data suggest that endoglin is not directly involved in the initiation process of EMT in EPDCs as is shown for endothelial cells [19,33], but might be beneficial for differentiation further on in the EMT-pathway.

Wilm's tumor suppressor WT1 and Pdgfrα

WT1 is a novel transcriptional activator of the *α4-integrin* gene and stimulation of *α4-integrin* expression by WT1 may promote cell adhesion in the epicardium [34]. Our data show that knockdown of *WT1* in human epicardial cells stimulated loss of epithelial

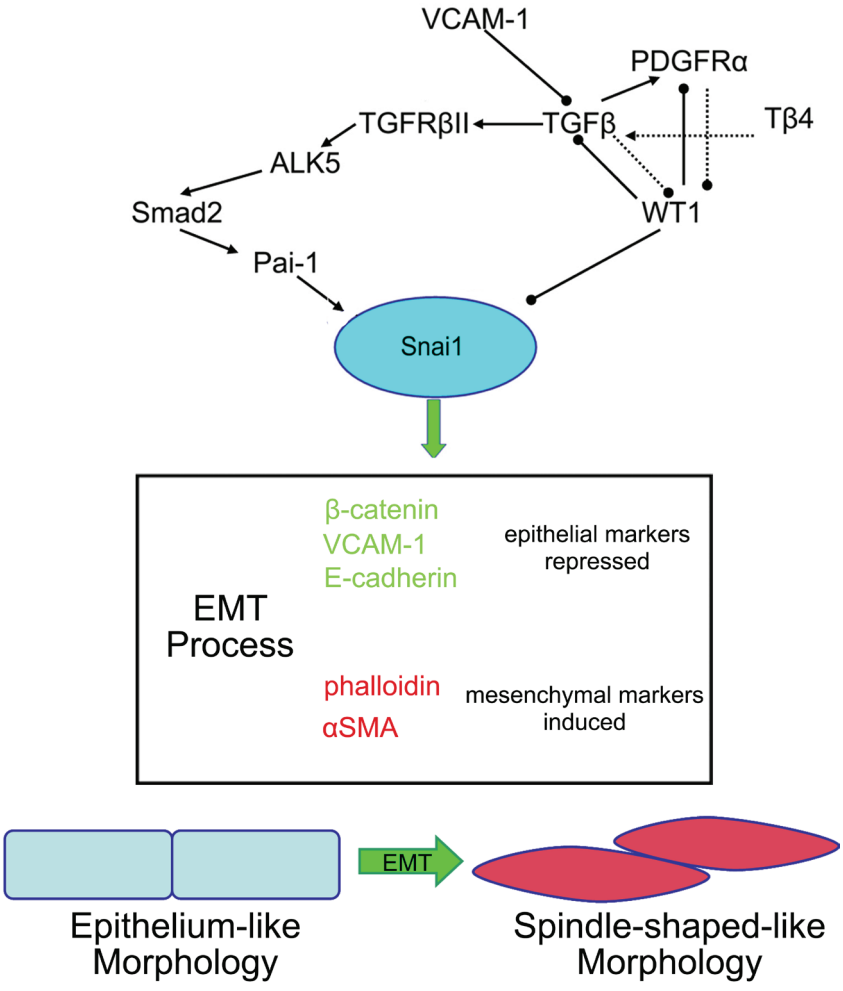


Figure 10. Epithelial-mesenchymal transformation (EMT) of human adult epicardial cells
Master regulator of EMT, Snai1 leads to dramatic changes in gene expression profile and cellular morphology. Snai1 represses expression of epithelial markers and triggers expression of EMT markers. Herewith we depicted a possible pathway which stimulates epicardial EMT after myocardial injury (Adapted from Kang *et al.*[47]). Arrows with arrowheads represent activation as arrows with bullets represent repression. Dashed arrow lines with arrowheads and bullets represent indicative pathways.

morphology and reduced expression of epithelial markers like E-cadherin, α4-integrin and VCAM-1 significantly, while the expression of EMT-marker Snai1 increased significantly. This suggests that WT1 is a repressor of the EMT process in adult epicardial cells. These findings are consistent with previous described data of Bergmann and Kirschner [34,35]. Bergmann *et al*. described that WT1 transcriptionally represses TGFβ [35] and Kirschner *et al*. described that WT1 transcriptionnally activates α4-integrin [34]. These data are similar to our observation but opposite to the data described by Martinez-Estrada *et al*. [36]. To determine whether WT1 has a direct and cell-autonomous role in epicardial EMT, Martinez-Estrada *et al*. generated tamoxifen-inducible WT1-knockout immortalized epicardial cells (*Cre+CoMEEC, E11.5*). CoMEECs had typical cobblestone morphology and showed robust WT1 expression. Loss of WT1 after tamoxifen treatment led to a robust increase in E-cadherin expression and was associated with downregulation of N-cadherin and αSMA. RT-PCR revealed downregulation of Snai1 after WT1 deletion [36]. The contradiction between our data and Martinez-Estrada *et al*. could be caused by the fact that EMT in human adult epicardial cells is regulated by other factors compared to EMT in mouse embryonic epicardial cells. Further research is needed to elucidate the exact role of WT1 in the process of EMT in human adult epicardial cells.

Knockdown of *WT1* and *Pdgfrα* [27] suggested that there is a possible relation between these two factors. Knockdown of *WT1* increased the expression of *Pdgfrα* significantly as downregulation of *Pdgfrα* increased the expression of *WT1* significantly in human adult epicardial cells [27]. PDGF-signalling is known to be important to support epicardial EMT. Both PDGF receptors, *Pdgfrα* and *Pdgfrβ* are expressed in the epicardium [11,37,38] and PDGF-BB, which can bind to both *Pdgfrα* and *Pdgfrβ*, is more potent in inducing cultured embryonic quail epicardial cells to undergo the initial steps of EMT [39] compared to both PDGF-AA and PDGF-AB, suggesting that this pathway is involved in epicardial EMT. Also our previous work showed an EMT-inducing role for *Pdgfrα* in mouse embryonic epicardial cells [27] as the receptor is able to repress expression of WT1. Unpublished data by Smith suggests that both receptors are required for development of a unique epicardial derivative, *Pdgfrα* for cardiac fibroblast and *Pdgfrβ* for smooth muscle cells. This would fit with earlier observations that show that *Pdgfrα* is more related to myocardial differentiation while is expressed during the stage of coronary artery formation [40]. Furthermore, PDGF-signalling stimulated the phosphorylation of Sox9 and combined with TGFβ induces a transcriptional complex with Smad2 and Snai1 (Smith *et al*. personal communication). A previous study shows that WT1 is required for Sox9 expression [41] and that the expression of Sox9 is involved in the process of EMT during cardiac cushion development [42]. We show that during the process of EMT both WT1 and *Pdgfrα* are involved and more elaborate studies will be necessary to further explore the link between both growth factors and TGFβ, Sox9, Smad2 and Snai1 and their potential roles in human adult epicardial EMT.

Another study showing involvement of PDGF-signalling in EMT is suggested by the role for PDGF in hepatocarcinogenesis, since PDGF-A ligand and PDGF receptor subunits were highly elevated upon TGFβ-induced EMT [25]. Our data shows that an increase of *Pdgfa* mRNA expression is accompanied by a decrease in *Pdgfrα* expression. This negative relation between PDGF-A and PDGFR-α is previously described in chicken embryos of which epicardial outgrowth was inhibited [11]. Furthermore, Paulsson *et al*. described that there is a density-dependent inhibitory effect of TGFβ on the expression of *Pdgfrα* [43]. Therefore, our observed TGFβ regulated decrease of *Pdgfrα* could also be due to an excess of TGFβ3-ligand. Future research on the role of *Pdgfrα* mediated by TGFβ and/or WT1 in the EMT process is needed to be able to fully understand this intriguing cascade.

Regenerative medicine

Epicardial cells are a promising tool with regard to cardiac regeneration therapy. Transplantation of exogenous human adult EPDCs into the ischemic mouse myocardium improved left ventricular (LV) ejection fraction. Adverse remodelling was also attenuated by EPDCs injection [12]. The importance of epicardial cells and EPDCs for cardiogenesis and regenerative medicine is related to their role in coronary vessel development and maintenance of myocardial architecture [12-16]. Activation of endogenous epicardium, stimulates the process of EMT by which cells can migrate into the myocardium and reactivation of the embryonic program of epicardial cells after myocardial injury, is of great importance for the process of intrinsic cardiac repair. Understanding more of the process of EMT and the involved pathways in human adult epicardial cells is important for the development of strategies to stimulate heart repair by endogenous cell sources.

Members of the TGFβ super family are markedly induced in the infarcted myocardium and through their potent effects are capable of playing a central role in infarct healing and cardiac repair [29]. Our data combined with previous studies suggest that TGFβ is not only a central mediator involved in the inflammatory and fibrotic phase of healing and may critically modulate many cellular steps in post-infarction cardiac repair [29] but can also induce EMT of epicardial cells.

Thymosin beta 4 (Tβ4) is reported to activate endogenous mouse adult epicardium after myocardial infarction [13,44]. This combined with the knowledge from previous studies that Tβ4 significantly increase the expression of TGFβ [45] and that Tβ4 increases the secretion of Pai-1 in endothelial cells [46], suggests that Tβ4 could be the initiator for the onset of the EMT-regulated pathway in adult epicardial cells (Figure 10). Therefore, Tβ4 might elevate TGFβ thereby inducing EMT of epicardial cells and it would be very interesting to see if the effects of TGFβ on VCAM-1, WT1 and *Pdgfrα* during EMT in human adult epicardial cells are initiatied by Tβ4. Future research in cardiac repair focussing on the potential of

the TGFβ/ALK pathway initiating EMT of endogenous human adult epicardial cells investigation via transcriptional regulation of *WT1* and *Pdgfrα*, might provide new treatment modalities.

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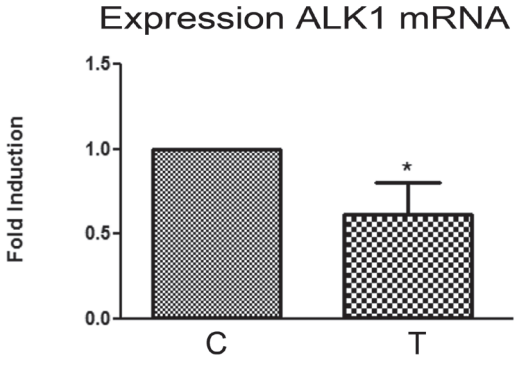
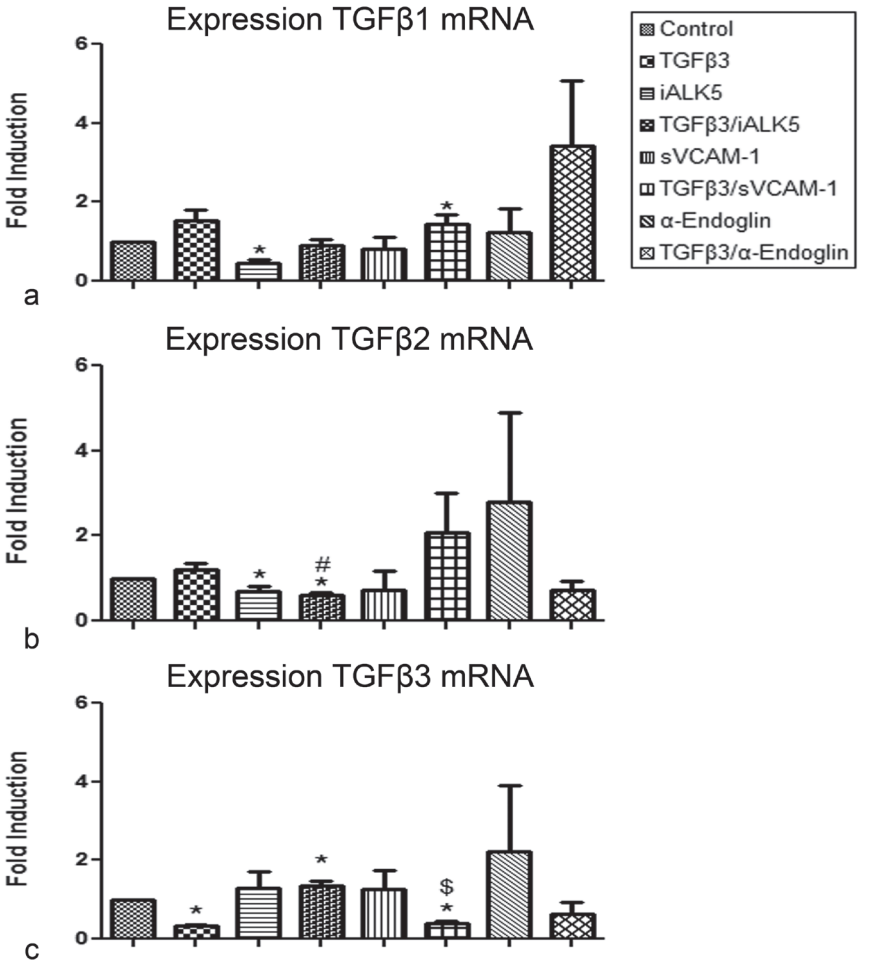
APPENDIX

RESULTS

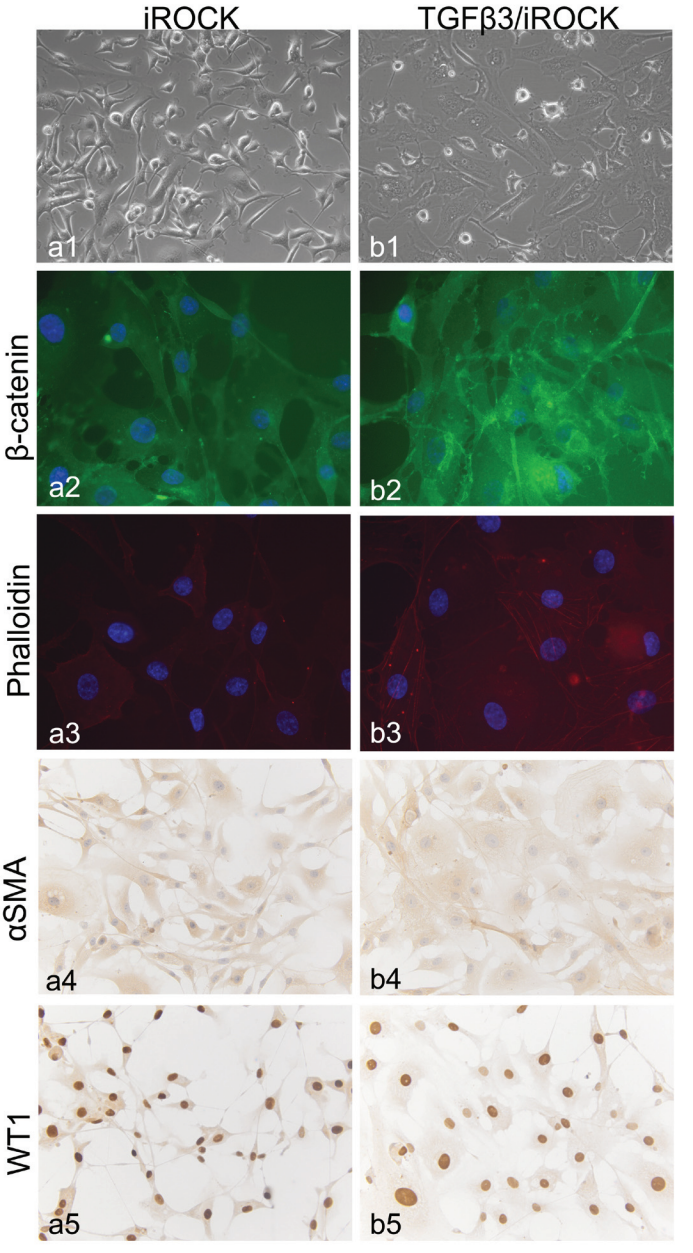


Appendix Figure 1. Expression of ALK1 in epicardial cells
Quantitative PCR on RNA of cEPDCs (control; C) and epicardial cells treated with 1ng/ml TGFβ3 (T) showed that the expression of *ALK1* was decreased by TGFβ3.

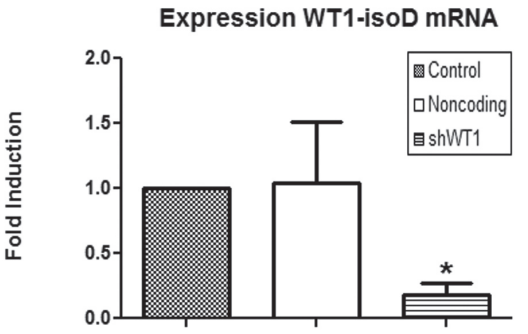


Appendix Figure 2. Expression of TGFβ-isoforms in epicardial cells

Quantitative PCR on RNA of epicardial cells and epicardial cells incubated with iALK5, α-Endoglin and sVCAM-1 in presence or absence of TGFβ3 showed that the expression of *TGFβ1* (a) and *TGFβ2* (b) mRNA were significantly reduced in the presence of iALK ($P<0.05$). The addition of TGFβ3 reduced the mRNA of *TGFβ3* mRNA significantly ($P<0.05$) (c). Addition of iALK5 in the presence of TGFβ3 was able to inhibited the decrease in *TGFβ3* mRNA expression stimulated by TGFβ3 as addition of sVCAM-1 was not.



Appendix Figure 3. The role of RhoA kinase EMT of adult epicardial cells
Inhibition of p160 Rho kinase activity caused elongation of epicardial cells (a1), which was accompanied by reduction in β-catenin (a2) staining bu there was no induction of stress fibers and αSMA (a3,4). Addition of iROCK in the presence of TGFβ was not able to prevent loss in epithelial character (b1), this was accompanied by increased expression of β-catenin and phalloidin (b2,3).



Appendix Figure 4. Role of WT1 in the process of EMT
Quantification of *WT1* isoform D mRNA expression in human adult epicardial cells after treatment with knockdown of *WT1* by shRNA results in significantly reduced expression of *WT1isoD*.