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General introduction

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Clinical Background

Myocardial infarction (MI) is the most common type of ischemic heart disease [1, 2]. Occlusion of a coronary artery results in deprivation of oxygen and the replacement of cardiomyocytes by a non-contractile fibrotic scar tissue [3, 4]. The endogenous regenerative capacity of the heart is insufficient due to the limited proliferation ability of cardiomyocytes [5] and the limited ability of resident cardiac progenitors to differentiate into cardiomyocytes [6-8]. Current therapy aims to restore cardiac perfusion by removing the coronary obstruction, but fails in renewing lost contractility. The last decade research has been focused on cell based-therapy. Although the improvement of ejection fraction after transplantation of progenitor cells, regeneration is insufficient to overcome cardiac progression toward heart failure [9, 10]. An alternative approach to improve cardiac regeneration is in situ activation of resident progenitor cells.

The heart develops from a differentiating population of progenitor cells. If we understand these differentiation processes occurring during development on cellular and molecular level, we might be able to induce efficient differentiation of resident progenitor cells during cardiac injury resulting in cardiac regeneration. Recent studies have identified the epicardium, the outer layer of the heart, as a likely source of cardiac progenitors [11-13].

1. The heart

The heart is a self-excitating, muscular organ situated in the thoracic cavity, just behind and slightly left of the breastbone. The heart functions as a pump, which pumps the blood, containing oxygen and nutrients, through the blood vessels of the cardiovascular system. In humans and other mammals the heart is divided into four chambers; two atria and two ventricles. The atria are the receiving chambers of the heart, in which the right atrium receives blood via the venae cavae from the systemic circulation and the left atrium receives oxygen rich blood via the pulmonary veins from the lungs. The ventricles are the output chambers of the heart, wherein the right ventricle pumps blood through the pulmonary artery to the lungs and the left ventricle pumps blood through the aorta to the systemic circulation (Figure 1).

The unidirectional blood flow through the heart is ensured by four valves. The tricuspid and mitral valve, are atrioventricular valves and are located at the junction of the atrium and ventricle of respectively the right and left side of the heart. The pulmonary and aortic valve, are semiluminar valves and are located at the junction of right ventricle and pulmonary artery

and left ventricle and aorta respectively. The tricuspid, aortic and pulmonary valve are composed of 3 leaflets, whereas the mitral valve is composed of 2 leaflets. The structure of the leaflets is highly organized and consist of collagen, elastin and proteoglycans [14, 15].

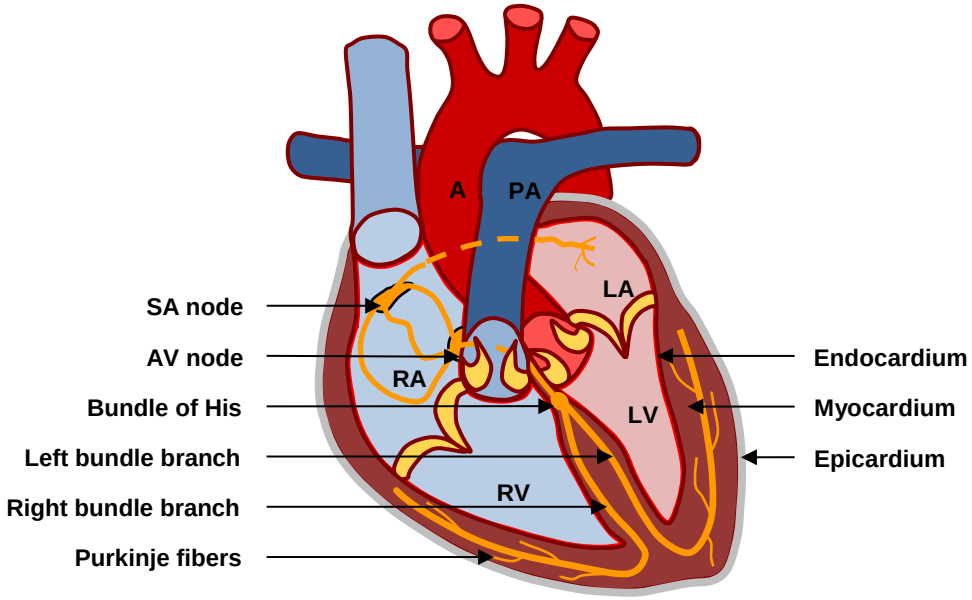

Figure 1. Schematic illustration of the heart. A. aorta; LA, left atrium; LV, left ventricle; PA, pulmonary artery; RA, right atrium; RV, right ventricle

The cardiac wall is made up of three layers; the endocardium, which is the inner layer of the heart, the myocardium and the epicardium, which is the outer layer of the heart. The myocardial layer consists of cardiomyocytes, cardiac fibroblasts, smooth muscle cells and endothelial cells [16]. Cardiomyocytes are the functional contractile units of the heart, fibroblasts play an important role in maintaining normal cardiac structure and smooth muscle cells and endothelial cells form the vessels of the coronary circulation. Two coronary arteries arise at the base of the aorta and ensure oxygen supply to the heart. The right and left coronary arteries branch into smaller vessels, capillaries and eventually become veins. The coronary veins fuse into the coronary sinus which delivers deoxygenated blood to the right atrium. Coronary arteries can be affected by atherosclerosis which can result in obstruction of coronary blood flow, causing acute myocardial infarction.

The heart has the unique feature of autorhythmicity by initiating a cardiac action potential in the sinoatrial node. This structure is located at the top of the right atrium. The cardiac conduction system (CCS) consists of specialized cardiomyocytes, identified by the expression of gap junctions [17]. The CCS is responsible for the initiation and propagation of the electrical signal leading to an organized contraction, first of the atria and subsequently the ventricles. Successively there is excitation of the SA-node, the AV-node, the bundle of His, the bundle branches and finally the Purkinje fibers (Figure 1). This cardiac electrophysiology can be modulated by the cardiac autonomic nervous system (cANS). The autonomic nervous system is divided in the sympathetic and the parasympathetic component and stimulation of the sympathetic component results in an increased heart rate, contraction force and conduction velocity, whereas stimulation of the parasympathetic component exerts inhibitory effects.

2. Early cardiac development

All bilaterian animals arise from the three primary germ layers; the ectoderm, the endoderm and the mesoderm. The ectoderm will contribute to the formation of e.g. the nervous system and skin, the endoderm will contribute to e.g. the epithelium of the digestive and respiratory system and the mesoderm will contribute to e.g. the cardiovascular system, smooth, cardiac and skeletal muscles, hematopoietic cells and connective tissue [18].

As there is a growing demand for oxygen and nutrients when the embryo is developing, the heart is the first organ to develop and is already functional in its most primitive structure. Cardiogenesis in the mouse starts at embryonic stage (E) 7.5 with the development of the cardiac crescent [19]. This cardiac progenitor population can be identified by the expression of cardiac transcription factors such as Nkx2.5, Tbx5 and GATA4 [20-23]. Cells from the cardiac crescent migrate to the midline of the embryo and fuse to form the primitive heart tube (E8) [22, 24]. This bilayered tubular structure starts contracting and is composed of a layer of cardiomyocytes on the outside and endothelial cells on the inside which are separated by cardiac jelly [25]. The linear heart tube elongates, which forces the heart to bend ventrally and to undergo a rightward looping, resulting in a C-shape formation. As the ventricular part of the heart tube moves caudally, the heart acquires a complex S-shape formation [24, 26]. As a consequence of this looping, future compartments of the heart become recognizable. The chamber forming regions, that will become atria and ventricles are situated at the outer curvatures of the tube [27, 28]. The inflow and outflow of the heart tube are brought together by a process called convergence [29].

During development endocardial cells in the atrioventricular canal and outflow tract will lose cell-cell contact and migrate into the cardiac jelly, forming the cardiac cushions [30]. This process is known as endocardial-to-mesenchyme transition (EndMT) [31]. These cushions are the precursors for the valves and septa of the heart and ensure a coordinated unidirectional bloodflow. In addition to the forming cushions, atrial and ventricular septation is also dependent of muscular growth of the atrial septum primum and ventricular septum. Fusion of these two septa with the atrioventricular cushions results in a four-chambered heart [32, 33] (Figure 2).

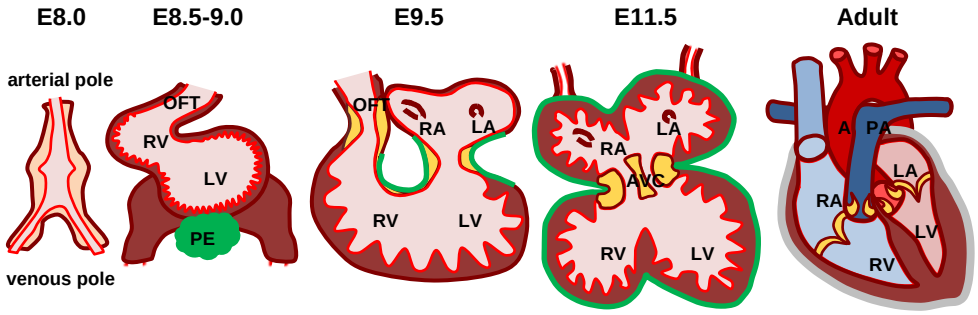

Figure 2. Cardiac development. E8.0 The primitive tube is formed by cardiomyocytes (brown) on the outside and endothelial cells (red) on the inside which are separated by cardiac jelly. E8.5 – E9.5 The primitive tube elongates and undergoes rightward looping. At the venous inflow tract of the developing heart the proepicardium arises. E11.5 Formation of septa and valves initiates the sepation of the heart into a four-chambered structure. Adult A fully maturated heart. The expression of WT1 throughout development is indicated in green. A, aorta; AVC, atrioventricular cushion; LA, left atrium; LV, left ventricle; OFT, outflow tract; PA, pulmonary artery; PE, proepicardium; RA, primitive right atrium; RV, right ventricle.

Even though the heart is already functional at an early stage of development, the growth and maturation of the heart are not completed until after birth [15]. Expansion of the heart in order to meet the required demand in oxygen and adaptation to the changing hemodynamics demands morphological changes of the heart after embryonic development.

3. Epicardial formation and epicardium-derived cells

At E8.5 a cauliflower-like cluster of cells becomes visible at the base of the inflow tract of the developing heart. This structure arises from the pericardial coelomic mesothelium and will form the proepicardium (PE). The proepicardium is a heterogeneous population of cells, characterized by the expression of WT1, Tbx18, Tcf21, Sema3D and Scx [34-37]. Cells from

the PE reach the heart by forming a physical bridge (chicken, mouse) and by free floating vesicles (mouse) [38], they attach and spread over the myocardium to form a mesothelial sheet. This outer layer of the heart is called the epicardium and is generally identified by the expression of the transcription factor Wilms' tumor-1 (WT1) [35, 39-41]. The first presence of epicardial cells at the outside of the heart is visible at the inner curvature of the heart around E9.5/E10. Subsequently covering of the heart proceeds in a dorsal to ventral manner. At E10.5 covering of the atria and ventricles is as good as completed, however, parts of the outflow tract are still largely depleted of epicardial cells [42, 43]. Complete coverage of the myocardium with epicardial cells is established in the mouse by E12.5 (Figure 2).

Once the heart is enveloped, a subset of epicardial cells lose cell-cell contact and undergo epithelial-to-mesenchyme transition (EMT), resulting in epicardium-derived cells (EPDCs) [44, 45]. Lineage tracing studies in mice have shown that EPDCs migrate into the myocardium and contribute to the formation of cardiac fibroblasts, endothelial and smooth muscle cells of the cardiac blood vessels, and cardiomyocytes [34-36, 46, 47], even though the contribution to the latter one is still under debate [43, 48-50]. Although the contribution of WT1-positive cells to endothelial cells is negligible [35], Sema3D- and Scx-positive cells of the proepicardium have a significant contribution to the formation of endothelial cells [34]. Experiments with quail-chicken chimeras, in which quail proepicardium was transplanted into the pericardial cavity of a chicken host, have also shown that EPDCs contribute to the formation of cardiac fibroblasts, endothelial, smooth muscle and endocardial cells, however no contribution to the formation of cardiomyocytes has been described [45, 51, 52] (Figure 3).

The epicardium plays a crucial role during the development of the heart. Inhibition of epicardial formation in chicken and mouse result in ventricular non-compaction and incomplete formation of the coronary vasculature [39, 53, 54]. In addition, the epicardium plays an essential role via paracrine signaling in myocardial compaction and cardiomyocyte proliferation [44, 55-57].

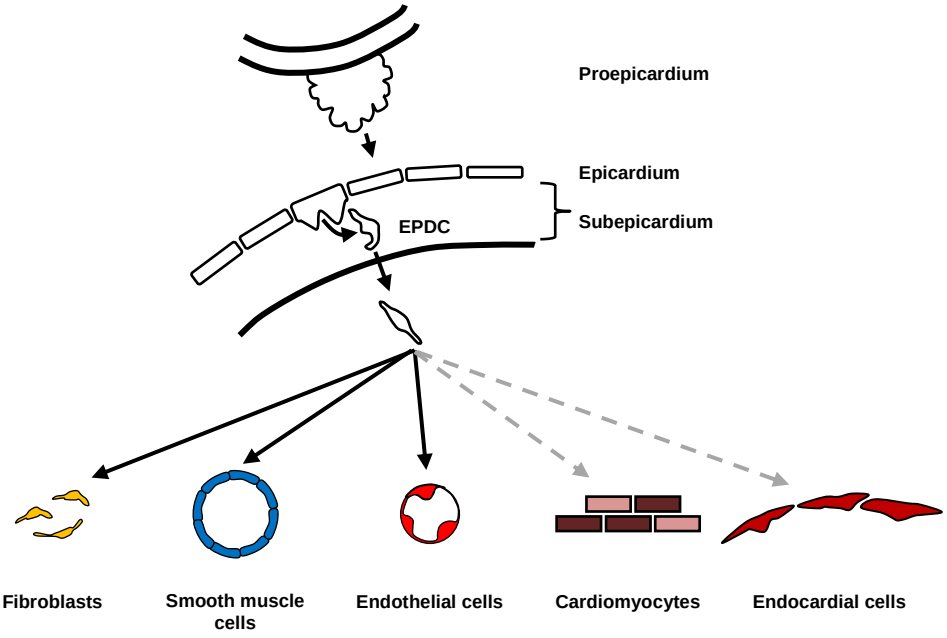

Figure 3. Formation of the epicardium and the fate of epicardial cells. The epicardium arises from the proepicardium. A subset of cells undergo EMT and migrate into the subepicardium and myocardium. EPDCs contribute to the formation of fibroblasts, smooth muscle cells and endothelial cells, however, the contribution to the formation of endocardial cells and cardiomyocytes is controversial. Cardiac fibroblasts can be subdivided into interstitial fibroblasts, perivascular fibroblast and valvular interstitial cells.

4. Myocardial infarction

Myocardial infarction (MI) is the most common type of ischemic heart disease [1, 2] and four phases can be distinguished in the cardiac wound healing process [4, 58].

In the first phase, cardiomyocytes die as a result of insufficient oxygen supply, due to coronary occlusion. In the second phase, an early inflammatory response to injury and cell dead leads to infiltration of inflammatory cells which remove the necrotic tissue [59]. This infiltration is facilitated by activation of latent matrix metalloproteinases (MMPs), which degrade the extracellular matrix (ECM) [60, 61]. In the third phase, which occurs approximately 3 days after MI, an increase in granulation tissue is observed in the infarcted area to replace the dead tissue. This is accompanied by the presence of activated fibroblasts and formation of new blood vessels [60, 62-64]. Transforming growth factor beta (TGFβ), a key growth factor during

development and injury, is upregulated after MI by immune cells and interstitial fibroblasts and [65-67] induces the differentiation of fibroblasts into myofibroblasts and the production of ECM [60, 63]. Remodeling and maturation of scar tissue characterize the fourth phase of the wound healing process and results in stabilization of the cardiac wall. Compensatory mechanisms to maintain cardiac output after scar formation results in ventricular dilatation and impairment of cardiac functioning, which ultimately lead to cardiac failure [3, 4]. Extensive deposition of ECM by activated myofibroblasts play an adverse key role during cardiac remodeling [68] (Figure 4).

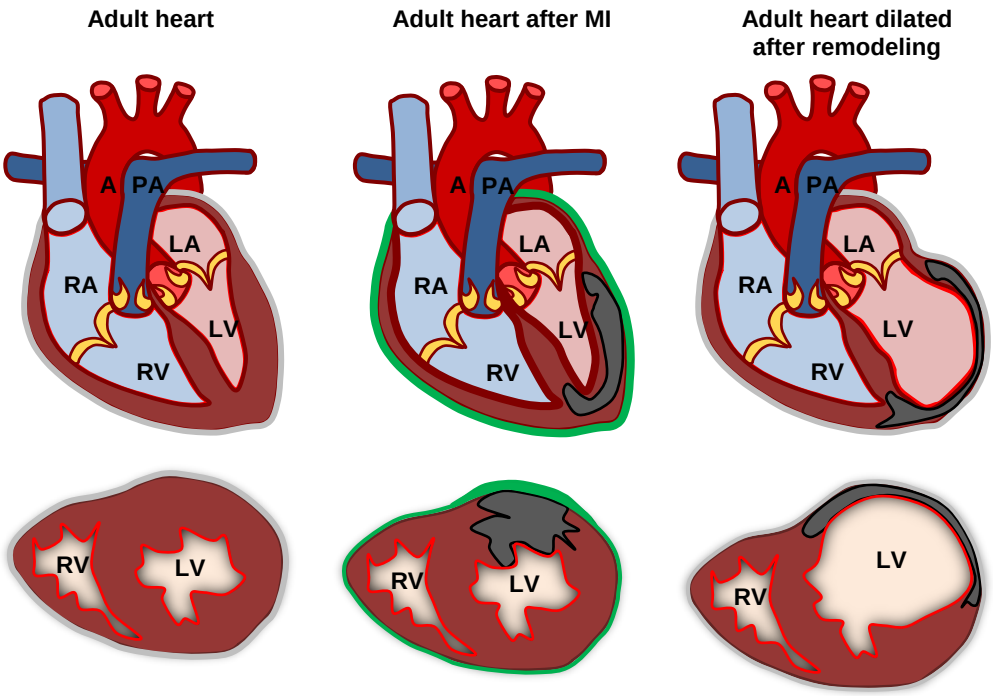

Figure 4. Myocardial infarction. The epicardial expression of WT1 is almost absent in the adult heart. Myocardial infarction results in the loss of cardiomyocytes and the replacement by fibrotic scar tissue. After myocardial infarction the epicardial expression of WT1 is reactivated in the entire heart. Cardiac remodeling following myocardial infarction results in ventricular dilatation and impairment of cardiac functioning. The expression of WT1 in the epicardium of the adult and infarcted heart is indicated in green.

In contrast to the quiescent appearance of the epicardium in the adult heart, after MI the epicardium is reactivated. This reactivation is illustrated by the upregulation of the embryonic gene expression program, thickening of the epicardium and recurrence of EMT [13, 69-72]. In

contrast to the mammalian heart, zebrafish are capable of myocardial regeneration by proliferation of existing cardiomyocytes [71-73]. In addition, the potential cellular contribution of the epicardium in cardiac repair was first described in the zebrafish [72]. After resection of the cardiac apex, or cryo-injury of the heart, epicardial cells proliferate, undergo EMT and migrate towards the site of injury [72, 74, 75]. The activated epicardial cells contribute to fibroblast, smooth muscle cells and endothelial cells, but do not give rise to newly formed cardiomyocytes [76]. In addition, as is seen during development, the epicardium has an important paracrine role during regeneration [69, 77]. The cellular contribution of the epicardium after MI in mammals is still under debate [11]. In addition, no migration of epicardial cells into the myocardium has been reported after infarction, unless the heart is pre-treated with thymosin beta-4 [13, 47, 69, 78]. Injection of EPDCs into the infarcted mouse heart resulted in preservation of left ventricular function, attenuation of cardiac remodeling, and increased vascular density. This indicates that EPDCs might be promising candidates for inducing regeneration when transplanted into the injured region [79].

5. The transcription factor Wilms' tumor-1

The transcription factor Wilms' tumor-1 (WT1) is strongly expressed in the epicardium during development and is re-expressed after infarction and is therefore used as a epicardial marker [35, 39-41, 46, 80]. WT1 was first discovered in renal tumors [81, 82] and contains four zinc-finger motifs at the C-terminus which are important for the binding of DNA. Besides its function to activate the transcription of genes, WT1 is also involved in posttranscriptional processes [83]. WT1 expression is essential during development of certain organs, including kidneys, gonads, spleen, and also for the proliferation of certain neuronal progenitors [54, 84].

WT1 is a pleiotropic molecule and multiple, often opposite biological roles have been described, depending on the location and moment of its expression. During kidney development WT1 is needed to induce mesenchymal-to-epithelial transition, whereas WT1 in the epicardium is required for epithelial-to-mesenchyme transition during cardiac development [80, 85]. The absence of WT1 leads to an increased apoptosis of cells in the kidney during development, suggesting WT1 plays a role in cell survival [86]. This is supported by the observation that WT1 in germ cells protects against apoptosis [87]. Cells lacking WT1 in the kidney showed an increased proliferation, whereas the proliferation of neuronal progenitors is reduced in the absence of WT1 [86, 88]. The contradictory WT1 functions during development are also present in tumors. Although it is generally known as a tumor suppressor gene in

Wilms' tumors it seems to play an oncogenic role in, e.g. breast and colorectal tumors, by supporting angiogenesis, which is required for carciogenesis [89, 90].

The expression of WT1 during the multipotential stages of the epicardium, both during development and after MI suggest WT1 to be an important regulator in the multipotency of cardiac cells and the epicardial cells in specific.

Scope of this thesis

Knowledge of normal development serves as a blue print for tissue regeneration. The epicardium has been identified as a potential source of cardiac progenitors, however, the exact contribution during regeneration is still under debate. The aim of this thesis is to gain more insight of the involvement of WT1 and the epicardium in the cellular and molecular processes of the developing heart and after cardiac injury.

In **Chapter 2**, the spatiotemporal expression pattern of WT1 during developmental stages and after cardiac injury in mice and a potential important role for WT1 in cardiac endothelial cell proliferation and vessel formation are described. **Chapter 3**, shows that the expression of WT1 is present during human cardiac development in cells that are crucial for the proper formation of the cardiac vessels and ventricular myocardium, i.e. epicardial, endothelial and endocardial cells. **Chapter 4**, a comprehensive book chapter is presented about the role of WT1 in cardiac development and after injury. In addition, this chapter presents a working model in which the role of WT1 in endothelial cells is based on results and observations from this thesis and previously published literature. In **Chapter 5**, we investigated the development of the epicardium in relation to cardiac innervation and we show for the first time that the epicardium plays a role in modulating the cardiac autonomic response prior to innervation.
In **Chapter 6**, the response of EPDCs to mechanical stimulation has been studied by transplantation of EPDCs in infarcted myocardium and by using an *in vitro* straining device.

Finally, the results and conclusions of the previous chapters of this thesis are discussed in **Chapter 7**, together with the future implications deriving from this work.

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2

Cardiac endothelial cells express Wilms' tumor-1;
Wt1 expression in the developing, adult and infarcted heart

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Abstract

Myocardial infarction is the leading cause of death worldwide. Due to their limited regenerative capacity lost cardiomyocytes are replaced by a non-contractile fibrotic scar tissue. The epicardial layer of the heart provides cardiac progenitor cells during development. Because this layer regains embryonic characteristics in the adult heart after cardiac injury, it could serve as a promising source for resident cardiac progenitor cells. Wilm's tumor-1 is associated with the activation and reactivation of the epicardium and therefore potentially important for the differentiation and regenerative capacity of the epicardium. To gain more insight into the regulation of WT1 we examined the spatiotemporal expression pattern of WT1 during murine development and after cardiac injury. Interestingly, we found that WT1 is expressed in the majority of the cardiac endothelial cells within the myocardial ventricular layer of the developing heart from E12.5 onwards. In the adult heart only a subset of coronary endothelial cells remain positive for WT1. After myocardial infarction WT1 is temporally upregulated in the endothelial cells of the infarcted area and the border zone of the heart. *In vitro* experiments show that endothelial WT1 expression can be induced by hypoxia. We show that WT1 is associated with endothelial cell proliferation: WT1 expression is higher in proliferating endothelial cells, WT1 knockdown inhibits the proliferation of endothelial cells, and WT1 regulates CyclinD1 expression. Finally, endothelial cells lacking WT1 are not capable to establish a proper vascular network *in vitro*. Together, these results suggest a possible role for WT1 in cardiac vessel formation in development and disease.

Keywords;

Wilm's tumor-1; endothelial cells; epicardium; cardiac development; cardiac regeneration; myocardial infarction

1. Introduction

Myocardial infarction (MI) is the most common type of ischemic heart disease and the leading cause of death in the Western world [1, 2]. Coronary occlusion leads to a reduced supply of oxygen to the cardiac muscle resulting in the loss of billions of cardiomyocytes, thereby impairing cardiac function, which can evolve into heart failure [3, 4]. The regenerative capacity of the mammalian adult heart is inadequate due to limited proliferation of cardiomyocytes [5] and the small number of cardiac progenitor cells present [6]. Current post-MI treatments focus on the recovery of cardiac vascularization to prevent progression of heart failure. Unfortunately, the only cure to effectively restore cardiac contractility is cardiac transplantation. However, the low number of available hearts for transplantation and the logistical and economic hurdles make the search for alternatives both necessary and interesting.

Stem cell-based therapy is emerging and intensively studied as regenerative treatment after MI, since this therapy aims to restore cardiac output by replacing lost cardiomyocytes and repair damaged vascular structures [7]. Although transplantation of cardiac stem cells into the infarcted heart leads to the formation of *de novo* cardiac tissue, the effect on cardiac regeneration is still marginal [8, 9]. This unsatisfying beneficial effect of cell transplantation might be due to *in vitro* culture conditions, which are necessary for cell expansion. Therefore the *in situ* activation of resident progenitor cells within the heart might be an attractive way to enhance the limited endogenous regenerative potential.

A promising niche for resident cardiac progenitor cells is the epicardium. Cells from this epithelial outer layer of the heart are known to play a key role in cardiac development [10-12]. A subset of epicardial cells undergoes epithelial-to-mesenchymal transition (EMT), transforms into epicardium-derived cells (EPDCs) and migrates into the myocardium. Lineage tracing studies have shown the contribution of EPDCs to fibroblasts, smooth muscle cells, endothelial cells and cardiomyocytes [13-18]. In the adult heart, the epicardium is reactivated upon injury and undergoes EMT, forms EPDC analogous to their embryonic counterparts and contributes to regeneration [15, 19-22].

Wilms' tumor-1 (WT1) is a transcription factor which is expressed in the embryonic and reactivated adult epicardium [14, 15, 23-25]. To understand more about the epicardium as a provider of cardiac progenitor cells more insight in the regulation of WT1 is needed. Therefore, as a first step the spatiotemporal expression pattern of WT1 is evaluated during

developmental stages and after cardiac injury. This study shows that cardiac expression of WT1 is not exclusive for epicardial cells and EPDCs. Cardiac endothelial cells in the developing, adult and post-infarcted heart express WT1 in a dynamic fashion. In addition, this study shows that hypoxia induces activation of WT1 in endothelial cells and that proliferation of endothelial cells is regulated by WT1. Together these observations suggest a role for WT1 in cardiac vessel formation in development and disease.

2. Material and methods

2.1. Animals

All Animal experiments were performed in wild type C57BL6 according to protocols approved by the local animal welfare committee of the Leiden University Medical Center. For embryo isolation, pregnant females were sacrificed and embryos were harvested and staged by number of days after vaginal plug, with day of plug considered as embryonic day (E) 0.5.

2.2. Myocardial infarction

Myocardial infarction was induced in 10-12 weeks old mice by ligation of the left anterior descending artery (LAD), as described previously [26]. Briefly, prior to surgery animals were injected intraperitoneal with buprenorphine as analgesic. Animals were anaesthetized with 2%isoflurane/98%oxygen and mechanically ventilated with a Harvard ventilator after intubation. Exposing of the LAD was done via left thoracotomy and opening of the pericardium. Myocardial infarction was induced by permanent ligation of the LAD, proximal to the branches, using a 7-0 prolene ligature. Hearts were isolated at 3, 7 and 28 days post-MI.

2.3. Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated with informed consent according to the protocol of Jaffe et al [27]. Cryopreserved HUVECs were cultured in Endothelial Cell Basal Medium 2(EBM2) medium, supplemented with EGM2 single aliquots (Lonza), on 1% gelatin-coated dishes at 37°C. Cryopreserved immortalized human microvascular endothelial cells (HMEC) were cultured in Endothelial Cell Medium, supplemented with Endothelial Cell Medium Supplement Kit (Cell Biologics), on 1% gelatin-coated dishes at 37°C.

2.4. Hypoxia assay

Endothelial cells were cultured in an atmospheric condition (20% O_2 and 5% CO_2) or hypoxic condition (1% O_2 and 5% CO_2) for 72 hours. WT1 expression was quantified by qPCR.

2.5. WT1 knockdown in endothelial cells

Cells were transduced with lentiviral clones for shRNA-mediated WT1 knockdown, or a scrambled sequence as a control as described earlier (MISSION-libary, Sigma) [28]. Cells were transduced with shRNA lentivirus in the presence of 4µg/ml polybrene for 6 hours and selected with puromycin (1 µg/ml) for 3 days. Confirmation of WT1 knockdown was shown by qPCR (Supplemental Figure S1).

2.6. Proliferation assay

The total of viable HUVECs, with and without WT1 knockdown, as a measure for proliferation was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl tetrazolium bromide (MTT; Sigma Aldrich) assay. HUVECs with and without WT1 knockdown were seeded in triplo in 96 wells plates with 5000 cells per cm^2, ensuring a non-confluent culture at the moment of seeding. At day 1, 2, 3 and 4 cultures were incubated for 3 hours with MTT (50µg/well) in fresh medium. Crystallized formazan dye in the cells was solubilised by adding dimethylsulfoxide (DMSO). Absorbance was measured at 490nm. Quantification was carried out by comparison absorbance between HUVEC with or without knockdown of WT1.

To acquire HUVECS with high and low proliferation, cells were seeded at a density of 5000 cells per cm^2 and cultured for 2 or 7 days respectively. After 7 days the cultures were confluent. WT1 expression was quantified by qPCR.

2.7. Matrigel Tube formation assay

Per well 80000 transduced HMECs were seeded in a Matrigel™ coated 24-well plate with 500µl serum reduced medium. Cells were incubated for 24 hours at 37°C, fixed with 4% paraformaldehyde/PBS at RT and imaged using brightfield microscopy.

2.8. qPCR analysis

Total RNA was isolated using TriPure followed by RNeasy Mini Kit (RNeasy, Qiagen) and treated with DNAse-I (Qiagen) according to the manufacturer's protocol. Subsequently 500ng of RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories). Quantitative real-time PCR for WT1 was performed using Sybr-green mastermix (Bio-Rad Laboratories). The primer sequences against human WT1 and annealing

temperature are available on request. Values were normalized for the housekeeping gene GAPDH. Results were compared using the two-tailed t-test and a significance level of $P<0.05$.

2.9. Luciferase reporter assays

HEK293T cells were seeded in 24-well plates, and transfected with the indicated plasmids. pGL3-CyclinD1-luc was described previously [29] and pGL3 empty served as a control. shRNA plasmids targeting WT1 (TRCN0000040063) and HIF1α (TRCN0000003809) were obtained from Sigma library. Twenty-four hours after transfection, cells were serum-starved for 24 hours and then stimulated with 10%FCS for 24 hours before harvesting. Harvested cells were assayed for luciferase activity with a Perkin Elmer luminometer. The internal control, β-gal expression plasmid, was used to normalize transfection efficiency.

2.10. Immunofluorescence

2.10.1. Sections

Isolated embryonic, neonatal and adult hearts were fixed overnight in 4% paraformaldehyde/PBS at 4°C. After dehydration through a graded series of ethanol, clearing in xylene, hearts were embedded in Paraclean II® (Klinipath), serially sectioned at 6µm and mounted on SuperFrost® Plus microscope slides (Thermo Fisher Scientific). After deparaffinization and rehydration, sections were boiled for 40 minutes in Vector® Antigen Unmasking Solution (Vector) using a pressure cooker. After blocking with 1% BSA in 0,1% Tween-PBS, sections were incubated overnight at 4°C with primary antibodies directed against WT1 (clone 6F-H2, Millipore; Santa Cruz; Calbiochem; see table in Supplemental Figure S3), platelet endothelial cell adhesion molecule (PECAM-1; M-20, Santa Cruz), VE-Cadherin (eBioscience), Von Willebrand factor (DAKO), alpha smooth muscle actin (αSMA, Sigma), proliferating cell nuclear antigen (PCNA, Sigma) and cardiac Troponin I (cTNI, HyTest Ltd). Tyramide Signal Amplification (PerkinElmer) was used, according to manufactory guidelines, to amplify the signal of the primary antibody against WT1, followed by visualisation of the biotin-conjugated Tyramid with Alexa Fluor® 488 streptavidin (Invitrogen). Alexa-conjugated fluorescent secondary antibodies (Invitrogen) were used to visualize the remaining primary antibody binding. All sections were mounted with ProLong® Gold antifade reagent (Invitrogen) containing DAPI.

2.10.2. Cells

After fixation of the cells for 10 minutes with 4% paraformaldehyde/PBS at RT, the cells were blocked in 5% FCS / 0.1% Triton X-100 in PBS for 30 minutes. Incubation with primary

antibodies directed against WT1 (Abcam), PECAM-1 (Sanquin) and PCNA (Sigma) was performed overnight at 4°C. Alexa-conjugated fluorescent secondary antibodies (Invitrogen) were used to visualize the primary antibody binding and cells were mounted with ProLong® Gold antifade reagent (Invitrogen) with DAPI.

3. **Results**

3.1. WT1 expression in the myocardial layer

Expression of the WT1 protein during cardiac development up until postnatal day 21 was mapped by immunofluorescent labelling (Figure 1). As described previously, WT1 expression was found in the proepicardium at embryonic day (E) 9.5 (Supplemental Figure S2), and from E10.5 onwards in the epicardium and EPDCs [14, 24]. The general consensus is that EPDCs loose the WT1 expression as they migrate from the subepicardium into the myocardial layer [14, 23]. We found persistent nuclear WT1 expression in the ventricular myocardial layer from E12.5 onwards starting in the outer rim and expanding towards the luminal site (Figure 1A-C). WT1 staining was confirmed by using multiple antibodies directed against different regions of the WT1 protein (Supplemental Figure S3). Before birth the WT1 expression was restricted to the compact layer (Figure 1B), whereas after birth, WT1 was also expressed in the trabeculae (Figure 1C). The expression of WT1 in the myocardial layer decreased in the second week after birth (data not shown).

3.2. Cardiac endothelial cells express WT1

The ventricular myocardial layer consists of cardiomyocytes, cardiac fibroblasts, smooth muscle cells and endothelial cells [30]. To identify the cell type that expresses WT1, double stainings of WT1 with cell-specific markers were performed (Figure 2). The WT1-positive cells do not express the myocardial specific marker cardiac Troponin I (cTnI) (Figure 2A,C). Surprisingly, the majority of the WT1 positive cells at E13.5 and virtually, all of the WT1-positive cells from E15.5 onwards within the myocardial layer expressed the endothelial cell marker, PECAM-1 (Figure 2A-C). Cardiac endothelial expression of WT1 has been confirmed by co-staining with VE-Cadherin and Von Willebrand factor (Supplemental Figure S4). This endothelial expression of WT1 was present both in the small capillaries and the larger coronary vessels (Figure 2B). WT1 was not observed in smooth muscles cells, as identified by alpha smooth muscle actin expression (Figure 2B). In the adult heart, the expression of

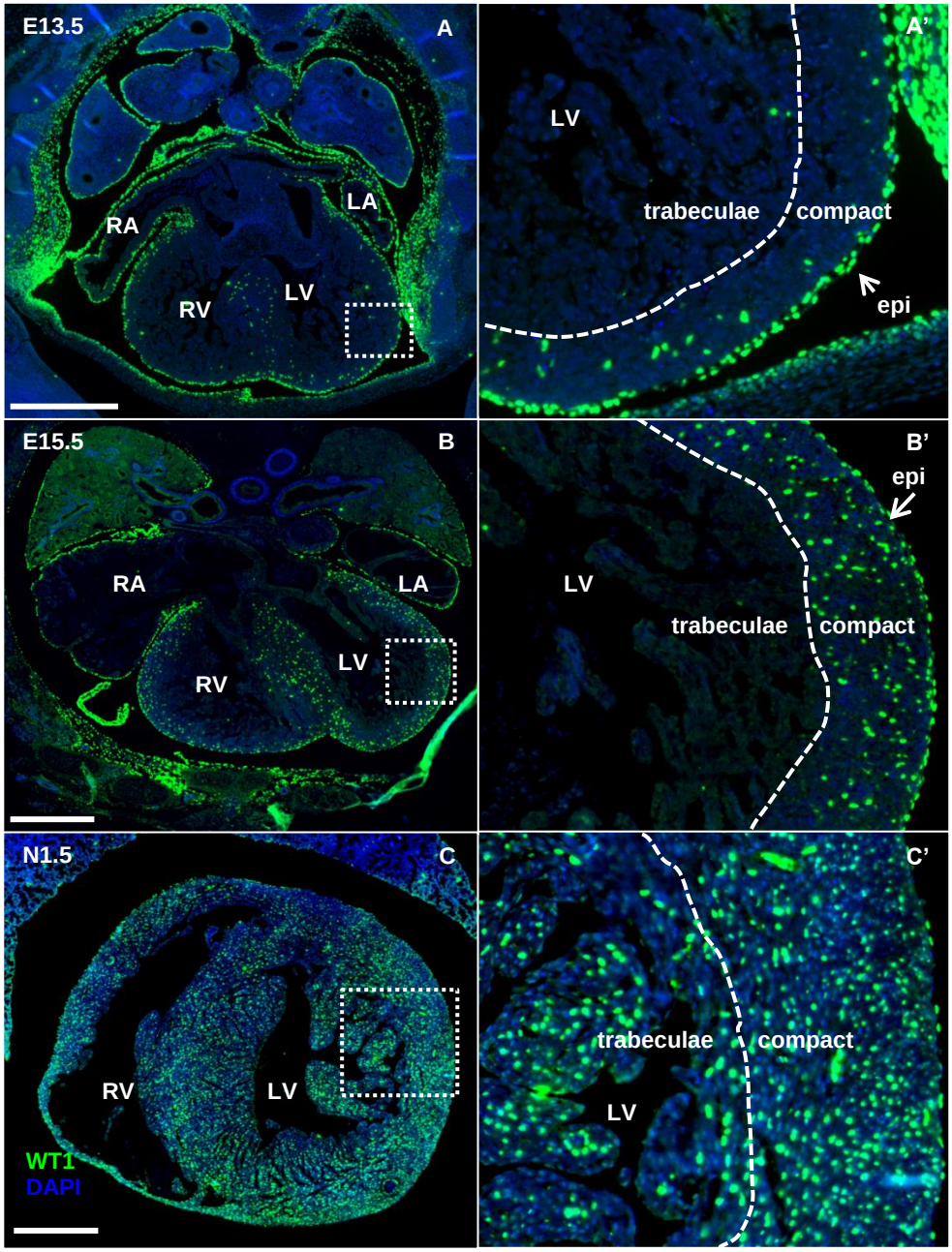


Figure 1. WT1 is expressed in the myocardial layer. a-c. Expression of WT1 is found in the myocardial layer of the embryonic heart from E12.5 onwards. **a'**, **b'**. The expression of WT1 is restricted to the compact zone of the myocardium before birth. **c'**. After birth, WT1 is also expressed in the trabeculae. Epi, epicardium; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle;. Scale bar: 500 um.

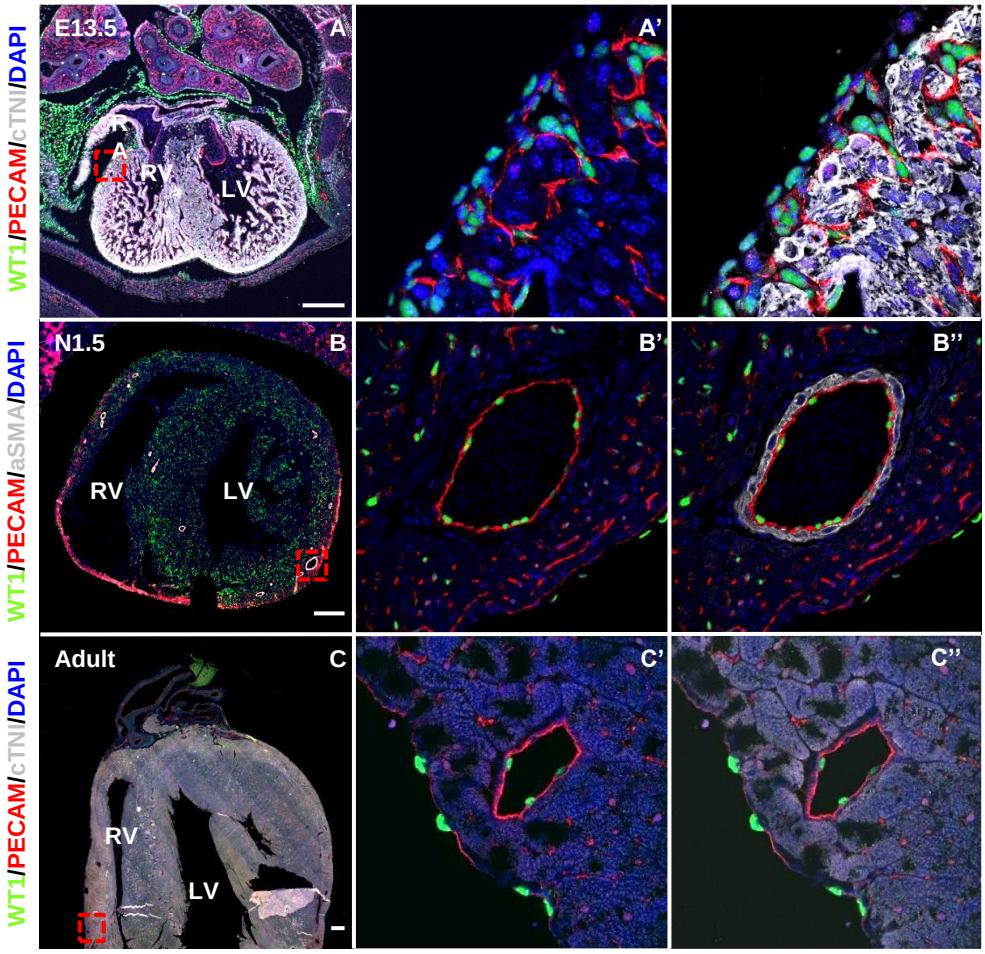


Figure 2. WT1 expression in endothelial cells of the heart. a, **c.** Costainings of WT1 with cTnI and PECAM show the expression of WT1 in the endothelial cells and not in the myocardial cells. **b-b'''**. WT1 and aSMA costaining shows that WT1 is not expressed in the smooth muscle cells of the coronary vasculature. **c-c'''**. During adulthood WT1 expression is restricted to the endothelial cells of the larger blood vessels of the heart. LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. Scale bar: 250 um.

WT1 was at much lower levels compared to embryonic and neonatal stages and appeared to be restricted to the endothelial cells of the larger blood vessels and hardly found in endothelial cells of the smaller vessels (Figure 2C). Our findings show that WT1 expression in the myocardial layer during cardiac development is restricted to the endothelial cells.

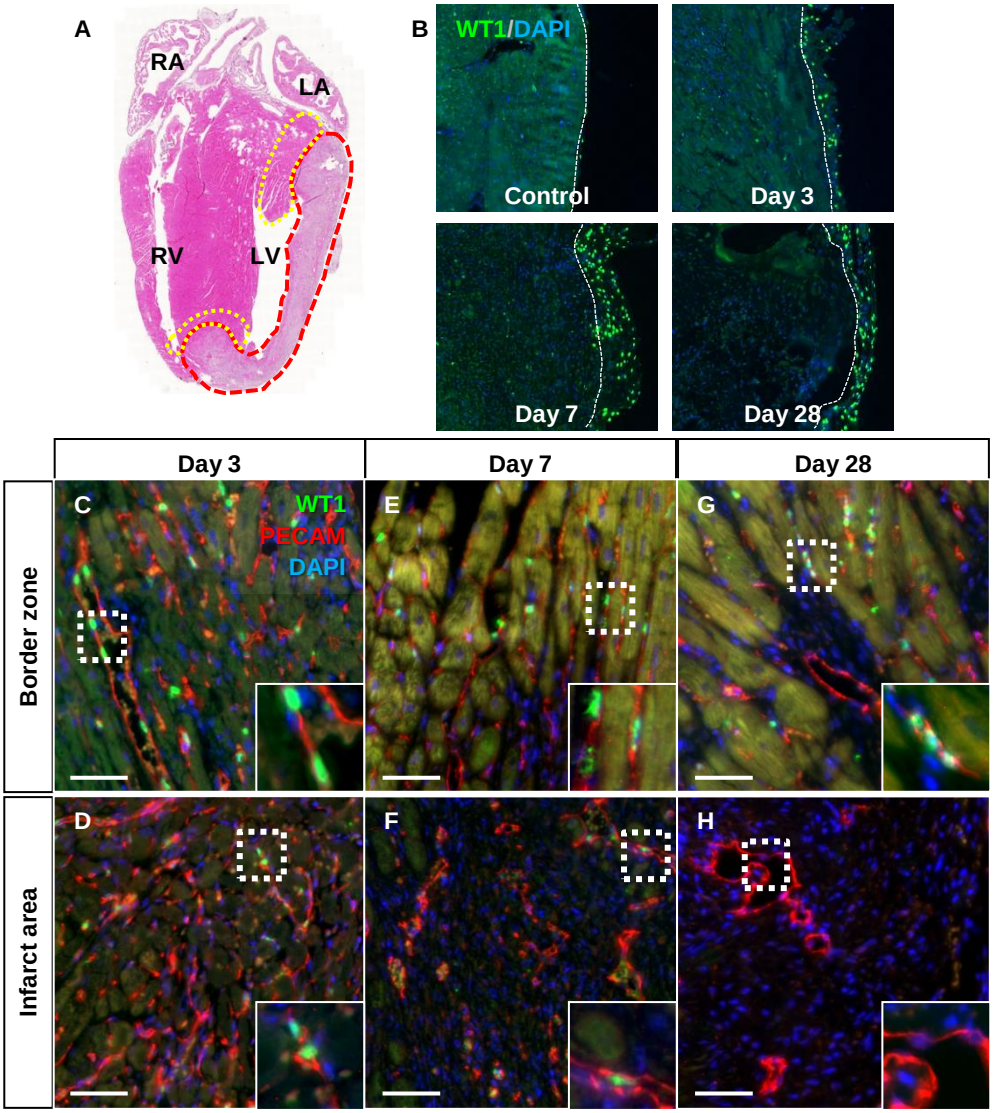


Figure 3. WT1 is temporally upregulated after MI in cardiac endothelial cells. a. H&E staining of an infarcted heart. The infarcted heart can be divided into an infarct area (red dashed line), border zone (yellow dashed line) and unaffected tissue. **b.** After MI the expression of WT1 within the epicardium is upregulated. **c-f.** 3 and 7 days after MI WT1 is upregulated in endothelial cells of both the border zone and the infarct area. **g**, **h.** 4 weeks after MI the expression of WT1 is lost in the endothelial cells in the infarct area, whereas the expression of WT1 in the endothelial cells in the border zone is still present. LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. Scale bar: 50 um.

3.3. Upregulation of WT1 in cardiac endothelial cells after MI

After MI the embryonic expression pattern within the epicardium is reactivated [25], which is illustrated by an upregulation of WT1 (Figure 3B). The expression of WT1 is also upregulated in endothelial cells, suggesting a similar reactivation (Figure 3C-H). Both at day 3 and 7 after MI, upregulation of WT1 was found in the infarct area as well as the borderzone of the heart (Figure 3A,C-F). The expression of WT1 within endothelial cells of the infarct area is lost 4 weeks after MI (Figure 3H). In the borderzone, the expression of WT1 remains present in the endothelial cells (Figure3G).

3.4. *Hypoxia induces WT1 expression in HUVECs*

After MI the infarct area is characterized by a hypoxic environment. To investigate the role of hypoxia on the expression of WT1 in endothelial cells, HUVECs, which express WT1 *in vitro* [31], were cultured and subjected to hypoxia. Culturing HUVECs under hypoxic conditions (1%O_2) for 72h resulted in a significant increase of WT1 mRNA expression compared to HUVECs cultured under normoxic conditions (20%O_2) (Figure 4A). This change in expression was also observed on protein level by fluorescent staining (Figure4B). The upregulation of WT1 in HUVECs, induced by hypoxia, was also found in HMECs (Supplemental Figure S5).

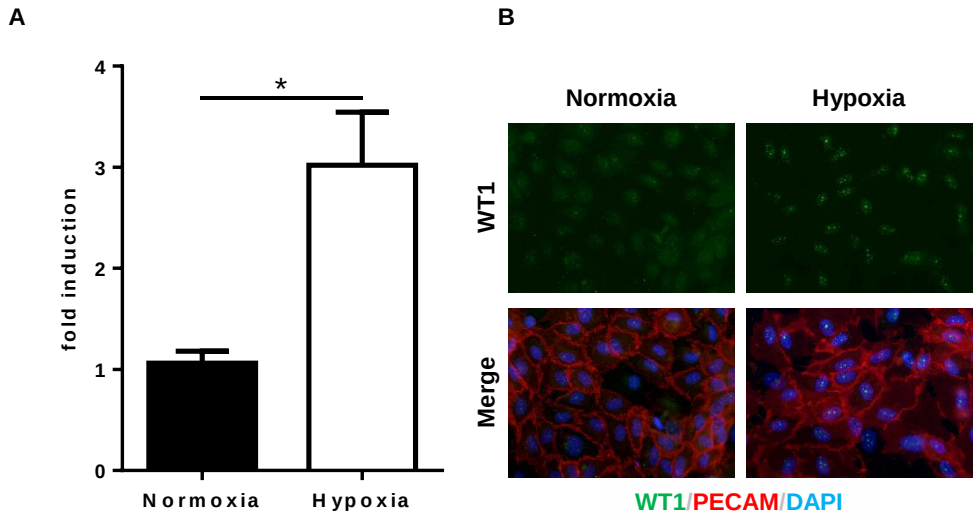


Figure 4. WT1 expression is induced in endothelial cells by hypoxia. HUVECs cultured under hypoxic conditions for 72 hours show an upregulation of WT1 expression on mRNA level (**a**) and protein level (**b**). *p<0,05.

3.5. Regulation of proliferation in HUVECs by WT1

To identify the functional role of WT1 expression in endothelial cells, HUVECs were transduced with shRNAs for WT1 as previously described [28]. To analyze the effect of WT1 inhibition on proliferation a MTT assay was performed. WT1 knockdown in endothelial cells resulted in significant lower MTT values, which indicates a role for WT1 in proliferation (Figure 5A). Based on these results, we hypothesized that HUVECs with high proliferation express higher levels of WT1 then HUVECs with lower proliferation. Confluent cultures were used to establish low proliferating HUVECs whereas non-confluent cultures were used to establish high proliferating HUVECs, which was confirmed by staining with the proliferation marker Ki67 (data not shown). A significant higher expression of WT1 was found in HUVECs with high proliferation compared to HUVECs with low proliferation as determined by qPCR (Figure 5B). Immunofluorescent staining shows co-labeling of WT1 and the proliferation marker PCNA in HUVECs with high proliferation, however, in HUVECs with low proliferation this is hardly detected (Figure 5C). Quantification of the PCNA+ and WT1+ cells showed a significant reduction of the percentage of double-positive cells in the low proliferating cultures compared to the high proliferating cultures (Figure5D). To determine if WT1 is expressed in proliferating endothelial cells *in vivo*, co-labelling of WT1 and the proliferation marker PCNA was performed (Figure 5E). The majority of PCNA expressing endothelial cells were positive for WT1. Previous studies have designated CyclinD1 as a direct target gene of WT1 [32]. A significant higher expression of CyclinD1 was found in control HUVECs compared to HUVECs with WT1 knockdown as determined by qPCR (Figure 5F). Luciferase activity of a CyclinD1 promoter-reporter plasmid was reduced in WT1 knockdown cells compared to control cells. Since WT1 expression is induced by hypoxia and HIF1α response elements are present in the WT1 promoter, we studied the effect of HIF1α knockdown in cells lacking WT1. HIF1α knockdown further decreased WT1-mediated CyclinD1 promoter activity in HEK293T cells (Figure 5G). Together, *in vitro* and *in vivo* data suggest a role for WT1 in endothelial cell cycle regulation.

3.6. WT1 regulates vascular network formation in vitro

To determine the role of WT1 expression in vascular network formation an *in vitro* tube formation assay on Matrigel using HMECs was performed. Knockdown of WT1 resulted in a poorly organized network formation (Figure 6A), suggesting a role for WT1 in angiogenesis. Quantification showed that the number of branching points was significantly reduced in endothelial cells with WT1 knockdown (Fi. 6B). In addition, the number of enclosed vascular circles, a measure of network formation, was strongly reduced in endothelial cells with WT1 knockdown (Figure 6C).

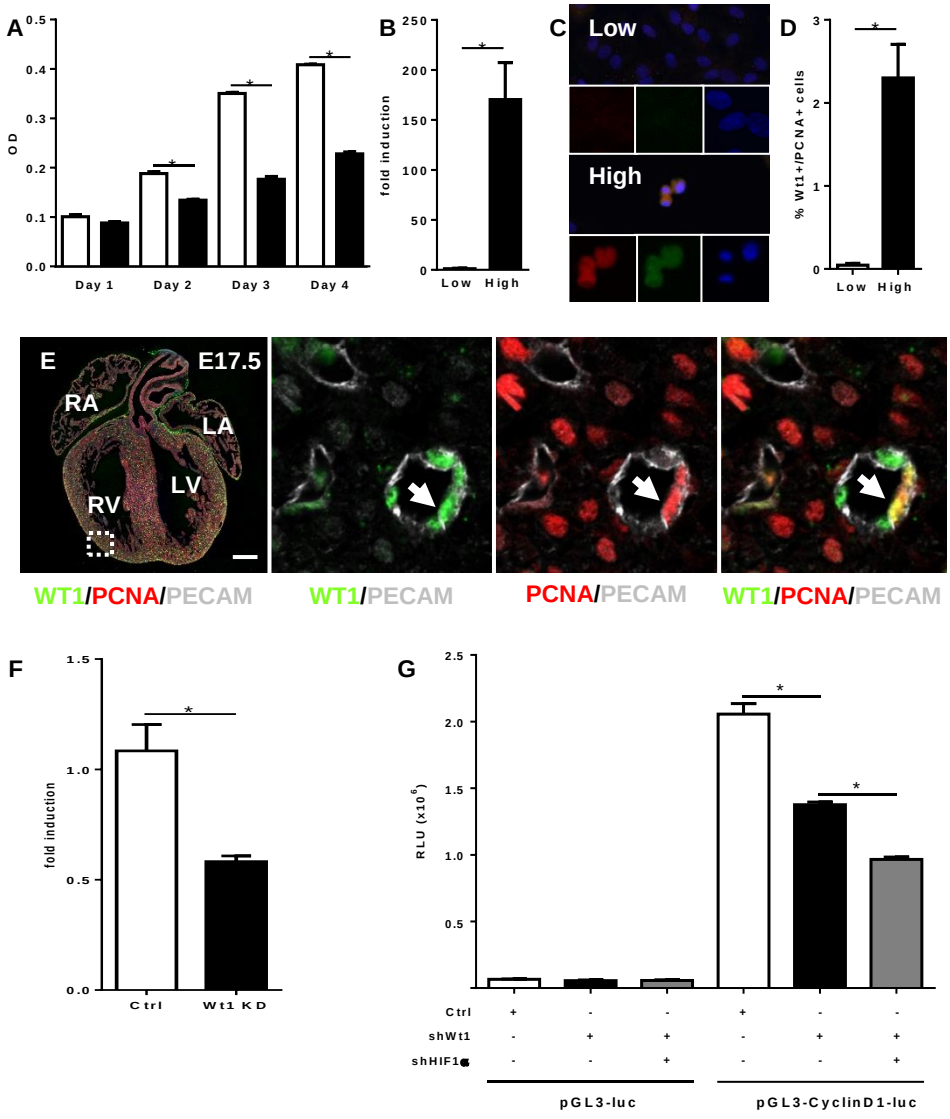


Figure 5. Endothelial cell proliferation is regulated by WT1. a. Quantification of the number of HUVECs by a MTT assay demonstrates that knockdown of WT1 (closed bars) results in a lower proliferation compared to control (open bars). **b.** HUVECs with high proliferation (closed bar) have a higher WT1 expression compared to HUVECs with low proliferation (open bar). **c.** *In vitro* immunofluorescent staining shows co-labeling of the proliferation marker PCNA with WT1 in HUVECs with high proliferation (High) but not in HUVECs with low proliferation (Low). **d.** Quantification of the PCNA+ and WT1+ HUVECs showed a significant reduction of the percentage of double-positive cells in the low proliferating (Low) compared to the high proliferating cultures (High) **e.** In vivo immunofluorescent staining shows co-labeling of the proliferation marker PCNA with WT1 in cardiac endothelial cells. **f.** HUVECs with WT1 knockdown (closed bar) show a reduced expression of CyclinD1 mRNA compared to control HUVECs (open bar). **g.** The CyclinD1 promoter-

reporter plasmid showed reduced induction of luciferase activity in WT1 knockdown HEK293T cells (closed bars) stimulated with FCS compared to control HEK293T cells (open bars). Additional knockdown of HIF1α further decreases CyclinD1 promoter activity in HEK293T cells (gray bars). LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. OD represents the optical density at wavelength 490nm. RLU represents relative luciferase units. *p<0,05, Scale bar: 500 um.

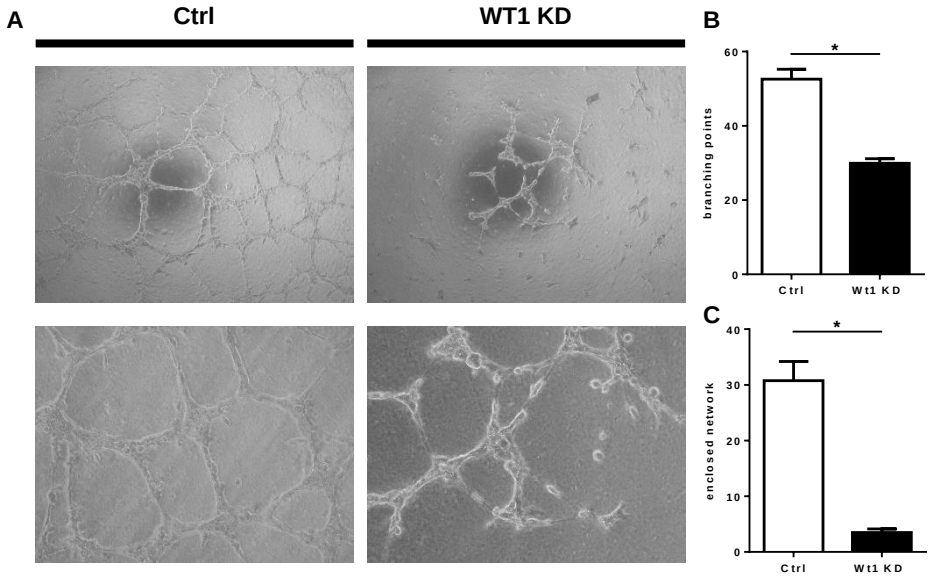


Figure 6. WT1 knockdown in endothelial cells reduces *in vitro* angiogenesis. a. Control HMEC (Ctrl) form tube like structures in Matrigel within 24 hours. WT1 knockdown HMEC (WT1 KD) result in a poorly organized network in Matrigel. **b.** The number of branching points was significantly reduced in WT1 knockdown HMEC (WT1 KD). **c.** The number of enclosed vascular circles was used as a measure of network formation, and was strongly reduced in WT1 knockdown HMEC (WT1 KD).

4. Discussion

This study clearly illustrates the unique expression of WT1 in the cardiac endothelial cells during heart development and after myocardial infarction. These observations broaden the potential involvement of WT1 during vascularization of the heart. In addition, these data demonstrate that WT1 is upregulated in endothelial cells by hypoxia and that endothelial cell proliferation is regulated by WT1.

In the epicardium, WT1 serves as a regulator of epicardial EMT [28, 33-35]. The role of WT1 in the cardiac endothelial cells is, however, less clear. The embryonic expression pattern and the transient upregulation of WT1 in cardiac endothelial cells after injury suggest a possible role for WT1 in formation of the cardiac vasculature. Previously, it has been shown that WT1 is upregulated in endothelial cells in a wide variety of tumors [31]. Suppression of WT1 expression in tumors reduced the angiogenic capacity *in vitro* and transplantation of WT1 knockdown tumor cells show a reduction in the growth rate compared to controls [36]. Further, WT1 has the capacity to directly enhance the expression of the potent angiogenic factor VEGF [37]. Those observations strengthen the hypothesis that WT1 might have an active role in vessel formation. The expression of WT1 might by regulated by hypoxia, which is one of the triggers that initiates coronary vessel formation during cardiac development [38, 39]. In tumor cell lines, WT1 is upregulated upon hypoxia and exposure of rats to hypoxia induced WT1 expression in coronary vessels [37, 40]. WT1 expression in vascular cells has been suggested to be part of the angiogenic response to ischemia [41]. This study shows that endothelial cells upregulate WT1 expression upon hypoxia, indicating that WT1 expression in the endothelial cells of the heart might indeed be triggered by hypoxia. In addition our results show that proliferation of endothelial cells is significantly reduced upon inhibition of WT1. Moreover, we show that knockdown of WT1 decreases CyclinD1 promoter activity. HIF1α further decreases WT1-mediated CyclinD1 promoter activity, confirming that WT1 is a down-stream target of HIF1α. Finally, the hypothesis that WT1 in endothelial cells play a role in vascular formation is supported by the poor formation of the vascular network *in vitro* in endothelial cells with WT1 knockdown.

Based on the concept that WT1 in the heart is exclusively expressed in the epicardium, WT1 is used for epicardial lineage tracing studies [14, 42, 43]. The description of the expression of WT1 in the cardiac endothelial cells appears contradictive to previous lineage tracing studies using WT1-Cre mice. These models showed that WT1-expressing cells give rise to fibroblasts, smooth muscle cells, potentially cardiomyocytes, but very limited to endothelial cells [14, 43]. The observation that activation of the inducible WT1-CreERT2 at E14.5, but not at E9.5, showed a significant labelling of endothelial cells in the reporter mice [44], together with the endogenous expression of WT1 in the cardiac endothelial cells (this study;[45]), suggests that the cardiac endothelial cells are not derived from the WT1-expressing epicardial cells. Other potential sources of the cardiac endothelial cells are the endocardium [46] sinus venosus [47] or non-WT1 expressing population of the epicardium [16]. The contradictory findings might be explained by low expression of WT1 in the endothelial cells of the developing heart compared

to the epicardium. Therefore, Cre expression using the WT1-Cre mouse might not be sufficient enough to induce reporter gene recombination. This study reveals that cardiac endothelial cells express WT1 themselves, explaining the labeling of the cardiac endothelial cells using the WT1-CreERT2 mouse, and hampering the study of the origin of the cardiac endothelial cells using WT1 regulatory regions to control Cre expression [44, 45].

The expression of WT1 in endothelial cells during cardiac development is restricted to the compact zone of the ventricular myocardial layer and appears to follow a gradual pattern towards the lumen of the heart. Only after the birth, WT1 positive cells are observed in endothelial cells of the trabeculae. Similar pattern has been observed tracing the fate of epicardial-derived cells using the WT1-Cre mouse, although these cells were postulated to be epicardial-derived cardiac fibroblasts [43]. Tracking simultaneously the cells fated to become the endothelial cells, smooth muscle cells and fibroblasts of the heart using specific markers would provide better understanding of the clonal and behavioral relationships of the components of the coronary vasculature.

Interestingly, the expression of WT1 is not a general feature of all endothelial cells, as in physiological conditions it is only expressed in endothelial cells of the heart (Supplemental Figure S6). WT1 expression in non-cardiac endothelial cells is observed under pathological conditions in some tumors [48] and *in vitro* conditions [31]. As a consequence of the contracting myocardium, the endothelial cells of the heart are subjected to continuous cycles of mechanical forces which are distinct than for other endothelial cells. High levels of mechanical forces can stimulate endothelial cell apoptosis [49, 50]. Potentially, WT1 also has a role in preventing endothelial cell apoptosis during the formation and maturation of the coronary vasculature as WT1 has been shown to be a regulator of apoptosis [51]. More studies are required to test this hypothesis.

Taken together, these data show endogenous cardiac endothelial expression of WT1 during development and after injury. The expression pattern of WT1 together with the effect of WT1 on the proliferation of endothelial cells demonstrate that, in addition to the essential role of WT1 in the epicardium, WT1 has an active role in cardiac endothelial cells.

Acknowledgements

We thank Kirsten Lodder for expert technical assistance and Drs. Beerend Hierck, Anke Smits and Noortje Bax for stimulating discussions. This study is supported by the Dutch Heart Foundation, the Netherlands Institute for Regenerative Medicine and Smartcare, part of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation.

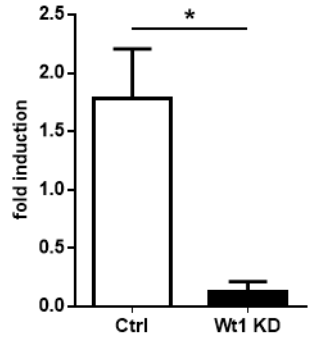
Disclosures

None

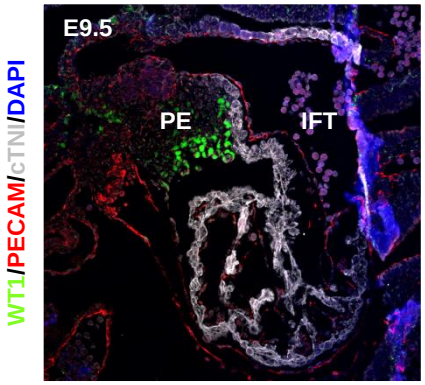
Abbreviations

αSMA	alpha smooth muscle actin
cTnI	cardiac Troponin I
E	embryonic day
EMT	epithelial-to-mesenchymal transition
EPDC	epicardium-derived cells
HUVEC	human umbilical vein endothelial cells
LAD	left anterior descending artery
MI	myocardial infarction
N	neonatal day
PCNA	proliferating cell nuclear antigen
PECAM-1	platelet endothelial cell adhesion molecule
VEGF	vascular endothelial growth factor
WT1	Wilms' tumor-1

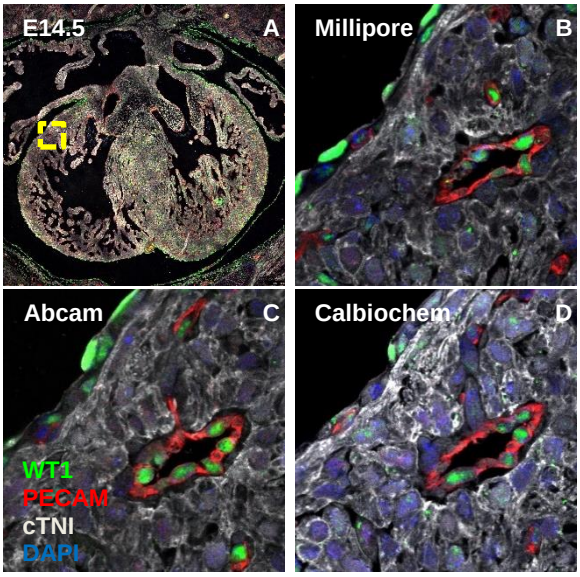
Supplementary Figures



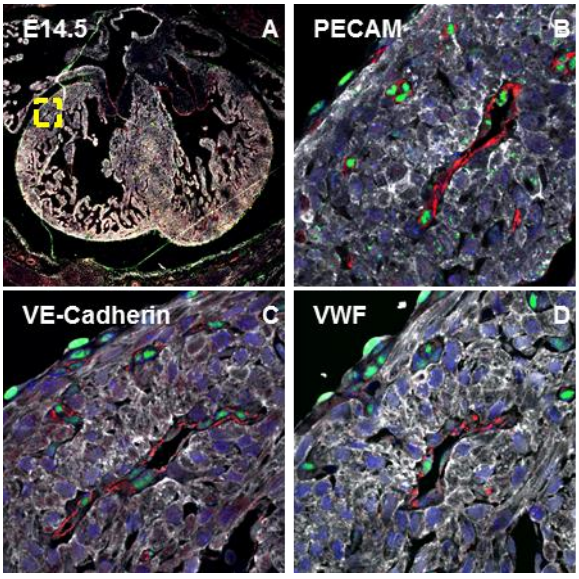
Supplementary Figure S1. Confirmation of WT1 knockdown in HMEC by qPCR.



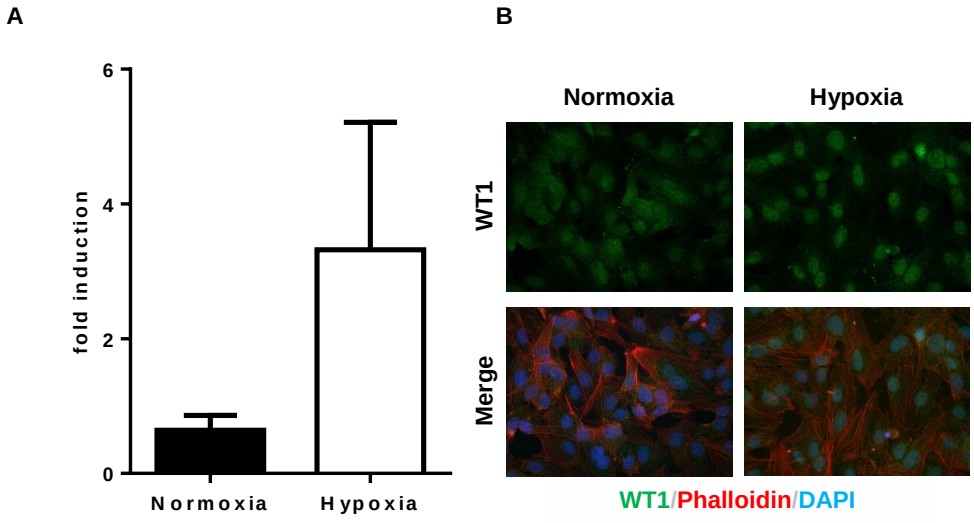
Supplementary Figure S2. WT1 is expressed in the proepicardium at E9.5. PEO, proepicardium; IFT, inflow tract.



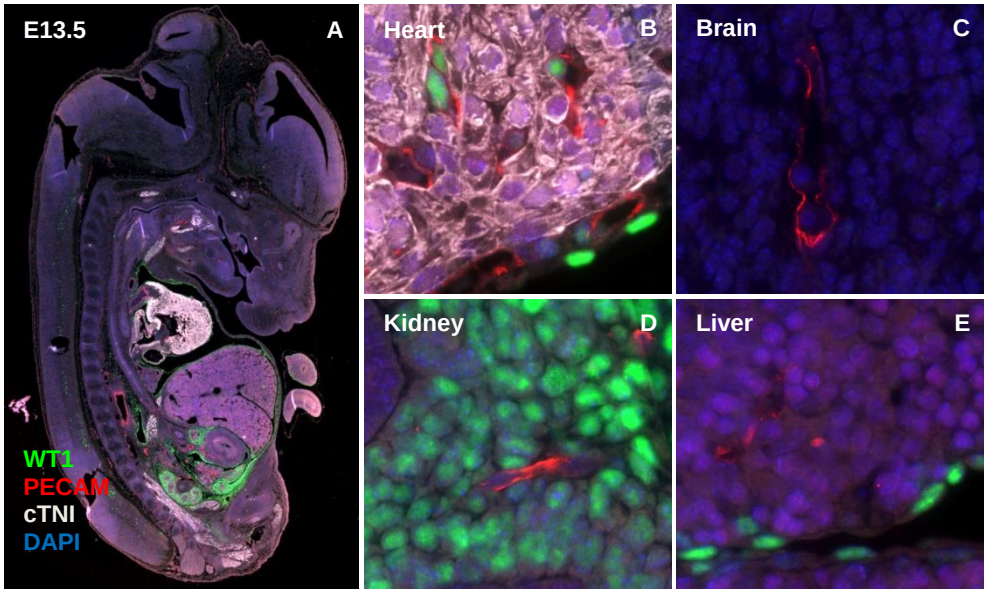
Supplementary Figure S3. Confirmation of the specificity of the WT1 antibody. a-d. Costainings of different WT1 antibodies with cTnI and PECAM show the expression of WT1 in the endothelial cells. **b.** WT1 (Millipore - Catalog # 05-753) and PECAM costaining. **c.** WT1 (Abcam - Catalog # ab89901) and PECAM costaining. **d.** WT1 (Calbiochem - Catalog # CA1026) and PECAM costaining.



Supplementary Figure S4. Confirmation of WT1 expression in endothelial cells of the heart. Costainings of WT1 (green) and cTnI (white) with **a**, **b.** PECAM (red) **c.** VE-Cadherin (red) **d.** VWF (red).



Supplementary Figure S5. WT1 expression is induced in endothelial cells by hypoxia. HMECs cultured under hypoxic conditions for 72 hours show an upregulation of WT1 expression on mRNA level (**a**) and protein level (**b**).



Supplementary Figure S6. WT1 expression during development is restricted to the endothelial cells of the heart. **a.** Costainings of WT1 with cTnI and PECAM show the expression of WT1 in the developing mouse embryo at E13.5. WT1 is expressed in the endothelial cells of the heart (**b**) but not in endothelial cells of the brain (**c**), kidney (**d**) and liver (**e**).

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3

The roadmap of WT1 protein expression in the human fetal heart

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Abstract

The transcription factor Wilms' Tumor-1 (WT1) is essential for cardiac development. Deletion of WT1 in mice results in disturbed epicardial and myocardial formation and lack of cardiac vasculature, causing embryonic lethality. Little is known about the role of WT1 in the human fetal heart. Therefore, as a first step, we analyzed the expression pattern of WT1 protein during human cardiac development from week 4 till week 20. WT1 expression was apparent in epicardial, endothelial and endocardial cells in a spatiotemporal manner. The expression of WT1 follows a pattern starting at the epicardium and extending towards the lumen of the heart, with differences in timing and expression levels between the atria and ventricles. The expression of WT1 in cardiac arterial endothelial cells reduces in time, whereas WT1 expression in the endothelial cells of cardiac veins and capillaries remains present at all stages studied. This study provides for the first time a detailed description of the expression of WT1 protein during human cardiac development, which indicates an important role for WT1 also in human cardiogenesis.

Keywords;

human cardiac development; epicardium; Wilms' Tumor-1; endothelial cell; endocardial cell

1. Introduction

The zinc-finger transcription factor Wilms' Tumor-1 (*WT1*), originally described as a tumor suppressor gene [1], has an essential role in proper formation of the heart [2]. The heart holds a unique position during development, since it is the first functional organ arising in the embryonic body. In humans, three weeks after fertilization, a hollow tube is formed consisting of cardiomyocytes at the outside and endocardial cells on the inside, separated by a layer of cardiac jelly [3]. By a complicated process of cardiac looping, the linear heart tube remodels into a four-chambered organ [3]. The outside of the heart will be covered by an epithelial layer known as the epicardium [4].

The epicardium arises from the proepicardium, a heterogeneous population of progenitor cells at the venous pole of the heart [5]. It forms by the migration of the epicardial cells over the developing tube, which results in a single cell layer covering the heart. A subset of epicardial cells undergoes epithelial-to-mesenchyme transition (EMT), and forms the epicardium-derived cells (EPDCs) [6, 7]. EPDCs migrate into the subepicardium and compact myocardium where they differentiate into fibroblasts and smooth muscle cells of the cardiac vessels [5, 6]. Avian and mouse embryos with hampered epicardial outgrowth exhibit severe cardiac developmental problems, including a thin myocardial wall and malformation of the coronary vasculature [8-12], demonstrating the essential role for the epicardium in cardiac development.

In mice, WT1 is often used as a marker for the epicardial layer, since nuclear expression of WT1 protein is found in embryonic and reactivated adult epicardium [10, 11, 13-15]. The first cardiac expression of WT1 protein is described in the proepicardium, followed by the presence in the epicardium [2, 16]. WT1 is also present in cardiac endothelial cells during development and after injury [17, 18], suggesting that the defects in the WT1 knockout models might not only be of epicardial origin.

The expression of WT1 protein is well documented for the developing mouse and avian heart [2, 10, 13-15, 18, 19]. Although the expression of WT1 protein has been described during human embryonic development, little is known in human cardiogenesis [4, 20, 21]. To reveal a potential role for WT1 in human cardiac development, we generated a detailed description of the expression pattern of WT1 protein in the hearts of well-staged human embryos.

2. Material and methods

2.1. Human tissue

All human fetal tissue was obtained after individual permission using standard informed consent procedures and conforms to the Declaration of Helsinki. Approval of the medical ethics committee of the Leiden University Medical Center was granted. Fetal hearts were collected after elective abortion at 4 to 20 weeks post fertilization. Isolated fetal tissue was fixed in 4% paraformaldehyde/PBS at 4°C, embedded in Paraclean II® (Klinipath) and serially sectioned at 6µm for analysis.

2.2. Immunofluorescence staining

The protocol used for immunofluorescence staining is described previously [18]. Briefly, slides were deparaffinised, rehydrated and subjected to heat-induced epitope retrieval with Vector® Antigen Unmasking Solution (Vector). Sections were incubated overnight at 4°C with primary antibodies directed against WT1 (CAN-R9(IHC)-56-2, Abcam), platelet endothelial cell adhesion molecule-1 (PECAM-1; M-20, Santa Cruz), vimentin (Cell Signaling), alpha smooth muscle actin (αSMA, Sigma) and cardiac Troponin I (cTnI, HyTest Ltd). Tyramide Signal Amplification (PerkinElmer) was used in order to amplify the WT1 signal. Alexa Fluor® 488 streptavidin (Invitrogen) ensured visualization of the biotin-conjugated Tyramid. All other primary antibodies were visualized with Alexa-conjugated fluorescent secondary antibodies (Invitrogen). Sections were mounted with ProLong® Gold antifade reagent (Invitrogen) containing DAPI.

3. Results

3.1. Epicardial covering of human hearts with WT1 positive cells

To unravel the expression pattern of WT1 protein during human cardiogenesis, multiple developmental stages were analyzed by immunofluorescent labelling. At 4 weeks of gestation the heart was largely covered by WT1 positive cells (Figure 1a-c). However, the absence of WT1 expressing cells at parts of the outer layer of the heart, as seen on the outflow tract (OFT) and ventricle (Figure 1a-c), suggests that the epicardium has not fully enveloped the myocardium yet. At week 5 of development the complete outer layer of the heart is now expressing WT1 (Figure 1d-f), indicating that epicardial covering of the heart is completed.

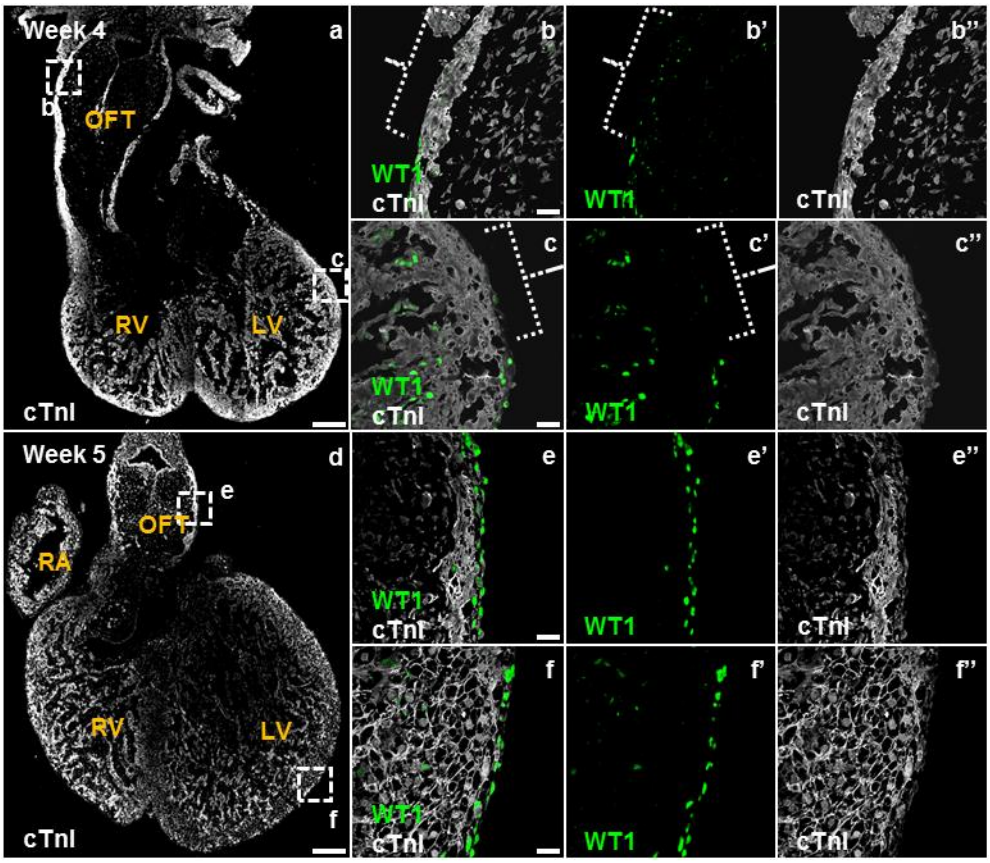

Figure 1. Epicardial covering of the human heart with WT1 positive cells is completed at week 5 of development. Sections are stained for WT1 (green) and the myocardial marker cTnI (gray). **a.** Overview of the human embryonic heart at week 4 of development. At this stage, parts of the OFT (**b**), and the ventricle (**c**) are not covered by WT1 positive cells (dashed line) **d.** Overview of the human embryonic heart at week 5 of development. At this stage the OFT (**e**) and ventricle (**f**) are fully covered by WT1 positive cells. LV, left ventricle; OFT, outflow tract; RA, right atrium; RV, right ventricle. Scale bar; a, d 250µm; b, c, e, f 50µm.

3.2. WT1 protein is dynamically expressed in the myocardial layer of the ventricles

While at week 5 of gestation WT1 is readily observed in the epicardial layer, it is only sporadically expressed in the compact myocardium (Figure 2a, d). In addition, WT1 expression is present in the trabecular region (Figure 2a, g), where it surrounds the myocardium of the trabeculae and co-labels with the endothelial cell marker PECAM-1 indicating that the cells expressing WT1 are the endocardial cells (Figure 2g). When development proceeds the expression of WT1 expands from the epicardium into the compact myocardium towards the trabeculae, where it starts at the base and extends towards the distal

tip of the trabeculae (Figure 2a-c, h, i and Supplemental Figure S1). Interestingly this coincides with vascularization of the ventricular wall (Figure 2a-c and Supplemental Figure S1). On the other hand, the endocardial WT1 expression has diminished over time and is almost absent at week 20 (Figure 2g, h, i). A marked thickening of the subepicardial space is observed, which contains strong WT1 expressing cells (Figure 2b, c, e).

To identify the cell type that expresses WT1 protein, we performed co-labelling with cell-type specific markers. Co-labelling shows that initially, WT1 positive cells in the compact and trabecular myocardium are mostly PECAM-1 negative (Blue arrow in Figure 2d, h). At later stages, however, increasing overlap of WT1 and PECAM-1 expression is observed, indicating that WT1 is expressed by endothelial cells (White arrow in Figure 2e, f, i). Although the majority of WT1 expressing cells within the myocardium are endothelial cells, a part of the WT1 positive cells at week 20 remains negative for PECAM-1 (Blue arrow in Figure 2f, i). These WT1+/PECAM-1- cells in the myocardial layer found at each stage are positive for the non-cardiomyocyte marker vimentin (Supplemental Figure S2), indicating that they are not cardiomyocytes. These cells exhibit overall a less intense expression of WT1 in comparison with the cardiac endothelial cells (Figure 2f, i).

3.3. WT1 protein is dynamically expressed in the myocardial layer of the atria

At week 5 of development, WT1 was virtually only observed in the epicardium and not in the myocardial layer or in the endocardial cells (Figure 3a, d, g). WT1 expression is observed in the myocardial layer of the atria and in some endocardial cells at week 10 (Figure 3b, e, h). The presence of WT1 within the myocardial layer of the ventricular side wall has expanded at week 20 (Figure 3c, f); this was seen to a lesser degree in the thinner free wall of the atrium (Figure 3i). Although WT1 was present in endocardial cells of the atria, this expression is not to the same extent as during the early development of the ventricles (Figure 3g, h, i). Similar as in the ventricles, the expression of WT1 is found in both endothelial cells (White arrow in Figure 3e, f) and cells that do not express PECAM-1 (Blue arrow in Figure 3e, f) with an overall higher WT1 expression in the endothelial cells (Figure 3e, f). In contrast to the homogeneous expression of WT1 throughout the myocardium of the ventricles, the atria display parts of myocardium which show hardly any expression of WT1, even in the last stages that have been analyzed in this study (Figure 3b, c).

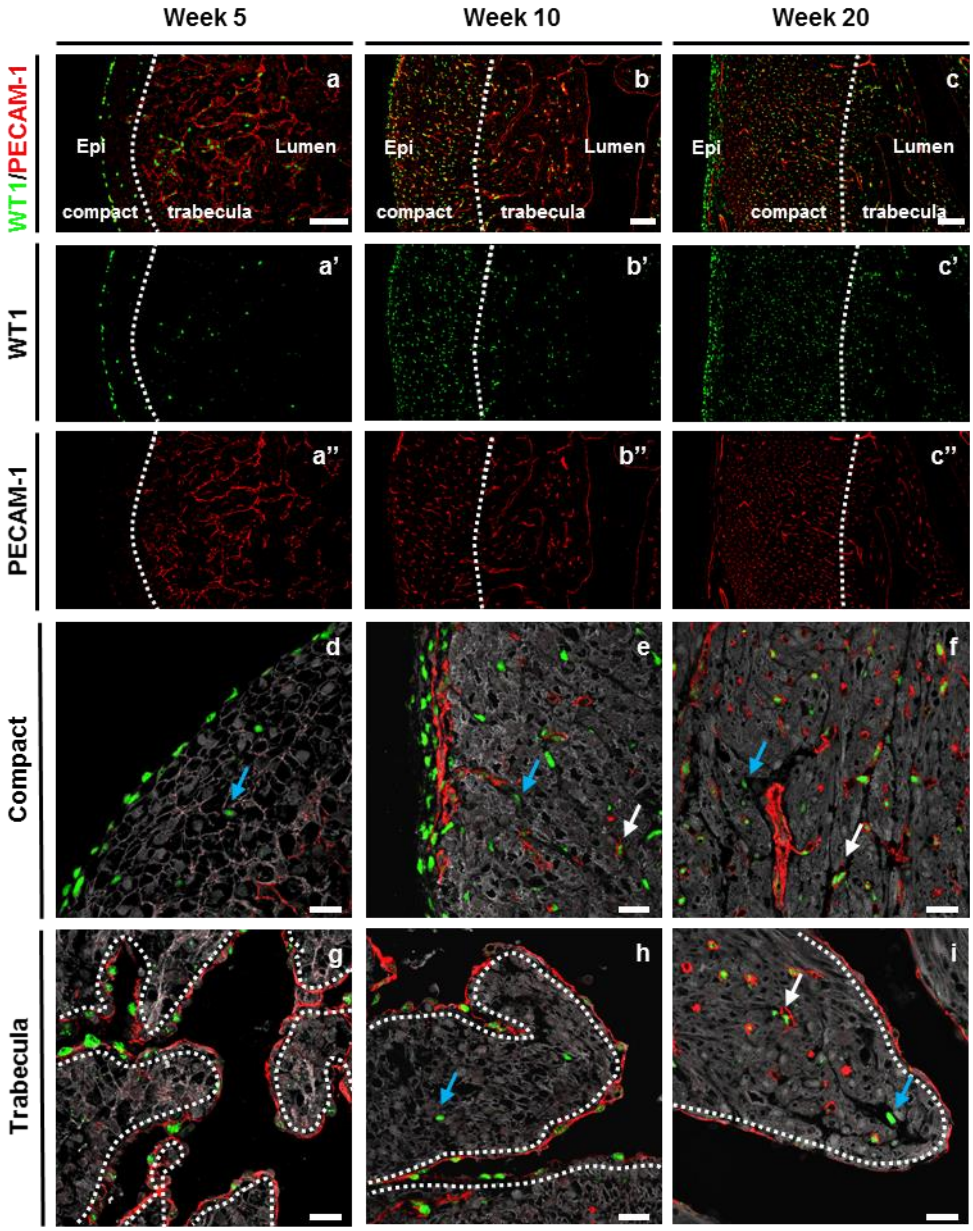

Figure 2. The dynamic expression of WT1 protein in the ventricle. a-c. Overview of the left ventricular wall showing the expression of WT1 (green) expanding from the epicardium towards the luminal side of the heart as development progresses, a similar pattern is seen with PECAM-1 expressing endothelial cells (red). **d-i**. Magnification of both the compact (**d-f**) and trabecular (**g-i**) layers of the myocardium. **a, d, g.** At week 5 the expression of WT1 is observed in the epicardium (**d**), sporadically in the compact layer of the myocardium (**d**) and the endocardial cells of the trabecular region of the heart (**g**). **b, e, h.** At week 10 the expression of WT1 is increased in the compact layer of the myocardium (**b, e**)

and less in the endocardial cells of the trabecular region of the heart (**h**). **c, f, i.** At week 20, the expression of WT1 is also observed in the myocardium of the trabeculae of the heart (**i**). As development proceeds increasing overlap of WT1 and PECAM-1 expression is observed. Arrows point to WT1 expression in PECAM-1 positive (White arrow) or PECAM-1 negative (Blue arrow) cells. The dashed lines in **g-i** indicate the outer borders of the trabecula. The PECAM-1 positive cells at the inner and outer side of the dashed lines are respectively endothelial and endocardial cells. Epi, epicardium. Scale bar; a-c 100µm; d-i 10µm.

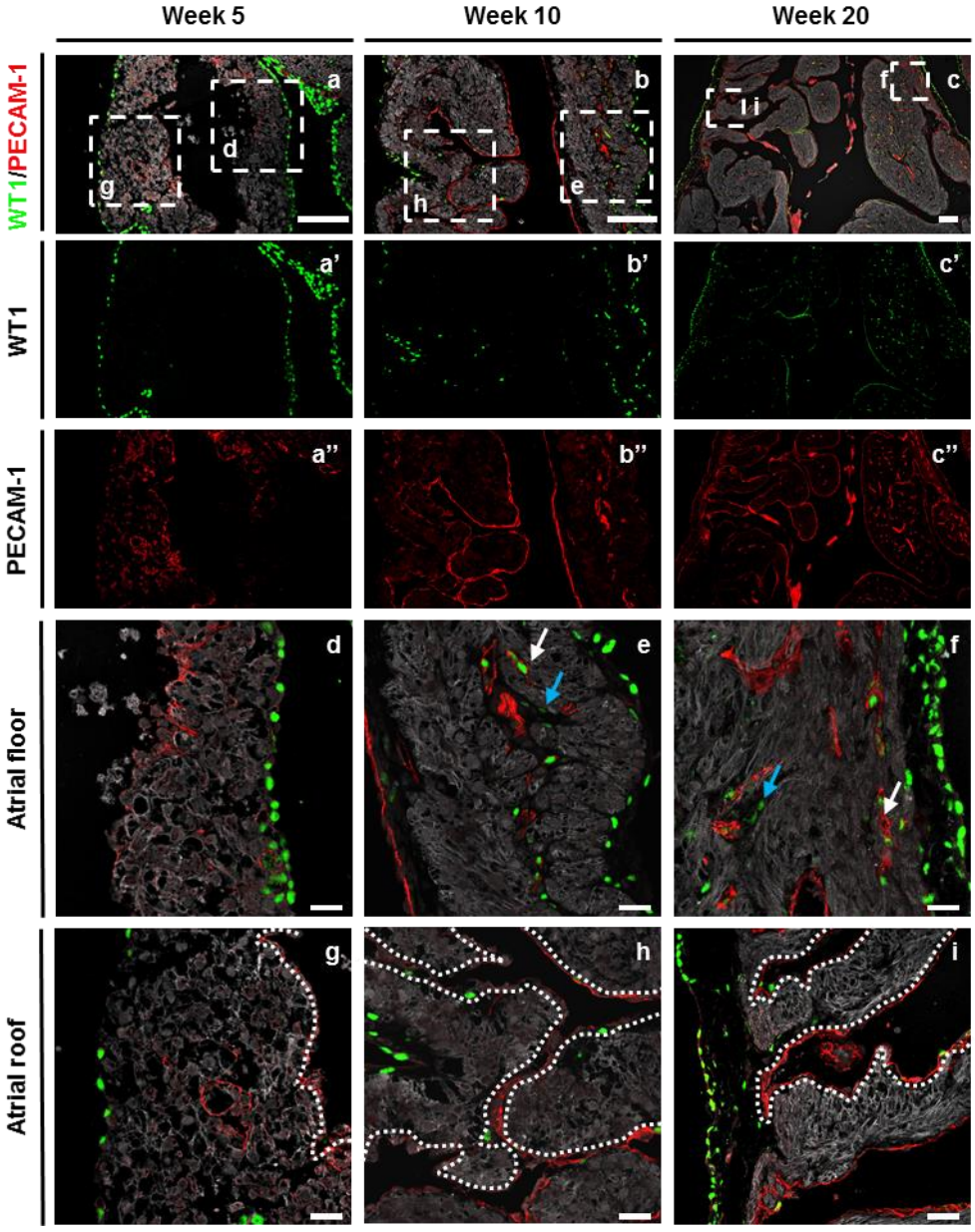

Figure 3. The dynamic expression of WT1 protein in the atrium. a-c. Overview of the left atrial wall showing the expression of WT1 (green) expanding from the epicardium towards the luminal side of the heart as development progresses, a similar pattern is seen with PECAM-1 expressing endothelial cells (red). **d-i.** Magnification of both the ventricular side wall (**d-f**) and free wall (**g-i**) of the atrium. **a, d, g.** At week 5 the expression of WT1 is observed in the epicardium. **b, e, h.** At week 10 the expression of WT1 is now also observed in the compact layer of the heart (**b, e**) and sporadically in the endocardial cells of the trabecular region of the free wall of the atrium (**g**). **c, f, i.** At week 20 the expression of WT1 in the myocardial layer of the ventricular side wall has expanded. As development proceeds, increasing overlap of WT1 and PECAM-1 expression is observed. Arrows point to WT1 expression in PECAM-1 positive (White arrow) or PECAM-1 negative (Blue arrow) cells. The dashed lines in **g-i** indicate the outer borders of the trabecula. The PECAM-1 positive cells at the inner and outer side of the dashed lines are respectively endothelial and endocardial cells. Note, no WT1 expression is found in the endocardium or myocardium at week 5 (**d, g**). Scale bar; a-c 100µm; d-i 40µm.

3.4. Expression of WT1 protein in developing coronary vessels during development

Since many WT1 cells express endothelial markers and WT1 has a significant role in proper vascular network formation [8, 9, 11, 18], we examined the presence of WT1 protein in the developing cardiac vessels in more detail. Coronary arteries are identified by an endothelial layer, surrounded by smooth muscle cells (Figure 4c, d). At week 10 of development WT1 is expressed in endothelial cells of both arteries and veins of the heart (Figure 4a, c). Expression of WT1 in cardiac arteries becomes less at later stages and at week 20 the expression is almost absent (Figure 4d). In contrast, the expression in veins and capillaries remains present (Figure 4b).

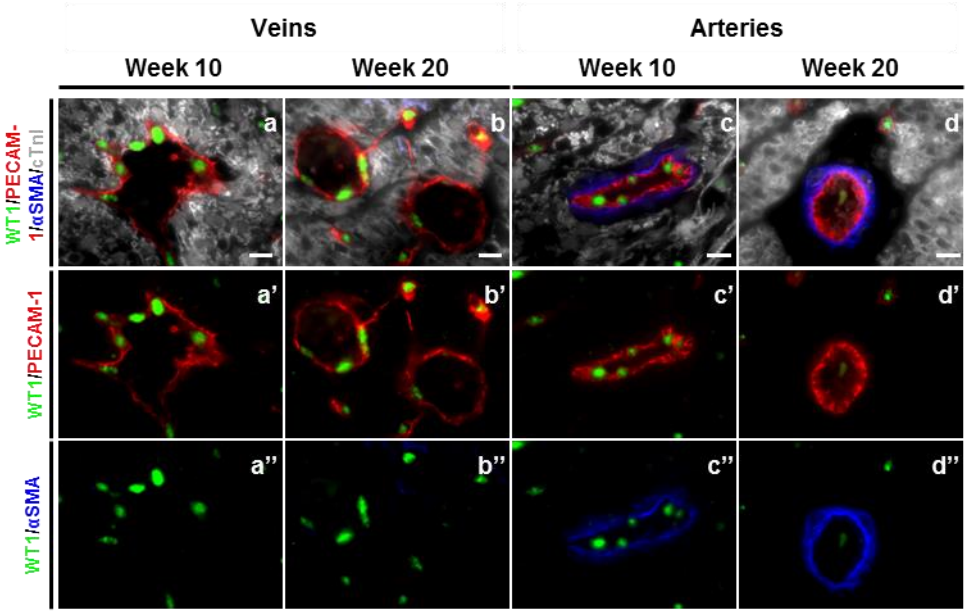

Figure 4. WT1 protein expression in cardiac arteries decreases with ongoing development. At week 10 of development expression of WT1 (green) is observed in endothelial cells (red) of cardiac veins (**a**) and in endothelial cells but not in smooth muscle cells (blue) around cardiac arteries (**c**). At week 20 of development expression of WT1 is still observed in endothelial cells of cardiac veins (**b**), whereas WT1 is not observed in endothelial cells of cardiac arteries anymore (**d**). Scale bar; 10µm.

4. Discussion

In this study we show for the first time a detailed description of the WT1 protein expression pattern in human cardiogenesis. The expression of WT1 enables us to follow the process of epicardial covering of the myocardium and supports that the formation of epicardium in human is completed before the 6th week of development [4]. In addition, we observed spatiotemporal expression of WT1 in endothelial and endocardial cells of the ventricles and atria (Figure 5).

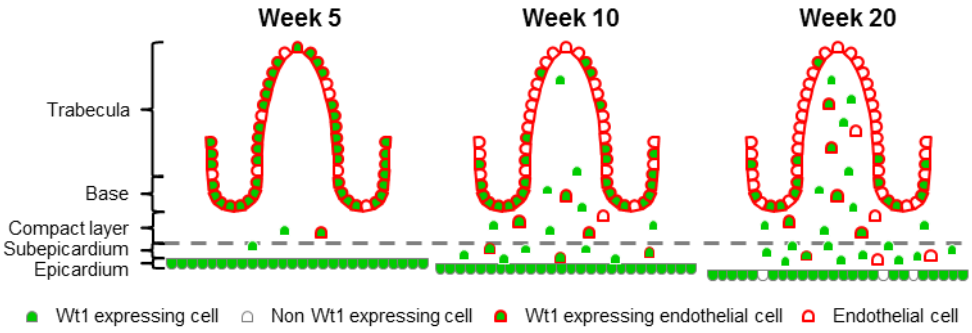

Figure 5. Working model of WT1 protein expression within the heart. The schematic overview shows that with ongoing development the expression of WT1 expands from the epicardium towards the lumen of the heart. WT1 is initially present in the endocardial cells of the trabeculae, but disappears over time. At the base of the trabeculae the expression remains visible. The first cells expressing WT1 within the myocardium do not co-localize with PECAM-1. With ongoing development more WT1 expression is observed in endothelial cells.

Apart from the differences in dimensions, the human and murine heart are anatomically highly comparable throughout cardiac development [22, 23]. We have shown that in both human and mouse the expression of WT1 is present in the epicardium, subepicardium and myocardial layer with a gradual expansion towards the luminal site (this study and [18]) (Figure 5). Similar to mouse, the WT1 expression in the human myocardial layer is predominantly present in endothelial cells.

A remarkable difference between mice and human is the endocardial expression of WT1 we observed in the human heart during early development. Whereas in mouse only sporadic expression can be observed in the endocardium ([18] and unpublished observation), in human many endocardial cells covering the ventricular trabeculae express WT1 in the initial stages of the development.

In addition, in mouse WT1+/PECAM-1- cells are only observed in the myocardial layer at the initial stages when WT1 expression starts to be seen in the myocardium. In human, even at the latest stage examined (week 20), WT1+/PECAM-1- cells were still present in the myocardial wall. The intensity of WT1 expression within the PECAM-1 negative cells appears to be less in comparison with the expression within PECAM-1 positive cells. Because WT1 is expressed in endothelial cells during angiogenesis [18, 24, 25] this observation might suggest that the WT1+/PECAM-1- cells are progenitor cells that eventually will differentiate into endothelial cells. Alternatively, WT1+/PECAM-1- cells are EPDCs that will gradually lose the expression of WT1 during migration from the epicardium into the myocardium and become cardiac fibroblasts or smooth muscle cells [15].

Another difference in cardiac expression of WT1 between human and mouse is the decrease in WT1 expression in endothelial cells of the cardiac arteries during human fetal development, whereas in mice, this happens after birth [18]. *In vitro* data showed that direct contact between endothelial cells and smooth muscle cells stimulates endothelial cell quiescence and maturation [26]. Arterial endothelial cells are surrounded by a solid layer of smooth muscle cells, which might be responsible for the downregulation of WT1. The timing differences in downregulation of WT1 in endothelial cells of the human and murine cardiac arteries might be caused by difference in maturation of the smooth muscle cells, which occurs in mouse after birth and in human before birth.

In the atria the expression of WT1 is delayed compared to the ventricles. This is illustrated by the absence of WT1 expression in the atria and the presence of WT1 in the ventricles both in the myocardium and endocardium at week 5 of development. Besides the delayed expression, also the number of WT1 positive endocardial cells in the atrium is less compared to the ventricle. We measured the thickness of the myocardial layers with and without WT1 expression and found that WT1 expression appears in myocardial layers thicker than approximately 70 µm (data not shown). Thickening of the myocardial walls of the ventricles is ahead of the atria, which might suggest earlier hypoxia in the myocardial layer of the

ventricles. In addition, the atrial walls eventually become less thick in comparison to the ventricles. As WT1 is induced by hypoxia [18, 27], the thickness of the myocardial layer might be a determined factor for the expression of WT1 in the myocardial wall.

It has been shown that WT1 is upregulated in endothelial cells of tumors and that suppression of WT1 reduces the angiogenic capacity [24, 25]. Further, knockdown of WT1 in endothelial cells reduces the network formation capacity *in vitro* [18, 28]. In addition, VEGF, a highly potent angiogenic factors, is one of the direct targets of WT1 [29]. Interestingly, the promoter of PECAM-1 contains multiple WT1-binding elements [30]. PECAM-1 ensures intercellular junction between endothelial cells and plays a role in the formation of vascular networks [30, 31] and vascular remodeling [32]. Remodeling implies changes in the extracellular matrix including the degradation of the matrix by matrix metalloproteases (MMPs) which enables vascular migration and network formation [33]. MMP9 is directly regulated by WT1 [24]. The expression of WT1 in cardiac endothelial cells during development in human suggests a conserved process in which WT1 plays an important role in the formation and remodeling of the cardiac vasculature.

The endocardium and the epicardium play an important role in myocardial compaction [34, 35]. The congenital cardiomyopathy left ventricular non-compaction might therefore be a result from the abnormal formation and signaling of the epicardium or endocardium. *Wt1* knockout mice develop an incomplete epicardial layer and suffer from ventricular non-compaction [2, 9]. Although both the endocardium and epicardium in human express WT1 (this study), a direct correlation between WT1 mutations and cardiac congenital anomalies has not been shown yet.

Taken together, WT1 is present in cells that are crucial for the proper formation of the cardiac vessels and ventricular myocardium, i.e. epicardial, endothelial and endocardial cells. These observations suggest an important role for WT1 in human cardiac development and could potentially contribute to an increased understanding of the pathogenesis of congenital heart defects.

Acknowledgements

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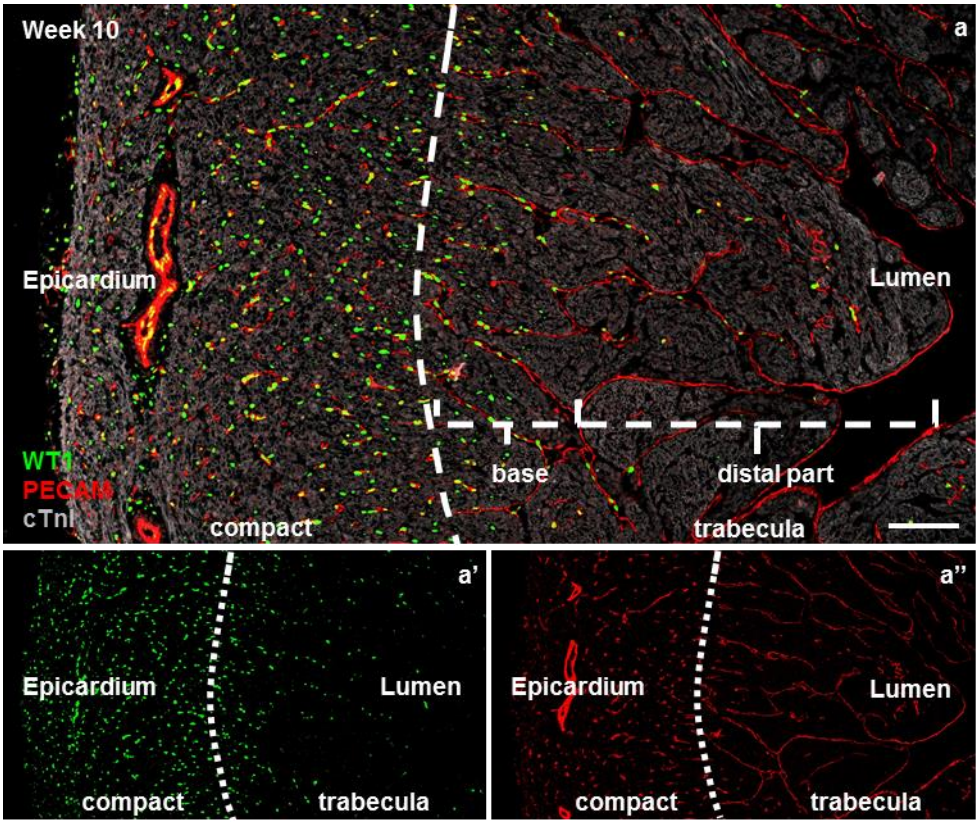
Disclosures

None.

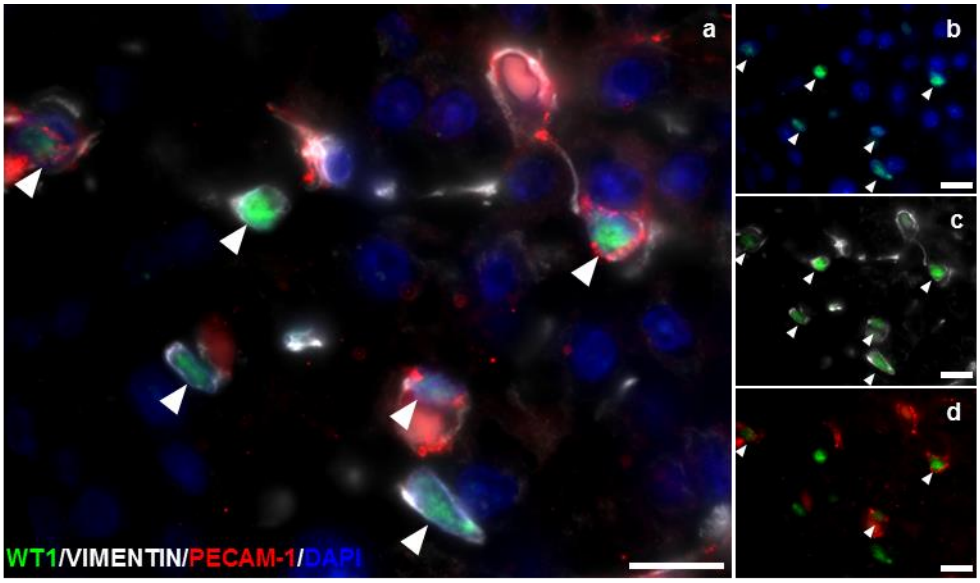
Abbreviations

αSMA	alpha smooth muscle actin
BSA	bovine serum albumin
cTnI	cardiac Troponin I
E	embryonic day
EMT	epithelial-to-mesenchymal transition
EPDC	epicardium-derived cell
MMP	matrix metalloprotease
PECAM-1	platelet endothelial cell adhesion molecule-1
WT1	Wilms' Tumor-1

Supplemental Figures



Supplemental Figure S1. WT1 protein expression at week 10 of development. The fetal heart is stained for WT1 (green), PECAM (red) and the myocardial marker cTnI (gray). The ventricle at week 10 shows a clear gradient of WT1 expression depicting the expansion of WT1 expression from the epicardial layer to the lumen of the heart during development. The majority of the WT1 positive cells in the compact layer of the myocardium are endothelial cells. At the base of the trabecula a few cells are positive for WT1, but do not express PECAM-1. The endocardial cells, on the other hand, do express WT1. In the distal part of the trabeculae WT1 and PECAM-1 expression is absent, and WT1 expression in endocardial cells is almost absent. Scale bar; 100μm.



Supplemental Figure S2. WT1 protein is expressed by non-myocardial cells. a-d. Representative image of the compact myocardium of a week 20 heart showing WT1 expressing cells (white arrowhead) within the myocardial layer co-labelled with the non-myocardial marker vimentin**.** A subset of WT1 expressing cells within the myocardial layer show co-labelling with the endothelial marker PECAM-1. White arrowhead, co-labelling with WT1. Scale bar; 25µm.

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4

WT1 in cardiac development and disease

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Abstract

The heart is essential for realizing the distribution of oxygen and nutrients throughout the body. Therefore, the heart is the first organ to develop and is already functional in its most primitive structure during embryogenesis. Recent studies indicate that the transcription factor Wilms' tumor-1 (WT1) is important for many aspects of cardiac development. WT1 expression is first observed in the proepicardium, a group of progenitor cells that give rise to a mesothelial sheet covering the heart, the epicardium. WT1 expression in epicardial cells is required for their epithelial-to-mesenchymal transformation forming epicardium-derived cells that will contribute to the formation of coronary vessels and interstitial fibroblasts. Endothelial cells within the heart also express WT1, whereas the endothelial cells in other parts of the embryo do not. The endothelial expression of WT1 during cardiac development is likely to be important for vascular formation. After cardiac injury, WT1 is temporally upregulated in the epicardium and in the endothelial cells in the infarcted area and border zone, which points to a potential important role for WT1 in cardiac repair and regeneration. In this chapter, we describe the many faces of WT1 within the heart.

Keywords

Cardiac development, Cardiac regeneration, Endothelial cells, Epicardium, Wilms' tumor-1

1. Introduction

The pleiotropic molecule Wilms' tumor-1 (WT1) is a transcription factor that was first discovered in renal tumors [1, 2]. It contains four zinc-finger motifs at the C-terminus which are important for the binding of DNA to activate gene expression. Besides its function to activate the transcription of genes, WT1 is also involved in posttranscriptional processes [3]. The expression of WT1 is essential during development of multiple organs, including kidneys, gonads, spleen, and the heart [4, 5]. In the developing heart, WT1 is strongly expressed in the outer layer, i.e. the epicardium, and in the cardiac endothelial cells. After myocardial infarction this WT1 expression reemerges in both lineages. In this chapter, we describe the dynamic expression of WT1 during development and after cardiac injury. We focus on the multiple roles of WT1 including epithelial-to-mesenchymal transformation and angiogenesis in cardiac development, repair and regeneration.

2. WT1 in cardiac development

2.1. The expression of WT1 in the epicardium during early cardiac development

Knockout of Wt1 in mice revealed that this transcription factor plays an essential role in cardiac development. In the absence of WT1, the vasculature of the heart is not formed, which disturbs proper formation of the heart and therefore results in prenatal death [3, 4]. Already in the developing embryo, the heart is essential for the supply of oxygen and nutrients. Therefore, it is the first organ to develop and function during embryogenesis. The heart has a mesodermal origin and is formed through gastrulation. The earliest recognizable structure is the primitive heart tube, which is formed at embryonic day (E) 8 in mouse, corresponding with day 21 post fertilization in human. This hollow structure consists of two cardiac cell populations, namely cardiomyocytes on the outside and endothelial cells on the inside which are separated by cardiac jelly [5] (Figure 1). The primitive tube elongates and undergoes rightward looping between E8.5 and E10.0 in mouse (day 23 and 28 in human) (Figure 1). Subsequent remodeling of the heart involves formation of septa and valves, formation and expansion of the chambers resulting in a septated four-chambered heart [6, 7] (Figure 1). Even though the heart is already functional at an early stage of fetal development, the growth and maturation of the heart is not completed until after birth [8].

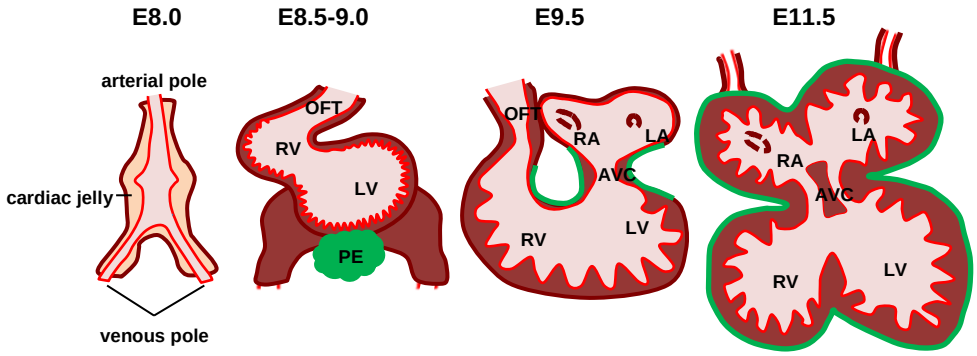

Figure 1. Early cardiac development and the expression of WT1. E8.0 The primitive tube is formed by cardiomyocytes (brown) on the outside and endothelial cells (red) on the inside which are separated by cardiac jelly. **E8.5 – E9.5** The primitive tube elongates and undergoes rightward looping. At the venous inflow tract of the developing heart the proepicardium arises. **E11.5** A septated four-chambered heart is created by formation of septa and valves. The expression of WT1 throughout development is indicated in green. AVC, atrioventricular cushion; LV, left ventricle; OFT, outflow tract; PE, proepicardium; RV, right ventricle.

A third population of cardiac cells envelopes the heart during development, the epicardium. The cells forming the epicardium are derived from the proepicardium, a heterogeneous transient cluster of cells [9], located at the base of the inflow tract of the developing heart (Figure 1). The earliest expression of WT1 in the heart is found in cells of the proepicardium, at E9.5 [10-12] (Figure 1 and 2a). Proepicardial cells reach the bare heart tube by formation of a tissue bridge or by free-floating vesicles and spread over the myocardium covering the complete heart, forming the epicardium. Recently, we have shown that the covering of the myocardium with WT1-positive cells occurs in a dorsal to ventral pattern between E9.75 and E10.5. In addition, the epicardial covering of the right ventricle is delayed and less dense compared to the left ventricle. Complete covering of the myocardium with WT1-positive cells is established in the mouse by E12.5 [11] (Figure 1). During human cardiac development, complete covering of the myocardium is observed at week 5 [13]. After enveloping the entire heart, the epicardium remains positive for WT1 both in mouse and in human during embryonic development [3, 10-18] (Figure 1 and Figure 2a).

After the formation of the epicardium, a subset of epicardial cells undergoes epithelial-to-mesenchymal transition (EMT), resulting in epicardium-derived cells (EPDCs) [19, 20] (Figure 2a). Lineage-tracing studies have shown that EPDCs migrate into the myocardium and

contribute to cardiac fibroblasts, endothelial and smooth muscle cells of the cardiac blood vessels, and cardiomyocytes [9, 12, 19, 21-23], although the contribution to the latter is still under debate [11, 14, 16, 24] (Figure 2b). The contribution of WT1-positive cells to endothelial cells however is minimal [12].

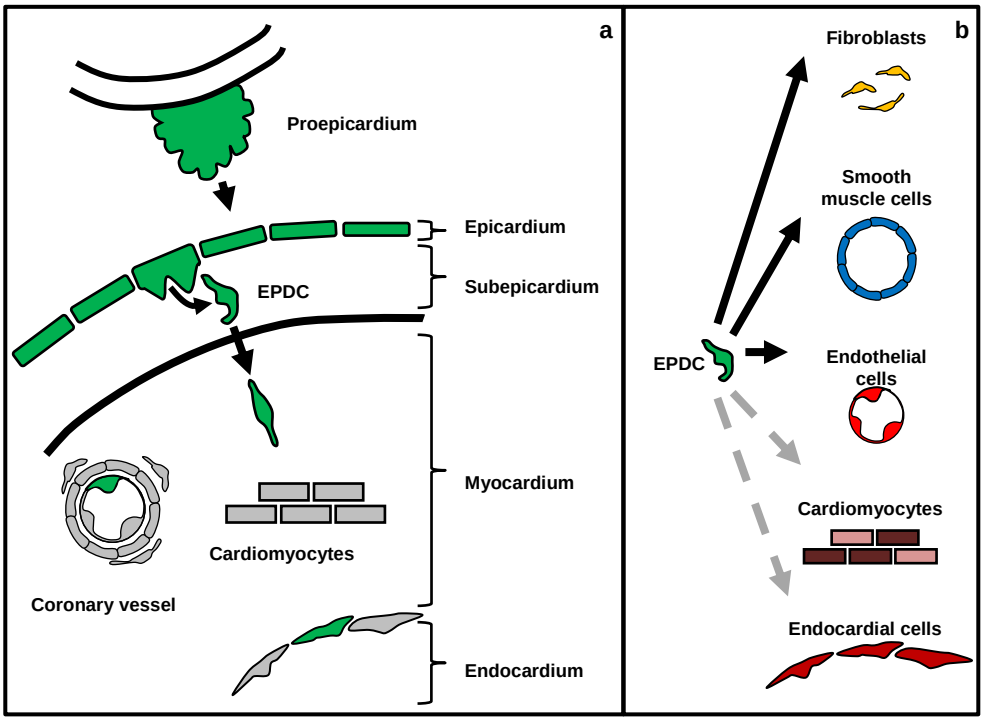

Figure 2. Expression of WT1 and the fate of epicardial cells. a. The epicardium arises from the proepicardium. A subset of cells undergoes EMT and migrates into the subepicardium and myocardium. WT1 (green) is expressed by cells in the proepicardium, epicardium, EPDCs, endothelial cells and endocardial cells. **b.** . EPDCs contribute to the formation of fibroblasts, smooth muscle cells and endothelial cells, however, the contribution to the formation of endocardial cells and cardiomyocytes is controversial. Cardiac fibroblasts can be subdivided into interstitial fibroblasts, perivascular fibroblast and valvular interstitial cells.

2.2. The role of WT1 in embryonic epithelial-to-mesenchyme transition

In addition to the essential role of WT1 in the formation of the epicardium [3, 4], several studies have shown that WT1 serves as a regulator of epicardial EMT. *Wt1*-knockout mice show a reduction of subepicardial mesenchyme [3]. In addition, epicardial cells are unable to detach from the epicardium, and EPDCs do not migrate into the subepicardium [18, 25].

Epicardial EMT is regulated by WT1 via multiple genes and pathways. Knockdown of *Wt1* in epicardial cells reduced the expression of SNAIL and SLUG, whereas the downstream target E-cadherin was upregulated. SNAIL and SLUG are key regulators of the EMT process and have an inhibitory effect on the expression of the epithelial marker E-cadherin [26]. WT1 directly promotes EMT by enhancing the expression of SNAIL and inhibiting the expression of E-cadherin [25]. Furthermore, WT1 was shown to be a positive upstream regulator of the Wnt pathway, which influences diverse aspects of cardiogenesis and is important for epicardial EMT [27]. Knockdown of *Wt1* resulted in a decrease of the downstream effectors of the Wnt pathway, *Ctnnb1* and *Lef1* [18]. WT1 directly regulates RALDH2, an enzyme involved in retinoic acid (RA) synthesis, and is expressed in the epicardium [18, 28, 29]. RA signaling is essential during embryonic development, and RA deficiency has been shown to result in cardiac abnormalities, similar to the phenotype of *Wt1*-knockout mice [20, 30]. In chicken, induction of RA signaling in EPDCs results in upregulation of WT1 [31], indicating a positive feedback loop between WT1 and RA.

Although most *in vivo* studies suggest an inducing role for WT1 in epicardial EMT, *in vitro* repression of WT1 induced the transformation of both human and mice cobblestone-like EPDCs into spindle-shaped cells, indicating a context-dependent and possibly, concentration-dependent function of WT1 [32, 33].

2.3. Expression of WT1 in cardiac endothelial cells

The classical consensus is that EPDCs lose their expression of WT1 during their migration from subepicardium into the myocardial layer [12, 34]. We have recently shown that the expression of WT1 is not restricted to the epicardium and subepicardium but is also present in the myocardial layer during development from E12.5 onward in mice and from week 5 after fertilization in humans [11, 13, 14] (Figure 2a and Figure 3). This expression of WT1 starts at the epicardial side and expands toward the luminal site of the heart [13, 14]. The myocardial wall of the heart consists of an outer condensed part, the compact layer, and an inner loosely arranged part, the trabecular layer. Before birth, the expression of WT1 within the myocardium in mice is restricted to the compact layer and expands into the trabeculae in the neonatal heart [14]. In human, the trabecular expression of WT1 is already observed before birth [13]. The cellular composition of the heart comprises cardiomyocytes, cardiac fibroblasts, smooth muscle cells, and endothelial cells [35]. Interestingly, in the myocardial layer of the heart, we found the expression of WT1 in endothelial cells of both small capillaries and the larger coronary vessels in mice and human [13, 14] (Figure 2a and Figure 3). In mice, the expression

of WT1 in endothelial cells is still present at neonatal stages but gradually decreases before adulthood [14]. In human, the expression of WT1 in endothelial cells of at least the arteries decreases before birth [13]. Another difference between mice and human is the widespread expression of WT1 in endocardial cells at early stages during human cardiogenesis (Figure 2a). The differences in WT1 expression between mouse and human can be explained by the difference in maturation time during pregnancy, as well as the differences in dimensions [36, 37].

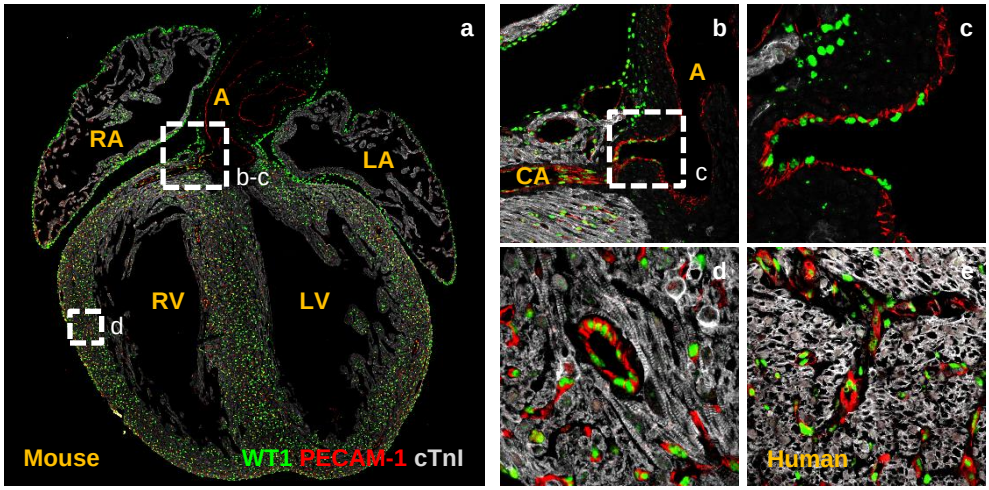

Figure 3. WT1 is expressed by cardiac endothelial cells during development. a. Overview of the mouse heart at E17.5. WT1 is expressed in the epicardium and endothelial cells (co-expression of WT1 (green) and PECAM-1 (red)). **b, c.** Magnification of the connection between aorta and coronary vessel show the specific cardiac expression of WT1 in endothelial cells. **d.** WT1 is expressed in the vasculature of the heart during murine development. **e.** WT1 is expressed in the vasculature of the heart during human development. A, aorta; CA, coronary artery; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle.

3. WT1 in the infarcted heart

The epicardial expression of WT1 decreases after birth and remains at low levels during normal homeostasis (Figure 4a). The endothelial expression of WT1 in the adult heart is low and mostly observed in some capillaries and cardiac veins [14]. In contrast to the quiescent appearance during adult physiological conditions, after myocardial infarction (MI) the epicardial and endothelial cells re-express WT1 (Figure 4d).

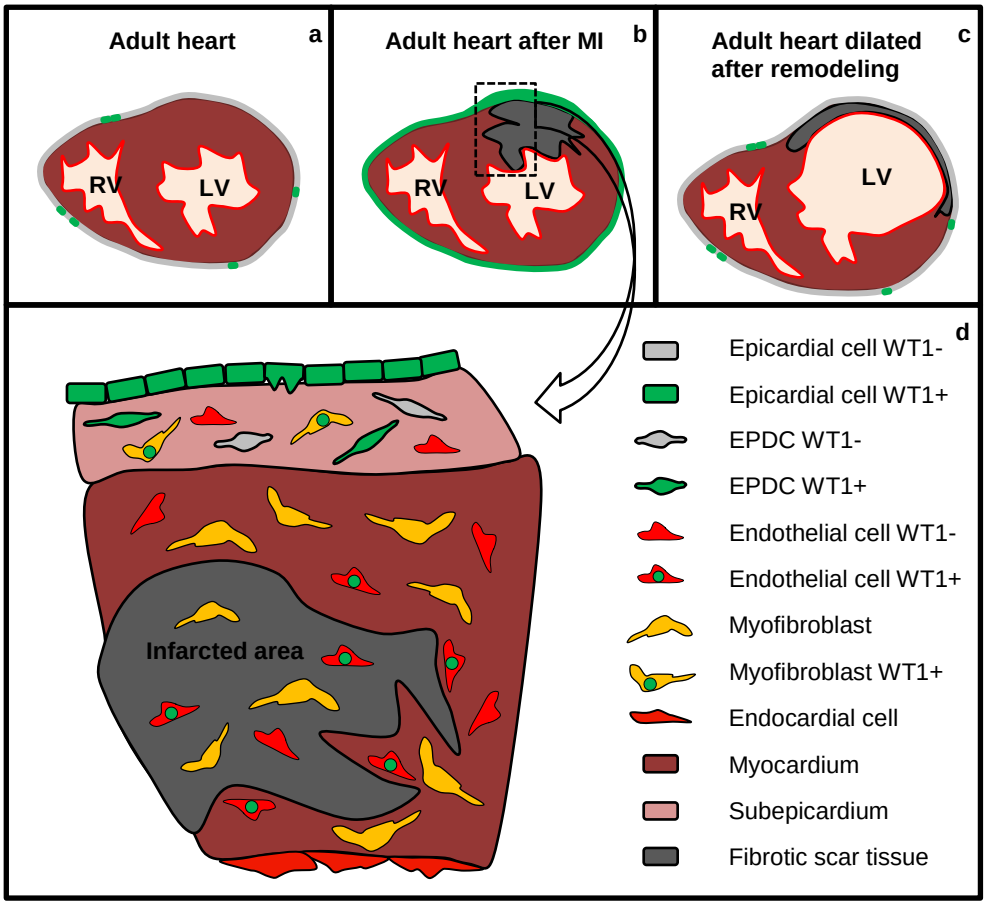

Figure 4. Expression of WT1 in the adult and injured heart. a. The epicardial expression of WT1 is almost absent in the adult heart. **b.** Myocardial infarction results in the loss of cardiomyocytes and the replacement by fibrotic scar tissue. After myocardial infarction the epicardial expression of WT1 is re-activated in the entire heart. The thickening of the epicardium is most pronounced at the infarcted area. **c.** Cardiac remodeling following myocardial infarction results in ventricular dilatation and impairment of cardiac pumping. **d.** Myocardial infarction results in re-expression of WT1 in the epicardium, epicardial EMT and migration of EPDCs into the subepicardium. Remodeling of the cardiac vasculature results in re-expression of WT1 in endothelial cells of the infarcted area and borderzone. The expression of WT1 throughout adulthood and after injury is indicated in green. LV, left ventricle; RV, right ventricle.

MI is the most common type of ischemic heart disease and the leading cause of death in the Western world [38, 39]. During MI, coronary occlusion leads to a reduced supply of oxygen to the cardiac muscle, resulting in massive cell death of cardiomyocytes. The extracellular matrix (ECM) is degraded ensuring infiltration of inflammatory cells, which remove the cellular debris generated during acute cardiac injury [40, 41]. The expression of WT1 in the epicardium is

already upregulated 1 day after infarction and is induced throughout the entire epicardial layer of the heart [42]. The subepicardium thickens by resurgence of the EMT process and is most pronounced at the infarcted area [43] (Figure 4b). The re-expression of WT1 and the revival of epicardial EMT suggest that WT1 regains its fetal role after MI. In the subepicardium, WT1 is expressed in the αSMA-positive cells but not in the endothelial cells [[44]; data not shown] (Figure 4d). Lineage tracing of epicardial cells indicated that the WT1-positive cells do not migrate into the infarcted area [44, 45]. Remarkably, priming the mouse heart before MI with Thymosin beta-4 resulted in the migration of epicardial cells into the myocardium and functional differentiation into cardiomyocytes after MI [22]. In the infarcted area, an increase in granulation tissue is observed approximately 3 days after MI, which is characterized by the presence of interstitial fibroblasts, myofibroblasts, and forming blood vessels [12, 14, 43, 46, 47]. As opposed to the expression in the subepicardium, upregulation of WT1 expression is present in endothelial cells and not in (myo)fibroblasts in the myocardial layer [[14]; data not shown; Figure 4]. Initially, upregulation of WT1 is observed in endothelial cells of the border zone and subsequently in the infarcted area, with a peak endothelial expression of WT1 at day 7 after MI [47]. As time progresses, vessels in the infarcted area become more mature, fibrotic scar forms, and the expression of WT1 disappears [14]. Interestingly, in the border zone, the expression of WT1 in endothelial cells is still detectable 4 weeks after MI, indicating that this region still undergoes active remodeling. The expression of WT1 in the epicardium gradual decreases after the first week to return to quiescent levels at 3 months after infarction [42] (Figure 4c). The remodeling and maturation of the fibrotic scar result in ventricular dilatation and impairment of cardiac functioning [48, 49] (Figure 4c).

The molecular mechanism causing WT1 reactivation in epicardial and endothelial cells is unclear. Inflammation might be a potential trigger for the activation of WT1 expression after MI. Proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are upregulated within the first few hours after injury [50]. These cytokines are able to activate the transcription of NF-κB [51], which is highly present after MI [52-54]. NF-κB upregulates WT1 expression [55], thereby potentially activating the epicardium after MI (Figure 5). The peak of NF-κB induction is found at day 3 after MI [56], the same day the first signs of angiogenesis, including the endothelial WT1 expression, are visible in the border zone [47]. Interestingly, WT1 has an anti-inflammatory role because it inhibits the expression of inflammatory cytokines by stimulation of IL-10 [57] (Figure 5).

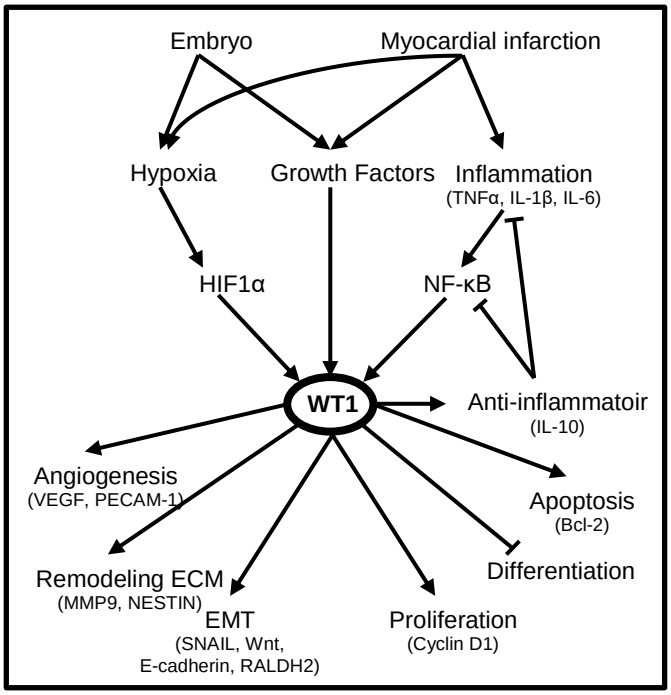

Figure 5. Working model of the regulation of WT1 and its target genes. The expression of WT1 is activated during embryonic development and in the adult heart after injury. Upregulation of WT1 is caused by hypoxia, growth factors and inflammation. WT1 in turn is able to activate multiple genes which are important for the regulation of different processes, including e.g. EMT, angiogenesis, remoedeling of the ECM.

The upregulation of epicardial WT1 after injury might also be caused by soluble factors released by the myocardium within the pericardial fluid (PF). Injection of PF from MI patients into the pericardial cavity induces the expression of WT1 in epicardial cells in mice in the absence of infarction [58, 59]. In addition, PF of patients affects gene expression in epicardial cells which are involved in EMT, among others the expression of SNAIL and TWIST are stimulated [59]. After MI, PF contains an increased number of exosomes, which are small extracellular micro-vesicles. These vesicles contain bioactive molecules and are important for intracellular communication and activation [60]. Recent proteomic analysis by Foglio et al. showed that clusterin is highly enriched in exosomes of PF of patients after MI [59]. Clusterin is involved in EMT in prostate cancer [61] and administration of clusterin in the pericardial cavity induced EMT in epicardial cells [59].

The upregulation of endothelial WT1 after injury might be caused by hypoxia (Figure 5). Hypoxia is a well-known condition that induces vascular formation during development and after MI via the hypoxia-inducible factor-1-alpha (HIF1α) [62]. The expression of WT1 can be directly upregulated by hypoxia through the HIF1α-responsive elements in the WT1 promoter [63] (Figure 5). *In vitro* exposure of human endothelial cells to hypoxia increased the expression of WT1 [14, 64]. Important for the response to hypoxia is that one of the downstream targets of WT1 is VEGF [65], one of the most potent angiogenic factors, both in embryonic vascular formation and in the growth of blood vessels after injury [66] (Figure 5).

4. The role of WT1 in endothelial cells

The expression of WT1 in endothelial cells is only found in the heart and not in other organs of the developing embryo [14] (Figure 3a-c). The cardiac-specific expression of WT1 is supported by a recent study that identified the unique gene expression profiles of endothelial cells, isolated form different organs [67]. In both human and mouse, the expression of WT1 in cardiac endothelial cells is significantly higher compared to non-cardiac endothelial cells, confirming cardiac-specific expression of WT1 in endothelial cells [67]. In fact, overexpression of WT1 was sufficient to differentiate endothelial cells into a more cardiac specialized population. The importance of WT1 for the development of blood vessels is highlighted by the re-expression in the cardiac vasculature after MI in mouse and after exposure of rats to hypoxia [14, 64]. A study by Coosemans et al. [68] claimed the expression of WT1 in cardiac endothelial cells of patients that died after MI. Although the expression of WT1 in cardiac endothelial cells is unique during normal conditions, the expression is also present in endothelial cells in other organs in a pathological condition. WT1 is found in endothelial cells of the skin in patients with chronic dermatitis [69], and WT1 has been observed in endothelial cells in a wide variety of tumors [68-72].

It is unclear why under physiological conditions WT1 expression is found only in cardiac endothelial cells. In contrast to other organs, the heart has the unique feature that it is exposed to cyclic strain [73]. It is known that mechanical forces during early development play an important role in cardiac morphology [74]. In addition, cyclic strain is able to regulate the process of EMT [75]. It is therefore tempting to speculate that stimulation of EMT by cyclic strain is regulated by an upregulation of the expression of WT1. Alternatively, WT1 might be induced by TGFβ, which is known to be upregulated by cyclic strain. TGFβ is able to

upregulate WT1 expression via Par-4 [76-78]. On the contrary, WT1 works as a negative feedback loop on TGFβ, by repressing its expression [79, 80].

At the very early stages of development, the fetal heart is predominantly dependent on glucose metabolism and shortly after birth the heart energy metabolism switches to fatty acid oxidation [81]. Facilitating the uptake of fatty acids is a unique feature of cardiac endothelial cells [67, 82]. WT1 expression is known to be essential for the cardiac endothelial fingerprint, therefore WT1 might be important for the repression of genes regulating the uptake of glucose.

Patients with Denys–Drash syndrome (DDS), carrying partial-loss-of-function mutations in the *WT1* gene, develop glomerulosclerosis. In addition, the capillaries of the glomeruli show abnormal development. A cause of these malformations is found in a strong decrease in the expression of platelet endothelial cell adhesion molecule-1 (PECAM-1) in endothelial cell of the glomeruli [83]. PECAM-1 is part of intercellular junctions and is present in mature vascular structures; additionally, its expression is upregulated during formation and remodeling of vascular networks [84, 85]. WT1 is a positive regulator of PECAM-1 [71], this may explain the poor organization of capillaries in patients with DDS. Knockdown of *WT1* in human endothelial cells confirms the importance of WT1 in the formation of vascular networks, as these cells are unable to form proper networks [14, 64]. The angiogenic role of WT1 is supported by a reduced sprouting capacity in an aortic ring angiogenesis assay from mice lacking WT1 expression in endothelial cells. In addition, the same study revealed that the vessel density in matrigel plugs after subcutaneously injection, in mice lacking WT1 expression in endothelial cells, is significantly reduced compared to wild-type animals [71]. Furthermore, deletion of WT1 in endothelial cells resulted in major reduction in cardiac vessel formation during mouse cardiac development supporting the presence of WT1 and the essential role of WT in cardiac endothelial cells [86].

Endothelial cells are anchored to a basement membrane that ensures structural and organizational stability. During vascular formation and remodeling, reorganization of the ECM is essential [87-89]. Degradation of the ECM is facilitated by an increase and activation of latent matrix metalloproteinases (MMPs). WT1 is able to directly upregulate the expression of MMP9 [70], thereby facilitating the degradation of ECM. The basement membrane is mostly made up of collagen IV, which is degraded by MMP9 [90]. Interestingly, the study of Johnson et al. [91] showed that in the absence of MMP9 revascularization of infarcted tissue is strongly impaired, confirming that remodeling is essential for angiogenesis. The role of WT1 in

remodeling is further strengthened by proteomic analysis in patients with DDS. Glomerular podocytes with WT1 mutations have a disturbed production of proteins forming the cytoskeleton [92]. Furthermore, the expression of intermediate filament Nestin is regulated by WT1 [93]. The expression of Nestin is increased in regenerating tissue and is believed to participate in cellular remodeling and angiogenesis [94-96]. Co-expression of WT1 and Nestin was found in the epicardium and endothelial cells of the embryonic heart in mice [93] and in vascular endothelium of patients who died after MI [97, 98].

Changes in the cytoskeleton are also required for cells to adapt to a less differentiated phenotype, allowing them to proliferate and migrate. Nestin is present in proliferating progenitor cells and positively regulates proliferation and migration [96]. Within the epicardium, a positive correlation was found between WT1 and proliferation [99]. In addition, WT1 plays a role in regulating the cell cycle. *In vitro* studies knocking down WT1 in human endothelial cells show reduced proliferation and migration [14, 72]. Proliferation of endothelial cells is directly regulated by WT1 via Cyclin D1 [14, 100], one of the many regulators of the cell cycle and present in the G1 phase [101]. Interestingly, the expression of WT1 is upregulated in embryonic stem cells during embryonic body differentiation, a proliferative period for mesenchymal cells. Upon cellular differentiation, the expression of WT1 was reduced [25]. The positive role of WT1 on migration might be the result of direct repression of the promoter of Cxcl10, an inhibitory chemokine preventing angiogenesis [17].

Finally, WT1 is known to play a role in apoptosis. This was already noticed in 1993 in *Wt1*-knockout mice; embryonic tissue of the kidney showed more cell death compared to wild-type littermates [4]. Over the last years, it has become clear that WT1 can directly regulate genes involved in apoptosis; however, it depends on the cellular context if WT1 has a pro- or anti-apoptotic effect [102]. Future research is required to investigate the role of WT1 in apoptosis of endothelial cells; potentially WT1 protects the forming and maturing blood vessels against cell death.

Together these observations suggest a role for WT1 in the remodeling, proliferation, and migration of cardiac endothelial cells and the formation of a proper vascular network (Figure 5). Stress factors such as hypoxia and inflammation are likely to play a role in the activation of WT1 both during cardiac development and in the response after injury (Figure 5). Future research, focusing on the molecular mechanisms, can hopefully reveal all pathways by which the angiogenic function of WT1 can be explained.

5. Clinical perspective and future research

Restoring the cardiac blood flow is the most important treatment of ischemic cardiomyopathy at this moment. To improve cardiac output, the cardiac wall consisting of cardiomyocytes, fibroblasts, endothelial and smooth muscle cells, has to be rebuild. Transplantation of cardiac stem cells after infarction improved the function [103-105], however, difficulties in acquisition of human tissue and *in vitro* expansion of cells limit the clinical applicability. An interesting approach would be to take advantage of the properties of WT1-expressing cells. The differentiation potential of WT1-expressing epicardial cells during development into the vasculature, fibroblasts and cardiomyocytes has positioned the epicardium as a promising target [20, 106, 107]. WT1-expressing stem cell-like cells are residing in the epicardium [58, 108]. In addition, Chong and colleagues [109] showed that cardiac colony-forming units originate from WT1-positive cells within the epicardium. Finally, activation of WT1 expression within the epicardium after injury revives the fetal differentiation potential in the epicardial cells. Facilitating the differentiation of these WT1-expressing cells towards cardiomyocytes, fibroblasts, endothelial and smooth muscles cells could provide a great tool to improve cardiac regeneration after injury. In addition, the indicated role of WT1 in the endothelial cells during development and injury has positioned the WT1-expressing endothelial cells as a potential target for improving angiogenesis in the diseased area.

A recent study indicates a role for the epicardium in autonomic modulation during early development. Within the initial stages of epicardial formation, WT1 in epicardial cells is co-expressed with the neuronal markers TUBB3 and NCAM [15]. Interestingly, the expression of WT1 was also found in the ventral region of the neural tube, as well as the roof of the 4th ventricle of the brain, supporting the neuronal phenotype of the epicardium. Dysfunction of the cardiac autonomic nervous system plays a role in the pathogenesis of arrhythmias [110] and hypertension [111] and is involved in disease progression in heart failure [112]. Understanding the mechanistic role of WT1 in the formation of the cANS might help to unravel processes that govern normal cANS development and opens possibilities for treatment after cardiac injury.

WT1 is associated with major generation processes during cardiac development like formation of epicardium, cardiac vasculature, valves, cANS, and also myocardial wall maturation, but also with major regeneration processes during cardiac repair like scar formation and angiogenesis. More knowledge on the upregulation of WT1 in cardiac cells and their subsequent response can contribute to the development and improvement of therapeutic strategies for cardiac repair, and thereby restoring a functional contractile cardiac wall.

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5

The epicardium as modulator of the cardiac autonomic response during early development

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Abstract

The cardiac autonomic nervous system (cANS) modulates heart rate, contraction force and conduction velocity. The embryonic chicken heart already responds to epinephrine prior to establishment of the cANS. The aim of this study was to define the regions of the heart that might participate in modulating the early autonomic response to epinephrine. Immunofluorescence analysis reveals expression of neural markers tubulin beta-3 chain and neural cell adhesion molecule in the epicardium during early development. In addition, expression of the β2 adrenergic receptor, the receptor for epinephrine, was found in the epicardium. Ex-ovo micro-electrode recordings in hearts with inhibition of epicardial outgrowth showed a significantly reduced response of the heart rate to epinephrine compared to control hearts. This study suggests a role for the epicardium as autonomic modulator during early cardiac development.

Keywords

epicardium; cardiac development; autonomic nervous system; autonomic modulation; cardiac conduction system

1. Introduction

The cardiac autonomic nervous system (cANS) is essential in modulating cardiac function by altering heart rate (chronotropy), conduction velocity (dromotropy) and force of contraction (inotropy)[1]. Dysfunctioning of the cANS plays a role in the pathogenesis of arrhythmias[1] and hypertension[2] and is involved in disease progression in heart failure[3]. Furthermore, normal functioning of the cANS is important for the prognosis of adult patients with congenital heart disease[4]. Understanding the processes that govern normal cANS development may help in unravelling the pathophysiology of abovementioned disease processes and in developing targeted treatment options.

The cANS can be divided into a sympathetic and parasympathetic component. In general, sympathetic stimulation results in an increase of heart rate, conduction velocity and force of contraction, while parasympathetic stimulation has an opposing effect. Sympathetic neurons have their cell bodies primarily in the paravertebral stellate ganglion, whereas parasympathetic cell bodies are located in the cardiac ganglia[5]. The cells contributing to the cANS are derivatives of neural crest cells (NCCs) and cells of the nodose placode[6,7]. Kroese *et al.* demonstrated that prior to cardiac sympathetic innervation of the developing chicken embryo, the heart already responds to the catecholamine epinephrine[8]. This neurotransmitter binds to beta (β) adrenergic receptors, thereby activating cAMP dependent signaling[9], resulting in an increase in heart rate, conduction velocity and force of contraction[5]. It is remarkable that expression of enzymes necessary for production of catecholamines was found throughout the myocardium during cardiac development in rat,[10] even before production is observed in the adrenal glands.[11,12] After addition of epinephrine in chick at Hamburger and Hamilton (HH[13]) stage 20-24, several hemodynamic parameters, including heart rate, increased significantly[8]. This supports an important role for catecholamines in the heart during early embryo development. Furthermore, stimulation of chick embryos with isoproterenol (β-agonist) at embryonic day 7 (HH30-31) resulted in an increase in cAMP.[14] In addition to responding to β-adrenergic stimulation, the early embryonic chicken heart was also shown to respond to β-adrenergic receptor blockade by reducing heart rate and cardiac output.[15] Thus prior to establishment of the cANS, the heart already responds to autonomic stimulation and blockade.

Interestingly, Kroese *et al.* showed that after treatment with all-trans retinoic acid (RA) the reaction to epinephrine, including the increase in heart rate, was significantly reduced[8]. Normal RA signaling has been shown to be important for proper development of the

epicardium[16]. This single layer of cells is derived from the proepicardial organ (PEO) and covers the initially bare primary myocardial heart tube. Cells derived from the epicardium are known to play an essential role during normal cardiac development and defects in epicardial development result in cardiac malformations (reviewed in[17]).

The aim of the current study was to identify which cell population in the developing heart plays a role in modulating the autonomic response during early development. Our studies reveal unanticipated expression of neuronal markers in the epicardium during early cardiac development. To investigate a potential role of the epicardium, electrophysiological experiments were performed with and without inhibition of epicardial outgrowth.

2. Material and Methods

2.1. Animals

Immunohistochemical analysis was performed in wild type mouse embryos with a mixed genetic background of different embryonic stages (E9.5-E17.5, mice described in[18]). The morning of the vaginal plug was considered E0.5. Pregnant mice were euthanized using CO_2 exposure and cervical dislocation. Animal care was in accordance with national and institutional guidelines and approved by the animal experiments committee of the Leiden University Medical Center.

To study protein expression in chick embryos, fertilized eggs of the White Leghorn chicken were incubated at 37°C and 80% humidity. Hearts were excised, and staged according to Hamburger and Hamilton (HH)[13]. Tissue was fixed in 4% paraformaldehyde for 24 hours and subsequently embedded in paraffin and sectioned (5 µm) for immunohistochemical analysis.

2.2. Human fetal tissue

A 5-week-old human fetal heart was collected after elective abortion based on individual informed consent procedures conforming to the Declaration of Helsinki. Furthermore, the study was approved by the Medical Ethics committee of the Leiden University Medical Center. Tissue was treated as described above.

2.3. Immunohistochemistry

The protocol used for immunohistochemical staining was described previously[19]. Briefly, slides were rehydrated, subjected to heat-induced epitope retrieval and incubated with the

following list of antibodies: anti-cardiac Troponin I (CTNI) (myocardial marker, 1:1000, 4T21/2, HyTest Ltd), anti-Wilms' tumor-1 (WT1) (expressed in the epicardium, 1:1000, ab89901, Abcam), anti-tubulin beta-3 chain (TUBB3) (neuronal marker, 1:200, AB78078, Abcam), Neural Cell Adhesion Molecule (NCAM) (neuronal marker, 1:250, AB5032, Merck), anti- β1 adrenergic receptor (β1AR) (receptor for epinephrine, 1:200, PA528808, Thermo Scientific), and anti-β2 adrenergic receptor (β2AR) (receptor for epinephrine, 1:200, ab61778, Abcam). To amplify WT1 expression Tyramide Signal Amplification (PerkinElmer) was used. Visualization was achieved by incubation with Alexa Fluor® 488 streptavidin (Invitrogen). The remainder of primary antibodies was visualized with Alexa-conjugated fluorescent secondary antibodies (Invitrogen) at a final concentration of 1:200. DAPI (D3571, 1/1000; Life Technologies) was used as a nuclear stain, after which slides were mounted with Prolong gold (Life Technologies).

2.4. Mechanical blocking of the proepicardial organ (PEO)

Mechanical inhibition of the epicardial outgrowth in the chicken embryo was performed as described previously[20]. At HH15, a window was created in the eggshell, after which the embryonic membranes were opened. Subsequently, a small piece of eggshell membrane was placed between the PEO and developing heart tube, after which the egg was re-incubated until the desired stage. In order to verify that outgrowth of the epicardial layer was hampered, hearts were sectioned and a Haematoxylin and Eosin staining was performed.

2.5. Ex-ovo extracellular micro-electrode recordings and epinephrine administration

To investigate the effect of epinephrine on heart rate, electrophysiological measurements were performed in embryonic chicken hearts at different developmental stages. After reaching the desired stage of development, embryos were extracted from the egg. The heart and some surrounding tissue were excised and placed in a temperature-controlled (37 ± 0.1°C) tissue bath containing Tyrode. Recordings were performed using a previously described protocol[21]. Recording electrodes were placed on the atrium and the ventricular apex, and a reference electrode in the tissue bath. The hearts of five groups of embryos were studied: 1. HH15 embryos (n=5), when the heart is not yet covered by epicardium; 2. HH19 embryos (n=3), when epicardial covering of the sinus venosus, atria and AV-canal has commenced; 3. HH21 embryos (n=3), when migration of epicardial cells around the heart is (nearly) complete[22]; 4. HH24-25 control embryos (n=9, no surgical manipulation), when epicardial covering has been completed and subepicardial mesenchyme is present; 5. HH24-25 embryos (n=9) after inhibition of epicardial outgrowth.

The hearts were allowed to reach a stable baseline heart rate (which was comparable between all studied groups, Supplemental Figure 1), after which 100 µl of pre-warmed epinephrine (1 mg/ml, Centrafarm, The Netherlands) was directly pipetted onto the heart. Pre-warmed Tyrode was administered as a negative control to HH24-25 hearts (n=6). The relative response to epinephrine was calculated by correcting the change in heart rate for the baseline heart rate. The heart rate was calculated every 10 seconds and plotted.

2.6. Statistical analysis

The Mann-Whitney U-test (two groups) or Kruskal-Wallis test (>two groups) were used, since the data was not normally distributed. $P < 0.05$ was considered statistically significant. Data shown is mean ± S.E.M. Statistical analysis was performed using the Graphpad Prism 6 software package (Graphpad Software).

3. Results

3.1. During early cardiac development the neuronal marker TUBB3 is expressed by the epicardium

In order to investigate which cell population plays a potential role in modulation of the early cardiac autonomic response, protein expression of the neuronal marker TUBB3 was analyzed during cardiogenesis.

At E9.5, the primary heart tube is not covered by epicardial cells and the PEO is recognizable (Figure 1a-d). Co-expression of neuron-specific TUBB3 and WT1 was observed in a subset of cells in the PEO (Figure 1a-d). WT1+/TUBB3- and WT1-/TUBB3+ cells were also present (Figure 1a-d), confirming the heterogeneity of the PEO[23]. At E10.5, the epicardial layer has started to envelope the heart and showed co-expression of TUBB3 and WT1 in most epicardial cells (Figure 1e-h). Co-expression of TUBB3 and WT1 was still clearly present at E11.5 in the majority of epicardial cells (Figure 1i-l). Strong expression of TUBB3 was observed in the mesenchyme of the endocardial cushions in the OFT and AV canal, shown at E11.5 (Figure 1i-l). TUBB3 expression in the epicardium decreased with ongoing development. At E12.5, only faint expression was observed in the epicardium (Figure 1m-p), disappearing at E13.5 (Figure 1q-t). At this stage, TUBB3 expression was only observed in subepicardial nerve fibers (Figure 1q-t) and no co-expression of TUBB3 and WT1 was observed in the epicardium or subepicardium.

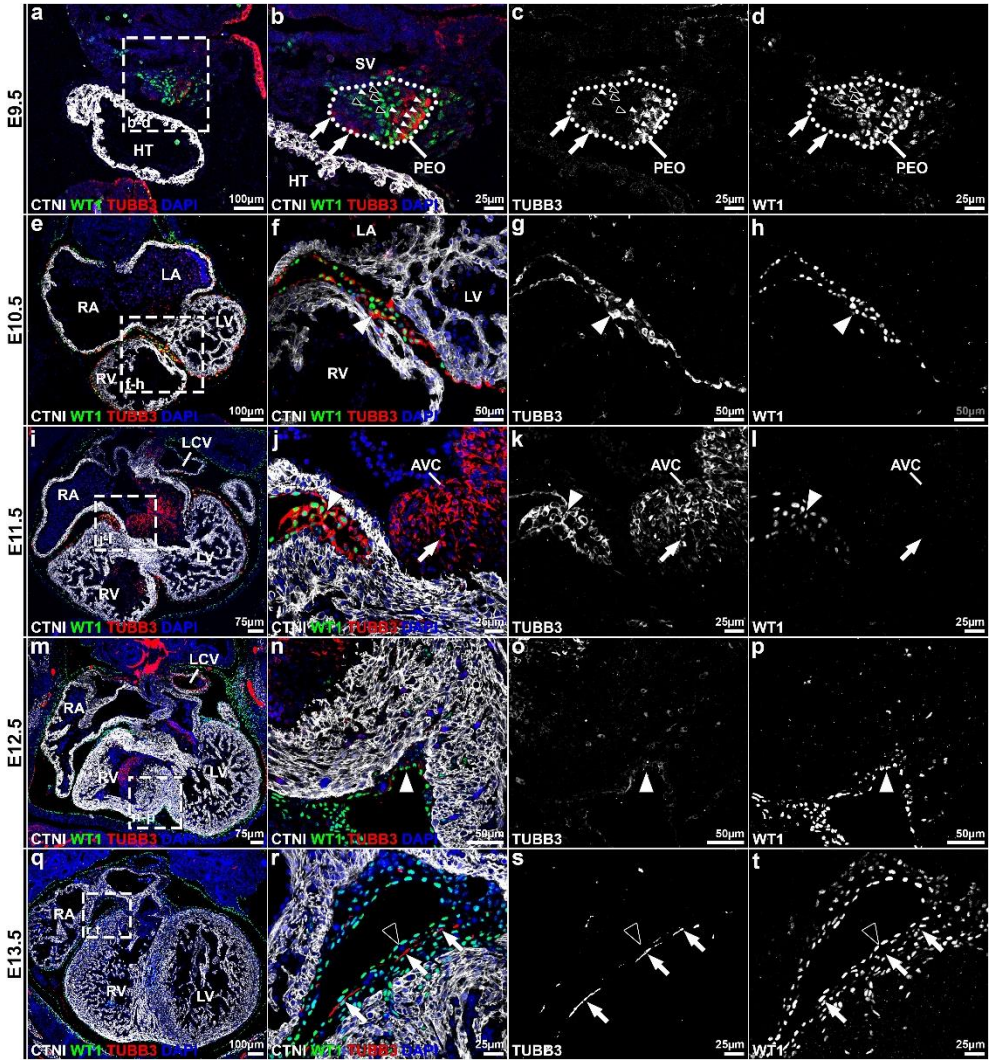

Figure 1. The neuronal specific marker TUBB3 is expressed in the epicardium and subepicardium during early development. a-d. At E9.5, the PEO (dotted line) is recognizable and the heart tube is not covered by epicardium. WT1+/TUBB3+ cells (white arrowheads in **b-d**), WT+/TUBB3- cells (black arrowheads in **b-d**) and WT1-/TUBB3+ cells (arrows in in **b-d**) are observed within the PEO. **e-h.** At E10.5 the heart is partially surrounded by epicardial cells and co-expression of TUBB3 with WT1 is seen in most epicardial cells (arrowheads in **f-h**). **i-l.** Co-expression of TUBB3 and WT1 in the epicardium is still present at E11.5 (arrowheads in **j-l**). TUBB3 expression is also seen in the endocardial cushions (arrows in **j-l**), shown here at E11.5. **m-p.** Co-expression of TUBB3/WT1 decreases from E12.5 (compare white arrowhead in **k** with white arrowhead in **o**). **q-t.** At E13.5 the expression of TUBB3 in the WT1+ epicardium (black arrowheads in **r-t**) is lost but is now present in subepicardial nerve fibers (arrows in **r-t**). HT, heart tube; SV, sinus venosus; PEO, proepicardial organ; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; LCV, left cardinal vein; AVC atrioventricular cushion.

To confirm the neuronal phenotype of the epicardium, NCAM (neuronal marker) expression was analyzed. At E11.5, co-expression of NCAM and TUBB3 was seen in the nervous system, including the neural tube and dorsal root ganglion (Supplemental Figure S2a-d). Furthermore, NCAM expression was seen in the epicardium, co-localizing with TUBB3 (Supplemental Figure S2e-h), thereby confirming the neuronal phenotype of the epicardium.

In addition, TUBB3 expression was analyzed during early human fetal development. A subset of epicardial cells and cells in the endocardial cushions showed expression of TUBB3 (Supplemental Figure S3b-c). Furthermore, cells in the subepicardial space showed TUBB3 expression (Supplemental Figure S3d-e).

3.2. A subpopulation of cells in the central nervous system co-express WT1 and TUBB3

The neuronal marker TUBB3 showed protein expression in the epicardium, co-expressing with WT1. In order to provide possible evidence that these proteins are involved in normal neuronal function, expression of TUBB3 and WT1 was studied in the nervous system. At E11.5, the developing nervous system showed expression of TUBB3 (Figure 2a-d). No WT1 expression was found (Figure 2a-d). Microscopic analysis at E13.5 revealed co-expression of TUBB3 and WT1 in the ventral region of the neural tube, as well as the roof of the 4th ventricle of the brain (Figure 2e-g). WT1 expression was observed along the entire length of the neural tube (Figure 2e). Co-expression of TUBB3 and WT1 in the spinal cord was still present at E17.5 (Figure 2h-k).

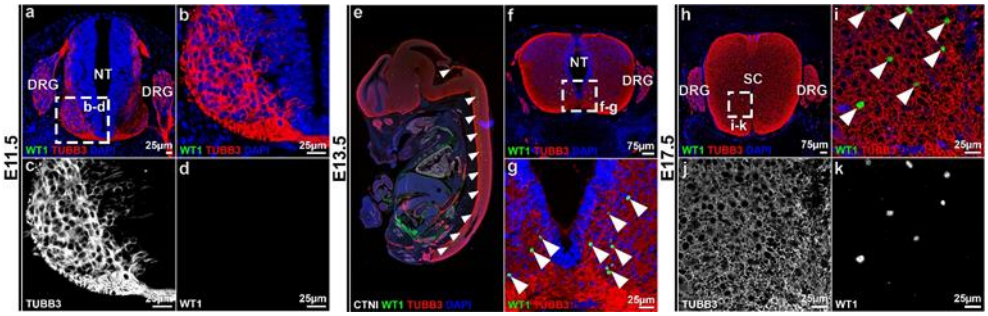

Figure 2. WT1 is expressed in the ventral horn of the spinal cord. a-d. At E11.5 TUBB3 is present in the neural tube, however, no co-expression with WT1 is found. **e-g.** From E13.5 co-expression of WT1 and TUBB3 is observed in the ventral horn of the neural tube and the roof of the 4th ventricle of the brain (arrowheads). **h-k.** Co-expression of TUBB3/WT1 is still present in the spinal cord at E17.5 (arrowheads in **i-k**). DRG, dorsal root ganglion; NT, neural tube; SC, spinal cord.

3.3. During early cardiac development the catecholamine receptor β2AR is expressed by the epicardium

To further confirm a potential neuronal phenotype of the epicardium, expression of the beta1 (β1) and beta2 (β2) adrenergic receptors (AR) was studied. To confirm neural co-expression of TUBB3 and β2AR, these markers were studied at E11.5 in the nervous system. Co-labeling of TUBB3 and β2AR was seen in the dorsal root ganglion and neural tube (Figure 3a-d).

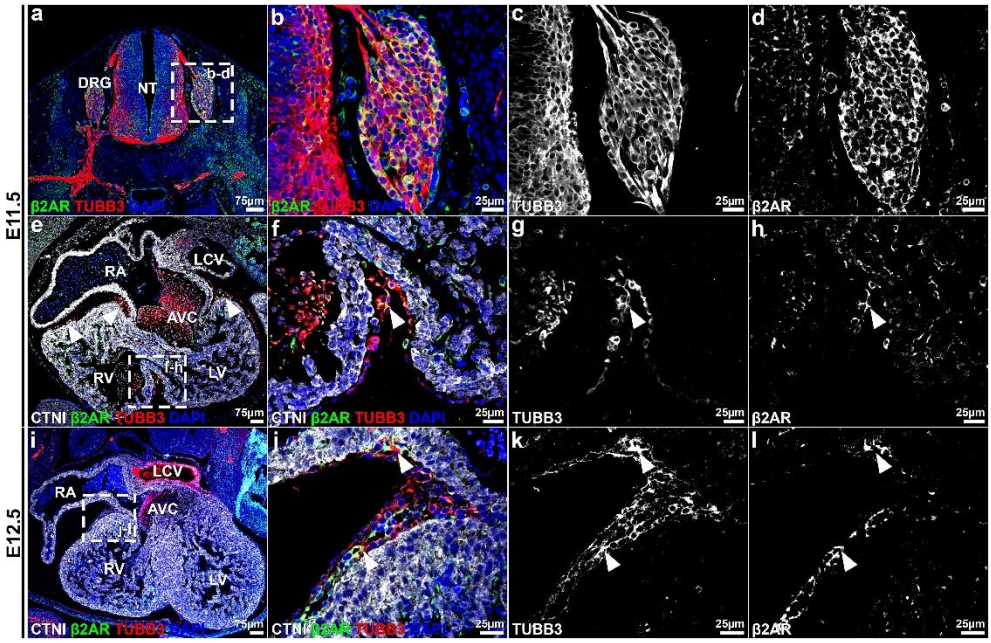

Figure 3. β2 adrenergic receptor is expressed in the epicardium and subepicardium during development. a-b. Co-expression of β2AR (green) and TUBB3 (red) is found in the dorsal root ganglia during murine embryonic development from E11.5 onwards. Separate grey values are shown for TUBB3 (**c**) and β2AR (**d**). **e-h.** At E11.5, a subpopulation of TUBB3+ epicardial cells is positive for β2AR (arrowheads in **f-h**). **i-l.** At E12.5 co-expression of TUBB3 and β2AR in the epicardium is lost. There is, however, expression of β2AR in the subepicardium of which a subset shows co-labelling with TUBB3 (arrowheads in **j-l**). DRG, dorsal root ganglion; NT, neural tube; RA, right atrium; LCA, left cardinal vein; AVC atrioventricular cushion; RV, right ventricle; LV, left ventricle.

In murine embryos at E11.5, most epicardial cells are TUBB3+, and a subpopulation of epicardial cells showed co-expression with β2AR (Figure 3e-h). β2AR+ cells were found throughout the epicardial layer, predominantly seen in the AV and interventricular sulcus (Figure 3e-h). Furthermore, expression of β2AR was seen in endocardial cells and a subpopulation of cells in the endocardial cushions (Figure 3e) Expression of TUBB3 and β2AR

was seen in the subepicardium at E12.5, with a subset of cells co-expressing both markers (Figure 3i-l). β1AR expression was not observed in these stages (not shown).

3.4. Inhibition of epicardial outgrowth results in a diminished response to epinephrine

At HH21 of chick development, expression of β2AR was found in the epicardium, PEO and endocardium (Supplemental Figure S4) which is in agreement with the β2AR expression found in mice. First, the role of the epicardial layer in the response of the heart to epinephrine was studied. For this purpose, chick hearts from embryos of subsequent stages of epicardial development were analyzed using *ex-ovo* micro-electrode recordings (Figure 4 and Supplemental Figure S5). The relative increase ((maximal heart rate – baseline)/(baseline)) * 100%) in heart rate after administration of epinephrine was calculated. HH15 hearts, which lack epicardial covering, showed no increase in heart rate after administration of epinephrine (Figure 4a and Supplemental Figure S5a). A significant increase in heart rate was observed in hearts of HH19 (when epicardial covering of the sinus venosus, atria and AV-canal has initiated), HH21 (epicardial covering has (nearly) been completed) and HH24 (the occurrence of subepicardial mesenchyme) embryos compared to HH15 (Figure 4b-e and Supplemental Figure S5b). This indicates that the presence of epicardium is required for the response to epinephrine during early developmental stages.

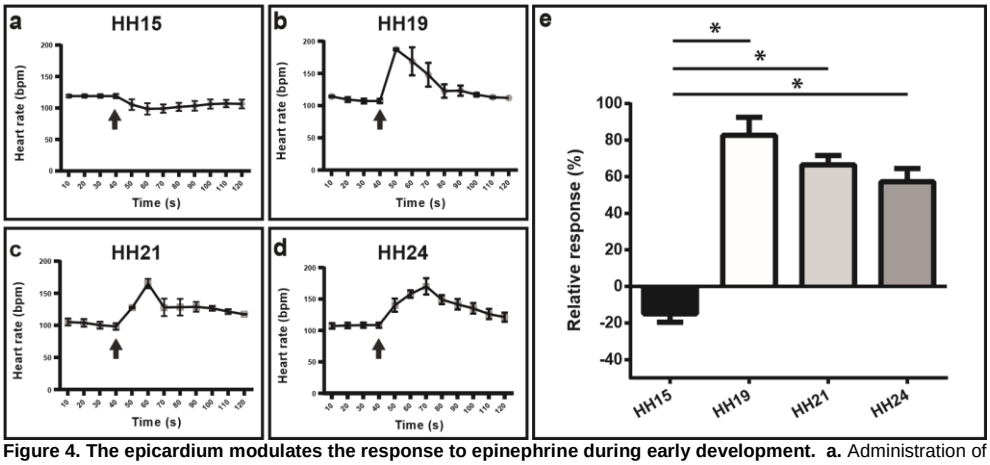

Figure 4. The epicardium modulates the response to epinephrine during early development. a. Administration of epinephrine to isolated hearts at HH15 results in a slight decrease in heart rate. **b-d.** Administration of epinephrine to isolated hearts at HH19, HH21 and HH24 results in a strongly increase in heart frequency. **e.** Relative response of hearts at HH19, HH21 and HH24 is significantly increased compared to the response of hearts at HH15.

To further substantiate the role of the epicardium in the response to epinephrine, an experimental model was used in which outgrowth of the epicardium is inhibited in chick embryos. To confirm epicardial inhibition, hearts were analyzed histologically, after the electrophysiological measurements. Analysis of the epicardial response to epinephrine was conducted at stage HH24, when the effects of the epicardial inhibition are clearly observed. At HH24, control hearts showed epicardial covering of the entire heart, with a cell-rich subepicardial space (Supplemental Figure S6). After epicardial inhibition, large portions of the heart were not covered by epicardium and the regions covered with epicardium showed less subepicardial cells (Supplemental Figure S6). Furthermore, compaction was hampered, as shown by a less dense compact myocardial layer compared to controls (Supplemental Figure S6).

Ex-ovo micro-electrode recordings were performed after administration of epinephrine in control and inhibited hearts. After administration of epinephrine to control hearts (n=9), a fast increase in heart rate was observed (Figure 5a and Supplemental Figure S5b). The relative increase in heart rate was 57,2 ± 7,20%. After epicardial inhibition (n=9) however, this response was decreased (Figure 5b,d p = 0,03 and Supplemental Figure S5d), with a relative increase in frequency of 24,8 ± 4,33%. Pre-warmed Tyrode served as a negative control. Upon administration a short decrease in heart rate was observed, after which the heart rate returned to the baseline frequency (Figure 4c and Supplemental Figure S5c).

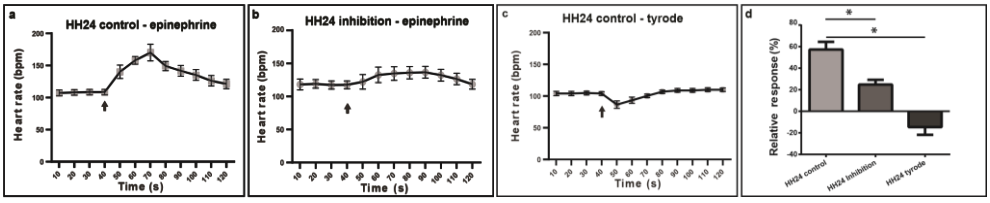

Figure 5. Repression of the epicardial covering of the heart reduces the cardiac response to epinephrine. a. Administration of epinephrine to isolated control hearts at HH24 result in a fast increase in heart frequency. **b.** Administration of epinephrine to isolated hearts after epicardial inhibition at HH24 results in a strongly reduced response in heart frequency. **c.** Administration of Tyrode to isolated control hearts results in a slight decrease in heart frequency. **d.** Relative response of hearts after epicardial inhibition is significantly reduced compared to the response of control hearts, *p=0,03.

The earliest electrical activity is generated in the myocardium of the sinus venosus (SV)[21]. Since the response to epinephrine was altered after epicardial inhibition, the next step was to evaluate epicardial covering of the SV myocardium in these hearts.

Hearts that were classified as successful inhibition of epicardial outgrowth in Figure. 4, were microscopically subdivided into two categories based on epicardial covering of the SV. Hearts were classified as “mild inhibition” (n=4) when the myocardium of the SV was covered by epicardium, but no subepicardial cells were present (Figure 6a,b). Hearts were classified as “severe inhibition” (n=5) when the myocardium of the SV was (largely) devoid of epicardium and subepicardial cells were not present (Figure 6d,e). Micro-electrode recordings revealed that the relative response to epinephrine was hampered in the mild inhibition group. The increase in heart rate was less pronounced and it took longer to return to the baseline heart rate after epinephrine administration compared to the control group (Figure 6c). In the severely inhibited group, virtually no response to epinephrine was observed (Figure 6f). The relative response to epinephrine was significantly decreased in the group classified as “severe inhibition” as compared to the “mild inhibition” group (p=0,016 Figure 6g).

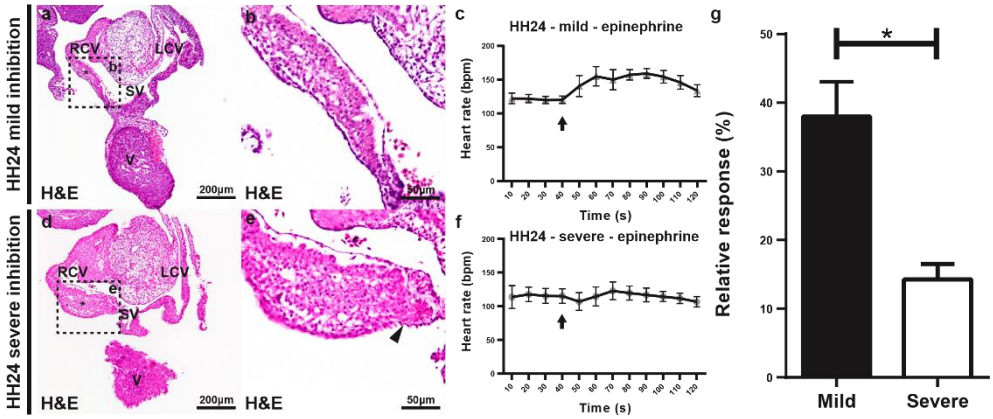

Figure 6. The severity of inhibition of epicardial covering of the sinus venosus correlates to the response to epinephrine. Hearts were microscopically subdivided into two categories based on covering of the sinus venosus (SV). **a-b.** Mild inhibitions show epicardial covering of the myocardium of the sinus venosus (SV), but subepicardial cells are not present. * in **a** shows the SAN, shown at higher magnification in **b. d-e.** Severe inhibition lack epicardial covering of the myocardium of the sinus venosus (SV) and subepicardial cells are not present.* in **d** shows the SAN, shown at higher magnification in **e.** Arrowhead in **e** shows the boundary of epicardial covering. **c.** Response to epinephrine after mild inhibition shows a hampered response to epinephrine, as compared to control hearts (compare with **4a**), as shown by a slower increase in heart rate and prolonged duration to return to baseline frequency. **f.** Administration of epinephrine to isolated hearts with severe inhibition at HH24 does not result in an increase in heart frequency. **g.** Relative response of hearts with severe inhibition (open bar) is significantly reduced compared to the response of hearts with mild inhibition (closed bar), *p=0,016. RCV, right cardinal vein; LCA, left cardinal vein; SAN, sinoatrial node; SV, sinus venosus; V, ventricle.

4. Discussion

Autonomic modulation is essential for proper functioning of the heart and contributes to the prognosis of patients with heart failure and congenital heart disease. Early in development, the heart already responds to sympathetic stimulation, even prior to the presence of sympathetic nerve fibers[8]. The exact mechanism behind this early response is poorly understood. The current study provides new evidence that can account for this response. Key findings of this study are; 1) The epicardium expresses TUBB3, NCAM and β2AR during early development, which are known neuronal markers; 2) Inhibition of epicardial outgrowth results in a disturbed response to epinephrine; 3) The severity of inhibition of epicardial covering of the sinus venosus myocardium correlates to the severity of disturbance in the response to epinephrine. Together, these results suggest a role for the epicardium in autonomic modulation of the heart during early development.

The current work describes expression of proteins known to be of importance in the nervous system in the epicardium. The tubulin isoform TUBB3 is primarily expressed in neurons and is important for axonal guidance and maintenance[24]. Expression however is not limited to neurons, since melanocytes (derived from the neural crest, as do neurons) also show TUBB3 expression[25,26]. To ensure the neuronal phenotype, staining of another neural marker, neural cell adhesion molecule (NCAM) was performed. NCAM expression was also present in the epicardium, confirming the neuronal phenotype of the epicardial layer. More indirect evidence suggesting a possible role for the epicardium in neural function is the co-expression of WT1 and TUBB3 in the central nervous system. Wt1, a transcription factor expressed in the embryonic PEO and epicardium, is necessary for normal epicardial and cardiac development[27]. The current manuscript shows WT1/TUBB3 co-expression in the central nervous system, which is in agreement with previous reports demonstrating an important role for Wt1 in neural functioning[28–32].

In addition to epicardial expression of NCAM and TUBB3, the receptor for epinephrine, β2AR, was expressed in the epicardium. Epinephrine binds to β2AR, which activates adenyl cyclase, resulting cAMP-dependant signaling[9], which leads to the sympathetic modulation of the heart rate, conduction velocity, and force of contraction.

To investigate the functional role of the epicardium in autonomic modulation, the response to epinephrine was analyzed in an experimental model in which normal outgrowth of the epicardium was inhibited. Results showed a significantly reduced response to epinephrine

after epicardial inhibition. To further validate these results, epinephrine was administered in control chicken embryos at HH15, a stage in which no epicardial cells are present on the heart tube. Interestingly, these embryos did not show a response to epinephrine, confirming the data seen after epicardial inhibition. These results show that epicardial covering of the heart is (at least partially) responsible for a normal response to epinephrine during early cardiogenesis.

Absence of epicardial covering of the sinus venosus myocardium, as was described in the "severe inhibition group", results in absence of the epicardial β2AR. If epinephrine cannot bind to this receptor, cAMP mediated signaling is likely to be hampered, resulting in an absent response to epinephrine. Furthermore, it was recently shown that the β2AR forms protein complexes with the funny-current ion channel HCN4, responsible for spontaneous depolarization of pacemaker cells. Disturbing the formation of the β2AR/HCN4 protein complexes results in a hampered response to sympathetic stimulation[33], a result also observed after epicardial inhibition (this study).

Kroese *et al* showed that disturbance of retinoic acid signaling results in a hampered response to epinephrine. Disturbing RA signaling in the epicardium by epicardial deletion of the retinoid X receptor-α (RXRα), results in defective epithelial-to-mesenchymal transition (EMT), thinning of the myocardium, disturbed coronary arteriogenesis and ventricular pre-excitation[34,35]. This phenotype is also seen after disturbance of epicardial outgrowth in avian embryos[36] and in *Wt1*-null mice[27,37]. Wt1 regulates RA signaling by activating RALDH2, the enzyme involved in RA synthesis[38], and Wt1-null mice show downregulation of RALDH2[27]. Vice versa, induction of RA signaling results in upregulation of Wt1 expression in chick epicardial-derived cells[39]. Therefore, disturbing the normal outgrowth of the epicardium could result in hampering of RA signaling. Previous data showed that RA treatment *in vitro* results in neuronal differentiation, with an increase in β2AR expression[40]. This indicates that RA signaling is important for normal β2AR expression. Disturbing this by epicardial inhibition (or administration of teratogenic concentrations of all-trans RA[8]) could therefore result in aberrant β2AR expression and an impaired response to its ligand, epinephrine.

Finally, recent work showed that normal development of the cardiac veins is required for normal development of the sympathetic nervous system of the heart[41]. This process is driven by nerve growth factor (NGF), secreted by vascular smooth muscle cells in subepicardial blood vessels[41]. Interestingly, the smooth muscle cells in the coronary vasculature derive from

the epicardium after EMT[42,43]. Disturbing epicardial outgrowth results in abnormal development of the coronary vasculature[36]. The hampered response to epinephrine described in the current study could possibly be explained by disruption of the earliest stages of blood vessel formation and NGF production, which is required for normal development of the autonomic nervous system[41].

The current study has several limitations. It cannot be excluded that inhibition of the outgrowth of the epicardium has secondary effects on the heart which can affect cardiac functioning, since epicardial cells are important for differentiation and maturation of cardiomyocytes.[44] However, our results show that epicardial covering is important for the response to epinephrine, since normal hearts without epicardium (HH15) do not respond to epinephrine. However, as soon as cardiac outgrowth of epicardial cells has commenced (HH19), the heart shows a rapid response to epinephrine. The early response seen at HH19, makes it unlikely that myocardial differentiation is responsible for the change in heart rate seen after administration of epinephrine. Furthermore, the response seen in normal hearts at HH15 was comparable to the response seen in epicardially inhibited hearts. This again shows that it is not myocardial differentiation, but the presence of epicardium which is responsible for the response to epinephrine. Although the chicken model demonstrates highly reproducible results, the results described in the current work could possibly be strengthened by electrophysiological testing in a mammalian model, which shows defects in epicardial covering. However, up to date, there is no mouse model specifically affecting outgrowth of the epicardium, since the genes commonly used for epicardium-specific expression of Cre are known to be expressed more broadly throughout the fetus during development.[19,45]

In conclusion, the current study provides evidence indicating a role for the epicardium in autonomic modulation during early development. Autonomic modulation is essential for proper cardiac functioning and dysfunctioning of the autonomic nerve system is implicated in several diseases, such as cardiac arrhythmias, heart failure, congenital heart disease and hypertension[1–3]. The current study provides evidence indicating a role for the epicardium in autonomic modulation during early development. Further research is required to investigate the role of the epicardium in autonomic dysfunction seen in common cardiac disorders, and to explore the mechanisms responsible for the early heart rate response mediated by the epicardium.

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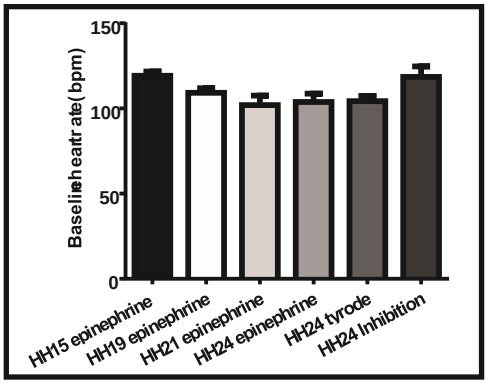
Disclosures

None

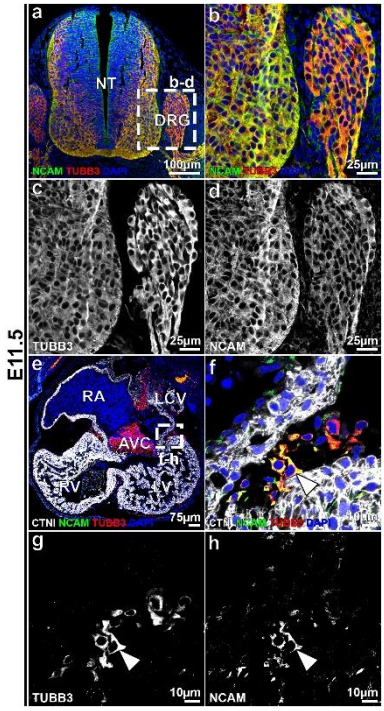
Abbreviations

β1AR	β1 adrenergic receptor
β2AR	β2 adrenergic receptor
cANS	cardiac autonomic nervous system
CTNI	cardiac Troponin I
E	embryonic day
EMT	epithelial-to-mesenchymal transition
HH	Hamburger and Hamilton
NCAM	Neural Cell Adhesion Molecule
NCC	neural crest cells
NGF	nerve growth factor
PEO	proepicardial organ
RA	retinoic acid
RXRα	retinoid X receptor-α
SV	sinus venosus
TUBB3	tubulin beta-3 chain
WT1	Wilms' tumor-1

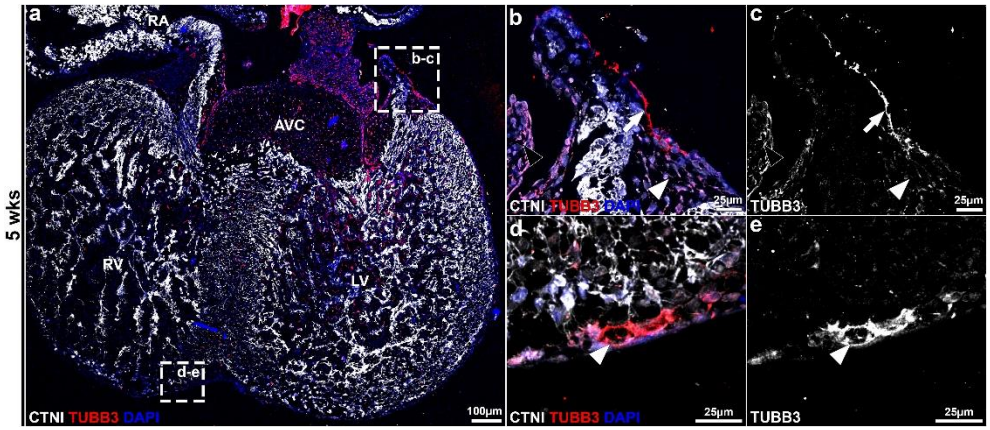
Supplementary Figures



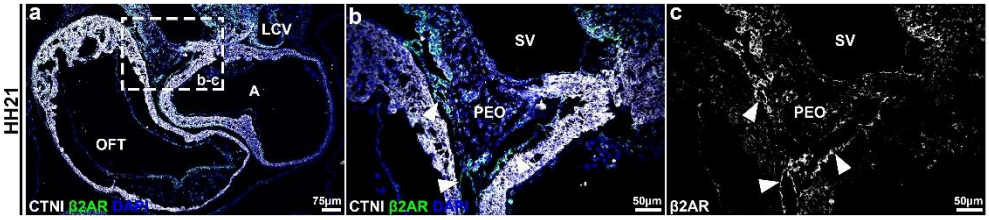
Supplemental Figure 1. Baseline heart rate of the experimental groups does not significantly differ between the groups.



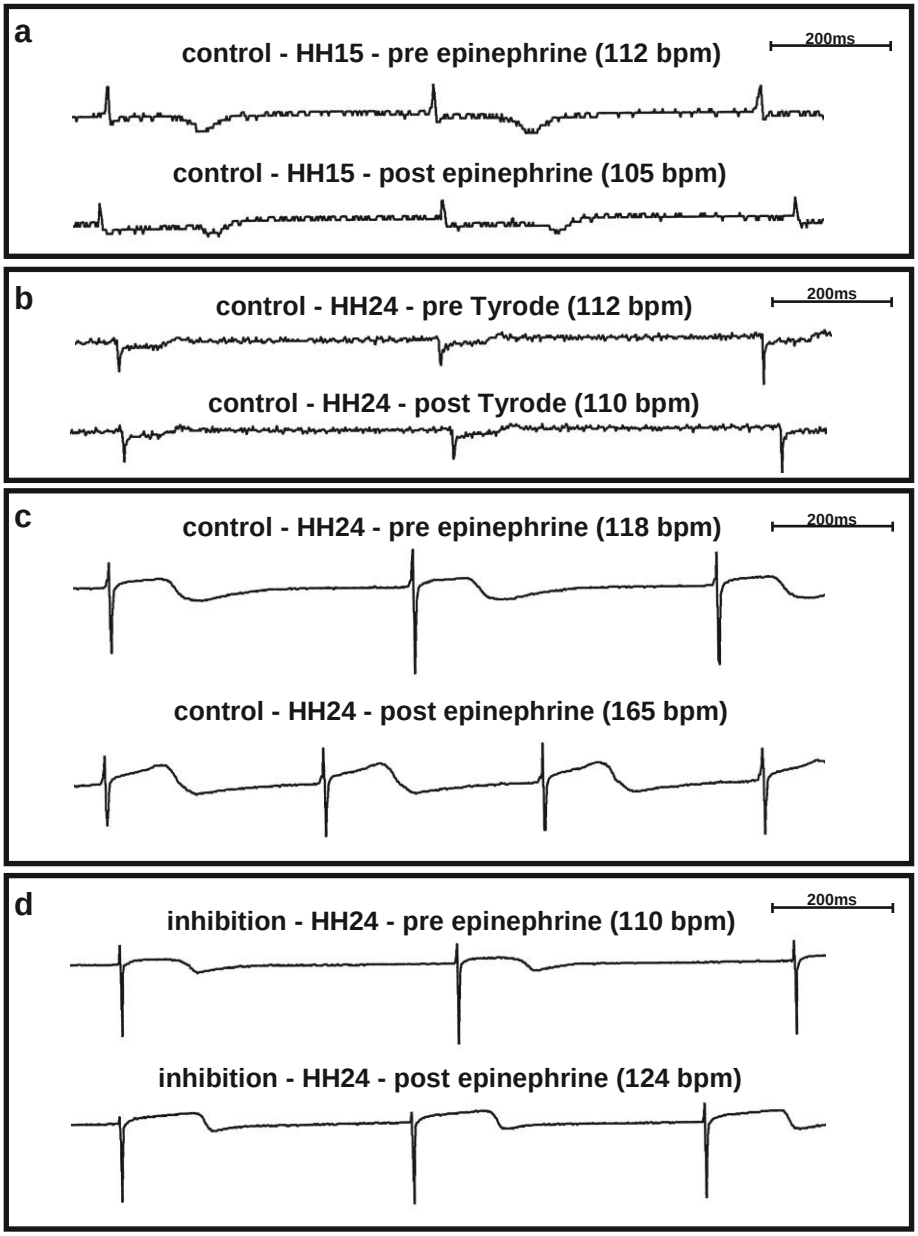
Supplemental Figure 2. Epicardial co-expression of the neuronal markers NCAM and TUBB3 confirms the neuronal phenotype of the epicardium. a-d. NCAM and TUBB3 are co-expressed in the nervous system, shown here in the neural tube (NT) and dorsal root ganglia (DRG). **e-h.** a subpopulation of epicardial cells co-express TUBB3 and NCAM (white arrowheads in **f-h**). AVC, atrioventricular cushion; LCV, left cardinal vein; LV, left ventricle; RA, right atrium; RV, right ventricle



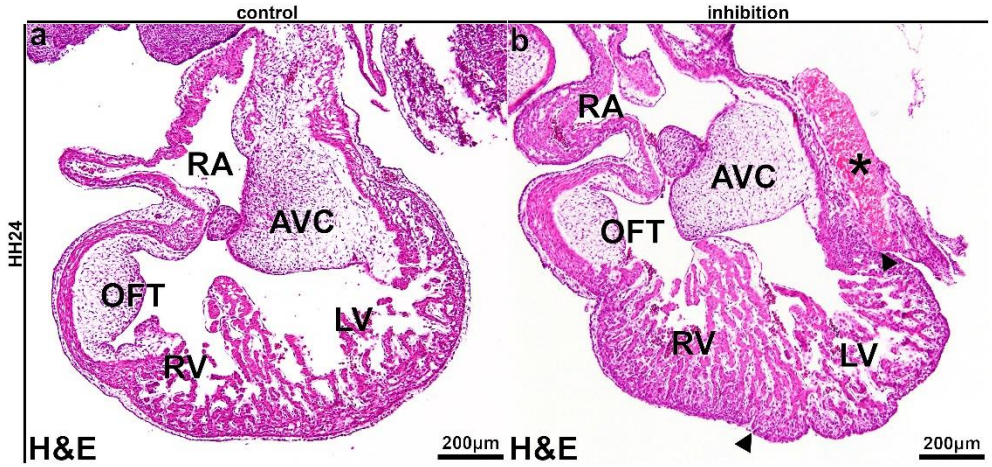
Supplemental Figure 3. The neuronal marker TUBB3 is expressed in the epicardium and subepicardium during early human development. a-c. Expression of TUBB3 is found in the epicardial layer (arrows in **b,c**), the subepicardium (white arrowheads in **b,c**) and the endocardial cushions (black arrowheads in **b,c**) of the human embryonic heart at week 7. **a,d-e.** Expression of TUBB3 is found in the subepicardial layer of the human embryonic heart (white arrowheads in **d,e**). AVC, atrioventricular cushion; RV, right ventricle; LV, left ventricle.



Supplemental Figure 4. β2AR expression in the epicardium in chick. a-c. At HH21 in chicken expression of β2AR is present in the proepicardial organ (PEO), epicardium (arrowheads in **b-c**) and endocardium. A, atrium; OFT, LCV, left cardinal vein; outflow tract; SV, sinus venosus; PEO, proepicardial organ.



Supplemental Figure 5. Representative electrode recordings. a. The heart rate at HH15 did not increase after administration of epinephrine. **b.** The heart rate at HH24 did not increase after administration of Tyrode. **c.** The heart rate at HH24 increased after administration of epinephrine. **d.** The heart rate at HH24 with epicardial inhibition did not increase after administration of epinephrine.



Supplemental Figure 6. Inhibition of epicardial outgrowth in chicken embryos. a. Control heart at stage HH24 showing epicardial covering and thick, cell-rich subepicardial space. **b.** Inhibition of epicardial outgrowth shows an incomplete covering of the heart and a thin subepicardial space at stage HH24. Black arrowheads point to the border of the epicardial lining. Note the abnormal shape of the ventricle with a thin compact myocardium in the region where epicardial covering is absent (between arrowheads). RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; OFT, outflow tract; AVC, atrioventricular cushion; * eggshell membrane.

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6

The mechanoresponse of hEPDC;
the effect of *in vitro* strain application

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Abstract

After myocardial infarction the disturbed balance between deposition and degradation of extracellular matrix results in adverse remodeling of the heart. Injecting human epicardium-derived cells (hEPDCs) after infarction improves cardiac function, however, the effect hEPDCs may have on matrix remodeling and the role of mechanical loading on this process has never been investigated. *In vivo* data show that hEPDCs contribute to matrix remodeling already early after injection by deposition of collagen. To investigate the effect of exposure to mechanical loading of hEPDCs on matrix remodeling, cells were cultured *in vitro* by exposing them to cyclic strain using a Flexcell FX-4000 T straining device. *In vitro* data show that cyclic strain alone does not have a major effect on the differentiation of hEPDCs into (myo)fibroblasts nor on the matrix remodeling behavior. Our experiments provide a first insight in the mechanoresponse of hEPDCs and show that exposure to cyclic stain alone is not sufficient to explain the contribution of these cells to matrix remodeling *in vivo*.

Keywords;

Epicardium, extracellular matrix, cardiac fibrosis, cardiac remodeling, mechanoresponse

1. Introduction

After myocardial infarction (MI), the damaged cardiomyocytes are replaced by non-contractile fibrotic scar tissue. This myocardial scarring immediately after heart injury is an important survival mechanism as it prevents rupture of the cardiac wall. Unfortunately, over time the fibrotic scar will lead to impaired function and ultimately to heart failure [1, 2]. This adverse cardiac remodeling is a consequence of a disturbed balance between deposition and degradation of extracellular matrix (ECM) by (myo)fibroblasts [3, 4].

After MI, the cardiac wound remodeling process can be divided in four phases [1, 5]. In the first phase, massive cardiomyocyte death occurs, followed by an early inflammatory response (phase 2). Cardiac cell death and influx of immune cells result in the release of cytokines, growth factors, and cell debris, inducing multiple cellular responses [1, 5-7]. Early infiltration of immune cells, which clean up the necrotic cells, is facilitated by degradation of the ECM produced by interstitial fibroblasts [7]. Interstitial matrix degradation is caused by an increase and activation of latent matrix metalloproteinases (MMPs) [8]. After approximately 3 days, the third phase of remodeling starts. The blood flow is restored and granulation tissue is formed by the infiltrated immune cells and interstitial fibroblasts. These cells release transforming growth factor beta (TGFβ), which stimulates activation and proliferation of quiescent fibroblasts into myofibroblasts [9]. The exact origin of cardiac myofibroblasts is still unclear [7, 10]. Myofibroblasts are (virtually) absent in a healthy heart and can be distinguished from fibroblast by the expression of alpha smooth muscle actin [11]. Myofibroblast are present during cardiac remodeling and scar formation and play a key role in the deposit of connective tissue [12]. Although myofibroblasts are necessary for the wound healing process, their presence within the heart can also negatively influence the organ's function. Myofibroblasts are not excitable and might thereby disturb the conduction velocity in the injured heart and the cardiac function. Remodeling and stabilization of the fibrotic scar characterize the fourth phase of the wound healing process. Extensive ECM turnover will affect the structure of the heart and thereby interfere negatively with the cardiac function.

Treatment of MI mainly focusses on the restoration of the cardiac circulation thereby restoring cardiac perfusion. More regenerative approaches attempt to focus on cardiomyocyte renewal and thereby preclude adverse remodeling. Stem cell therapy aims to recover cardiac function by restoring cardiovascular tissue by replenishing the myocardial wall with new cardiomyocytes and the formation of new blood vessels. The proper deposit of ECM is essential to restore the integrity of the cardiac wall and thereby improve function. Adult cardiac

progenitors are the most feasible cells to use since they are committed to cardiac differentiation, can be obtained autologous and have no ethical objections.

Recent studies positioned the epicardium, the outer layer of the heart, as a potential source of cardiac progenitors [13-16]. The epicardium is essential during cardiac development as it plays a role in the development of the vasculature and the connective tissue of the heart [17-19]. Furthermore, epicardium-derived cells (EPDCs) enhance proliferation, cellular maturation and alignment of cardiomyocytes thereby contributing to synchronized beating [20-22]. During development a subset of activated epicardial cells lose cell-cell contact and undergo epithelial-to-mesenchymal transition (EMT), resulting in EPDCs [13, 23]. EPDCs migrate into the subepicardium and contribute to the formation of cardiac fibroblasts, smooth muscle and endothelial cells [23-27]. The potential differentiation of EPDCs into cardiomyocytes is still under debate [15]. During adult life, epicardial cells are reactivated after myocardial infarction (MI), making them an interesting cell source for regeneration [14, 16, 28, 29]. Injecting human EPDCs (hEPDCs) after MI results in an improvement of the cardiac function, though, the effect on cardiomyocyte regeneration remains limited [14]. An additional drawback of cell transplantation therapy is that engrafted hEPDCs demonstrate a myofibroblast phenotype, which might adversely affect cardiac remodeling and function [12].

The objective of this study is to investigate the matrix remodeling behavior of hEPDCs *in vivo* and the effect of mechanical loads on the behavior of hEPDCs *in vitro*. Therefore, hEPDCs were injected in the infarcted mouse heart and the expression of human collagen was analyzed. Furthermore, hEPDCs were exposed to cyclic strain *in vitro* with the use of a Flexcell system and the differentiation and matrix remodeling behavior of hEPDCs was determined. Despite the suggestions from the literature [30], our results show that cyclic strain does not have a major effect on the differentiation of hEPDCs toward myofibroblasts. The triggers for hEPDCs to contribute to matrix remodeling *in vivo* are therefore multifunctional.

2. Material and methods

2.1. Immunofluorescent staining in vivo material

The protocol used for immunofluorescent staining is described previously [31]. Briefly, slides were deparaffinised, rehydrated and subjected to heat-induced epitope retrieval with Vector® Antigen Unmasking Solution (Vector). Sections were incubated overnight at 4°C with primary antibodies. The antibodies and their suitable dilutions are listed in Supplemental Table 1. All

primary antibodies were visualized with Alexa-conjugated fluorescent secondary antibodies (Invitrogen). Sections were mounted with ProLong® Gold antifade reagent (Invitrogen) containing DAPI.

2.2. Culture of epicardial derived cells

Cultures of human epicardial cells were prepared from human adult auricles, considered as surgical waste as previously described [32]. Briefly, the layer of epicardium was stripped from the auricle, after which the tissue was placed in a gelatin coated culture disk and capped with a round coverslip to prevent the tissue from floating. Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) and medium 199 (M199) (Invitrogen) 0.5% penicillin (Invitrogen), 0.5% streptomycin (Invitrogen) and 10% inactivated fetal calf serum (Invitrogen). Seven days after culturing, when outgrowths of epithelium-like cells were visible the coverslips and remaining tissue were removed.

2.3. Culture conditions

To elucidate the role of strain on differentiation and remodeling behavior of hEPDCs cells were either cultured under static conditions or exposed to 8% cyclic strain. In addition, cells of both conditions were cultured either in the presence of TGFβ or SB-431542, an Alk5 inhibitor.

2.4. Flexcell® Tension Systems

Epicardial derived cells were seeded on UniFlex® Culture Plates, coated with collagen I (Dunnlab). After attachment and 24 hours adaptation the cells were subjected to uniaxial cyclic strain for 48 hours by using a Flexcell FX-4000 T straining device (Flexcell) to study the effects of cyclic strain (8%) and the effects of static culture (0%). A strain magnitude of 8% and a frequency of 1 Hz were chosen as a suitable strain condition based the physiological mechanical loads on the cardiac wall [33]. Strain experiments were performed with cells from multiple patients in two independent experiments per patient.

2. 5. mRNA isolation and quantitative PCR analysis

Total RNA was isolated using TriPure and treated with DNAse-I (Qiagen) according to the manufacturer's protocol. Subsequently 250ng RNA per sample was reverse transcribed into cDNA using the MMLV based cDNA synthesis kit (Bio-Rad). Primers for quantitative PCR (qPCR) were designed with Beacon Designer 7.0 (Premier Biosoft International). Primer sequences and annealing temperatures are presented in Supplemental Table 2. cDNA

samples were subjected to qPCR using iQTM SYBR@ Green Supermix (Bio-Rad) and the Bio-Rad IQ™5 detection system (Version2.0).

2.6. Immunofluorescent staining in vitro material

EPDCs cultured on UniFlex® Culture Plates were fixated in 4% paraformaldehyde (Merck) in PBS at RT for 15 minutes, permeabilised with 0.5% Triton X-100 (Merck) in PBS for 10 minutes and blocked with 5% BSA in PBS for 30 minutes. Subsequently, incubation with primary antibodies were performed overnight at 4°C in NET-gel [50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% NP40, 0.25% gelatin] with 0.5% BSA. The antibodies and their suitable dilutions are listed in Supplemental Table 1. Incubation for 2 hours with Alexa-conjugated fluorescent secondary antibodies (Invitrogen) were used to visualize the primary antibody binding. Nuclear counterstain was achieved by DAPI (1:500) in NET-gel incubation for 5 minutes. Finally, cover glasses were mounted on microscope slides with Mowiol.

2.7. Statistical analysis

The paired t-test was used to compare the grouped samples. $P<0.05$ was considered statistically significant. Data shown is mean ± S.E.M. Statistical analysis was performed using the Graphpad Prism 6 software package (Graphpad Software). In addition, the conditions within one patient are shown within the figures in order to correct for the variations between patients although no statistics has been performed on this data.

3. Results

3.1. In vivo evaluation of the production of collagen by hEPDCs after infarction

In previous studies, injection of hEPDCs after MI improved left ventricular function and attenuation of adverse remodeling [14]. To investigate the initial contribution of the injected hEPDCs after MI to matrix remodeling, the expression of human collagen I was visualized by immunofluorescent labelling. Already two days after injection, expression of human collagen was observed (Figure 1a). The produced collagen was not only present in the cells, but also outside the cells, suggesting secretion of human collagen. Over time, at both day 4 and day 7 after MI the production and deposition of human collagen was slightly increased (Figure 1b, c). Injected hEPDCs were still present after 6 weeks, as shown by the presence of eGFP positive cells. The amount of human collagen I, however, was strongly decreased after 6 weeks although the total amount of collagen I was higher in the infarcted area compared to earlier time points (Figure 1d).

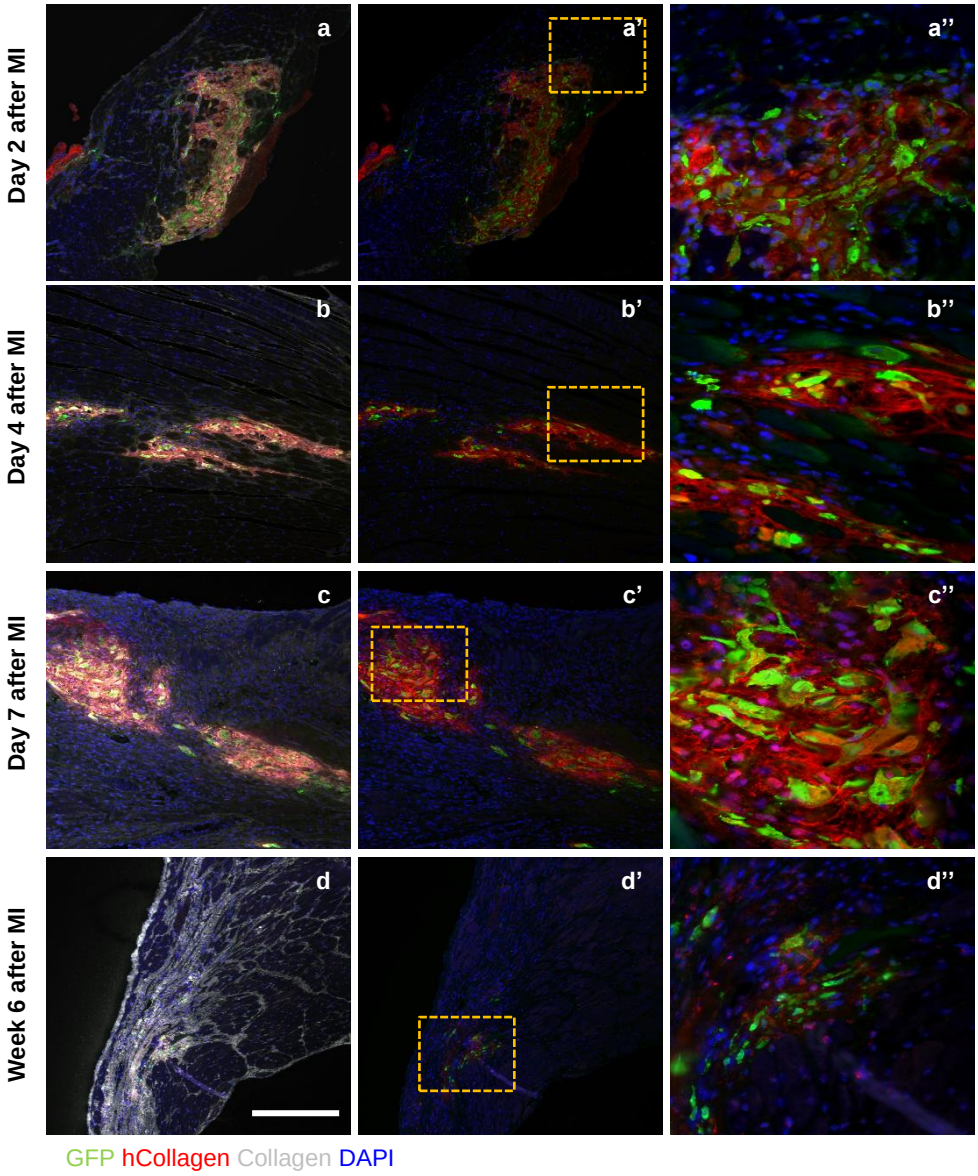


Figure 1. Collagen is expressed by human EPDCs after injection. a. 2 days after injection hEPDCs (green) express human collagen (red). **b, c.** The expression of human collagen (red) increased at day 4 and 7 after MI. **d.** hEPDCs (green) were still present in the infarcted heart. The total amount of collagen (white) was very prominent, however, the amount of human collagen (red) was greatly reduced. Scale bar 250um.

3.2. Exposure of hEPDCs to cyclic strain in vitro does not result in deposition of ECM proteins

The ECM consists primarily of collagens to provide structural support to cells. The elasticity, is mainly determined by elastin and the binding of cells to the ECM is facilitated by fibronectin [34]. When cells are injected, their environment changes from a static environment to an environment in which they encounter mechanical loads. Therefore, we cultured hEPDCs under cyclic strain to analyze the effect of mechanical loading on the production of ECM.

Exposure to strain for 48 hours revealed no significant increase in neither *collagen I* nor *III* gene expression (Figure 2a, b). The expression of *fibronectin* mRNA was not changed either. Expression of *elastin* was not observed in the strained and unstrained cells (data not shown). On protein level, no change in the expression levels of collagen I or III was found upon strain. In both strained and unstrained conditions the collagens were present around the nucleus and in the cytoplasm, but not extracellular (Figure 2c, d).

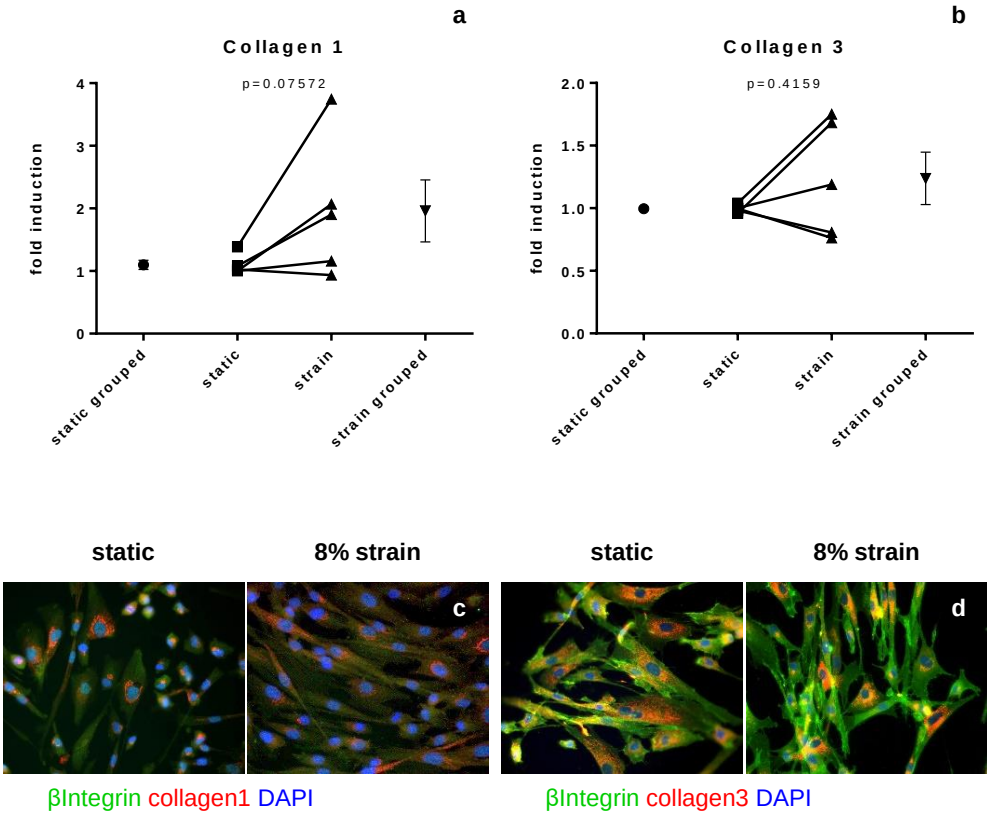
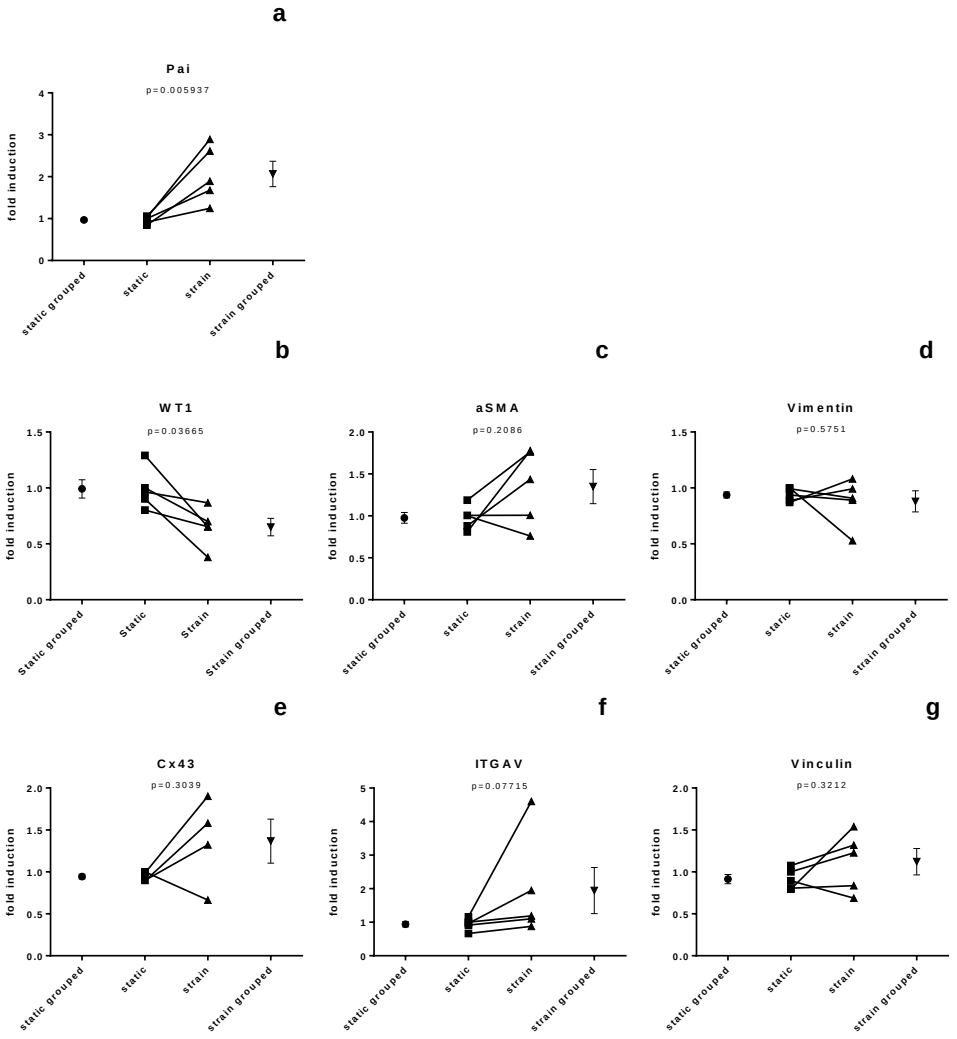


Figure 2. Cyclic strain does not induce the expression of collagen in EPDCs. hEPDCs exposed to stain showed no upregulation of collagen 1 or 3 expression on mRNA level (**a, b**) or protein level (**c,d**). βIntegrin was used to display the contours of the cells. Pictures were taken with a 40x objective.

3.3. Cyclic strain does not induce differentiation or proliferation of hEPDCs

EPDCs can differentiate into fibroblast and transplantation of EPDCs in the ischemic environment after MI is known to drive their direction towards myofibroblasts due to elevated levels of TGFβ in the ischemic environment [23, 26]. Levels of TGFβ are believed to raise upon cyclic strain and thereby stimulate differentiation of the cells [30]. Therefore, in this study we elucidate whether cyclic strain is a factor which can stimulate hEPDCs to differentiate toward (myo)fibroblasts and as a result to adverse remodeling by analyzing the expression of various markers.



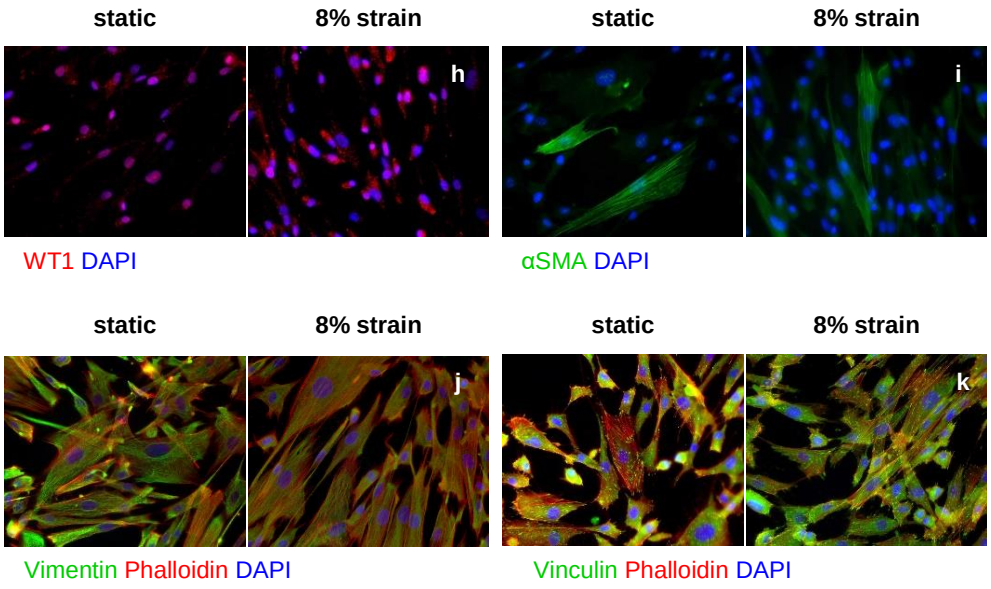


Figure 3. Cyclic strain does not induce the differentiation of hEPDCs into fibroblasts. hEPDCs exposed to stain show a significant upregulation of Pai-1 (**a**) and a downregulation of WT1 expression on mRNA level (**b**) and protein level (**h**). The differentiation markers aSMA and vimentin show no upregulation on mRNA level (**c, d**) or protein level **(i, j).** The connective markers Cx43, ITGAV and vinculin are not upregulated on mRNA level (**e, f, g**). The expression of vinculin on protein level is more organized and concentrated at the focal adhesions (**k**)**.** Pictures were taken with a 40x objective.

Analysis of mRNA expression showed that exposure to cyclic strain for 48 hours resulted in a significant downregulation of the epicardial marker *WT1* and upregulation of the TGFβ downstream target gene *PAI-1* (Figure 3a, b). However, the expression levels of *αSMA* and *vimentin*, markers for fibroblasts differentiating into myofibroblasts, were not significantly increased (Figure 3c, d). Also, *Cx43*, *ITGAV* and *vinculin* mRNA levels, markers for cell-cell and cell-matrix interaction, were not significantly upregulated when applying strain (Figure 3e, f, g).

Although, the expression level of most proteins did not show major changes (Figure 3h-k), vinculin seems more structured and more concentrated at the site of focal adhesions when hEPDCs were exposed to strain. The localization of vinculin on focal adhesions coincides with the organization of the stress fibers, which were visualized with phalloidin (Figure 3k).

Mechanical stimulation is known to activate cellular proliferation [35-38], therefore cellular turnover of hEPDCs was analyzed by visualization of the expression of Ki67 (Figure 4a).

Interestingly, exposure of hEPDCs to strain resulted in a lower expression of Ki67, suggesting that cyclic strain retained hEPDCs in a quiescent state (Figure 4b).

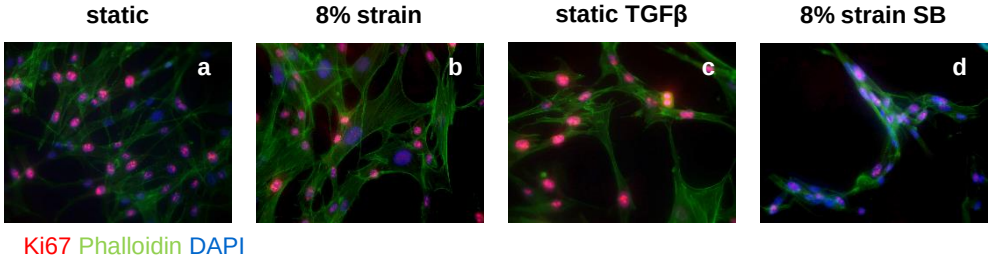


Figure 4. Cyclic strain does not induce proliferation in hEPDCs. a. Cellular proliferation of hEPDCs was visualized by the expression of the proliferation marker Ki67 **b.** Exposure of hEPDCs to cyclic strain did not increase the expression of Ki67. **c.** Exposure of hEPDCs to TGFβ did increase the expression of Ki67. **d.** The decreased expression of Ki67, when strain and SB were combined, confirmed the TGFβ mediated effect of strain. Pictures were taken with a 40x objective.

3.4. SB reduces the differentiation in hEPDCs

Although application of cyclic strain did not influence the production of matrix components by hEPDCs nor did it influence their differentiation potential, the expression of *αSMA* and *collagen I* in both static and strained conditions suggests the involvement of TGFβ signaling. TGFβ is known to induce, both the differentiation of fibroblasts into myofibroblasts, and the production of ECM [7, 10]. To study the involvement of the TGFβ signaling pathway, SB-431542 (SB), which inhibits the TGFβ signaling pathway by inhibiting the TGFβ type I receptor serine/threonine kinase ALK5 [39], was added to hEPDCs cultures and the expression of differentiation markers and ECM proteins were analyzed. The application of cyclic strain in combination with SB treatment resulted in a significant decreased expression of *PAI-1* mRNA in hEPDCs, confirming the inhibition of the TGFβ signaling pathway (Figure 5a).

After 48 hours, the expression of the *WT1* was significantly upregulated in hEPDCs exposed to both strain and SB compared to hEPDCs that were only exposed to strain (Figure 5b). The expression of *αSMA* mRNA, on the other hand, was significantly downregulated in hEPDCs exposed to both strain and SB (Figure 5c, d). Cell-cell interactions, indicated by the expression of *Cx43* and *ITGAV*, were significantly reduced after exposure to both strain and SB (Figure 5e, f). The expression of *vinculin*, however, was not different between both groups (Figure 5g). Blocking the TGFβ pathway with SB-treatment did significantly inhibit the production of *collagen I* whereas the expression of *collagen III* was unaffected (Figure 5h, i). The changes on mRNA level for both differentiation as well as matrix proteins were confirmed by fluorescent staining (Figure 6).

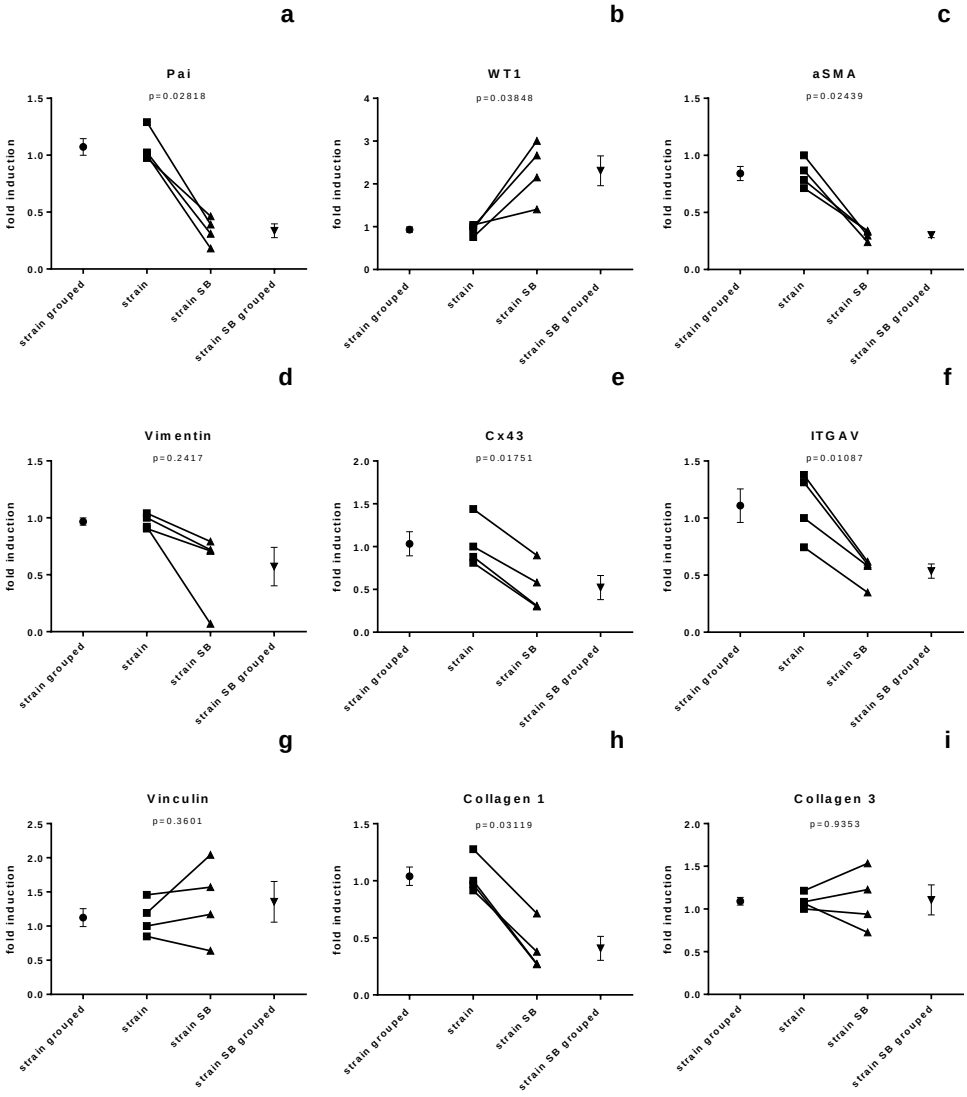


Figure 5. SB reduces the differentiation in hEPDCs. hEPDCs exposed to stain showed a significant downregulation of the expression of the TGFβ downstream target Pai1 on mRNA level when cultured in the presence of SB (**a**). The inhibition of the TGFβ signaling with SB resulted in a significant upregulation of WT1 (**b**) and downregulation of aSMA (**c**), Cx43 (**e**), ITGAV (**f**) and collagen 1(**h**). No significant change was found in the expression of vimentin (**d**), vinculin (**g**) and collagen 3 (**i**).

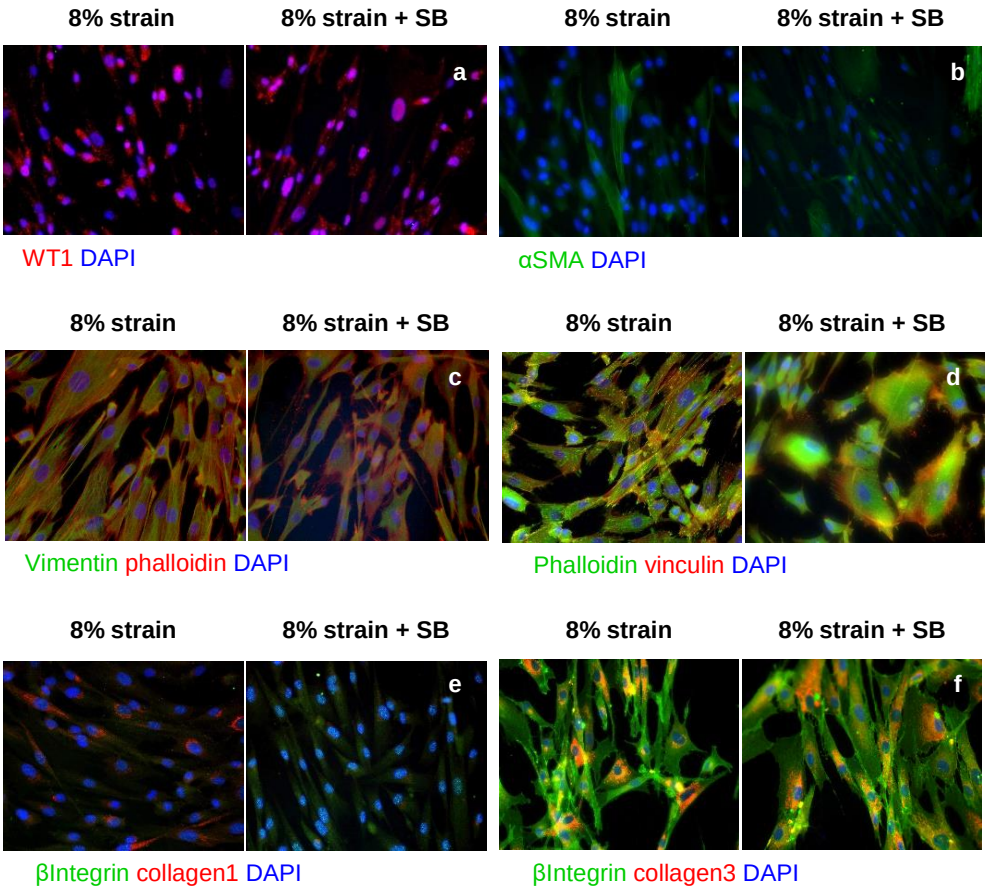


Figure 6. Inhibition of the TGFβ pathway reduces the expression of fibroblast differentiation markers on protein level. The inhibition of the TGFβ signaling with SB results in a upregulation of WT1 (**a**) and downregulation of aSMA (**b**) and collagen 1(**e**). No visible change was found in the expression of vimentin (**c**), vinculin (**d**) and collagen 3 (**f**). Pictures were taken with a 40x objective.

Although strain did not stimulate proliferation of hEPDCs (Figure 4b), TGFβ in static condition stimulated hEPDC proliferation (Figure 4c). This proliferation was inhibited in hEPDCs cultured in static condition with SB, as observed by a decreased expression of Ki67 (Figure 4d), indicating that hEPDC proliferation is regulated by the TGFβ signaling pathway.

3.5. Addition of TGFβ has no effect in the presence of strain

So far, our data suggest that application of cyclic strain does not influence the differentiation potential of hEPDCs. Reduction of hEPDC differentiation into (myo)fibroblasts by culturing these cells in the presence of SB in combination with strain showed involvement of the TGFβ signaling pathway. The experiment was repeated in the presence of both strain and TGFβ in order to determine if these cells have the capacity to respond to TGFβ stimulation.

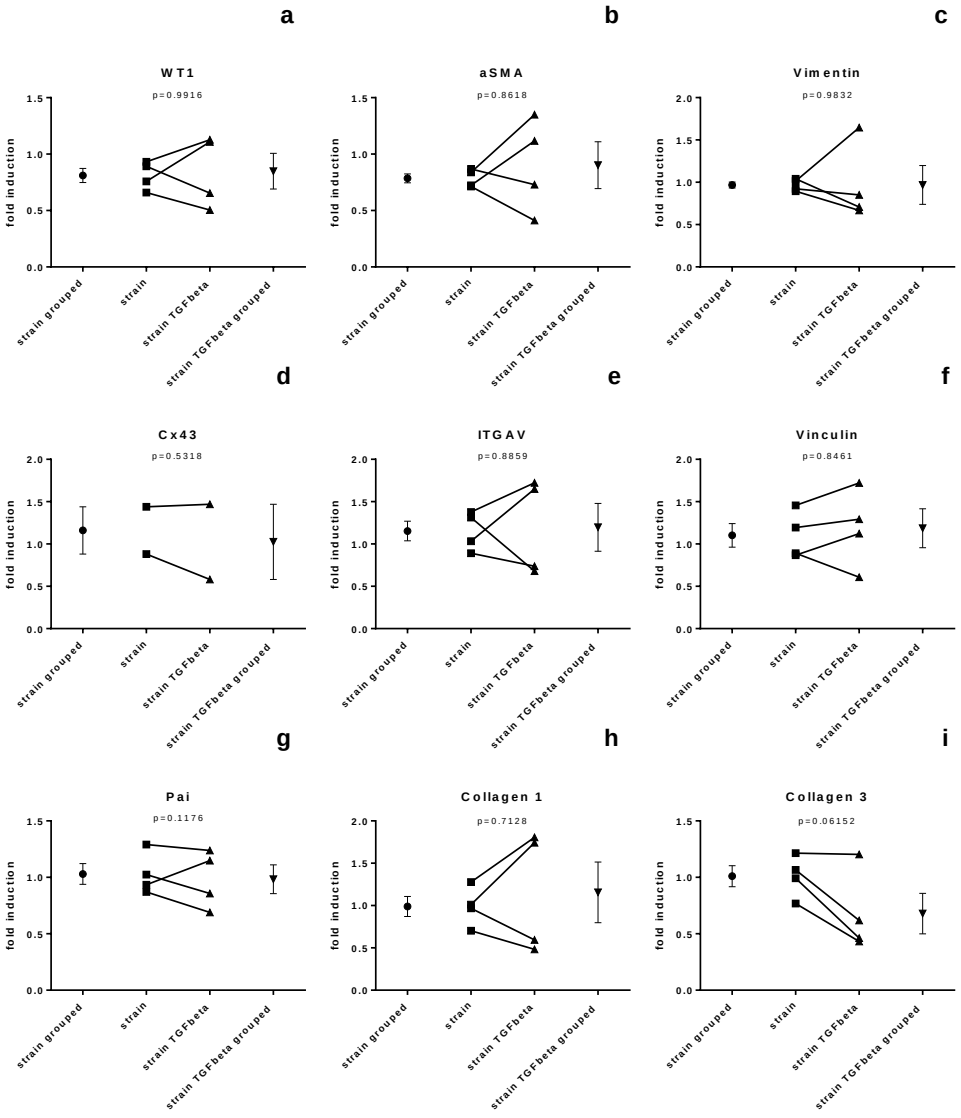


Figure 7. Cyclic strain and TGFβ do not have an synergistic effect on hEPDCs. No significant difference was found in the expression of the TGFβ downstream target Pai-1 (**g**). Expression of the differentiation markers WT1 (**a**), aSMA (**b**) and vimentin (**c**) were not significantly different when hEPDCs were exposed to both cyclic strain and TGFβ. No significant differences were found in the expression of the connective markers Cx43 (**d**), ITGA (**e**) and vinculin (**f**). No significant differences were found in the expression of the ECM proteins collagen 1 (**h**) and 3 (**i**).

We did not observe an effect of the addition of TGFβ to hEPDCs which are being exposed to cyclic strain. No significant difference was found in the expression of the TGFβ downstream target *PAI-1* by qPCR analysis (Figure 7g). Also the differentiation markers (*WT1*, *αSMA*, *vimentin*), cell-cell markers (*Cx43*, *ITGAV*), cell-matrix interaction marker (*vinculin*) and ECM proteins (*collagen I, III*) did not show any significant changes compared to only exposure of cyclic strain or TGFβ (Figure 7a-i). Therefore adding TGFβ to the hEPDCs strain culture did not have an effect on differentiation and matrix remodelling behaviour of hEPDCs.

4. Discussion

Stem cells are a potential source for cardiac regeneration after infarction to restore the cellular structure of the cardiac wall. Stem cells should ensure an organized wound healing process with as end result a “healthy” contractile tissue with minimal adverse fibrosis. Its crucial role during development and the differentiation potential of EPDCs has positioned the epicardium as a promising source of resident stem cells for cardiac regeneration [13]. Already 2 weeks after transplantation of hEPDCs into the infarcted heart preservation of the left ventricular function and attenuated post-ischemic remodeling is observed. In addition, the ventricular wall showed diminished ventricular dilatation [14]. The positive effect of the epicardium on myocardial injury is partially mediated by secreted paracrine factors [40]. In this current study, we show for the first time that hEPDCs contribute to matrix remodeling already after 2 days after MI by the deposition of human collagen I. Although the amount of secreted human collagen I seems minimal, the produced collagen becomes more organized within the first week after MI. Potentially the production of human collagen I by the injected hEPDCs will contribute to stiffening of the cardiac wall. The produced human matrix might function as a natural scaffold which protects the heart from rupture and thereby reducing adverse remodeling and dilatation of the heart as previously described [14]. Additional experiments should clarify if hEPDCs after injection can secrete more than one ECM protein, such as collagen III, elastin and fibronectin, in order to create a mature organized ECM. Alternatively, the presence of hEPDCs and the newly produced collagen may prevent the surrounding cardiomyocytes and endothelial cells from cell death and thereby preserve cardiac function.

Future research needs to determine whether injection of hEPDCs induces the expression of proliferation markers, and/or reduces apoptosis in the adjacent cells *in vivo*. Although hEPDCs are still present 6 weeks after injection, the amount of human collagen is strongly decreased. This suggests that hEPDCs are only able to respond in the acute phase after MI and that the secreted human collagen is replaced by murine collagen during remodeling of the cardiac wall.

Cell based therapy requires a large number of cells. However, only a limited number of cells from the adult heart can be obtained, which implies *in vitro* expansion of cells. Therefore hEPDCs are taken from their natural environment, which is exposure to mechanical loads, and cultured under static conditions [32, 41]. After injection of the cultured cells into the injured heart, hEPDCs encounter mechanical loading again, to which they have to adapt and respond. The effect of mechanical stimuli on the differentiation and matrix remodeling behavior of expanded hEPDCs was investigated *in vitro* by application of cyclic strain with the use of a Flexcell system. Our data show that *WT1* and *PAI-1* expression significantly change upon strain indicating that the EPDCs are sensing and responding to mechanical stimuli. This is supported by the concentration of vinculin at the site of focal adhesions when hEPDCs are exposed to strain. Significant changes are, however, not found in expression of *collagen*, *αSMA* and other markers, like *Cx43*, after 48 hours of cyclic strain. Some of the individual samples do show rather an upregulation than downregulation of these genes, suggestion a potential trend of differentiation from hEPDCs towards myofibroblasts. This trend is illustrated by a slight increase of *Cx43*, which is known to be increased during the differentiation of fibroblasts to myofibroblasts [11, 42]. However, the large variation between the samples prevents a significant outcome.

The lack of unambiguous data is possible due to patient variation, as hEPDCs are derived from multiple donors. Furthermore, the lack of a mechanically induced response in some of the samples might be due to an already active status of hEPDCs in static culture. Adult hEPDCs are mostly fibroblast-like cells, which are known to develop into myofibroblasts *in vitro* [23, 26, 43]. The transformation into myofibroblasts that occurs *in vitro* for some samples, might prevent the detection of a further increase in the number of myofibroblasts upon cyclic strain. Myofibroblast differentiation might be induced by the stiff surface the EPDCs are cultured on initially [43]. It is known that cellular behavior, including differentiation, is influenced by matrix stiffness [44-46]. As a result, experiments might have been performed with a relatively heterogeneous population of cells; hEPDCs, fibroblasts, and myofibroblasts. So, in future experiments it is important to take the local matrix stiffness on cells into account.

In addition, although the experiments were performed with heat-inactivated serum, it is possible that the amount of TGFβ in the culture medium is already high enough to elicit a maximal response of EPDCs. In this case, the addition of extra TGFβ will have no additional effect on the response of EPDCs *in vitro*. This might be prevented by repeating the experiment with culture medium without, or with the minimum amount of serum. Furthermore, the autocrine release of TGFβ by hEPDCs during the *in vitro* culture might contribute to making these cells already activated [47]. Together this could explain the lack of response by stimulation of the TGFβ signaling pathway, but the presence of response after inhibition of the TGFβ signaling pathway. Therefore, it is important to determine the homogeneity and the status of activation of the cell population, prior to start of the experiment.

The lack of a clear effect of cyclic strain induced with the Flexcell system might be explained by the simplicity of the *in vitro* setting, since many features of the native *in vivo* environment are missing. First of all, since we only work with a monolayer of hEPDCs, the cell-cell interaction with different cell types as present in the native heart is absent. The cardiac wall consists of cardiomyocytes, cardiac fibroblasts, smooth muscle cells, endothelial cells and a highly organized extracellular matrix, all of which interact with each other [48] and in our *in vitro* setting we only have a mix of hEPDCs, fibroblast and myofibroblast depending of the patient. In addition, the cardiac wall is a three dimensional structure and our experiments are performed a two-dimensional model. Lastly, *in vivo*, cytokines, hypoxia, growth factors, and cell debris after infarction are among the many factors that potentially contribute to the differential response of EPDCs and in this study we only incorporated TGFβ.

In summary, we show that injected hEPDCs are able to produce and deposit collagen early after MI, which might contribute to the reduced deterioration of the heart. Our preliminary results suggest that application of cyclic strain does not have a major effect on the differentiation of hEPDCs toward (myo)fibroblasts on the short term, which might be due to inter donor variations and the chosen culture conditions. Although many aspects of the microenvironment of the heart after MI are missing, application of cyclic strain with the Flexcell system can provide first insight in the mechanoresponse of cells. Increasing the number of samples and further optimization of the *in vitro* protocol might allow illustrating the effect of cyclic strain and thereby may have great value for the development of new therapeutics for the regeneration of the heart after MI.

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Disclosures

None

Abbreviations

αSMA	alpha smooth muscle actin
Cx43	connexin 43
ECM	extracellular matrix
EMT	epithelial-to-mesenchyme transition
EPDC	epicardium derived cell
ITGAV	integrin alpha-V
MI	myocardial infarction
MMPs	matrix metalloproteinases
PAI-1	plasminogen activator inhibitor-1
TGFβ	Transforming Growth Factor beta
WT1	Wilm's tumor-1

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7

General discussion

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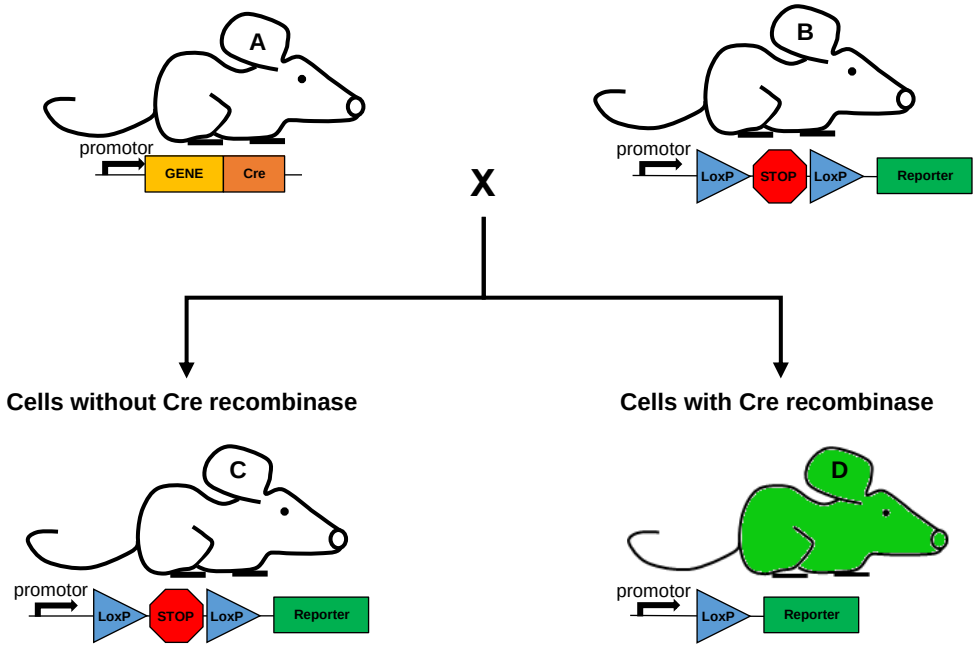
Formation of the heart involves the integration of multiples cell types that interact with each other to create a highly organized myocardial wall. This structure consists of cardiomyocytes supported by fibroblasts, a network of blood vessels and extracellular matrix (ECM). Due to cardiac diseases the organization and interaction of cells and the ECM are disrupted which leads to (adverse) remodeling of the myocardial wall. The aim of this thesis is to increase our understanding of the involvement of WT1 and the epicardium in the cellular and molecular processes of the developing heart and after cardiac injury. Our studies focused on (1) the cardiac expression and potential role of WT1 during, both human and murine, development and after cardiac injury, (2) the functional role of the epicardium in the modulation of the cardiac autonomic response before cardiac innervation and (3) the effect of strain on epicardium-derived-cells.

Impact of the endogenous expression of WT1 in endothelial cells of the heart for fate mapping studies

Fate mapping through lineage tracing aims to identify all progeny of a single cell population, based on a common characteristic. The Cre/lox system is a powerful tool to study lineage tracing in tissue development, homeostasis, and disease [1, 2]. It involves a Cre-mediated site-specific recombination, used to carry out deletions, insertions, translocations and inversions at specific sites in the DNA of cells [3]. Loxp sequences highlight specific binding sites which can be recognized by Cre recombinase. For lineage tracing purposes, the Cre-mediated recombination results in removal of a stop codon, thereby enabling the transcription of a reporter gene. The permanent staining of cells, which is the result of this technique, allows visualization of these cells and their progeny [1] (Figure 1). The expression of the Cre recombinase determines which cell type is traced and should therefore be controlled by regulatory elements of a gene that is exclusively expressed by the cell type of interest. The activation of the Cre-recombinase in this specific cell type can also be controlled if the inducible CreERT2 is used, in which the Cre is kept outside the nucleus, unable to recombine the DNA. Upon tamoxifen treatment, Cre recombinase moves into the nucleus, resulting in time- and tissue-specific activation of Cre [4].

The initial observation that the expression of WT1 in the heart is restricted to the (pro)epicardium [5-7] has resulted in the use of regulatory elements of the WT1 gene to specifically label the epicardial lineage to follow its progeny [8-10].



Schematic of the Cre–Lox system used for lineage tracing. Transgenic mouse A contains a Cre recombinase under the control of a gene specific promotor. The Cre recombinase is only expressed in cells in which the gene is expressed. Transgenic mouse B contains a reporter sequence which is driven by a ubiquitous promotor that is normally terminated by an inserted stop codon, which is flanked by LoxP sites. Crossbreeding mouse A and B can produce transgenic mouse C or D. In mouse D the LoxP sites are recognized by the Cre-recombinase.Cre-mediated recombination results in removal of the stop codon, thereby enabling the transcription of the reporter gene and permanent labeling of cells.

In the study of Zhou and colleagues, using WT1-driven lineage tracing, labeling of endothelial cells was found, although minimal [8]. Recently another lineage tracing study, using a different WT1-Cre model, found labeling of approximately 20% of the endothelial cells in the heart [11, 12]. Both studies concluded that the WT1-expressing epicardial cells contribute to the formation of endothelial cells in the heart. Data presented in this thesis show that cardiac expression of WT1 is not restricted to the (pro)epicardium, but is also expressed in endothelial cells of the forming coronary vasculature [13, 14] and might be responsible for labeling of the cardiac endothelial cells in WT1-driven lineage tracing studies. Caution should therefore be exercised in the interpretation of using WT1 promoter to label epicardial cells. Whereas we observed WT1 expression in virtually all endothelial cells during cardiac development, only limited number of labeling is found in the lineage tracing. This is possibly due to the low expression levels of WT1 in these endothelial cells causing insufficient expression of Cre

recombinase to efficiently recombine, or to the poor recombination efficiency of some models [15]. That regulatory elements of WT1 expression in endothelial cells can drive recombination is shown by a significant labelling of endothelial cells after activation of Cre at E14.5 when the inducible WT1CreERT2 is used, and in our *ex vivo* studies (not shown) [16].
The WT1-driven lineage tracing thus appears not to be specific enough to draw conclusions about the ability of the epicardium to contribute to the cardiac endothelial cell population. Other epicardium-specific Cre models have been used to address this issue. The TBX18-Cre, Sema3d-Cre, Scx-Cre drive recombination in the (pro)epicardium and label endothelial cells in lineage tracing studies [17, 18]. The significant contribution of the Semaphorin3d and Scleraxis lineage traced cells to the endothelium versus the minimal contribution of WT1 lineage traced cells can be explained by the heterogeneity of the epicardial cells [17]. We did, however, find that virtually all epicardial cells have high expression levels of WT1, which should result in sufficient levels of Cre recombinase and high percentage of recombination in the WT1 expressing epicardial cells and significant labelling of the endothelial cells which are derived from the epicardium. Possibly, Semaphorin3d and Scleraxis positive cells contribute less to the formation of the epicardium, and migrate directly into the myocardium where they contribute to the cardiac endothelial population. As all epicardial cells are WT1 positive, this would suggest that the Semaphorin3d and Scleraxis lineage traced cells are not all derived from the epicardium but from another source. Indeed, Semaphorin3d and Scleraxis lineage traced cells are also found in the sinus venosus (SV) and endocardium respectively, which both have been shown to potentially contribute to endothelial cells (see below). TBX18 lineage-traced endothelial cells on the other hand are very sparse [18].

Experiments in chicken did suggest that the (pro)epicardium contributes to the pool of cardiac endothelial cells. Injection of a lacZ-expressing retrovirus into the precardiac mesoderm as well as lineage tracing of proepicardial cells after direct labelling resulted in incorporation of the label into endothelial cells [6, 19, 20]. Studies using an elegant technique in which a quail proepicardium was transplanted into the pericardial cavity of chicken embryo's, in which the proepicardium was removed, showed that EPDCs contributed to the formation of coronary vessels [21-24]. In addition, quail proepicardial explants cultured on Matrigel showed the ability of EPDCs to differentiate into endothelial cells [6]. The question of purity of the proepicardial tissue used in the experiments might be relevant, considering that the proepicardial tissue might contain liver tissue that can contribute endothelial cells to the SV. Chicken-quail chimera studies in which proepicardium transplants without liver tissue were used did not lead to endothelial cell formation [21]. In summary, although many studies point

to a possible contribution of (pro)epicardial cells to endothelial cells, conclusive evidence has not been presented yet. In addition, the percentage of endothelial cells derived potentially from the epicardium is not defined, but appear to be low in all different models and studies.

The observation that the epicardium is not the only source of endothelial cells in the heart and the identification/recognition of several additional cell populations that can contribute to the formation of coronary endothelial cells [25] complicates the discussion about the origin of the cardiac endothelial cells. As mentioned before, the SV is one of these possible sources. This transient embryonic structure is the venous pool of the heart and receives blood from the vitelline vein, umbilical vein and common cardinal vein before it flows into the atrium [26]. Red-horse et al. used apelin-cre mice to label the apelin-expressing endothelial cells in the SV. Coronary endothelial cells in most of the heart were found to be positive for the reporter, which suggested to the authors that these cells are SV derived [27]. However, the specificity of apelin for the SV is challenged, because at E11.5 and E12.5 coronary vessels in the heart are also positive for apelin expression [28], which does not allow to draw strong conclusions about SV derived endothelial cells. Arita et al. showed that the SV is populated by a heterogeneous population of endothelial cells and suggest that the more immature population of the SV contributes to the coronary vasculature [29].
Another potential source for the endothelial cells might be the endocardium, the inner lining of the heart. Studies in which Nfatc1-Cre mice have been used to follow the fate of endocardial cells [30-33], showed labeling of the majority of coronary arteries [34]. Nfatc1, however, is not only expressed by endocardial cells during cardiac development, but also by the endothelium of the SV [35]. The natriuretic peptide receptor 3 (Npr3) is suggested to be a more specific marker for the endocardium, and lineage tracing using the Npr3-CreER mouse showed limited labeling of the coronary endothelial cells in the free wall, but a significant contribution to the coronary endothelium of the septum is seen [35].

Together, these observations suggest 3 potential sources that provide cardiac endothelial cells to the heart in a spatial restricted manner during development [28]. The SV and epicardium provide endothelial cells from the outside of the heart, whereas the endocardium contributes to the formation of endothelial cells that cannot be easily reached from the outside, i.e. the ventricular septum of the heart. The location of the different potential sources is closely associated with the location of its endothelial cell progeny, which suggests that the source is more determined by its closest anatomical position, rather than by its specific nature. Given the likely multiple sources of cardiac endothelial cells, quantitative approaches might help to

define the predominant origin(s) of these cells at specific locations within the heart. This thesis shows that it is a challenging quest to specifically label these predominant origin(s) during development at this moment.
The 3 suggested sources, the epicardium, the SV and the endocardium are closely connected. The SV is continues with the proepicardium outside the heart and the endocardium inside the heart. Cells from the proepicardium might contribute to the SV, so it can be questioned how different these 3 sources actually are. The environment of cells at their destination is likely to play a more important role to determine the endothelial phenotype and function. The importance of cellular environment is also illustrated by the culture of endothelial cells from different organs as they lose their specific gene expression profile *in vitro*. However, overexpression of WT1 was sufficient to differentiate endothelial cells into a more cardiac specialized population [36], indicating that the environment is more important than origin in determining their fate and function.

Epicardial contribution to cardiac innervation

The migration of cardiac nerves from the subepicardium into the myocardium coincident with the sprouting of coronary vessels [37]. Results presented in this thesis suggest a role for the epicardium in the autonomic modulation of the developing heart prior to the formation of the cardiac nervous system, a role in the formation of the cardiac nervous system itself and might even suggest the possibility that the epicardium has a structural contribution in the formation of nerves.

The idea that the epicardium acts as a receptor for epinephrine derives from the observation that the catechol receptor β2AR is expressed by WT1-positive epicardial cells. Furthermore, these cells also express the neural markers tubulin beta-3 chain (TUBB3) and Neural Cell Adhesion Molecule (NCAM), indicating that the epicardium has a temporarily neuronal phenotype. Noteworthy is the expression of WT1 in known neuronal structures along the central region of the mouse spinal cord during development, suggesting that the expression of WT1 is important for the neuronal phenotype of cells [38-40]. This hypothesis is supported by the observation that WT1 is involved in neuronal functioning of the olfactory system and retina [38, 41]. Only a limited number of cells in the spinal cord co-express WT1 and TUBB3, suggesting that WT1 is only expressed by a subpopulation of the neural cells [39]. Although the exact role of WT1 during neuronal development is unclear, WT1 might be responsible for keeping the cells in a more undifferentiated state by regulating the expression of intermediate

filament Nestin [42]. Nestin is a neural stem/progenitor cell marker, during development of the central nervous system (CNS) and in adult CNS stem/progenitor cells [43-45], and neuronal differentiation is associated with a downregulation of Nestin in neurons and glial cells [46, 47]. In addition, co-expression of WT1 and Nestin is found in the epicardium [42], suggesting that Nestin is a target of WT1 in neuronal cells and thereby inhibiting differentiation.

The functional role of the epicardium in the response of the heart to epinephrine was confirmed by inhibition of epicardial outgrowth in chicken [39]. This inhibition resulted in a significant reduction of the increase in heart rate after administration of epinephrine. In mice, knockout of PDGFbeta resulted in impaired formation of the epicardium, EPDCs and coronary vessels [48, 49]. Interestingly, lack of maturation and hypoplasia of cardiac nerves was also observed in PDGFbeta knockout embryos, supporting the potential association between the epicardium and cardiac innervation [48].

Many cardiac neurons are of neural crest origin, however, cardiac neurons can also originate from non-neural crest sources, such as, for example, the neurons that innervate the sinus node [50, 51]. The co-localization of WT1 and neuronal markers in the subepicardium during development and the subsequent loss of WT1 expression in cells expressing TUBB3, might suggest that some of the WT1-positive subepicardial cells differentiate into neurons. As mature nerves are very thin, differentiation of EPDCs into nerve fibres might never been observed in lineage tracing studies. Careful re-examination might be required to discern a possible epicardial contribution in these studies.

The discrepancy between the behaviour of EPDCs *in vivo* and *in vitro*

The role of the epicardium during development and its reactivation after myocardial infarction identifies the epicardium as a potential source for cardiac progenitors. It is known that epicardial cells play an essential role in myocardial compaction and cardiomyocyte proliferation during development and regeneration via paracrine signaling [52-57]. Winter et al. showed that transplantation of human EPDCs into ischemic myocardium in mice preserves left ventricular function, which was already detectable two weeks after transplantation. In addition, the ventricular wall showed diminished dilatation, increased vascular density and increased PCNA expression in the infarcted area and border zone [58]. Based on the results presented in chapter 7, we suggest that transplantation of human EPDCs possibly contributes to a reduced deterioration of the heart after infarction by the immediate deposition of collagen.

This deposition creates a scaffold for the heart and protects the heart initially against rupture. In addition, the collagen may protect the cardiomyocytes against the inflammatory environment. As PCNA is a well-known marker for DNA repair, this marker might indicate cellular survival rather than cellular proliferation and this characteristic accounts for the expression of PCNA in the infarcted area and border zone. [59].

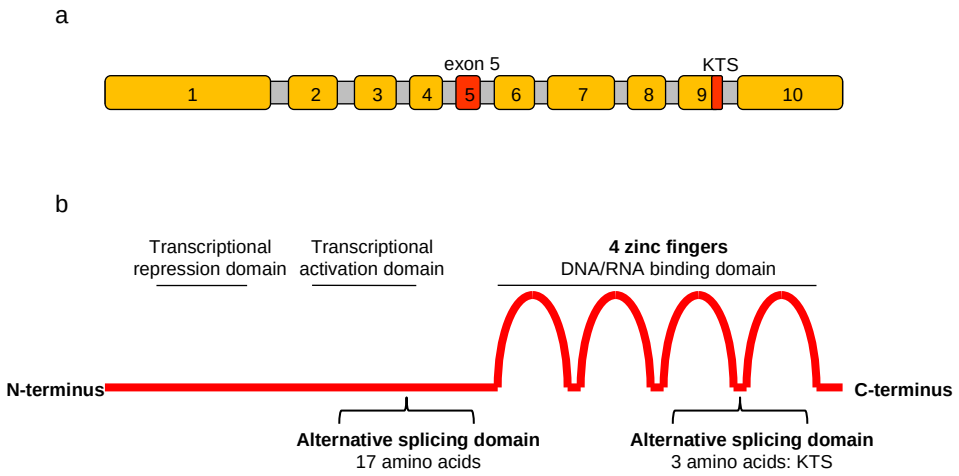
While *in vivo* EPDCs show deposition of collagen already 2 days after injection, *in vitro*, no significant increase in collagen is found after 48 hours strain. Even after the addition of TGFbeta, a factor that is known to be upregulated after infarction [60-62], no increase in the expression of collagen was found. These results indicate that strain and the presence of TGFbeta alone is not enough to recapitulate the *in vivo* behavior of EPDCs and support the hypothesis that the total set of environmental impulses on cells determine their fate and behavior. The contracting myocardium, the extracellular matrix, the three dimensional environment, cytokines, hypoxia, growth factors, and cell debris after infarction are among the many factors that potentially contribute to the differential response of EPDCs *in vitro* versus *in vivo*. The *in vitro* model provides the possibility to add one factor at the time and thereby determine the minimal set of factors that is needed to induce a certain behavior of cells. Faithfull recapitulation of the *in vivo* response of cells in an *in vitro* model, however, is challenging.

Isoforms WT1

The human WT1 gene contains 10 exons and expands approximately 50kd [63, 64]. Transcription leads to a gene product with four C-terminal zinc-finger domains for nucleic acid binding and transcriptional activation and repressing domains at the N-terminus [65, 66]. WT1 is known as a pleiotropic molecule, through alternative splicing, RNA editing and alternative translation start sites, as many as 36 isoforms are already known [16, 66]. Each different isoform of this transcription has a unique DNA-binding domain, which can affect the activation or repression of target genes. The presence of WT1 plays an important role in cell survival, proliferation, differentiation, angiogenesis and apoptosis, however, its function has been reported to dependent on the isoform and the organ where it is expressed [66-68]. In addition to its function as a transcription factor, there are also isoforms which have a higher affinity for RNA and thereby may fulfil a role in posttranscriptional regulation [69].

The first major class of WT1 isoforms, and the only isoforms that are conserved between all vertebrates, are designated as WT1(+KTS) and WT1(-KTS). These isoforms are the result of variable splicing of exon 9, leading to the insertion/omission of three amino acids lysine(K),

threonine(T) and serine(S) between zinc-finger 3 and 4 of the protein [70]. The WT1(+KTS) isoforms show a higher affinity to RNA compared to the WT1(-KTS), which exhibits a higher affinity to DNA. The ratio between WT1(+KTS) and WT1(-KTS) is conserved among tissues and a misbalance during development leads to malformations [70]. A second class of well-known WT1 isoforms is the insertion or exclusion of exon 5, resulting in the insertion or exclusion of 17 amino acids at the N-terminal site of the zinc-finger domains [70] (Figure 2).



Organization of the WT1 locus (a) and basic structure of the WT1 protein (b). The human WT1 gene contains 10 exons and expands approximately 50kd and is located on chromosome 11p13. Two major isoforms are generated by alternative splicing events at exon 5 and 9. Alternative splicing at exon 5 results in insertion or exclusion of 17 amino acids. Alternative splicing at exon 9 results in insertion/omission of three amino acids lysine(K), threonine(T) and serine(S) between zinc-finger 3 and 4 of the protein. Nowadays a total of 36 isoforms of WT1 are known, generated by translation from variable start codons, RNA editing, and posttranslational modification. The numerous functions attributed to WT1 are likely a consequence of the many isoforms of this protein.

The numerous functions that have been assigned to WT1 (Chapter 5) are likely a consequence of the many isoforms of this protein. Whether each isoforms has its own specific function or whether it also depends on the organ and time-window in which WT1 is expressed is unclear. The function of WT1 appears to correlate with the regulation of the epithelial-mesenchymal balance [71, 72]. However, whereas in the kidney WT1 is required for mesenchymal-to-epithelial transition, in the heart WT1 is required for epithelial-to-mesenchymal transition [66, 73, 74]. Additionally, the function of WT1 can also be influenced by ratio between isoforms [70, 75].

To determine the expression of WT1 we used an antibody which assumedly recognizes all isoforms and therefore we were not able to discriminate between the different isoforms. Our findings however would suggest that multiple different isoforms are present. The WT1(+KTS) isoform has been shown to be required for olfactory neuron formation [41]. Similar isoform might be present in the subepicardium during development where we see co-localisation with neuronal markers (Chapter 6). In epicardial cells, the WT1(-KTS) has an repressive effect on E-cadherin, which is lost upon EMT [74] and might be the isoform present in the epicardial cells. WT1 has been observed in tumor endothelial cells in a wide variety of tumors [76-80]. It is known that WT1 stimulates the expression of VEGF [81], however, the biological role for the various isoforms is unclear. Angiogenesis was promoted by WT1 (-Ex5/-KTS) isoform in an *in vivo* ovarian cancer model [82], whereas the highest upregulation of VEGF was seen in Ewing Sarcoma cells expressing the WT1 (+Ex5/+KTS) isoform [81]. In conclusion, it is very likely that different isoforms of WT1 might be present in the epicardium, in cardiac endothelial cells, in epicardial cells that express neuronal markers and in EPDCs, whereby various isoforms of WT1 account for their postulated different functions in each cell type. Elucidating the expression and function of the different isoforms in the heart might provide great new insight in the regulation of cardiac development and disease. Our experiments show that the epicardium and the expression of WT1 are very dynamic, both during development and after injury. This thesis contributes to our understanding of the heart, but also shows that there is still much research needed before we know exactly how this complex but fascinating organ works.

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English summary

In **Chapter 1** we provide an overview of the heart in general and about development and myocardial infarction in more detail. In addition, we describe the development of the epicardium, the cell layer which is situated around the heart and characterized by the expression of the transcription factor Wilms' tumor-1 (WT1) during development.

In **Chapter 2** of this thesis we studied the spatiotemporal expression pattern of WT1 during developmental stages and after cardiac injury. Our results show that WT1 is expressed in cardiac endothelial cells in the developing, adult and post-infarcted heart in a spatial and temporal expression pattern and is not exclusively for epicardial cells and EPDCs as has been suggested previously. In vitro experiments show that WT1 plays an important role in endothelial proliferation and formation of the vascular network. We suggest a role for WT1 in angiogenesis and conclude that, in addition to the essential role of WT1 in the epicardium, WT1 has an active role in cardiac endothelial cells.

In **Chapter 3** of this thesis we provide a detailed description of the expression of WT1 protein during human cardiac development. we studied the expression of WT1 during human cardiogenesis. We show that human expression of WT1 is present in cells that are crucial for the proper formation of the cardiac vessels and ventricular myocardium, i.e. epicardial, endothelial and endocardial cells [3].

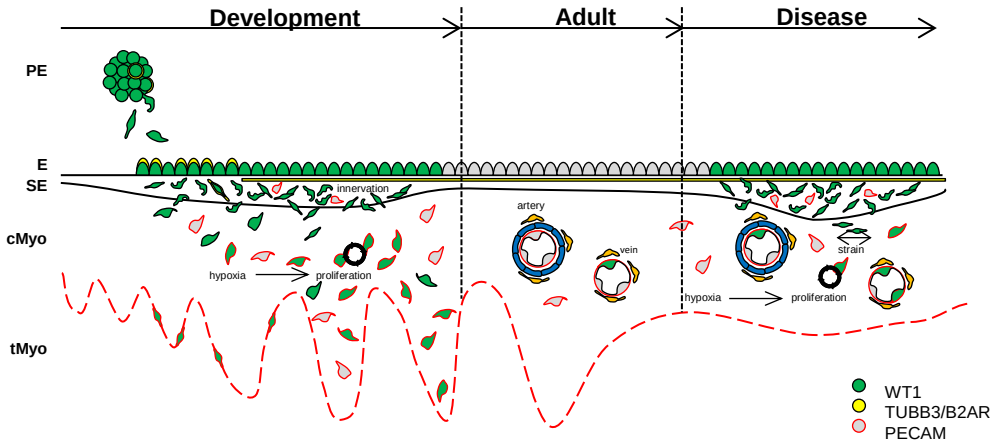
In **Chapter 4** of this thesis we provide a review of the many aspects of WT1 within the heart during development and after injury and propose a working model for the role of WT1 in endothelial cells, based on our own experiments, which are described in this thesis and previous literature.

In **Chapter 5** of this thesis we studied the role of the epicardium in autonomic modulation of the developing heart prior to innervation. Our results show that the epicardium expresses the neuronal markers TUBB3, NCAM and β2AR during early development. Using an ex-ovo model we show that inhibition of epicardial outgrowth results in a disturbed response to epinephrine. Together, these results show for the first time that the epicardium plays a role in modulating the cardiac autonomic response prior to innervation.

In **Chapter 6** of this thesis we studied the response of EPDC to mechanical stimulation. Using an *in vitro* straining model, we show that mechanical stimulation slightly stimulates the differentiation of EPDCs into myofibroblasts, but does not result in the deposition of ECM. On

the other hand, injection of EPDCs in the heart after MI results in a fast deposition of collagen, indicating that factors additional to stretch determine the behavior of EPDCs.

Finally, the results and conclusions of the previous chapters of this thesis are discussed in **Chapter 7** (Discussion), together with the future implications deriving from this work.



The expression of WT1 in the heart during development, in the adult heart and after cardiac injury. The expression of WT1, during different cardiac conditions, as described in this thesis is illustrated in green. PE, proepicardium, E; epicardium, SE; subepicardium, cMyo; compact myocardium, tMyo; trabeculated (non-compacted) myocardium.

Nederlandse samenvatting

Het hart is het belangrijkste en meest essentiële orgaan van ons lichaam. En ondanks de levensnoodzakelijke functie die dit orgaan vervult, zijn er nog altijd prangende vragen over het hart waarnaar dagelijks onderzoek wordt gedaan. De belangrijke vraag waar onderzoekers op het gebied van het hart zich op dit moment mee bezig houden is; hoe kunnen we het hart weer gezond, fit en vitaal krijgen nadat er schade is ontstaan?

Het hart is een autonoom orgaan waarin de aansturing vanuit pacemakercellen ervoor zorgt dat het bloed met zuurstof, voeding- en afvalstoffen door onze bloedvaten wordt gepompt. Het volwassen hart bestaat uit vier afzonderlijke hartkamers, twee atria (boezems) en twee ventrikels (kamers). Tijdens de embryonale ontwikkeling is de eerste herkenbare structuur van het hart echter een lineaire, holle buis. Kort na de vorming zal deze structuur de bloedstroom op gang brengen door middel van een peristaltische beweging. De hartbuis zal zich door (1) een gecompliceerd proces dat cardiale-looping wordt genoemd, (2) de toevoeging van cellen en (3) expansie ontwikkelen tot de gekende vier-kamer structuur. Tijdens de embryonale fase ontwikkelt zich een bloemkoolachtige structuur nabij het hart welke het proepicard wordt genoemd. Cellen van deze structuur bekleden het hart en vormen zo de buitenlaag die het epicard wordt genoemd. Deze laag cellen rondom het hart speelt een zeer belangrijke rol tijdens de ontwikkeling aangezien het zowel een paracrien effect heeft als structureel bijdraagt aan het hart.

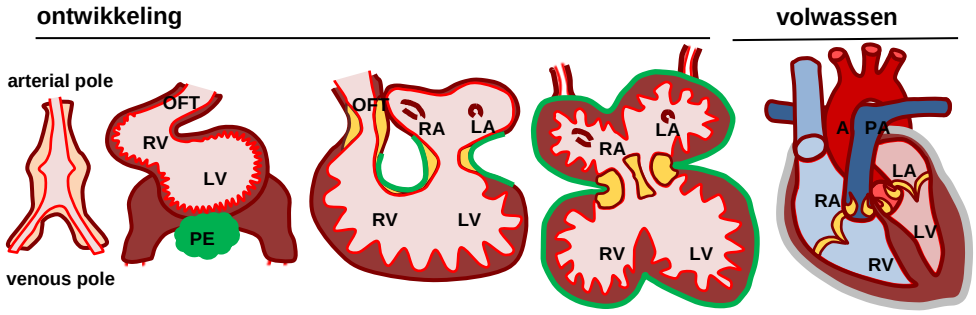


Figure 1. Hartontwikkeling. De primitieve hartbuis wordt gevormd door hartspiercellen (bruin) aan de buitenkant en endotheelcellen (rood) aan de binnenkant, welke gescheiden worden door cardiale gelei. De primitieve hartbuis verlengt en ondergaat een complexe structuurverandering, cardiale-looping genaamd. Aan de veneuze kant van het hart ontstaat het proepicard. Dankzij de vorming van septa otstaan er twee atria en twee ventrikels en ontstaat een volwassen vier-kamer structuur. Het hart wordt omgeven door het epicard. De expressie van WT1 tijdens de ontwikkeling is weergegeven in groen. A, aorta, LA, linker atrium; LV, linker ventrikel, OFT; uitstroom hart; PA, pulmonale arterie; PE, proepicard; RA, rechter atrium; RV, rechter ventrikel.

Epicardiale cellen kunnen via het proces, epitheliale naar mesenchymale transitie, los komen van het epicard en de spierlaag van het hart binnendringen. Deze cellen worden epicard afgeleide cellen (EPDCs) genoemd en eenmaal in de spierlaag van het hart differentiëren deze cellen naar fibroblasten, gladde spiercellen en endotheelcellen. De bijdrage van EPDCs in de vorming van hartspiercellen staat echter ter discussie. Na een myocard infarct wordt het epicard geactiveerd en speelt het een belangrijke rol in het herstel en de minimale regeneratie van het hart.

WT1 is een transcriptiefactor die tot expressie komt in het proepicard en het epicard tijdens de ontwikkeling van het hart. Dit eiwit speelt een belangrijke rol in de transformatie van epicardcellen van epitheliaal naar mesotheliaal waardoor ze uit het epicard het myocard in kunnen migreren. De expressie van WT1 verdwijnt in het volwassen hart, maar wordt opnieuw geactiveerd na een infarct. Dankzij het expressiepatroon is WT1 lang als marker gebruikt voor het epicard.

Het myocard infarct (hartinfarct) is de belangrijkste oorzaak van ziekte en sterfte in de westerse wereld binnen de groep van hart- en vaatziekten. Een infarct ontstaat door een tekort aan bloedtoevoer in een deel van de hartspier als gevolg van het afsluiten van een van de coronairen (kransslagaders). Het gevolg is dat er geen zuurstof en voedingsstoffen bij de spiercellen van het hart worden afgegeven en een deel van deze spiercellen zal hierdoor afsterven. De ontstekingsreactie die hierop volgt zorgt ervoor dat de dode cellen worden opgeruimd en dat er een robuust litteken ontstaat waardoor het scheuren van het hart voorkomen wordt. De gevolgen van dit litteken zijn echter een verminderde pompfunctie van het hart en men is daarom ook naarstig op zoek naar een behandeling die dit moet voorkomen.

De afgelopen jaren is het onderzoek vooral gericht geweest op de behandeling van hart- en vaatziekten en naar de beste populatie aan cellen die nodig is voor celtherapie. In dit proefschrift gaan we terug naar de ontwikkeling van het hart en proberen we de lessen van de ontwikkelingsbiologie met betrekking tot het bouwen van een hart beter in kaart te brengen zodat we in de toekomst gerichter kunnen zoeken naar mogelijke behandelingen.

Hoofdstuk 1 geeft een algemene introductie over de context waarin dit proefschrift is geschreven. Dit hoofdstuk is een korte inleiding over de opbouw en functie van het hart, de belangrijkste processen tijdens de hartontwikkeling en de pathofysiologie van een myocard infarct. Daarnaast geven we aan welke kennis er is op het gebied van het epicard en zijn belangrijkste merker tot op heden, WT1.

Mijn scriptie heeft zich voor een groot gedeelte gefocust op het bestuderen van het expressiepatroon van WT1 en de rol van het epicard tijdens de ontwikkeling en na schade aan het hart.
In **Hoofdstuk 2** hebben we op een uitgebreide manier de expressie van WT1 in het hart van de muis in kaart gebracht tijdens de embryonale ontwikkeling en na een myocard infarct door middel van immunofluorescentie. De experimenten die beschreven staan in dit hoofdstuk laten zien dat de expressie van WT1 niet beperkt blijft tot het epicard tijdens de ontwikkeling, maar dat deze ook aanwezig is in de spierlaag van het hart. Een uitgebreide analyse toont aan dat het de endotheelcellen van de coronairen zijn die WT1 tot expressie brengen. Daarnaast laten we zien dat WT1 opnieuw tot expressie komt in de endotheelcellen na een myocard infarct en dat dit patroon tijdelijk van aard is. Experimenten met behulp van celkweek hebben laten zien dat WT1 in endotheelcellen en belangrijke rol speelt bij de celdeling en de vorming van een vasculair netwerk. WT1 lijkt dus een belangrijke factor te zijn wanneer het gaat om de aanleg van nieuwe bloedvaten, ook wel bekend als angiogenese.

De expressie van WT1 is uitgebreid gedocumenteerd voor de ontwikkeling van het hart in de muis en vogel. Er is echter weinig gekend over de expressie van WT1 tijdens de humane embryonale ontwikkeling van het hart. In **Hoofdstuk 3** wordt het expressie patroon van WT1 uitgebreid beschreven tijdens de ontwikkeling van het humane hart tussen week 4 en week 20. WT1 expressie bleek in epicardiale, endocardiale en endotheliale cellen aanwezig te zijn op een plaats en tijd specifieke manier. De expressie van WT1 volgt een patroon waarbij het zich uitstrekt vanaf het epicard naar het lumen van het hart. Er zijn echter wel verschillen in timing en niveau van expressie te onderscheiden tussen de atria en de ventrikels. Bovendien laten de endotheelcellen van de coronaire arteriën een vermindering van de expressie van WT1 zien gedurende de ontwikkeling, waar de expressie aanwezig blijft in de endotheelcellen van de venen. De gelijkenis in het WT1 patroon tussen mens en muis ondersteunt het gebruik van de muis als model voor het bestuderen van de humane ontwikkeling en hartziekten.

In **Hoofdstuk 4** beschrijven we de veelzijdigheid van WT1 binnen het hart zowel tijdens de ontwikkeling als na een infarct. Daarnaast hebben we een model opgezet waarin de rol van WT1 in endotheelcellen wordt beschreven, gebaseerd op literatuuronderzoek en de bevindingen die gedaan zijn tijdens dit promotieonderzoek.

In **Hoofdstuk 5** hebben we een studie uitgevoerd waarin we de relatie tussen het epicard en de ontwikkeling van het autonome zenuwstelsel van het hart bestuderen. In het verleden heeft men laten zien dat kippenharten tijdens de ontwikkeling gevoelig zijn voor adrenaline, nog voor er sprake is van innervatie van zenuwen. Dit hormoon komt vrij op het moment dat er lichamelijke inspanning vereist is en zorgt onder meer voor het verhogen van de hartslag en het krachtiger samentrekken van de hartspier. Wij hebben laten zien dat het epicard een belangrijke structuur is die ertoe bijdraagt dat het hart kan reageren op de neurotransmitter adrenaline alvorens er innervatie is. In dit hoofdstuk beschrijven we de expressie van de neurale markers TUBB3, NCAM en β2AR. Voornamelijk deze laatste, de β2AR, ofwel de bèta-2-adrenerge receptor is van belang omdat het adrenaline bindt. De functionele rol van het epicard is vervolgens onderzocht door de uitgroei van het epicard te verstoren tijdens de ontwikkeling van een kip. We hebben hartjes met een verstoorde epicardvorming en een normale epicardvorming met elkaar vergeleken en zagen een sterk verminderde toename van de hartfrequentie na toediening van adrenaline in de hartjes met een verstoorde epicardvorming. De conclusie die wij hieruit hebben kunnen trekken is dat het epicard vroeg tijdens de ontwikkeling een tijdelijke rol vervult als modulator van de autonome respons. Met andere woorden, het epicard is belangrijk voor de verhoogde hartslag als respons op het toedienen van adrenaline.

Tijdens de ontwikkeling zullen er cellen vanuit het epicard de hartspierwand in migreren, dit zijn epicard afgeleide cellen (EPDCs). Deze EPDCs zijn de voorlopercellen van de vaten en bindweefsel in het hart en daardoor potentieel een geschikte kandidaat om gebruikt te worden voor celtherapie. In een eerdere studie, waarbij deze cellen zijn ingespoten in een muismodel met een myocard infarct, zag men een verminderde afname van de hartfunctie. Het exacte werkingsmechanisme achter deze toename ten opzichte van de onbehandelde groep is echter nog niet volledig gekend. In **Hoofdstuk 6** suggereren wij dat het uitscheiden van collageen door de ingespoten EPDCs na een infarct ertoe bijdraagt dat het hart minder snel verslechtert. Daarnaast hebben we gekeken aan de hand van een *in vitro* model of de rek waaraan de cellen worden blootgesteld na het inspuiten effect heeft op de productie van extracellulaire matrix eiwitten, de differentiatie van EPDCs naar fibroblasten en de proliferatie

van deze cellen. Onze voorzichtige conclusie is dat rek een minimaal effect heeft op deze biologische processen in EPDCs. Dit is gunstig voor het gebruik van deze cellen met betrekking tot celtherapie. Doordat deze cellen geen sterk prolifererende fibroblasten worden die een overmatige remodelling van de hartwand veroorzaken hebben ze mogelijk eerder een positieve bijdragen aan het proces van wondherstel.

Hoofdstuk 7 omvat de algemene discussie van dit proefschrift. We concluderen dat WT1 geen exclusieve marker is voor het epicard tijdens de hartontwikkeling en dat dit eiwit tot expressie komt in de endotheelcellen van de coronairen. Daarnaast tonen we aan dat de muis een goed model is voor onderzoek wanneer we willen kijken naar de ontwikkeling van het hart. In dit proefschrift wordt tevens een nieuwe functie aan het epicard toebedeeld in de modulatie van de autonome respons nog voor de aanwezigheid van innervatie. Een meer algemene conclusie is dat de omgeving van cellen uiteindelijk een belangrijkere rol speelt in het uiteindelijke lot en functioneren van deze cellen dan hun oorsprong.

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Curriculum Vitae

Sjoerd Duim was born on February 23th, 1987 in Dirksland. He graduated from secondary school (Regionale Scholengemeenschap 'Hoeksche Waard') in 2005. In September that same year he moved to Leuven, Belgium, where he started his study Biomedical Sciences at the Catholic University of Leuven. Under the supervision of Professor Sampaolesi at the University of Pavia, Italy, he completed his master research project. The research focused on the use of stem cells as a possible treatment for muscular dystrophy and during this period he became interested in the potential role of stem cells in muscular repair.

After graduation in 2011 he started his PhD at the department of Molecular Cell Biology in the Leiden University Medical Center in the group of prof. dr. Marie-José Goumans and under supervision of dr. Boudewijn Kruithof. Since July 2016, Sjoerd is working as a Clinical Chemistry Resident at the Haaglanden Medisch Centrum in Den Haag.

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