Endothelial WT1 and the epicardium in cardiac development and disease

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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.

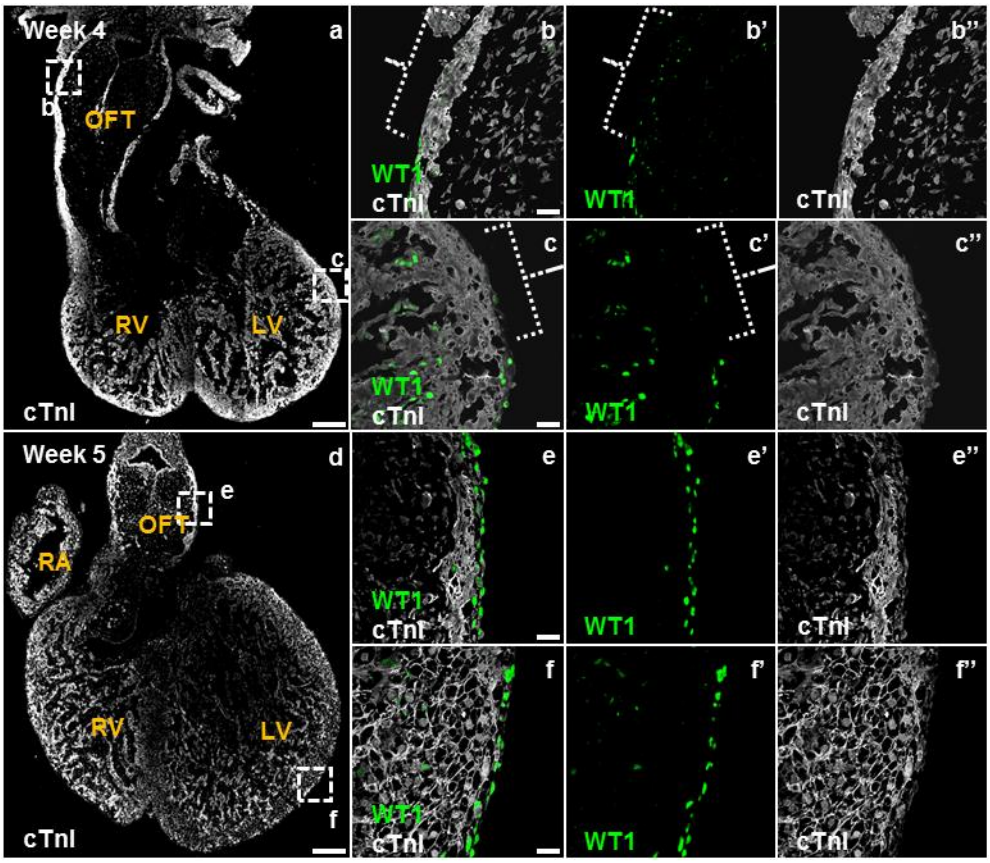

Figure 1. Epicardial covering of the human heart with WT1 positive cells is completed at week 5 of development. Sections are stained for WT1 (green) and the myocardial marker cTnI (gray). **a.** Overview of the human embryonic heart at week 4 of development. At this stage, parts of the OFT (**b**), and 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).

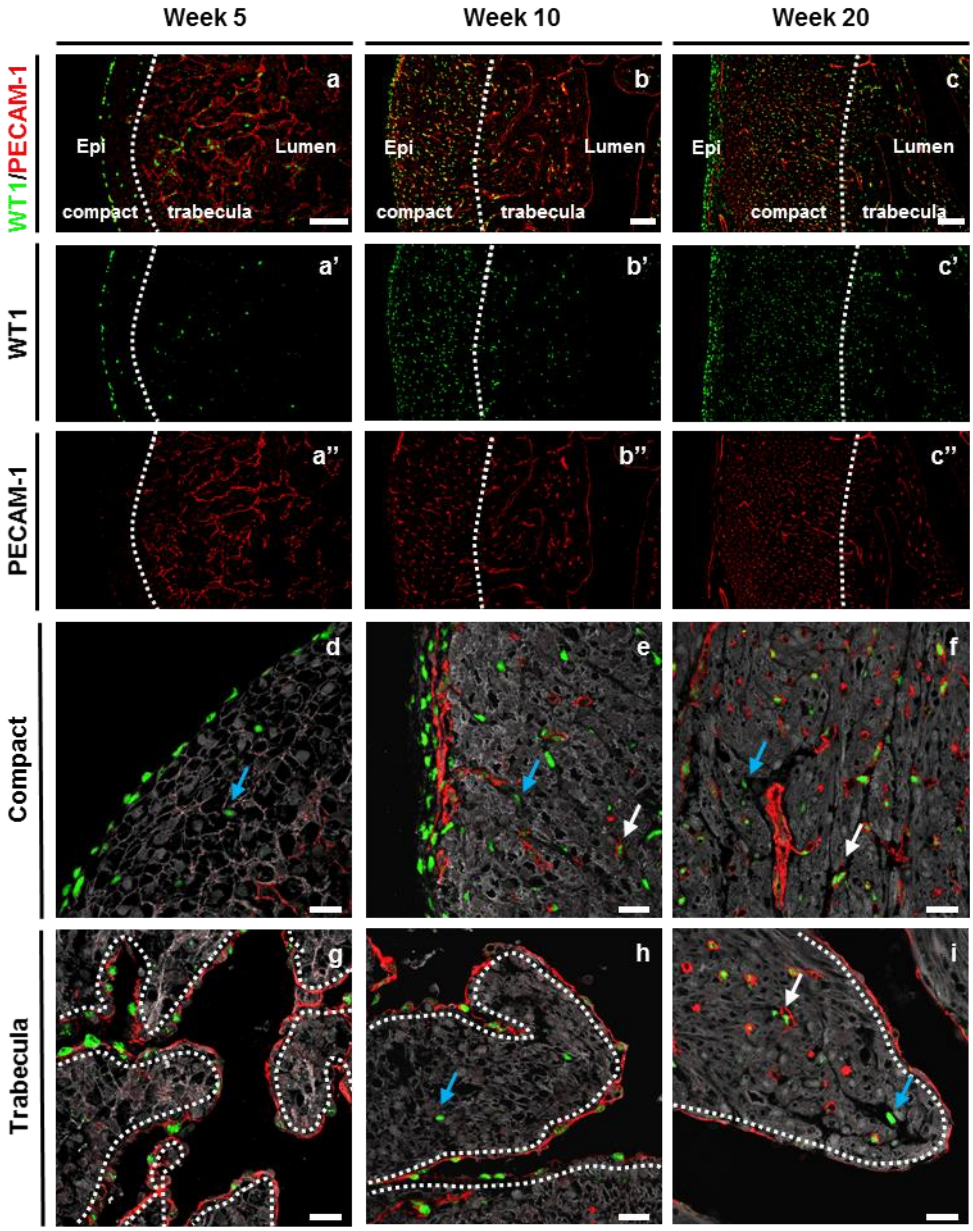

Figure 2. The dynamic expression of WT1 protein in the ventricle. a-c. Overview of the left ventricular wall showing the expression of WT1 (green) expanding from the epicardium towards the luminal side of the heart as development progresses, a similar pattern is seen with PECAM-1 expressing endothelial 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.

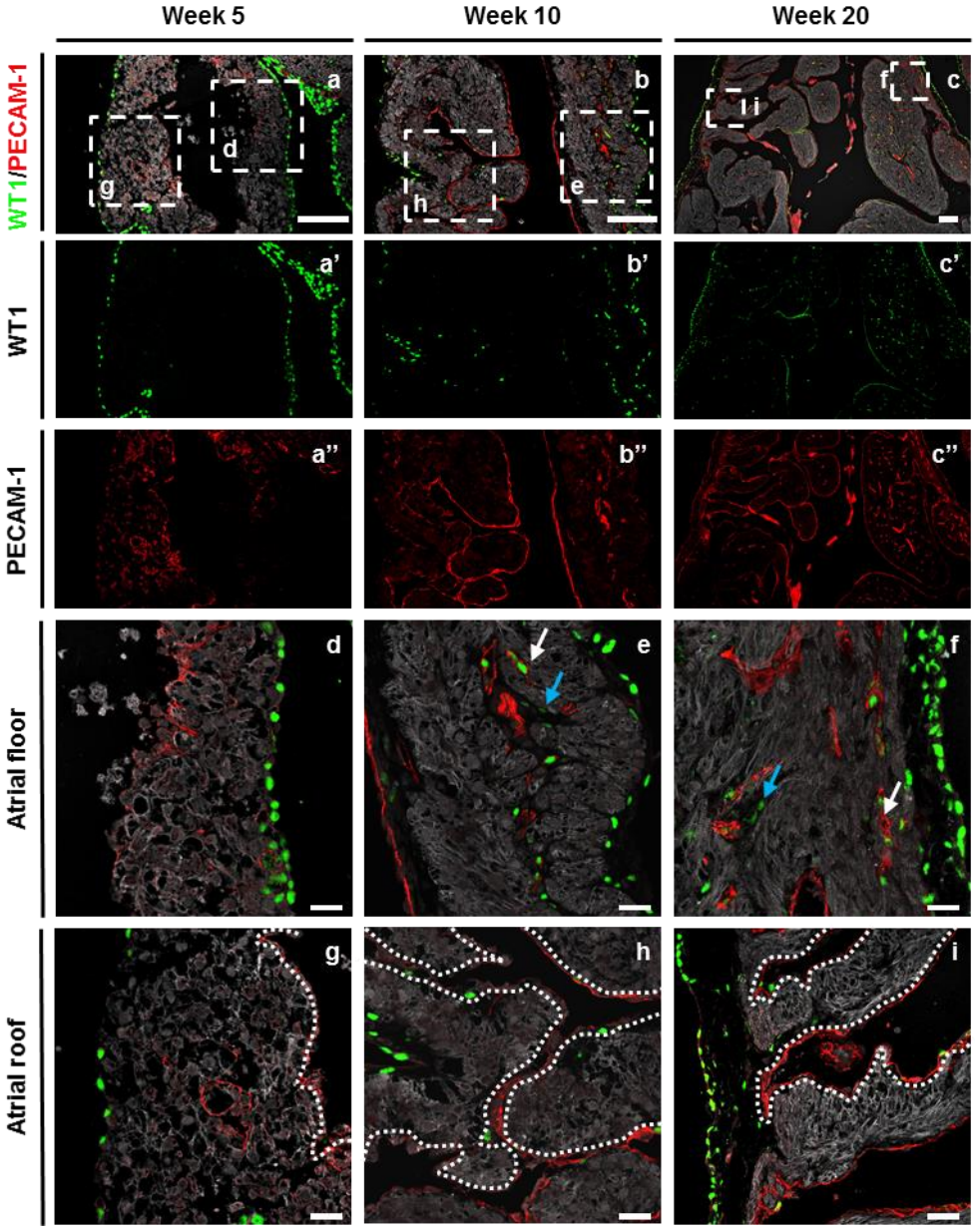

Figure 3. The dynamic expression of WT1 protein in the atrium. a-c. Overview of the left atrial wall showing the expression of WT1 (green) expanding from the epicardium towards the luminal side of the heart as development progresses, a similar pattern is seen with PECAM-1 expressing endothelial cells (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).

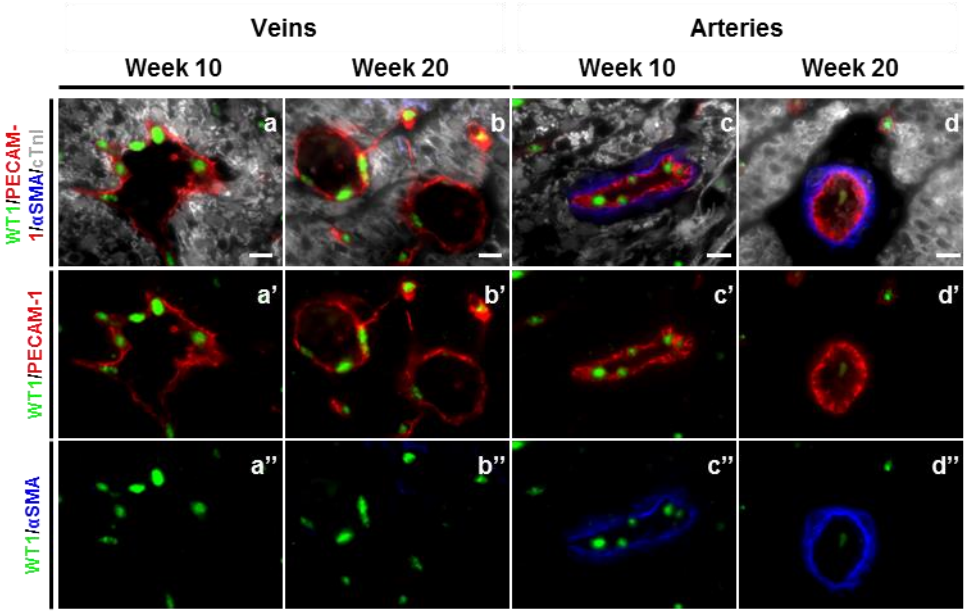

Figure 4. WT1 protein expression in cardiac arteries decreases with ongoing development. At week 10 of development expression of WT1 (green) is observed in endothelial cells (red) of cardiac veins (**a**) and in endothelial cells but not in smooth muscle cells (blue) around cardiac arteries (**c**). 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).

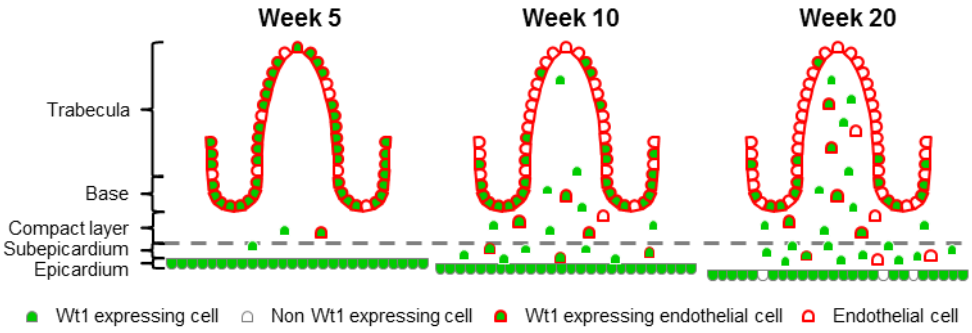

Figure 5. Working model of WT1 protein expression within the heart. The schematic overview shows that with ongoing development the expression of WT1 expands from the epicardium towards the lumen of the heart. WT1 is initially present in the endocardial cells of the trabeculae, but disappears over time. 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.

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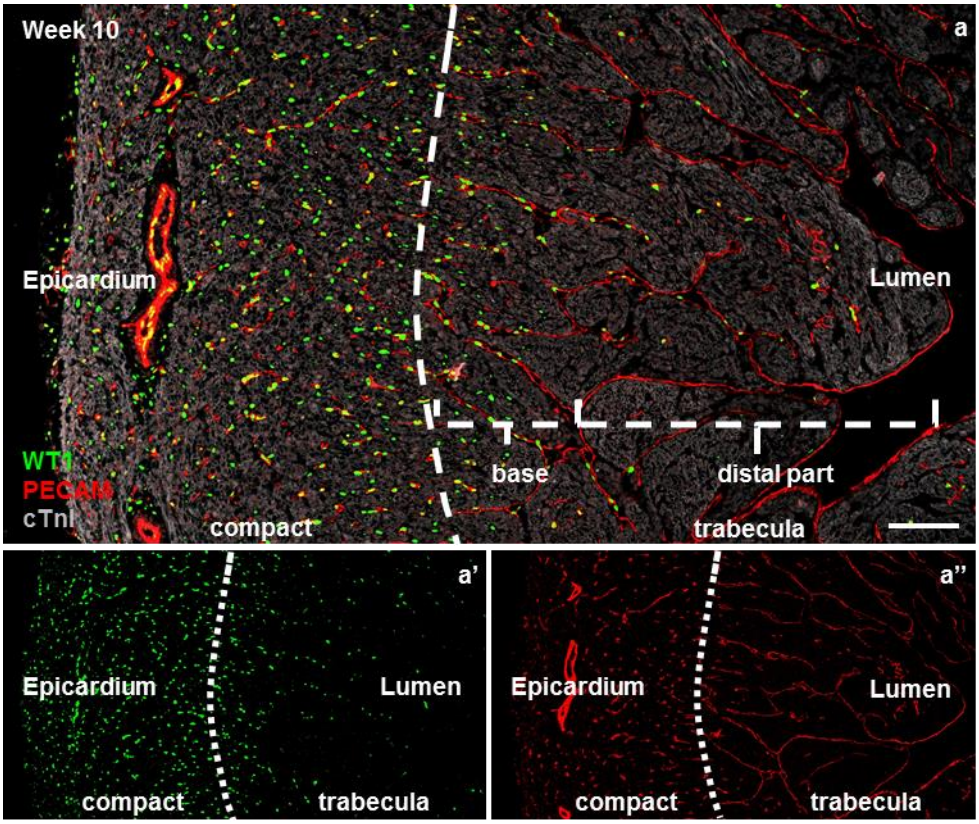
Disclosures

None.

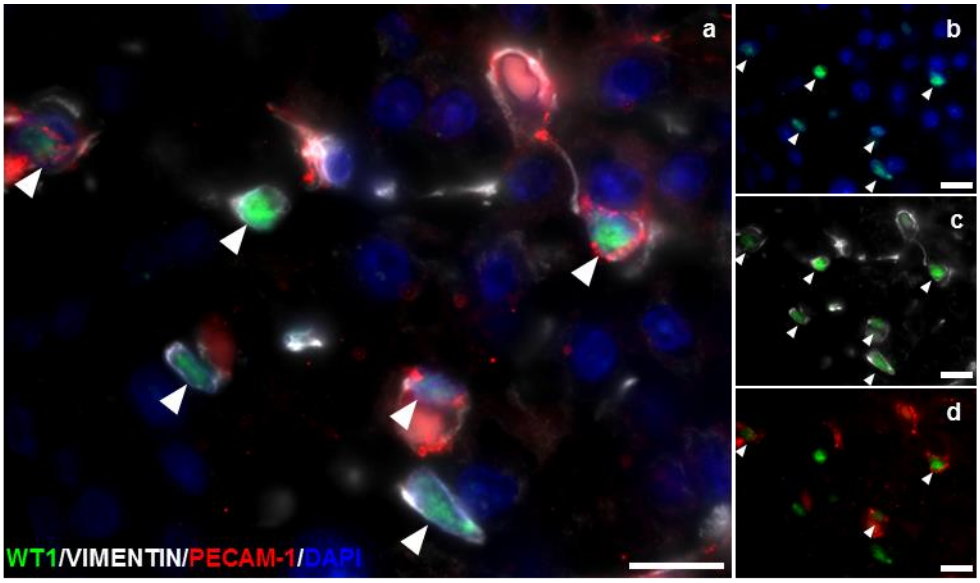
Abbreviations

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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