Wt1 and Retinoic Acid Signaling in the Subcoelomic Mesenchyme Control the Development of the Pleuropericardial Membranes and the Sinus Horns

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Rationale: The cardiac venous pole is a common focus of congenital malformations and atrial arrhythmias, yet little is known about the cellular and molecular mechanisms that regulate its development. The systemic venous return myocardium (sinus node and sinus horns) forms only late in cardiogenesis from a pool of pericardial mesenchymal precursor cells.

Objective: To analyze the cellular and molecular mechanisms directing the formation of the fetal sinus horns.

Methods and Results: We analyzed embryos deficient for the Wt1 (Wilms tumor 1) gene and observed a failure to form myocardialized sinus horns. Instead, the cardinal veins become embedded laterally in the pleuropericardial membranes that remain tethered to the lateral body wall by the persisting subcoelomic mesenchyme, a finding that correlates with decreased apoptosis in this region. We show by expression analysis and lineage tracing studies that Wt1 is expressed in the subcoelomic mesenchyme surrounding the cardinal veins, but that this Wt1-positive mesenchyme does not contribute cells to the sinus horn myocardium. Expression of the Raldh2 (aldehyde dehydrogenase family 1, subfamily A2) gene was lost from this mesenchyme in Wt1−/− embryos. Phenotypic analysis of Raldh2 mutant mice rescued from early cardiac defects by retinoic acid food supply revealed defects of the venous pole and pericardium highly similar to those of Wt1−/− mice.

Conclusions: Pericardium and sinus horn formation are coupled and depend on the expansion and correct temporal release of pleuropericardial membranes from the underlying subcoelomic mesenchyme. Wt1 and downstream Raldh2/retinoic acid signaling are crucial regulators of this process. Thus, our results provide novel insight into the genetic and cellular pathways regulating the posterior extension of the mammalian heart and the formation of its coelomic lining. (Circ Res. 2010;106:1212-1220.)

Key Words: sinus horn ■ venous pole ■ sinoatrial node ■ Tbx18 ■ Raldh2

The systemic venous return of the mature mammalian heart, which terminates in the right atrium, consists of multiple anatomic components including the myocardial sleeves of the right superior and inferior caval veins, the sinoatrial node (SAN), the coronary sinus (persisting left caval vein in the mouse), and the sinus venarum.1 The systemic venous return is a focus of congenital malformations and atrial arrhythmias;2,3 necessitating insight into the cellular and molecular programs by which it arises during cardiac development. Most myocardial components of the heart are not represented in its initial anlage, but are continuously added by recruitment and differentiation of precursor cells.4 The sinus horns, the myocardial parts of the common cardinal veins upstream of the venous valves that bulge into the pericardial cavity, form by pericardial precursors that differentiate into sinus venosus myocardium around the systemic venous connection to the atrium.5 They form only after embryonic day (E)9.5, when outflow tract, left and right ventricle, and the common atrium have already been established. In adults, most of the right sinus horn myocardium is incorporated into the right atrium to form the sinus venarum. In humans, the left sinus horn will lose its connection to the body and form the coronary sinus, whereas in mouse it will persist as the left superior caval vein.

Few genes regulating venous pole development have been characterized, including the Tbx18 (T-box transcription factor 18) that marks the sinus horn lineage.6,7 Wt1 (Wilms tumor 1) was initially identified as a tumor suppressor gene involved in the etiology of Wilms’ tumor in humans. Genetic analyses in the...
mouse subsequently uncovered essential roles for the transcription factor Wt1 in the development of numerous organ systems.7 In the heart, Wt1 is required for the mesenchymal transition of epicardial cells. Failure to provide cellular constituents to the developing coronary vessels results in pericardial bleeding and midgestational lethality.8,9 Here, we present data demonstrating an additional function of Wt1 in the formation of the common cardinal veins and the pleuroperticardial membranes (PPMs). We implicate retinoic acid (RA) signaling in the etiology of these defects and show a primary requirement of Wt1 and Raldh2 (aldehyde dehydrogenase family 1, subfamily A2) in the sub-coelomic mesenchyme from which the PPMs are released.

Methods

Animal care was in accordance with national and institutional guidelines. Mice carrying a null allele of Wt1 (Wt1tm1Jae)5,6 Tbx18GFP (Tbx18tm2Akis)5 and Raldh2 (Aldh1a1tm1Dea)11,12 R26loxP and tetO3LacZ-GFP reporter mice,12,13 and the RARE-Hsp68LacZ [synonym: Tg[RARE-Hspa1b/lacZ]12Jrt/J] reporter transgenic line,14 which harbors a tetrameric repeat of the RARβ2 RARE linked to the Hsp68 minimal promoter used to determine RA signaling, were all described before. For the Wt1BAC-IRES-EGFPCre transgenic line, the BAC clone RP23–266M16 was modified by inserting an IRES/EGFP-Cre cassette 17bp downstream of the translation stop site of the Wt1 gene. (The generation and evaluation of this line will be described elsewhere.) In Wt1f/f mice, the coding sequence of an improved tetracycline-dependent transactivator TTA2S5 was introduced into the Wt1 locus by gene targeting (E Lausch, S Fees, C Steinwender, C Spangenberg, L Eshkind, E Bockamp, B Zabel, manuscript in preparation). All mouse lines were maintained on an outbred (NMRI or FvB) background.

An expanded Materials and Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Defects in the Systemic Venous Return of Wt1-Deficient Hearts

Wt1−/− mice maintained on an NMRI outbred background died during midgestation presenting defects reported on earlier, including lack of kidneys, diaphragmatic hernia, and defects in coronary vessel formation the latter of which may underlie embryonic lethality. Yet, histological inspection of surviving embryos at E14.5 revealed an undescribed variation in the systemic venous return that prompted us to analyze the cardiac venous pole more carefully (Figure 1). In wild-type embryos of this stage, the sinus horns bulged into the pericardial cavity (Figure 1A). These sinus horns were completely myocardIALIZED (cardiac troponin I–positive; Figure 1C). Pericardial and pleural cavities were separated by the PPMs that were stretched out as thin tissue layers between their roots in the lung bud and their distal attachment points in the ventrolateral body wall (Figure 1A and 1C). In contrast, Wt1 mutant hearts featured thinner, nonmyocardialized cardinal veins (Figure 1B and 1D). These occupied an abnormal lateral position within the PPMs that also tethered the atrial roofs to the body wall. The pleural cavity was not fully expanded, leaving the pericardium ventrally attached to the body wall by a mesh of loose

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
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<tr>
<td>E</td>
<td>embryonic day</td>
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<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>PPM</td>
<td>pleuroperticardial membrane</td>
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<td>RA</td>
<td>retinoic acid</td>
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<td>RARE</td>
<td>retinoic acid response element</td>
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<tr>
<td>Raldh2</td>
<td>aldehyde dehydrogenase family 1, subfamily A2</td>
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<td>SAN</td>
<td>sinoatrial node</td>
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<td>Tbx18</td>
<td>T-box transcription factor 18</td>
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<td>Wt1</td>
<td>Wilms tumor 1</td>
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Figure 1. Sinus horn defects in Wt1-deficient hearts. Histological, molecular and morphological analyses of sinus horns (A through F) and the SAN (G through N) were carried out on transverse sections of the venous pole region of E14.5 wild-type and Wt1−/− hearts. A through F, Histological stainings with hematoxylin/eosin (A and B), in situ hybridization analysis of cTnI expression (C and D), and 3D reconstructions of serial sections stained for cTnI in a dorsal–posterior view. Atrial myocardium is shown in green, cardinal vein myocardium in gray, the lumen of the cardinal veins in brown, and the pulmonary vein myocardium in red (E and F). Asterisks (B and D) mark persisting subcoelomic mesenchyme. G through N, In situ hybridization analyses of sections through the base of the cardinal veins for SAN marker genes with probes as indicated (G through I and K through M), and 3D reconstructions of serial sections stained for Tbx3 expression through the cardinal veins (in brown) to visualize the SAN (in gray) (J and N). icv indicates inferior cardinal vein; la, left atrium; lu, lung; lscv, left superior cardinal vein; pc, pericardial cavity; ple, pleural cavity; ppm, pleuroperticardial membrane; pv, pulmonary vein; ra, right atrium; rscv, right superior cardinal vein; rsh, right sinus horn.
mesenchymal cells (Figure 1B). More anteriorly on the left side, pleural and pericardial cavities communicated by a thin canal leaving space for the atria to touch the lung (arrow in Figure 1D). Because sections planes between different embryos were sometimes difficult to match, we performed serial sections and subsequent 3D reconstruction analysis. This confirmed our histological findings and showed that in Wt1−/− hearts, the nonmyocardialized superior cardinal veins were thin and lying lateral to the atria near the body wall. The defects were restricted to the sinus horns, as the myocardium of pulmonary vein and the dorsal atrial wall myocardium appeared unchanged (Figure 1F).

The SAN is an elongated “comma-shaped” structure at the junction of the right venous entrance and the atrium expressing Tbx3, the Hcn4 gene (hyperpolarization-activated, cyclic nucleotide-gated K+), and Tbx18 (Figure 1G through 1I).6 In Wt1−/− hearts, these markers were detected at E14.5 but the domain of expression protruded away from the right superior cardinal vein (Figure 1K through 1M). Three-dimensional reconstruction of the SAN from serial sections stained for Tbx3 expression confirmed that the SAN myocardium was not wrapped around the sinus horn as in the wild type (Figure 1J) but had an open “wing”-like structure (Figure 1N). Volume measurements revealed that the SAN cell mass was not significantly reduced in Wt1-deficient embryos (data not shown), arguing that SAN morphology rather than myocardial differentiation and growth are affected by loss of Wt1.

Sinus Horn Defects of Wt1−/− Embryos Arise Early in Development

To determine the onset of venous pole defects in Wt1-deficient hearts, we analyzed embryos at earlier developmen-
tal time points (Figures 2 and 3). In Wt1−/− embryos, the venous pole region appeared unchanged at E9.5 (data not shown). At E10.5, protrusions of the cardinal veins into the pericardial cavity generated short sinus horns that, however, failed to expand and myocardialize at subsequent stages (Figure 2A through 2L). Analysis of Hcn4 expression indicated presence of dispersed pacemaker tissue at E12.5 (Online Figure I). The subcoelomic mesenchyme around the common cardinal veins was present but loosely organized at E11.5 and 12.5 (asterisks in Figure 2F and 2J). At E12.5, a mesothelial lining separated the posterior pleural and pericardial cavities in the wild type (Figure 3). These PPMs originated laterally in the body wall at the height of the developing ribs, and projected medially to connect to the hilus of the lung bud at more anterior levels (Figure 3A). In subsequent stages, the lateral insertion points moved ventrally following the distal extension of the ribs to become loosely connected to the sternum at E14.5. During this growth phase, the PPMs were stretched out to thin epithelial sheets. The pleural cavity dramatically gained in volume. Loose mesenchymal tissue initially filling the pleural cavity at the onset of its expansion was completely cleared by E14.5 (Figure 1A; Figure 3B and 3C). In Wt1−/− embryos, PPMs were not apparent as distinct mesothelial linings separating the 2 cavities at E12.5 and E13.5. Instead, the pericardium was tethered to the atrial roof and cardinal veins on one side and to a mesenchymal mesh that was continuous with the lateral body wall on the other side (Figure 3D and 3E). Inflation of this subcoelomic mesenchyme was delayed and clearance not achieved by E14.5 (Figures 1B and 3F). Thus, defects in sinus horn formation and PPM/subcoelomic mesenchyme organization arise early in sinus horn/caval vein development and may be linked.

**Wt1 Is Not Expressed in Sinus Horns but in Mesothelia and the Underlying Mesenchyme**

To determine the temporal and spatial requirement for Wt1 in sinus horn development more carefully, we analyzed expression of Wt1 from E10.5 to E14.5 in the venous pole region (Figure 4). We compared Wt1 expression to that of Tbx18, which is restricted to the mesenchyme and myocardium of the sinus horns. **Figure 4. Wt1 expression in the subcoelomic mesenchyme. A through L,** Comparative in situ hybridization analysis of Wt1 and Tbx18 expression on transverse sections of the venous pole region of E10.5 to E14.5 hearts of wild-type (A through H, K, and L), Tbx18−− (I) and Wt1−− (J) embryos. **Black arrowheads** point to Tbx18 expression in the mesenchyme and myocardium of the sinus horns. **M through P,** Immunofluorescence analysis of Wt1 and Tbx18 protein at the cardiac venous pole. **Q through T,** Comparative immunofluorescence analysis of Wt1 and β-galactosidase protein expression in the venous pole region in Wt1BAC-IRES-EGFP-Cre/H11001; Rosa26R -galactosidase/+ embryos. cTnI immunofluorescence (red) marks sinus horn and atrial myocardium. Stages, genotypes, and probes are as indicated. Abbreviations are as in the legends of Figures 1 and 2.
From E10.5 to E12.5, Wt1 was expressed in the developing mesothelia, including the epicardium, pericardium and pleura, and in the subcoelomic mesenchyme of the lateral body wall including the one surrounding the common cardinal veins, but surprisingly, not in the sinus horns and the SAN where Tbx18 was strongly expressed (Figure 4A through 4H). Coinmunofluorescence analysis of Wt1 and Tbx18 in the venous pole region at E12.5 revealed patterns of protein expression identical to mRNA domains, confirming mutual exclusion of the 2 expression domains (Figure 4M through 4P). Complementary expression of Wt1 in the mesenchyme surrounding the common cardinal veins and Tbx18 in the sinus horn myocardium, prompted us to examine the possibility of negative cross-regulation of these transcription factors. However, Tbx18 was not derepressed in the Wt1 mutant and vice versa (Figure 4I and 4J). At E14.5, Wt1 was maintained in the mesothelia whereas Tbx18 expression persisted in the sinus horn myocardium (Figure 4K and 4L).

The sinus horn defect in Wt1 mutants suggests that the Wt1-positive mesenchyme surrounding the common cardinal veins may provide precursor cells for the Wt1-negative sinus horn myocardium. To test this hypothesis, we used genetic lineage tracing systems to determine the fate of Wt1-expressing cells. First, we used a binary system with mice carrying a knock-in of the tTA gene in the Wt1 locus and a reporter line with a tetO6:LacZ-GFP transgene.13 Expression of tTA under Wt1 control elements leads to transcription of the LacZ gene and production of the β-galactosidase protein whose longev-
ity provides a tool to trace cells initially expressing Wt1. We did not find β-galactosidase positive cells in the sinus horn myocardium at E12.5 and E14.5 using this system (Online Figure II). We next crossed a Wt1BAC-IRES-EGFPCre line, in which the expression of the Cre recombinase gene mimics that of the Wt1 gene, with R26LacZ reporter mice.12 This system irreversibly labels Wt1-expressing cells and their daughters by LacZ expression (β-galactosidase activity). Again, we did not find β-galactosidase positive cells in the sinus horn myocardium at E12.5 and E16.5 (Figure 4Q through 4T), providing further evidence that the Wt1-positive mesenchyme of the lateral body wall does not represent a cellular source for the sinus horn myocardium.

Collectively, these analyses suggest Wt1 is not required within the sinus horn mesenchyme like Tbx18 is, but that Wt1 expression in the mesothelia and/or subcoelomic mesenchyme controls sinus horn formation by an indirect process.

**Cellular Changes of the Lateral Body Wall Mesenchyme in Wt1−/− Mice**

We next analyzed whether changes of proliferation and apoptosis may underlie the failure of sinus horn formation and the persistence of the subcoelomic body wall mesenchyme. Cell proliferation appeared unaffected in Wt1−/− embryos at E11.5 (wild type 0.214±0.0064 versus mutant 0.211±0.0067; P=0.78). At E12.5, we detected a significant but small increase of cell proliferation in the Wt1−/− lateral body wall mesenchyme (wild type 0.151±0.0031 versus mutant 0.183±0.0035; P<0.0001) (Figure 5A through 5E). Using TUNEL assays, we did not detect changes of the low levels of apoptosis in the subcoelomic mesenchyme at the venous pole region at E11.5 (we analyzed 3 wild-type and 3 Wt1 mutant embryos) and E12.5 (4 embryos of each genotype were analyzed) in Wt1−/− hearts (Figure 5F, 5G, 5J, and 5K). At E13.5, we observed clusters of...
apoptotic cells in the mesenchyme underlying the PPM insertion point in the body wall in 8 of 11 tested wild-type embryos (Figure 5H and 5I). These clusters of apoptotic cells were not detected in Wt1−/− embryos (n=5) at this stage (Figure 5L and 5M). Thus, loss of apoptosis in this region may contribute to the nonrelease of the PPMs from the lateral body wall and the persistence of the underlying mesenchyme.

**Loss of Raldh2 Expression in the Lateral Body Wall Mesenchyme**

To identify molecular changes that may underlie and explain the defects of the venous pole in Wt1 mutant hearts, we analyzed expression of genes that represent markers for the myocardium of the atria, the SAN and the sinus horns. Expression of these markers was unchanged in the Wt1−/− hearts. Thus, unchanged expression of these markers may reflect the persistence of the underlying mesenchyme.

Our analysis uncovered a number of novel markers for the subcoelomic mesenchyme of the lateral body wall that were coexpressed with Wt1 in this region (Figure 6). The Osr1 (odd-skipped related 1), the gene encoding sFRP2 (secreted frizzled-related protein 2), and sulfatase 1 (Sulf1) were additionally expressed in the dorsal mesocardium (Figure 6A, 6C, and 6E), whereas Sulf1 showed additional expression in epicardium (black arrow Figure 6E) and pericardium (black arrowhead in Figure 6E). Expression of Osr1, sFRP2, Sulf1, and Gata6 (GATA binding protein 6) was maintained (albeit at seemingly lower levels) in the loose Wt1−/− lateral body wall mesenchyme, independently confirming the persistence of cells formerly expressing Wt1 in this region (asterisks in Figure 6A through 6H). Raldh2 was coexpressed with Wt1 in the epicardium, pericardium and subcoelomic mesenchyme from E11.5 to E14.5 in the entire pericardial cavity (Online Figure IV). Intriguingly, expression of Raldh2 was specifically lost in the lateral body wall mesenchyme (asterisks in Figure 6J) but persisted in the epicardium (black arrow in Figure 6J) and pericardium (black arrowhead in Figure 6J). Raldh2 is an enzyme that converts retinaldehyde to RA. To study which tissues respond to this compound in this region, we analyzed LacZ expression of a transgenic reporter line for RA signaling (RARE-Hsp68LacZ). LacZ expression was detected in a dorsal to latero-ventral wave in the subcoelomic mesenchyme of the lateral body wall at E12.5 and E13.0 strongly arguing that RA signaling does not act onto the developing sinus horns (Figure 6K through 6N).

Because of the similarity of the cardinal vein phenotypes in Wt1−/− and Tbx18−/− mice, we tested whether loss of Tbx18 affects expression of marker genes in the adjacent subcoelomic mesenchyme. However, unchanged expression of these genes (including Raldh2) in Tbx18−/− embryos excluded a paracrine Tbx18-dependent signal from the sinus horn myocardium/mesenchyme onto the subcoelomic mesenchyme (Online Figure V) and implied that sinus horn defects in Wt1- and Tbx18 mutants are of different etiology.

**Loss of Raldh2 Results in Defects in Sinus Horn Formation**

Loss of Raldh2 expression in Wt1 mutants suggested that Raldh2 is a downstream effector of Wt1 in sinus horn formation.

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**Figure 6. Absence of Raldh2 expression in the Wt1−/− lateral body wall mesenchyme.** A through J, In situ hybridization analysis of marker expression carried out on transverse sections of the venous pole region of E12.5 wild-type and Wt1−/− hearts. Genotypes and probes are as indicated. Asterisks (A through K) mark the subcoelomic lateral body wall mesenchyme. Black arrows and arrowheads (E, F, I, and J) mark epicardium and pericardium, respectively. J, Loss of Raldh2 expression in the subcoelomic mesenchyme of Wt1−/− embryos. K through N, In situ hybridization analysis of Raldh2 and LacZ expression carried out on transverse sections of the venous pole region of E12.5 (K and L) and E13.0 (M and N) hearts of RARE-Hsp68LacZ/+ transgenic embryos (RARE). Arrowheads in L and N mark RA signaling. Abbreviations are as in Figures 1 and 2.
Figure 7. RA signaling is required for cardinal vein formation after E9.5 in the mouse heart. Histological, molecular, and morphological analyses of sinus horns (A through F) and the SAN (G through J) were carried out on transverse sections of the venous pole region of E14.5 wild-type (left column) and Raldh2-deficient embryos food supplied with RA between E7 and E9 (RA E7-E9) (right column). A through F, Histological stainings with hematoxylin/eosin (A and B), in situ hybridization analysis of cTnl expression (C and D), and 3D reconstructions of serial sections stained for cTnl in a dorsal–posterior view. Atrial myocardium is shown in green, cardinal vein myocardium in gray, the lumen of the cardinal veins in brown, and the pulmonary vein myocardium in red (E and F). Asterisks (B and D) mark persisting subcoelomic mesenchyme. G through J, In situ hybridization analysis of sections through the base of the cardinal veins for expression of SAN marker genes with probes as indicated. Abbreviations are as in Figures 1 and 2.

Discussion

Our study has established that the transcription factor Wt1 is required for the correct formation of the cardiac venous pole. We suggest that Wt1 functions primarily in the subcoelomic mesenchyme of the lateral body wall to mediate the release of the PPMs by apoptosis. This, in turn, mediates the correct repositioning of the cardinal veins medially to the lungs and the dorsal mesocardium. Wt1 function in the subcoelomic mesenchyme may be mediated by RA signaling. Tbx1/8 acts independently in the sinus horn mesenchyme to warrant its growth and myocardial differentiation.

Sinus Horn Defects Are Secondary to Changes in PPM Formation

Our analysis of Wt1−/− embryos revealed that sinus horn defects are tightly associated with pleuropericardial abnormalities suggesting that alterations in mesothelial development may accompany or cause cardinal vein malformations. These pericardial defects are similar to congenital defects in human that have been described as rare anomalies. They may or may not be symptomatic, with symptomatic patients often experiencing chest pain. In the more frequently occurring partial pericardial absence, surgical intervention may be required to prevent strangulation of the heart. The etiology of these human defects is not well understood, but descriptive studies of human embryos have pointed to an important role of the PPMs.17,18 The proximal aspects of the common cardinal veins (the ducts of Cuvier) are embedded in the PPMs. Thus, failure of one or both PPMs to close will result in lateralization of cardinal veins. At this point, it remains unclear why myocardialization of these lateralized cardinal veins fails, but physical separation from sources of differentiation signals (eg, the sinoatrial region) may be involved. Different opinions for the causative factor for the nonclosure of PPMs have been put forward.18 In 1909, Perna proposed that the premature atrophy of the left duct of Cuvier results from insufficient blood supply to the PPMs.19 Alternatively, altered growth of the heart may inappropriately stretch and tear the PPMs.20 To our knowledge, normal and abnormal pericardial development has not been described in any detail in the mouse. Our phenotypic characterization of Wt1- and Raldh2-deficient embryos pinpoint to the decisive role of the subcoelomic mesenchyme of the lateral body wall in the etiology of pericardial defects. The persisting subcoelomic mesenchyme in these mutants tethers the PPMs to the lateral body wall, thus, preventing appropriate expansion and cardinal vein relocalization. It also hampers the inflation of the pleural cavity that may rely on a dorsal to ventral wave of PPM release by the swelling and subsequent disappearance of the underlying mesenchyme.
RA Signaling May Mediate Wt1 Function in the Subcoelomic Mesenchyme

RA has been identified as an important signaling factor in numerous developmental processes.\(^2\) RA also controls heart morphogenesis and differentiation,\(^6\) and acts as an epicardial factor for myocardial differentiation.\(^2\) Our analysis of the expression and function of the main RA biosynthetic enzyme Raldh2 expands the role of RA signaling to pericardial development and sinus horn formation. Raldh2 mutant embryos rescued from early cardiac defects by RA food supply exhibited a spectrum of phenotypic changes, including reduced PPMs, left-sided pericardial–pleural communication, persistence of the body wall mesenchyme, and lateralized cardiac veins, that are compatible with a primary requirement of RA for pericardial development. Using a RA reporter mouse, we detected RA signaling in the subcoelomic mesenchyme of the lateral body wall adjacent to the developing PPMs. RA signaling was spatially dynamic because it shifted ventrally preceding PPM detachment. Localized apoptosis of the RA signaling positive mesenchyme may mediate PPM release from the lateral body wall. This situation is somehow reminiscent of the role of RA signaling in vertebrate limb development where it mediates apoptosis of the interdigital mesenchyme to release the digits.\(^3\) Noteworthy, administration of all-trans RA in patients with acute promyelocytic leukemia can lead to an RA syndrome that features pleural and pericardial effusion.\(^4\) Loss of the RA-metabolizing enzyme Cyp26a1 in zebrafish results in defects in common cardinal vein formation.\(^5\) Together, this suggests that RA is important for normal development and function of mesothelia and cardiac veins. Colocalization of Wt1 and Raldh2 expression in the lateral body wall mesenchyme, loss of Raldh2 expression in this domain in Wt1\(^{-/-}\) embryos, and phenotypic similarities of the pericardial defects of either mutant strongly suggests that RA signaling acts downstream of Wt1. We tried to rescue pericardial defects of Wt1-deficient mice by feeding pregnant mice with RA starting from E8.5, ie, shortly before onset of sinus horn formation, until E14.5 when this process ends. A concentration of 0.25-g RA per gram of food, which was efficient to rescue sinoatrial defects of Raldh2 mutant mice, did not rescue sinus horn and PPM defects in Wt1\(^{-/-}\) mice. However, cardinal vein defects were not rescued in the Raldh2\(^{-/-}\) embryos supplied with RA until E14.5 either, suggesting that exogenous RA does reach its target tissue after E9.5 in the embryo (Online Figure VIII). Hence, it remains open whether Wt1 regulates additional genetic circuits that synergize with RA to mediate its downstream functions.

A link between Wt1 and Raldh family members has already been described in other developmental contexts. Raldh1 expression is downregulated in the urogenital ridge of Wt1\(^{-/-}\) embryos.\(^6\) Wt1 and Raldh2 are coexpressed in the epicardium and epicardiially derived cells of the developing avian heart.\(^7\) A direct regulation of Raldh2 expression by Wt1 has also been proposed during liver development.\(^8\) Here, Wt1 and Raldh2 expression coincide in the coelomic lining, and in Wt1\(^{-/-}\) mice Raldh2 expression is downregulated in these mesothelial cells, leading to defects in liver morphogenesis. Additionally, the left pleuropertitoneal membrane is not fully formed. The regulation of Raldh2 expression by Wt1 may therefore represent a general mechanism during the development of murine mesothelia.

Cellular Contributions at the Cardiac Venous Pole

We have recently shown that the myocardialized proximal aspects of the superior and inferior caval veins form only after heart looping by recruitment and subsequent myocardial differentiation of Tbx18-positive pericardial precursors.\(^9\) Wt1 expression in the subcoelomic mesenchyme directly abuts the Tbx18 expression domain at the venous pole, suggesting that Wt1-positive precursors feed into the pool of Tbx18-positive sinus horn cells. However, our lineage tracing experiments negate such a possibility, but instead stress that Tbx18 exclusively marks the sinus horn myocardial lineage throughout heart development.\(^6\) In Tbx18\(^{-/-}\) mice, the cardinal veins are positioned inside the PPMs, but appear less lateralized compared to Wt1-deficient embryos. Delay but not failure of myocardialization of proximal cardinal veins in Tbx18\(^{-/-}\) embryos\(^6\) may relate to the different topological situation of the PPMs that are not tethered to the lateral body wall as in Wt1\(^{-/-}\) embryos. Expression of Wt1 and Raldh2 is unchanged in Tbx18\(^{-/-}\) mice, indicating that Wt1 and Tbx18 regulate different cellular and molecular programs during pericardial and caval vein development. Although the sinus horns fail to form in Wt1\(^{-/-}\) embryos, the volume of the SAN is largely unchanged. This contrasts the complete lack of the large SAN head structure in Tbx18\(^{-/-}\) embryos\(^6\) and suggests that Wt1 controls the formation of myocardialized sinus horns but not of the region destined to form the SAN head that is regulated by Tbx18. Altered SAN morphology in Wt1\(^{-/-}\) embryos, however, argues that positioning of the developing SAN next to the myocardialized sinus horns is critical for correct morphogenesis of this structure.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**

- The sinus horns, the myocardIALIZED parts of the intrapericardial aspects of the common cardinal veins, form late in cardiac development, from a pool of pericardial cells that is distinguished from precursors of the other cardiac components by the presence of Tbx18 and absence of Nkx2-5 expression.

- The sinus horns contribute to the mature systemic venous return system but also to the pace maker tissue of the heart, the sinoatrial node (SAN), which is a common focus of congenital malformations and atrial arrhythmias.

**What New Information Does This Article Contribute?**

- The WT1 (Wilms tumor 1) gene is expressed in the subcoelomic mesenchyme of the lateral body wall surrounding the cardinal veins, but this WT1-positive mesenchyme does not contribute cells to the sinus horn myocardium.

- WT1 expression in the subcoelomic mesenchyme is required for sinus horn formation, release and growth of the pleuropericardial membranes, and expansion of the pleural and pericardial cavities.

- Retinoic acid signaling may mediate WT1 function in the subcoelomic mesenchyme for pleuropericardial membrane release, as well as relocalization and myocardialization of cardinal veins.

The cardiac venous pole is a common focus of congenital malformations and atrial arrhythmias, yet little is known about the cellular and molecular mechanisms that regulate its development. Here, we have identified expression of the WT1 gene and downstream retinoic acid signaling in the subcoelomic mesenchyme of the lateral body wall as a crucial requirement for the development of the myocardial components of the venous pole and of the pericardium. Our study uncovers the critical role of the subcoelomic mesenchyme in this program. Unexpectedly, this tissue does not represent a cellular source for sinus horn myocardium but controls sinus horn formation indirectly. We suggest that relocalization and myocardialization of cardinal veins depends on the correct temporal release of the pleuropericardial membranes in which the cardinal veins are embedded from the underlying body wall mesenchyme. Hydration and apoptotic removal of the mesenchyme may be cellular mediators in this process. Our results provide novel insight into the genetic and cellular pathways regulating the posterior extension of the mammalian heart, and the formation of its coelomic lining. Our study suggests that congenital malformations of the cardiac venous pole and pericardial defects present together and share a common etiology.
Wt1 and Retinoic Acid Signaling in the Subcoelomic Mesenchyme Control the Development of the Pleuropericardial Membranes and the Sinus Horns

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“Wt1 and retinoic acid signaling in the subcoelomic mesenchyme control the development of the pleuropericardial membranes and the sinus horns”

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Supplemental Online Materials and Methods

Mice and genotyping
In Wt1\textsuperscript{tTA}\textsuperscript{\textsuperscript{\textsuperscript{TA}}}, knock-in mice, the coding sequence of an improved tetracycline-dependent transactivator tTA\textsuperscript{2S\textsuperscript{1}} was introduced into the Wt1 locus by gene targeting (Lausch et al., manuscript in preparation). This exchanged exon 1 and 360 bp of 3' downstream sequence of Wt1 for the coding sequence of tTA2S, substituting the Wt1 translational start site with the ATG of tTA2S. Expression of tTA2S under endogenous Wt1 control elements faithfully recapitulates the pattern of native Wt1, both in adult mice and during development. Heterozygous Wt1\textsuperscript{tTA}\textsuperscript{\textsuperscript{\textsuperscript{TA}}} knock-in mice are phenotypically normal, showing no anatomical or histopathological abnormalities up to an age of fifteen months. Wt1\textsuperscript{tTA}/tTA embryos, however, died prior to day E11.5 of gestation as described for Wt1\textsuperscript{-/-} mice\textsuperscript{2}. Administration of the tetracycline derivative doxycycline (which has a high affinity for tTA2S) to Wt1\textsuperscript{tTA} mice at a concentration of 0.1 mg per ml drinking water was sufficient to stringently suppress transcription of tetracycline-responsive transgenes in all Wt1-positive tissues in vivo, as analyzed by qPCR, histochemistry, immunohistochemistry, and chemiluminescence. For fate mapping of Wt1-positive cells in situ, Wt1\textsuperscript{tTA} knock-in mice were crossed with reporter strains carrying a tet\textsubscript{O\textsubscript{7}}LacZ-GFP transgene.\textsuperscript{3} In double-transgenic animals maintained without doxycycline (-DOX), Wt1-controlled tTA2S drives expression of both the green fluorescence protein (GFP) and ß-galactosidase from a tTA-responsive bidirectional promoter, composed of seven tet-repressor binding sites (TetO\textsubscript{7}) immediately upstream of an RNA polymerase II transcriptional start site of the cytomegalovirus immediate early promoter. For the generation of mutant embryos, heterozygous mice were intercrossed. For timed pregnancies, vaginal plugs were checked in the morning after mating, noon was taken as embryonic day (E) 0.5. Embryos were harvested in PBS, fixed in 4% paraformaldehyde overnight and stored in 100% methanol at –20°C before further use. Wildtype littermates were used as controls. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR (protocols upon request). H. Hedrich, state head of the animal facility, approved the care of animals and experiments at Medizinische Hochschule Hannover.

Food supply of retinoic acid
Pregnant females were treated with retinoic acid (RA) to rescue venous pole development in Raldh2- and Wt1-deficient embryos similar to described protocols.\textsuperscript{4,5} All-trans-RA (Sigma) from a 5 mg/ml ethanol stock suspension was diluted in 50 ml water and mixed with 50 g powdered food (irradiated PicoLab rodent diet 20) to a final concentration of 100 mg/g food. The RA-containing food mixture was left in the cage for ad libitum feeding and renewed twice a day until the day of sacrifice.

Histological analyses
For histological stainings embryos were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned to 5 or 10 µm. Sections were stained with haematoxylin and eosin, following standard procedures.

\textit{In situ} hybridization analysis
\textit{In situ} hybridization analysis with digoxigenin-labeled antisense riboprobes followed a published protocol.\textsuperscript{6} Details of used probes upon request.

Immunohistochemistry
For immunohistochemistry rabbit polyclonal antibody against Wt1 (C-19, Santa Cruz Biotechnology, 1:200), goat polyclonal antibody against Tbx18 (C-20, Santa Cruz
Biotechnology, 1:200), monoclonal antibody against cardiac Troponin I (1:500 MAB1691, Millipore),\(^7\) and rabbit IgG fraction against β-Galactosidase (Cappel, 1:4000) were used as primary antibodies. Alexa488 goat-anti-rabbit (Invitrogen, 1:250) and biotinylated donkey-anti-goat (Dianova, 1:200) were used as secondary antibodies. Nuclei were stained with 4',6-Diamidino-2-phenylindol (DAPI) (Roth) or Topro3 (Invitrogen). For staining with the Tbx18 antibody, paraffin sections were pressure cooked for 3 min in antigen unmasking solution (H-3300, Vector Laboratories Inc). The signal was amplified using the Tyramide Signal Amplification (TSA) system from Perkin-Elmer (NEL702001KT, Perkin Elmer LAS). For double staining with Wt1, Alexa488 goat-anti-rabbit secondary antibody was added during the streptavidine-HRP step of the TSA protocol. For β-Galactosidase, the primary antibody was amplified using the VECTASTAIN ABC Kit (Peroxidase rabbit IgG, Vector Laboratories, PK-4001). The detection of this antibody was performed with the DAB Peroxidase Substrate Kit from Vector Laboratories (SK-4100).

**Documentation**
Sections were photographed using a Leica DM5000 microscope with Leica DFC300FX digital camera. Immunofluorescence for Wt1\(^{BAC-ires-EGFPCR/\pm}; R26\(^{lacz}/\pm\) lineage tracing was detected using a Leica SPE confocal microscope. All images were processed in Adobe Photoshop CS.

**Three-dimensional reconstruction**
Three-dimensional visualization and geometry reconstruction of patterns of gene expression determined by *in situ* hybridization was performed as described previously.\(^8\) Shortly, serial sections were used for *in situ* hybridization and were documented as described. The remaining analysis was done with the help of the software “Amira” (Version 4.1.1, Mercury Computer Systems Inc). The pictures were aligned, and regions for reconstruction were labeled. After surface conversion a three-dimensional model was obtained.

**Proliferation and apoptosis assays**
Cell proliferation in the E11.5 and E12.5 embryos was investigated by detection of incorporated 5-bromo-2-deoxyuridine (BrdU) on 5-µm sections of paraffin-embedded specimens similar to previously published protocols.\(^9\) Ten sections each of three embryos of each genotype at E11.5 and 15 sections each of four embryos of each genotype at E12.5 were used for quantification. The BrdU-labeling index was defined as the number of BrdU-positive nuclei relative to the total number of nuclei, as detected by DAPI counterstain, in the Wt1 expressing region next to the cardinal veins. Statistical analyses were performed using the 2-tailed Student's t-test. Data were expressed as mean ± SEM. Differences were considered significant when the P-value was below 0.05.

Detection of apoptotic cells in 10-µm paraffin sections of E10.5 to E13.5 embryos was based on modification of genomic DNA utilizing terminal deoxynucleotidyl transferase (TUNEL assay) and indirect detection of positive cells by fluorescein-conjugated anti-Digoxigenin antibody. The procedure followed exactly the recommendation of the manufacturer (Serologicals Corp.) of the ApopTag kit used. Three sections each of two embryos of each genotype at E10.5, of three embryos of each genotype at E11.5, of four embryos of each genotype at E12.5 and of six wildtype and five Wt1-mutant embryos at E13.5 were analyzed. Additionally three sections of two RARE-LacZ embryos of E12.5 and E13.0 each were used. The detection of apoptotic cells in the Raldh2-deficient mice was performed with three embryos of each stage. Wildtype and Raldh2-deficient embryos were littermates and fed with retinoic acid from embryonic day E7 to E9.
References


Online Figure I. SAN morphology is changed in Wt1-deficient hearts. In situ hybridization analysis for sinoatrial node (SAN) expression of hyperpolarization-activated, cyclic nucleotide-gated K+ 4 gene (Hcn4) in E12.5 hearts of wildtype (wt) and Wt1-deficient (Wt1−/−) embryos on transverse sections through the base of the cardinal vein. rsh, right sinus horn; ra, right atrium; rcv, right common cardinal vein.
Online Figure II. Wt1-positive subcoelomic mesenchyme does not contribute to the sinus horns. Analysis of LacZ expression by *in situ* hybridization (A,C) and of β-galactosidase protein (β-Gal) by immunohistochemistry (B,D) in Wt1<sup>TTA</sup> x tetOβiLacZ-GFP embryos at E12.5 and E14.5 on transverse sections through the cardiac venous pole. Black arrowheads point to the border of the LacZ expression between the Wt1-positive subcoelomic mesenchyme of the lateral body wall and the absence of LacZ expression in the myocardium of the sinus horns. rcv, right common cardinal vein; lcv, left common cardinal vein.
Online Figure III. No changes of marker gene expression for atrial, sinoatrial node (SAN) and sinus horn myocardium in Wt1-deficient hearts. In situ hybridization analysis was carried out on transverse sections of the venous pole region of E12.5 hearts of wildtype and Wt1-deficient embryos. Genotypes and probes are as indicated. Nkx2.5 (A,B) and Connexin40 (Cx40) (C,D) were confined to the atrial myocardium and were excluded from the myocardium of the sinus horns and the SAN. Expression of Tbx5 (E,F) and Gata4 (G,H) comprised the myocardium of the SAN in addition to that of the atria. Isl1 expression (I,J) was confined to the SAN myocardium. The expression domain of Tbx18 (K,L) comprised the epicardium and pericardium, the myocardium of the sinus horns and the SAN. Shox2 (M,N) is found in the SAN myocardium and the venous valves, similar to bone morphogenetic protein 4 (Bmp4) (O,P) that however appeared patchier in its expression. Asterisks in (A) mark the subcoelomic lateral body wall mesenchyme. dm, dorsal mesocardium; la, left atrium; lcv, left common cardinal vein; ra, right atrium; rcv, right common cardinal vein.
Online Figure IV. Wt1 and Raldh2 are coexpressed in epicardium, pericardium and subcoelomic mesenchyme of the lateral body wall during heart development. In situ hybridization analysis of Wt1 and Raldh2 gene expression carried out on transverse sections of wildtype embryos from E11.5 to E14.5 on different section planes. Probes and stages are as indicated. A-D', expression of Wt1 and Raldh2 at the posterior part of the pericardial cavity; E-H', expression of Wt1 and Raldh2 at the venous pole region; I-L', expression of Wt1 and Raldh2 at the anterior part of the pericardial cavity. icv, inferior cardinal vein; lu, lung; li, liver; la, left atrium; lv, left ventricle; lcv, left common cardinal vein; lsh, left sinus horn; pc, pericardial cavity; ppm, pleuropericardial membrane; ra, right atrium; rv, right ventricle; rcv, right common cardinal vein; rsh, right sinus horn.
**Online Figure V.** Markers for the subcoelemic mesenchyme of the lateral body wall are unchanged in *Tbx18*-deficient embryos. *In situ* hybridization analysis of marker gene expression carried out on transverse sections of the venous pole region of E12.5 hearts of wildtype and *Tbx18*−/− embryos. Genotypes and probes are as indicated. Asterisks in (A) mark the subcoelomic lateral body wall mesenchyme. dm, dorsal mesocardium; la, left atrium; lcv, left common cardinal vein; ra, right atrium; rcv, right common cardinal vein.
Markers for the subcoelomic mesenchyme of the lateral body wall are unchanged in *Raldh2*-deficient embryos. *In situ* hybridization analysis of marker gene expression carried out on transverse sections of the venous pole region of E12.5 hearts of wildtype and *Raldh2*−/− embryos food supplied with RA between E7 and E9 (RA E7-9). Genotypes and probes are as indicated. Asterisks in (A) mark the subcoelomic lateral body wall mesenchyme. dm, dorsal mesocardium; la, left atrium; lcv, left common cardinal vein; ra, right atrium; rcv, right common cardinal vein.
Online Figure VII. Absence of apoptotic cell clusters in the lateral body wall mesenchyme of Raldh2-deficient embryos. Analysis of apoptosis in the Wt1-positive subcoelomic mesenchyme on transverse sections trough the venous pole in wildtype and Raldh2-deficient embryos food supplied with RA between E7 and E9 (RA E7-E9) by TUNEL staining. Dorsal is oriented up. Stages and genotypes are as indicated. Asterisks in (A) mark the subcoelomic lateral body wall mesenchyme. The white arrow (B) points to the cluster of apoptotic cells in E13.5 wildtype embryos. rcv, right common cardinal vein; ra, right atrium; lcv, left common cardinal vein; la, left atrium; ppm, pleuropericardial membrane.
Online Figure VIII. Exogenous retinoic acid (RA) does not rescue Raldh2-deficiency in venous pole development after E9.5. Histological, molecular and morphological analyses of sinus horns (A-H,M,N) and the sinoatrial node (I-L) were carried out on transverse sections of the venous pole region of E14.5 wildtype, Wt1- and Raldh2-deficient embryos food supplied with RA between E7 and E14 (RA E7-14). A-H,M-N, Histological stainings with hematoxylin and eosin (A-D), in situ hybridization analysis of cTnI expression (E-H), and 3D-reconstructions of serial sections stained for cTnI in a dorsal-posterior view. Atrial myocardium is shown in green, cardinal vein myocardium in grey, the lumen of the cardinal veins in brown, and the pulmonary vein myocardium in red (M,N). Asterisks in B,D,F,H mark persisting subcoelomic mesenchyme. I-L, in situ hybridization analyses of sections through the base of the cardinal veins for expression of marker genes for the sinoatrial node with probes as indicated. icv, inferior cardinal vein; la, left atrium; lu, lung; lscv, left superior cardinal vein; pc, pericardial cavity; plc, pleural cavity; ppm, pleuropericardial membrane; pv, pulmonary vein; ra, right atrium; rscv, right superior cardinal vein; rsh, right sinus horn.