Chapter 2

Differentiation of the Left and Right Ventricle Governed by Interaction with Epicardium-Derived Cells as Studied in the TGFbeta2 Mutant Mouse

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Submitted

ABSTRACT

Background

In the adult heart, morphological and functional differences of right ventricle (RV) and left ventricle (LV) are apparent. We hypothesized that variability in contribution of epicardium-derived cells (EPDCs) to the developing RV and LV might explain these differences. The aim of this study was to assess normal and disrupted formation of the compact myocardial layer of the RV and LV in mice.

Methods and Results

Epicardial sheet formation and contribution of EPDCs were studied in wildtype and TGFβ2-null embryonic mice (E9.5–14.5) using expression patterns of WT1 and a Cre-activated WT-1 reporter model. After epicardial covering of the heart tube, EPDCs were observed first in the inner curvature and RV wall. WT-1 expressing cells were abundantly observed in the wall of both ventricles at E13.5, more pronounced within the LV correlating with more pronounced thickening of the LV compact myocardial layer. In TGFβ2-null mice, formation and migration of EPDCs was severely diminished, although an epicardial sheet was formed. Differences in RV and LV myocardial thickness as observed in wildtype, were absent in TGFβ2-null mice.

Conclusions

Spatio-temporal differences in contribution of EPDCs to RV versus LV myocardium were observed during development. Compact myocardial layer formation starts upon migration of EPDCs into the ventricles and is more pronounced in the LV. Disruption of EPDC migration results in absence of normal myocardial thickening, only significant in the LV. This suggests that EPDC-myocardial interaction in the LV differs from that in the RV, which may explain occurrence of lateralized cardiomyopathies as isolated LV non-compaction and may prove relevant for development of cell-based and drug-based therapies.

INTRODUCTION

Right ventricular (RV) function is an important determinant of survival in a large number of cardiovascular diseases.[1–3] Therapies aimed at long-term improvement of RV function are scarce.[4] In addition, medical therapies which proved to be beneficial in left ventricular (LV) disease are generally not useful in the dysfunctional RV.[5, 6] Therefore, dedicated cell-based therapy might be of interest for the treatment of RV disease.[7, 8] Proper understanding of the differences between the LV and RV on a morphological and molecular level is important for development of these therapies.

In the past decade the identification of the second heart field (SHF) showed that the ventricles have a different developmental origin.[9–11] Early in cardiovascular development, the heart exists of a primary heart tube with a venous and an arterial pole.[12] Through migratory processes from the SHF, cells are added to the arterial (and venous) pole of the heart.[9] Whereas the primary heart tube provides the majority of cells of the LV, the SHF provides most cellular components of the RV.[10, 13] This different origin (primary versus SHF) may provide a developmental explanation for the observed differences between the adult LV and RV.[14] The epicardium has concerted interactions with both ventricles during specific stages of development, which is bound to have implications for functioning of the adult heart.[15, 16] The epicardium is derived from the pro-epicardial organ (PEO) which is an element of the caudal part of the SHF and expresses, amongst others, WT-1, TGFβ2, Id2 receptor and PDGFα receptor.[16–18] Epicardial cells migrate from the PEO, cover the ventricles in an orchestrated pattern and form an epithelial sheet. The epicardial cells that cover the distal vascular part of the outflow tract of the heart probably originate from the arterial pole of the heart.[19, 20] After spreading over the heart, epicardial cells go into epithelial-to-mesenchymal transition (EMT) forming a subepicardial layer, in interaction with the underlying myocardium,[21] and migrate into the ventricular wall as epicardium-derived cells (EPDCs).[22, 23] Inside the myocardium EPDCs contribute, amongst others, to vessel formation, differentiation of the Purkinje network and partly differentiate into interstitial fibroblasts.[15, 24, 25] It was demonstrated that the latter cell-population induces normal LV growth.[25, 26] Experimental knock-out of epicardium-associated genes in mice showed abnormal epicardium and abnormal development of the ventricular myocardium.[18, 27–29] Knock-out of TGFβ2, which is expressed in epicardium and is required for EMT, is associated with thin uncompacted myocardium,[27] probably due to a lack of EPDCs. In vitro studies demonstrated the essential role of EPDCs in myocardial proliferation, maturation and alignment.[30] The crucial role of the epicardium for normal ventricular development and compaction is evident, as is the propensity for clinical cases of specific cardiomyopathies, like non-compaction cardiomyopathy and arrhythmogenic right ventricular dysplasia, to occur in a lateralized fashion (i.e. left vs. right sided, respectively.[31, 32] We hypothesized that EPDCs interact differently with the LV as compared to the RV. As a consequence, EPDCs may contribute to the difference in function and morphology of the postnatal LV and RV. This

is particularly relevant since recent studies identified the potential of EPDCs to reactivate embryonic differentiation programs in the adult ischemic heart.[33–36]

We are the first to analyse the differences in development of the RV and LV related to the formation of epicardium, the timing of EMT and the migration of EPDCs. The aim of the current study was 1: To assess whether the timing of epicardial sheet formation and EPDC migration is different in the RV as compared to the LV, and 2: To investigate the effect of disrupted epicardial development on formation of the compact layer in the RV versus the LV using the TGFβ2-null mouse model.

METHODS

Mice

For the study of normal development, wild type mouse embryos were obtained from the CLB-Swiss strain. To study the fate of EPDCs, $WT1^{CreERT2/+}$ and the Cre-activated reporter $Rosa26^{fsLz}$ mice were used.[37] Wt1CreERT2/+ mice were generated by gene targeting followed by Flp-mediated removal of a Neo resistance casette, as described previously (Zhou et al, 2008, also see **Figure 2F**). CreERT2 is a fusion protein composed of Cre recombinase and a modified variant of the estrogen receptor hormone binding domain. CreERT2 recombines floxed targets in the presence, but not the absence of tamoxifen (Feil et al., 1997). 2 mg tamoxifen (Sigma) was injected peritoneally into pregnant mice at E10.5 to induce Cre activity. Rosa26fsLz (Soriano, 1999) and Rosa26mTmG (Muzumdar et al., 2007) mice were used as Cre-dependent reporters. These mice express LacZ and membrane localized GFP, respectively, after Cre-mediated recombination. Upon maternal injection with tamoxifen, this reporter expresses B-galactosidase upon Cre-mediated recombination and thus provides a means to follow EPDCs after differentiation even when WT-1 is downregulated.

To investigate the effect of abnormal EPDC formation and function, the TGFβ2-null mouse was studied.[27] TGFβ2 is abundantly expressed in epicardium[38] and plays an important role in epicardial cell differentiation and invasion.[39] This model provides an effective means to study the effect of abnormal EPDC formation and function, simultaneously for LV as well as RV development. The handling of all animals and embryos was according to the Guide for Care and Use of Laboratory Animals, as published by the NIH. The day the vaginal plug was detected, was designated embryonic day (E) 0.5. Pregnant female mice were sacrificed on consecutive days from E 9.5-E 14.5 and, per day, three embryos were harvested for the study.

Immunohistochemical procedures

After fixation in 4% paraformaldehyde in phosphate buffered saline (0.1 M, pH 7.2) and subsequent dehydration embryos were embedded in paraffin, sectioned transversely (5µm) and serially mounted on glass slides. Immunohistochemical staining was performed with antibodies against MLC-2a (1/6000, kindly provided by S.W. Kubalak, Charleston, SC); Nkx2.5 (1/4000, Santa Cruz Biotechnology Inc., CA, United States, SC-8697) and WT-1 (1/1000, Santa Cruz Biotechnology Inc., CA, United States, SC-192). The slides were first incubated for 45 min using ABC-reagent (Vector Laboratories, Burlingame, United States, PK 6100), and then with 400 µg/ml 3–3'di-aminobenzidin tetrahydrochloride (DAB, Sigma-Aldrich, St Louis, United States, D5637) dissolved in trismaleate buffer pH 7.6 to which 20 µl H_2O_2 was added. The latter incubation was done 5 min for MLC-2a and 10 min for Nkx 2.5 and WT1. Counterstaining was done using 0.1% hematoxylin (Merck, Darmstadt, Germany) for 5 sec, and the slides were subsequently rinsed with tap water for 10 min. Finally, slides were dehydrated and mounted with Entellan (Merck, Darmstadt, Germany).

Three-dimensional reconstruction and quantification

To address the timing of epicardial covering of the ventricles, the expression of WT-1 from E9.5 to E11.5 was 3D visualized. On each embryonic day, micrographs were made (magnification 10X) of serial sections to cover the whole embryonic heart from the proximal part of the great vessels on the cranial side, down to the inferior cardinal veins on the caudal side. The micrographs were processed using the AMIRA software package (Template Graphics Software, San Diego, CA, United States) as described previously.[40] First, the myocardium, the arterial and the venous pole were reconstructed in MLC-2a stained sections. Then, the expression of WT-1 was superimposed. To this purpose, the nucleus of each WT-1 positive cell, staining brown after incubation with WT-1 antibody, was separately indicated. Thus, the location of WT-1 positive cells in the epicardial sheet and in the extra-epicardial structures in relation to the underlying and surrounding tissue is provided.

Wall thickness was assessed in the apical and mid-lateral free wall of the LV and RV. Per stage (E10.5–14.5), micrographs were made of at least three different wild type and TGFβ_2-null mouse embryonic hearts. In each heart, a section was selected at approximately the same level that included the LV and RV, the interventricular septum and the atrioventricular transition. Wall thickness (apical and mid-lateral, LV and RV) was measured in each section by 2 measurements at the mid lateral wall, and at the apical wall, respectively. Measurements were performed by two observers in consensus (MRMJ, RVS), blinded from the phenotype of the hearts at the time of measurement, using the analysis software ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2011). Results were depicted and significance of differences between RV and LV in wildtype hearts, and in knockout

hearts, and between wild type and knockout embryos, was calculated with GraphPad Prism 4.00 for Windows (GraphPad Software, La Jolla, CA, United States).

RESULTS

Epicardial sheet formation

At E9.5, the PEO expressed WT-1 and protruded into the coelomic cavity at the caudal part of the heart at the sinus venosus (Figure1A). Differentiation of the sinus venosus myocardium had only just been initiated and the cardinal veins were still devoid of myocardium. The expression of WT-1 was not confined to the PEO but was also observed in the mesenchyme surrounding the left and right cardinal veins (Figure 1A, arrows). As became apparent from the three- dimensional reconstruction at this stage (Figure 1E), these WT-1 positive cells enclosed the left and right cardinal veins and the proximal part of the sinus venosus in a semicircular fashion. This area of WT-1 positive cells was also observed along the dorsal coelomic cavity wall and was continuous with the PEO (Figure 1). Small clusters of WT-1 positive cells were already detached from the PEO (Figure 1C, D) and were found at the inner curvature of the looping heart tube. The LV was larger in size as compared to the RV and in both ventricles, trabeculations were observed. The trabeculations of the LV were coarser and more pronounced compared to the RV. The outer layer of myocardium was thin, as no compact myocardium had developed, yet.

At E10.5, cardiac looping had progressed, however the heart was still in an unseptated state consisting of a common atrium, primitive LV and the outflow tract. Only remnants of the PEO were visible at the original caudal site of the heart and most WT-1 positive cells now attached to the heart tube to form the epicardial sheet (Figure 2D). The early epicardial sheet covered the primitive (common) atrium, the primitive LV and the part of the RV adjacent to the primary fold. Distal parts of the RV were sparsely covered with WT1-positive cells and the outflow tract was for the greater part uncovered (Figure 2). Pronounced expression of WT1 in the coelomic cavity lining was observed. Furthermore, an epicardial sheet extended from the coelomic cavity wall covering the distal end of the outflow tract (Figure 2A-C). Expression of WT1 was more prominent on the right side (putative aorta) of the outflow tract, whereas on the left side (putative pulmonary trunk) the expression of WT1 was almost absent (Figure 2A).

At E11.5, the PEO-derived epicardial sheet extended towards the outflow tract, leaving the left side uncovered, whereas on the right (aortic) side the PEO-derived and arterial pole-derived epicardium were connected and formed a continuous sheet (Figure 2E-G).

From E12.5 the heart was fully covered, however, a lower density of WT-1 expressing cells was observed on the RV compared to the LV surface (Figure 2H, I), as observed from WT1Cre-ERT2/+ at ED 13.5.

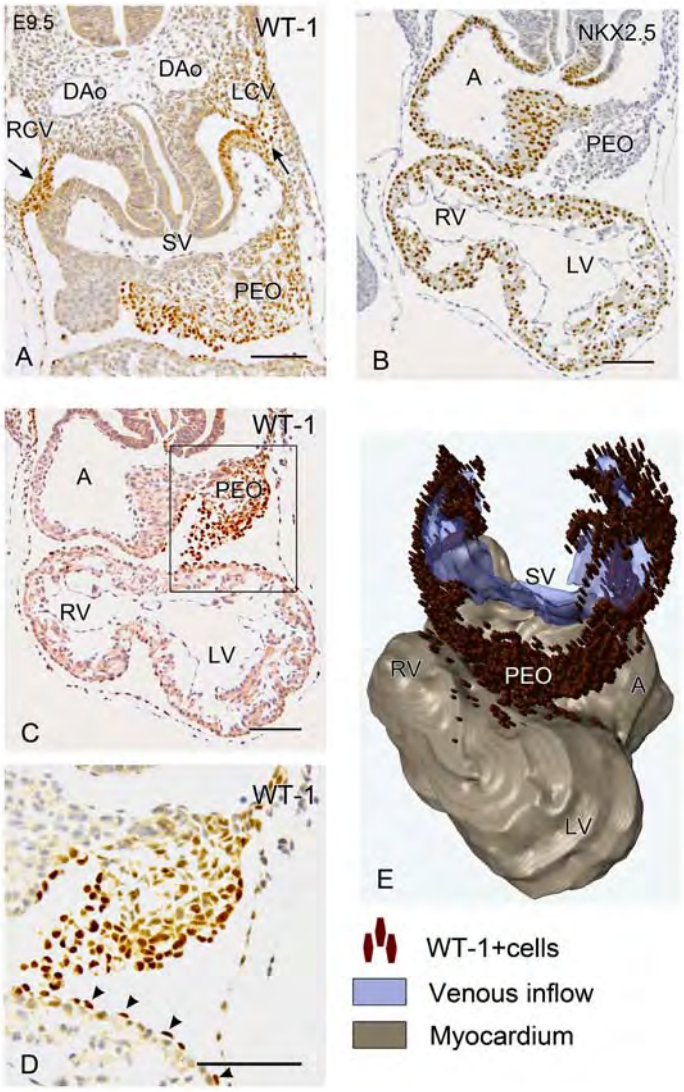

Figure 1. E9.5. WT-1 expression in the pro-epicardial organ and putative sinus venosus. A,B: Caudal sections approximately at the level of entrance of the cardinal veins in the sinus venosus (SV). Cells of the pro-epicardial organ (PEO) express WT-1 (A) and not Nkx2.5 (B). WT-1 positive cells are also observed surrounding the right cardinal vein (RCV) and left cardinal vein (LCV, arrows in A) C,D: Epicardial cells traverse the coelomic cavity and attach to the myocardial surface of the ventricle (boxed area in C, which is enlarged in D) Arrowheads in D indicated WT-1 positive epicardial cells. The first epicardial cells are observed at the inner curvature and on the left ventricular surface. E. 3D-reconstruction (left-antero-caudal view) indicating WT-1 expression (indicated by the brown cones) at the inflow part of the heart. Besides the PEO, WT-1 expression is seen in the tissue surrounding the LCV and RCV, that are indicated in blue. Myocardium of the atria and ventricles is indicated in grey. The area of WT-1 expression around the cardinal veins is continuous with the PEO. Abbreviations: A: primitive atrium, DAo: dorsal aorta, LV: primitive left ventricle, RV: primitive right ventricle. Bars: 100 µm

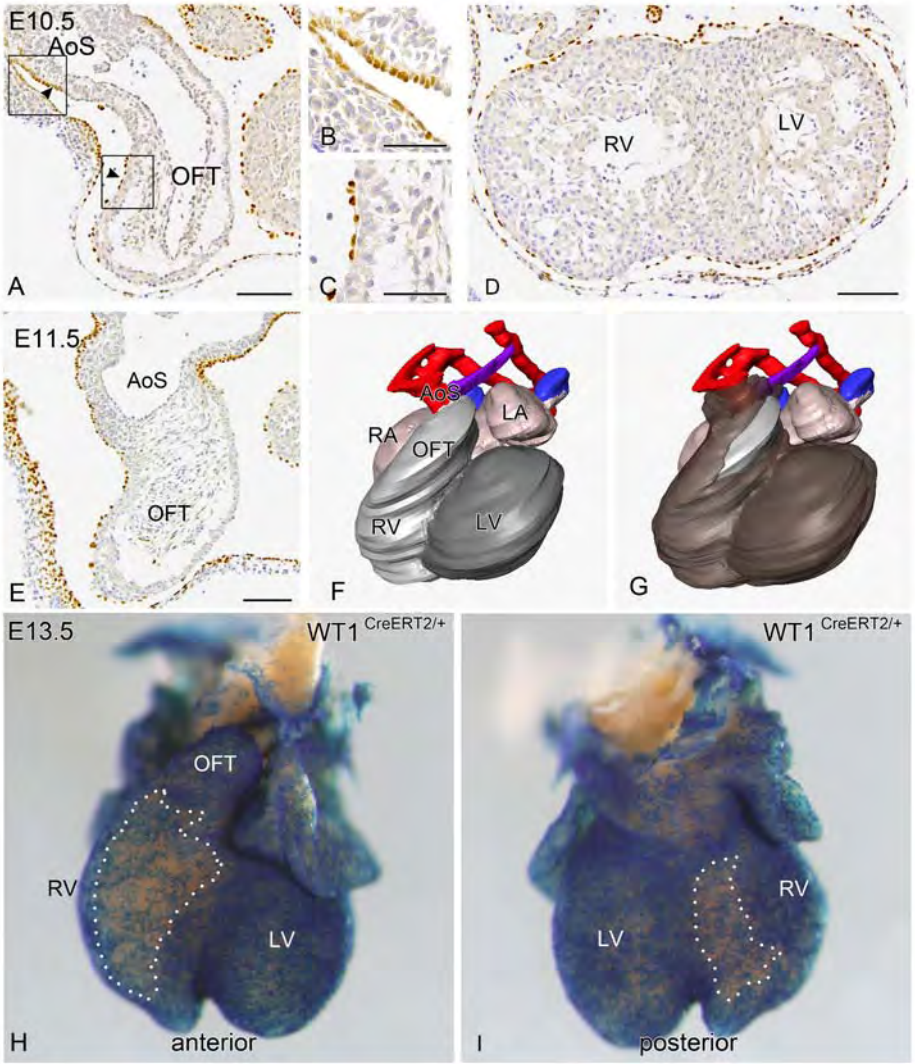

Figure 2. Epicardial covering of the heart at E10.5, E11.5 and E13.5. A-C. E10.5. The outflow tract (OFT) is covered with WT-1-expressing epicardial cells on the right side (putative aorta), whereas on the left side (putative pulmonary trunk), no epicardial cells are observed. Cells of the arterial pole epicardium have a cuboidal shape (arrowhead in upper boxed area in A, which is enlarged in B). Epicardial cells covering the more proximal right part of the OFT (that are pro-epicardial organ derived) have a flat, epithelial morphology (arrowhead in lower boxed panel in A, shown enlarged in C). D. Towards the apex both the right ventricle (RV) and left ventricle (LV) have an epicardial covering. The left ventricle (LV) is fully covered by epicardium at E10.5. E. At E11.5 the left side of the OFT is still uncovered by epicardium. F,G: 3D-reconstructions of myocardium (light grey for RV and OFT myocardium, dark grey for LV myocardium) and epicardium (dark brown) demonstrating that the left side of the OFT (the putative pulmonary trunk (purple), is the latest to be covered by epicardium. The atria are depicted in pink. H,I: At E13.5 the PEO-derived ventricular epicardium is fully developed, however the density of epicardial cells is lower at the RV surface as compared to the LV surface as can be observed in WT1$^{CreERT2/+}$. AoS: aortic sac. Bars: panels B,C: 50µm, panels A,D,E: 100 µm

EPDC formation and migration

From E12.5 onwards, generalized EMT throughout the epicardium of the ventricles resulted in a subepicardial layer covering the LV and the RV and formation of EPDCs was extensive (Figure 3). Migration of EPDCs was observed into RV myocardium but not into the LV (Figure 3A-C). The expression pattern of WT1CreERT2/+ confirmed the presence of EPDCs in the thin myocardial layer of the RV, whereas epicardium-derived cells could not be seen in the LV (Figure 3G-L).

Myocardial compaction

Figure 4 summarizes the process of myocardial compaction. At E11.5, both ventricles had a thin and uncompacted myocardial wall with loosely organized cells, while the RV was slightly smaller in size. The LV was the first in which reorganization of the compact myocardial layer could be observed at E12.5 (Figure 4A-D). This compact layer was characterized by a lower intensity of MLC-2a expression compared to the trabecular myocardium. At E13.5, MLC2a expression was of lower intensity within the compact outer layer of LV myocardium, whereas reduction of MLC2a expression occurred only at E14.5 in the RV (Figure 4E-H). Simultaneous with reduced expression of MLC2a, cellular organization changed within the LV wall. Structured layers of myocardium started to form in the LV, opposed to the loose organization in RV myocardium. The difference in compaction between the LV and RV was also reflected in myocardial thickening. Since both ventricles were thin-walled and consisted of a single or double layer of cells, no significant difference was observed in myocardial thickness between the LV and RV at E11.5 (Figure 4I). Thereafter, the LV was already significantly thicker at E12.5 in the apical wall and from E14.5 also at mid-lateral level, increasing considerably during development (Figure 4I).

Simultaneous with myocardial compaction, expression of WT-1 within the ventricles increased, specifically in the LV where EPDCs were abundantly present in the compact myocardial layer (Figure 4J-M). In the RV, some EPDCs were present in the compact myocardium, but also in the trabeculae, which was not seen in the LV. Differences in density of EPDcs were as also observed in the expression pattern of WT1CreERT2/+ at E14.5 when compaction had commenced in both ventricles (Figure 4N-P).

Disruption of epicardial cell migration

TGFβ2 plays an important role in EMT and migration of EPDCs,[38] and expression is associated with regular formation of an epicardial sheet (Figure 5). Morphology of the myocardium

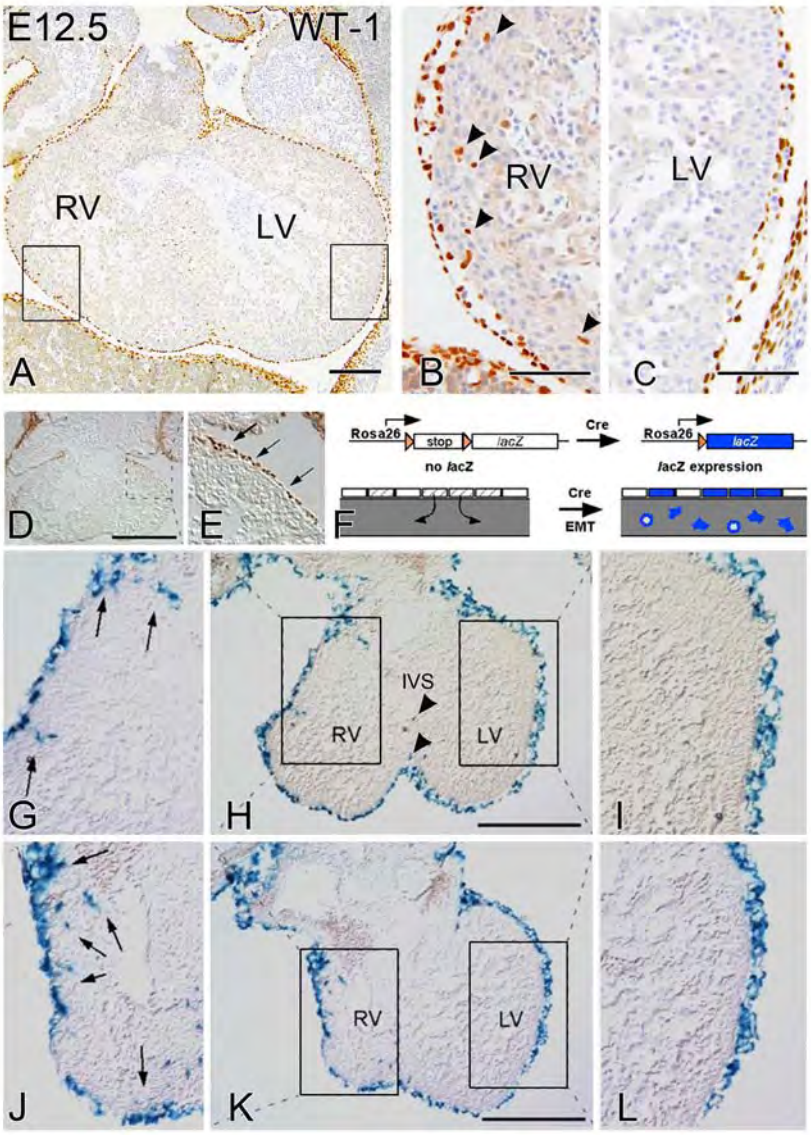

Figure 3. E12.5 EPDC formation and migration starts in the right ventricle. A: Overview section of the right ventricle (RV) and left ventricle (LV) at stage E12.5. The ventricles are completely covered by a WT-1 positive epicardial layer. B-C: Enlargements of the boxed areas in A, showing the right ventricle (RV) (B) and left ventricle (LV) (C). A subepicardial layer has started to form in both the RV and LV. EPDCs are found in solely in the myocardium of the RV (B, arrowheads) and not the LV (C). D,E: To confirm this observation, epicardial cells were followed using WT1$^{CreERT2/+}$. D: overview section, E: enlargement of the boxed area in E, at the level of the LV. No EPDC's are found in the myocardium. F. Schematic depiction of the *Wt1*$^{CreERT2/+}$ mouse model. For description see the Methods section. G-L: Results in WT1$^{CreERT2/+}$, stage E12.5. A subepicardial layer is present in both ventricles and migration of EPDCs into myocardium is observed in the RV (arrows in G and J), but not the LV (I and L). Some EPDCs were also present in the RV part adjacent to the interventricular septum (IVS) (J), and in the IVS itself (arrowheads in H). Bars: B,C: 50µm, other bars: 100 µm

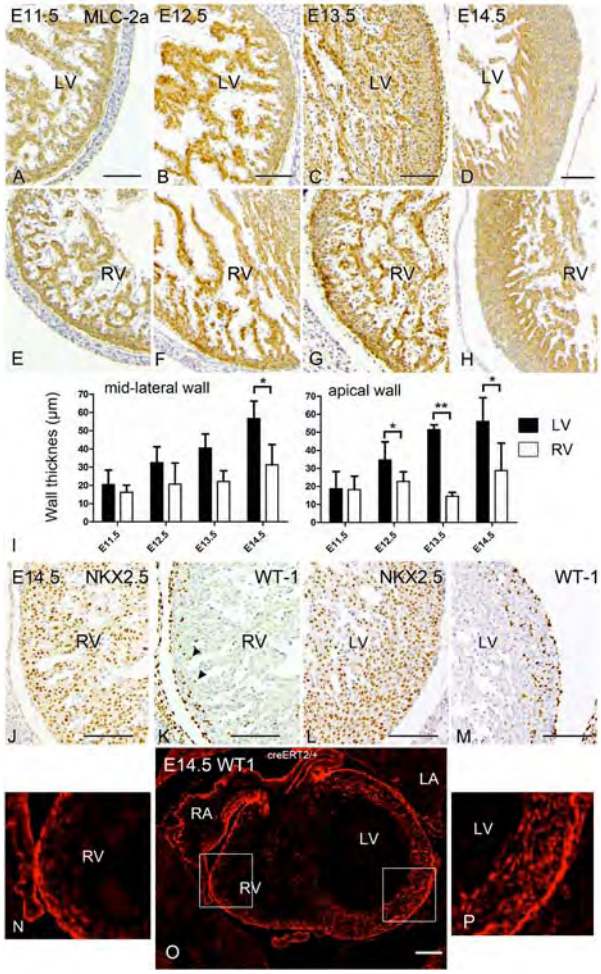

Figure 4. EPDCs and myocardial compaction at stages E11.5- E14.5. A-D. Myocardial compaction and thickening in the left ventricle (LV) at stages E11.5-E14.5. Development of a compact myocardial layer can observed starting from E12.5 and increasing in subsequent stages. E-H: Myocardial compaction and thickening in the right ventricle (RV) at stages E11.5-E14.5. A compact myocardial layer can be observed starting only at E14.5 in the RV. I. Quantification of myocardial thickening. Measurements at the level of mid-lateral wall are indicated in the left panel, and measurements at level of the apical wall are indicated in the right panel. There is a significant increase in wall thickness of the left ventricle during these developmental stages, whereas this increase in wall thickness is not significant for the RV. At the level of the mid-lateral wall, the LV wall is significantly thicker than the RV wall at E14.5, whereas at the apical wall, the LV is already significantly thicker at E12.5. J-M: E14.5: Structural differences are observed comparing the RV and LV. In the RV (J,K), the compact myocardium is still relatively loosely organized as compared to the LV (L,M). Abundant WT1 expressing EPDC's are observed specifically within the compact myocardial layer of the LV (M). In the RV some EPDC's are also observed in the compact myocardial layer, as well as at the base of the trabeculae (K, arrow heads). N-P: WT1$^{CreERT2/+}$. O is an overview section at the level of the RV and LV. N and O are enlargements of the boxed areas in O, at the level of the RV (N) and LV (P). Results in WT1$^{CreERT2/+}$ confirmed the abundant presence of EPDCs in the LV compact myocardial layer (P) and the presence of some EPDCs within the compact RV myocardium (N). *p<0.05; **p<0.005. Bars: 100 µm

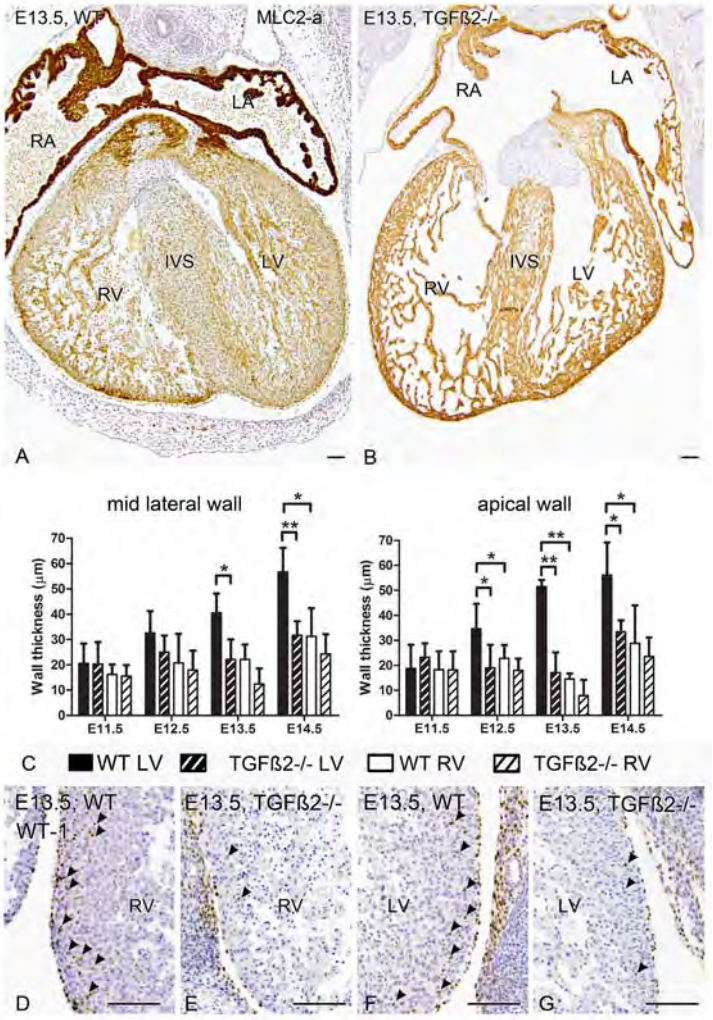

Figure 5. E13.5. TGFβ2 is required for normal EPDC migration and ventricular compaction. A,B: Overview sections of the heart at stage E13.5 in WT (A) and TGFβ2 knockout (B) embryos. In TGFβ2 knockout embryos thin myocardium is observed in both the left ventricle (LV) and right ventricle (RV). The myocardium of the interventricular septum (IVS) has a spongeous appearance. C-F: Overview of myocardial compaction from E11.5 to E14.5 in wildtype (C,E) and TGFβ_2-null (D,F) mouse embryos. In TGFβ_2-null mouse embryos (D,F), both the LV and RV myocardium is thin. Migration of EPDCs into the ventricles is severely reduced, and only a few EPDCs can be observed in the RV and LV (D, F, arrow heads). G: Quantification of myocardial thinning. Measurements at the level of mid-lateral wall are indicated in the left panel, and measurements at the level of the apical wall are indicated in the right panel. In contrast to wildtype, no significant difference in wall thickness between the RV and LV was measured in the TGFβ2 knockout embryos. The increase in LV wall thickness throughout development observed in wildtype, is lacking in TGFβ2 knockout embryos. In the midlateral wall, a significant difference in wall thickness of the LV was observed between wildtype and TGFβ2 knockout embryos at stages E13.5 and E14.5. In the measurements of the apical wall, a significant difference in wall thickness in the apical wall of the LV was observed between wildtype and TGFβ2 knockout from E12.5 onwards. *p<0.05; **p<0.005. RA: right atrium, LA: left atrium. Bars: 100 µm.

was severely abnormal in TGFβ2-null embryos. Both ventricular vascular walls were thin and uncompacted, while the LV was most prominently affected (Figure 5A,B). In TGFβ2-null mice, expression of WT1 was confined to the epicardium and could only sparsely be found within myocardium. (Figure 5C-F).

In knockout embryos no significant differences between RV and LV ventricular wall (apical or mid lateral) were observed throughout development. The increase in LV apical wall thickness observed in wildtype, was lacking in the knockout animals (Figure 5G). Comparing wildtype and knockout embryos, significant differences in wall thickness between wildtype and TGFβ2-null embryos were observed in the apical wall of the LV and from E13.5 onward this difference was also significant for thickness measurements in the LV mid-lateral wall (Figure 5G).

DISCUSSION

We evaluated the difference in timing of epicardial sheet formation, EMT and migration of EPDCs between the LV and RV in wildtype and TGFβ2-null mouse embryos. WT1$^{CreERT2/+}$ and the Cre-activated reporter Rosa26^{fsLz} were used in addition to the protein expression patterns of WT-1. WT-1 was expressed within the mesenchyme surrounding the sinus venosus, the PEO and epicardium. Epicardial covering in the RV is completed later and is less dense compared to the LV. In the RV free wall, migration of EPDCs occurs earlier compared to the LV free wall. EPDCs, having an essential role in normal myocardial compaction development, are more abundant within LV compared to RV myocardium. Finally, due to lack of normal EMT in TGFβ2-null mouse embryos the number of EPDCs is severely reduced in both ventricles, but more pronounced in the LV, resulting in myocardial thinning which is more prominent in the LV.

A number of studies indicate the relevance of preserved RV function for survival in patients with cardiac disease.[41–44] Although a wide range of therapeutic options is available for LV failure, treatment possibilities are still limited in case of RV dysfunction.[4] Many differences between the LV and RV on morphological as well as molecular level are founded in embryonic development.[45] The recognition of developmental processes, such as addition of cell-populations to the embryonic heart e.g. SHF, EPDCs and neural crest cells, have contributed to understanding differences between the LV and RV.[9, 10, 15, 22, 46] However, little attention has been paid to right/left differences in interaction of these cell populations. This is of particular interest since specific cardiomyopathies have a propensity to occur lateralised.[31, 32]

EPDCs and ventricular compaction

The nuclear transcription factor WT-1 is expressed in the PEO and epicardium and expression is down-regulated upon differentiation of EPDCs.[47] Therefore, the use of reporter constructs

is essential to trace the fate of EPDCs after EMT.[37] The current study demonstrates that WT-1 is expressed in epicardium, but also abundantly in the venous pole of the heart at early developmental stages, in the area that surrounds the lumen of the left and right cardinal veins, where the sinus venosus myocardium will form. As a consequence WT-1 is not entirely epicardium-specific, but it is generally accepted that with care WT-1 can be used to study EPDCs.[48, 49]

EPDCs contribute to smooth muscle cell formation, development of the cardiac conduction system, composition of the annulus fibrosus and cardiac valves and formation of interstitial fibroblasts.[22, 25, 50–53] During ventricular development, epicardium and EPDCs interact with underlying and surrounding myocardium on different levels depending on the stage of development.[16] EPDCs that differentiate into interstitial fibroblasts contribute to myocardial proliferation and compaction.[25] Since the LV and RV have a distinct developmental origin (first heart field vs. SHF) and a different timing in terms of growth, it is postulated that the interaction between epicardium and EPDCs on the one hand and myocardium on the other hand varies between both ventricles.

The current study shows that epicardial covering is completed later and is less dense in the RV compared to the LV. At E9.5, epicardial covering of the ventricles commences, and the RV expands quickly as a result of proliferation and addition of SHF-derived myocardium.[54] At this stage, the RV is thin-walled and the RV myocardium is loosely organized. The epicardial sheet expands gradually and maintains a contiguous network of epithelial cells.[16] As a consequence, RV expansion, which is most prominent on the left side of the OFT, surpasses extension of the epicardial sheet. This may explain why specifically the left side of the OFT is the latest to be covered by epicardium.

Invasion of EPDCs into the myocardium occurred earlier as compared to the LV, although epicardial covering was completed later in the RV. Furthermore, EPDCs were observed near the trabecular surface of the RV and not the LV. In a previous chick-quail chimera study, it was demonstrated that permissiveness for EPDCs is variable within the embryonic heart.[55] The exact mechanism that regulates this variation in myocardial permissiveness is still unclear. We postulate that the loose organization of RV myocardium facilitates early invasion of EPDCs into the RV.

From E13.5 the number of myocardially located EPDCs increased dramatically, specifically within the LV. Previous studies demonstrated that this invasion process is essential for normal development of the compact myocardial layer.[25, 26] EPDCs induce myocardial alignment and proliferation through cell-cell interaction.[30] EPDCs are the primary source of cardiac interstitial fibroblasts[22] regulating myocardial proliferation and compaction through fibronectin and collagen synthesis in a process that requires $\beta 1$ Integrin signalling.[26] Therefore, EPDCs are important for the difference in proliferation between the LV and RV. The current study shows that proliferation and myocardial thickening starts at E12.5 in the LV, simultaneous with the invasion of EPDCs. Although EPDCs were observed in the free wall of the RV, their density was

far more prominent in the LV. The spatio-temporal difference in EPDC invasion in the RV may explain why RV myocardium remains thinner as compared to LV myocardium. Alternatively, the lack of an epicardial contribution, more specifically EPDCs, during ventricular development would exert strongest effects in the LV.

TGFβ2 and myocardial proliferation

Normal EMT, required for the formation of EPDCs , is a tightly regulated process that involves expression of several genes during specific time-intervals.[16] Most of these genes serve multiple functions during cardiovascular development and experimental knock-out of epicardial genes resulted in severe malformations and embryonic lethality as observed in WT-1 knock-out.[56] TGFβ2, expressed by epicardium, is required for normal EMT and migration of EPDCs.[57] TGFβ2-null embryos have multiple cardiac defects, but embryonic lethality was not observed.[27] In TGFβ2 null embryos the compact myocardial layer of the ventricles was abnormally thin and spongy. Hence this model is well-suited for the study of abnormal EPDC contribution to ventricular development. Our evaluation shows that knock-out of TGFβ2 results in a severely reduced densities of EPDCs within the myocardium of the ventricles. The morphological changes were most prominent in the LV, which was expected since the contribution of EPDCs was more important for the LV in wildtype embryos. In the knockout animals, the normal difference in myocardial thickness between RV and LV disappeared, as quantification of myocardial thickening in the RV demonstrated similar thickness in wildtype and TGFβ_2-null embryos, underscoring that the number of invading EPDCs is instrumental in myocardial architecture.

EPDCs and the difference between LV and RV myocardium

Whereas the LV largely originates from the first heart field-derived primary heart tube, the RV develops as a result of the addition of cellular components from the SHF.[9–11] This requires regulated proliferation and differentiation which is partly mediated by Wnt, Hedgehog, bone morphogenetic protein and fibroblast growth factor (FGF) signaling pathways.[58] FGF specifically is important for proliferation within the SHF, lengthening of the OFT and normal RV development.[59] To date, a role for FGF in early development of the LV has not been identified. However, FGF signaling is required for normal invasion of EPDCs and for myocardial growth in both ventricles.[25, 60] It is postulated that the multiple functions of FGF during RV development result in responses to EPDC-mediated FGF signaling that differ between the RV and LV.

The LV and RV express different sarcomeric genes during cardiac development.[61] The distinctive origins of the LV and RV (first heart field vs. SHF) may account for these differences in

expression patterns, and could also be relevant for the growth potential of the ventricles.[62] Although EPDCs induce myocardial organization and proliferation, the characteristics of the underlying and surrounding myocardium are bound to be important.

Functional relevance of EPDCs for the treatment of cardiac disease

RV dysfunction is a recognized problem in adult patients with congenital or acquired heart disease.[4] Most medical therapies have no or limited effect on RV function in situations of RV overload. In patients with transposition of the great arteries and a systemic RV, treatment with angiotensin receptor blockers does not improve ventricular function, whereas this effect is beyond dispute in patients with LV disease.[5] Similarly, beta-blockade has adverse effects in patients with pulmonary hypertension and an overloaded RV.[6]

In recent investigations, it was suggested that the multipotent EPDCs may have potential for cell-based therapies.[33, 34, 63, 64] More importantly, the paracrine function of EPDCs during cardiac development may be preserved in adult life, which can be relevant for the development of novel treatment strategies in cardiovascular disease.[36, 65] Our evaluation indicates that the interaction between myocardium and epicardium follows different patterns in the RV and LV. Therefore, future studies need to explore the mechanisms that guide EPDC-myocardium interaction in the RV and LV separately.

CONCLUSION

WT-1 can be used to investigate the fate of EPDCs. However, data interpretation should be performed with diligence since WT1 is also expressed in non-epicardial cell populations. The RV is covered later and less densely by epicardium compared to the LV. Invasion of EPDCs occurs slightly earlier and with a different patterning in the RV. Myocardial thickening occurs from E12.5, upon migration of EPDCs into myocardium, being more prominent in the LV and requires TGFβ2. In TGFβ2-null mouse embryos, migration of EPDCs into myocardium is severely reduced resulting in thin, uncompacted ventricles, most obvious in the LV.

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