*Podoplanin* deficient mice show a Rhoa related hypoplasia of the sinus venosus myocardium including the sinoatrial node.
ABSTRACT

We investigated the role of podoplanin in development of the sinus venosus myocardium comprising the sinoatrial node, the dorsal atrial wall and the primary atrial septum as well as the myocardium of the cardinal and pulmonary veins. We analyzed podoplanin wildtype and knockout mouse embryos between 9.5-15.5 days post conception (dpc) using immunohistochemical marker podoplanin, sinoatrial node marker HCN4, myocardial markers MLC-2a, Nkx2.5 as well as Cx43, coelomic marker WT-1 and epithelial-to-mesenchymal transformation markers E-cadherin and RhoA. 3D reconstructions were made and myocardial morphometry were performed. Podoplanin mutants showed hypoplasia of the sinoatrial node, the primary atrial septum and dorsal atrial wall. Myocardium lining the wall of the cardinal and pulmonary veins was thin and perforated. Impaired myocardial formation is correlated with abnormal epithelial-to-mesenchymal transformation of the coelomic epithelium due to upregulated E-cadherin and downregulated RhoA, which are controlled by podoplanin. Our results demonstrate an important role for podoplanin in development of sinus venosus myocardium.

INTRODUCTION

During early embryogenesis the lateral plate mesoderm splits into two layers: the somatic and the splanchnic mesoderm forming the outer and the inner layer of the coelomic cavity.1,2 The somatic mesoderm is involved in development of the body wall and extremities while myocardial precursors are restricted to the splanchnic mesoderm.3,4 Subsequently, the left and right primary heart fields (cardiogenic plates) fuse at the ventral midline resulting in the primary linear heart tube, which starts looping at 8.5 days post conception (dpc).1,2,5

Previous studies of heart development5-7 have shown that further development of the heart tube is related to the addition of cells at both the arterial and venous pole of the heart, forming the outflow and the inflow tract myocardium, respectively. These early observations on the addition of the secondary myocardium have recently been supported by several studies describing the addition of myocardium at the arterial pole, being secondary8 and anterior heart fields9-12 and the posterior heart field (PHF) at the venous pole12-15 of the developing heart. The complete length of the splanchnic mesoderm contributing to the addition of myocardium at both poles of the heart was called second heart field14 or second lineage.10,12,14

A number of genes and proteins, considered as early markers of the second heart field at the outflow and inflow tract, have been reported, such as MesP1,16 Fibroblast growth factor (Fgf)
8 and 10, 9 BMP-2 and Nkx2.5, 8,13,15 Isl1, 14 inhibitor of differentiation Id2, 17 GATA factors targeting Mef2c, 18,19 Tbx1, Tbx3 and Tbx18 15,20 and Shox2. 21 Recently we have added podoplanin to this list as a novel gene in cardiac development.

As a coelomic and myocardial marker, podoplanin is specifically expressed in the mesenchyme and in the myocardium at the venous pole. 13,22 Studying the mesenchymal population, podoplanin expression was observed in the proepicardial organ and epicardium. 13,22,23 In the cardiomyocyte population podoplanin staining was seen in major parts of the developing atrioventricular cardiac conduction system, in sinus venosus myocardium including the sinoatrial node, the venous valves, the dorsal mesocardium, the dorsal atrial wall and primary atrial septum. Also the myocardium surrounding the cardinal veins and the common pulmonary vein belongs to this population. 13,22 In earlier publications, podoplanin, a 43-kd mucin type transmembrane glycoprotein, first named E11 antigen as a new marker for an osteoblastic cell line, 24 was also reported in the nervous system, the epithelia of lung, eye, oesophagus and intestine, 25 the mesothelium of the visceral peritoneum, 24 the coelomic wall (pericardium) lining the pericardial cavity 13 and the epicardium, 22 the podocytes of the kidney 26 and the lymphatic endothelium. 27

To elucidate a possible functional role of podoplanin in cardiac development and more specifically in the development of the sinus venosus myocardium, we studied podoplanin knockout mouse embryos and used several immunohistochemical markers. To investigate the sinus venosus myocardium including the sinoatrial node we have used hyperpolarization-activated, cyclic nucleotide-gated cation 4 (HCN4). 28-30 In addition, atrial myosin light chain 2 (MLC-2a), NK2 transcription factor related locus 5 (Nkx2.5) and connexin 43 (Cx43) were used. Furthermore, we studied E-cadherin, a cell to cell adhesion protein, and RhoA important for epithelial-to-mesenchymal transformation (EMT) of the coelomic epithelium, a process that allows epithelial cells to become mobile mesenchymal cells. 31 To visualize the epithelium and mesothelium of the coelomic cavity, the epicardium and sites of active EMT we have used Wilm’s tumor suppressor protein (WT-1). 32-34 It has been described that the PHF and resulting myocardium are derived from the epithelial lining of the coelomic cavity (splanchnic mesothelium) by EMT. 13 During normal development, loss of E-cadherin is needed for proper EMT resulting in loss of epithelial features 35 and consecutive development into migratory mesenchymal cells. During abnormal development, an upregulated state of E-cadherin, by e.g. lack of podoplanin, causes an altered EMT. 22,36 Podoplanin can, therefore, be considered as an inhibitor of E-cadherin stimulating EMT. In addition, RhoA activation by podoplanin and ezrin interaction have been described to lead into podoplanin-induced EMT. 37 Similar to E-cadherin, lack of podoplanin might lead to downregulated RhoA and altered EMT.
In the current paper we studied the role of podoplanin in the development of the sinus venosus myocardium derived from the PHF, which is a part of the more extensive second heart field. We demonstrate that knocking out the podoplanin gene leads to myocardial abnormalities in sinus venosus myocardium at the venous pole of the developing mouse heart.

MATERIAL AND METHODS

Generation of podoplanin⁻/⁻ mice and harvesting of embryos
The podoplanin knockout mice were generated by homologous recombination in embryonic stem cells from the 129S/v mouse line by inserting a neomycin phosphotransferase cassette in a 7.7kb genomic fragment encompassing exons II to V. The complete description of this model was reported previously. Briefly, the heterozygous ES cell clones were test-bred for germline transmission with Swiss mice to generate podoplanin⁻/⁻ offspring (50% 129S/v: 50% Swiss genetic background) using standard procedures. These mice were crossed to obtain podoplanin⁻/⁻ embryos and podoplanin⁺/+ (wildtype) littermates. The morning of the vaginal plug was stated 0.5 dpc. Pregnant females were sacrificed and embryos were harvested.

General description
We investigated the lining of the coelomic cavity and the morphology of the sinus venosus myocardium of the heart in 27 wildtype mouse embryos of 9.5 dpc (n=4), 10.5 dpc (n=4), 11.5 dpc (n=3), 12.5 dpc (n=4), 13.5 dpc (n=5), 14.5 dpc (n=4) and 15.5 dpc (n=3) and compared these with 37 podoplanin knockout mouse embryos of 9.5 dpc (n=4), 10.5 dpc (n=4), 11.5 dpc (n=6), 12.5 dpc (n=8), 13.5 dpc (n=6), 14.5 dpc (n=5) and 15.5 dpc (n=4). All embryos were fixed in 4% paraformaldehyde (PFA) and routinely processed for paraffin immunohistochemical investigation.

Immunohistochemistry
Immunohistochemistry was performed with antibodies against MLC-2a (1/6000, kindly provided by S.W. Kubalak, Charleston, SC, USA), Nkx2.5 (1/4000, Santa Cruz Biotechnology, Inc., CA, USA, SC-8697), podoplanin (clone 8.1.1., 1/500, Hybridomabank, Iowa, USA), WT-1 (1/1000, Santa Cruz Biotechnology, Inc., CA, USA, sc-192), E-cadherin (1/150, Santa Cruz Biotechnology, Inc., CA, USA, SC-7870), HCN4 (1/1000, Alomone labs, The Netherlands, APC-052), RhoA (1/2000, Santa Cruz Biotechnology, Inc., CA, USA, SC-418) and Cx43 (1/200, Sigma-Aldrich Chemie, USA, C6219). The primary antibodies were dissolved in phosphate buffered saline (PBS)-Tween-20 with 1% Bovine Serum Albumin (BSA, Sigma Aldrich, USA). Between subsequent incubation steps all slides were rinsed in PBS (2x) and PBS-Tween-20 (1x). The slides were incubated with the secondary antibody for 45 min: for
MLC-2a, WT-1, E-cadherin, HCN4 and Cx43 with 1/200 goat-anti-rabbit-biotin (Vector Laboratories, USA, BA-1000) and 1/66 goat serum (Vector Laboratories, USA, S1000) in PBS-Tween-20; for Nkx2.5 with 1/200 horse-anti-goat-biotin (Vector Laboratories, USA, BA-9500) and 1/66 horse serum (Brunschwig Chemie, Switzerland, S-2000) in PBS-Tween-20; for podoplanin with 1/200 goat-anti-Syrian hamster-biotin (Jackson Imunno research, USA, 107-065-142) and 1/66 goat serum (Vector Laboratories, USA, S1000) in PBS-Tween-20 and for RhoA with 1/200 horse-anti-mouse-biotin (Santa Cruz Biotechnology, Inc., CA, USA, SC-9996-FITC) and 1/66 horse serum (Brunschwig Chemie, Switzerland, S-2000) in PBS-Tween-20. The slides were incubated with ABC-reagent (Vector Laboratories, USA, PK 6100) for 45 min. For visualization the slides were incubated with 400 μg/ml 3-3’-di-aminobenzidin tetrahydrochloride (DAB, Sigma-Aldrich Chemie, USA, D5637) dissolved in Tris-maleate buffer pH 7.6 to which 20 μl H₂O₂ was added: MLC-2a, and E-cadherin 5 min; Nkx2.5, HCN4, Cx43, WT-1 and podoplanin 10 min. Counterstaining was performed with 0.1% haematoxylin (Merck, Darmstadt, Germany) for 5 sec, followed by rinsing with tap water for 10 min. All slides were dehydrated and mounted with Entellan (Merck, Darmstadt, Germany).

3D reconstructions

We made 3D reconstructions of the sinus venousus myocardium based on MLC-2a, Nkx2.5 and HCN4 stained sections of wildtype as well as podoplanin knockout embryos of 12.5 dpc in which the morphological differences were shown. The reconstructions were made as previously described using the AMIRA software package (Template Graphics Software, San Diego, USA).

Morphometry of the myocardium

Based on HCN4, MLC-2a and Nkx2.5 stained sections, sinus venosus and separately sinoatrial node myocardial volume estimation was performed of 12 wildtype mouse hearts of 11.5 dpc (n=3), 12.5 dpc (n=3), 13.5 dpc (n=3), 14.5 dpc (n=3) and 12 podoplanin knockout mouse hearts of 11.5 dpc (n=3), 12.5 dpc (n=3), 13.5 dpc (n=3), 14.5 dpc (n=3) based on Cavalieri’s principle as described previously. Statistical analysis was performed with an independent sample-t-test (P<0.05) using the SPSS 11.0 software program (SPSS Inc, Chicago, III). In summary, regularly spaced points (100 mm² for sinus venosus myocardium and 49 mm² for sinoatrial node myocardium) were randomly positioned on the HCN4 stained myocardium. The distance between the subsequent sections of the slides was 0.075 mm for sinus venosus myocardium and 0.025 mm for the sinoatrial node. The volume measurement was done using the HB2 Olympus microscope with a 100x magnification objective for the sinus venosus myocardium and 200x for the sinoatrial node.
RESULTS

General description
We studied the embryonic phenotype of the podoplanin knockout mice, which show an increased embryonic death of approximately 40% of the homozygote embryos between 10-16 dpc. Additionally, 50% of the neonatal homozygote knockout mice die within the first weeks of life, while heterozygous mutants reach sexual maturity. The cause of embryonic death has been correlated to the cardiac defects\textsuperscript{22} while the cause of neonatal death is still unknown.

Figure 1. Transverse sections showing EMT sites of the coelomic cavity epithelium in the hearts of 10.5 dpc wildtype (WT) and podoplanin knockout (podoplanin\textsuperscript{\textminus\textminus}) mouse embryos. To demarcate the coelomic mesothelium and sites of active EMT, we studied WT-1 staining (a, b, e and f) and E-cadherin staining (c and g). WT-1 positivity was found in the mesothelium (asterisk in b and f) and epithelium (CE) of the coelomic cavity and epicardium (EP) of the WT embryos (a and b). WT-1 staining was more extensive at the corners of the coelomic cavity (asterisk) below the left cardinal vein (LCV), where the CE appeared to be cuboidal and well organized (b). In the podoplanin\textsuperscript{\textminus\textminus} embryos WT-1 was also present in these regions (e and f), however, the CE was disorganized and the cells were irregular in shape and size (compare a and b with e and f). E-cadherin staining appeared stronger in both the EP and CE of the podoplanin\textsuperscript{\textminus\textminus} embryos (g) as compared to the WT mouse embryos (c). Remarkably, in the podoplanin\textsuperscript{\textminus\textminus} E-cadherin staining was not only upregulated in the CE but also in the underlying mesothelial cells (c and g). In WT embryos RhoA expression was seen in the coelomic mesenchyme, CE and epicardium (d). In the mutants overall expression of RhoA was downregulated (h). LA indicates left atrium. Scale bars: (a), (e) = 60μm; (b-d), (f-h) = 30μm; magnification box in (c), (d), (g) and (h) = 20μm.
Several marked morphological cardiac abnormalities were observed in the knockout mouse embryos. In the younger stages a hypoplastic proepicardial organ (10.5 dpc) as well as ventricular myocardium were observed with discontinuous epicardium, a thin layer of the subepicardial mesenchyme and a diminished amount of epicardium derived cells (EPDCs). These hearts also presented with outflow tract abnormalities such as severe dextroposition of the aorta, coronary artery abnormalities, spongious myocardium of the developing interventricular septum and impaired formation and fusion of the atrioventricular cushions. At the sinus venosus region, which is the area of our focus, myocardial hypoplasia and morphological abnormalities were observed. In general, the sinus venosus myocardial abnormalities, mentioned below, were seen in the sinoatrial node, the dorsal atrial wall, the atrial septum as well as the myocardium of the cardinal and pulmonary veins.

Morphology and immunohistochemical expression patterns of the sinus venosus myocardium will be described in the knockout embryos and compared to wildtype embryos, in subsequent stages of heart development.

**Podoplanin expression in the heart**

In wildtype mice the first expression of podoplanin can be recognized as early as 9.5 dpc in the coelomic mesothelium and in the proepicardial organ. Moreover, podoplanin was observed in the myocardium of the medial wall of the left cardinal vein shortly before entering the sinus venosus. At 10.5 dpc podoplanin staining in this region was more extensive and extended into the dorsal mesocardium. At the right side the staining is evaluated for the first time at the level of the future sinoatrial node, in the medial wall of the right cardinal vein. The venous valves showed also podoplanin positivity. At 12.5 dpc podoplanin was clearly observed in the pericardium, epicardium, sinoatrial node, venous valves, dorsal mesocardium, myocardium of the pulmonary vein, atrial septum and ventricular conduction system. Remarkably, podoplanin staining was also observed at the left side bordering the medial contour of the left cardinal vein. The pattern and intensity of this region was similar to the sinoatrial node region at the right side, although the left-sided region was smaller at this stage.

**Sites of epithelial-to-mesenchymal transformation**

To demarcate the coelomic mesothelium and sites of active EMT, we used WT-1 staining as a coelomic mesothelial marker, E-cadherin staining as a cell-to-cell adhesion marker and RhoA playing a crucial role in EMT at specific sites of the venous pole. WT-1 was observed in both the coelomic mesothelium and proepicardial organ at 9.5 dpc in the wildtype. In the mutants, compared to the wildtype embryos, these regions were smaller and E-cadherin was upregulated. At 10.5 dpc in wildtype embryos WT-1 positivity was found in the single layer of mesothelium of the coelomic cavity and epicardium (Figure 1a and b). Marked staining was seen at the
corners of the coelomic cavity at both sides adjacent to the cardinal veins, where the expression of WT-1 was more extensive and the cells of the coelomic mesothelium appeared to be cuboidal and well organized (Figure 1b). In the *podoplanin* knockout embryos WT-1 was also present in these regions (Figure 1e and f), however, the coelomic mesothelium was disorganized, the cells were irregular in shape and size and seemed to have maintained their epithelial confinement (Figure 1, compare a and b with e and f). The defective spreading of the epicardium at several locations was shown after WT-1 staining that followed this pattern and did not normally cover the myocardium in the knockout mouse embryos (Figure 1a and e). Increased E-cadherin staining has been observed clearly in both the epicardium and mesothelium lining the coelomic wall in the *podoplanin* knockout embryos (Figure 1g) compared to the wildtype mouse embryos (Figure 1c). Remarkably, in the mutants E-cadherin staining was not only upregulated in the epithelium of the coelomic cavity but also in the underlying mesenchymal cells (Figure 1c and g), supporting the observation of disturbed EMT. At 10.5 dpc in wildtype embryos major RhoA expression was seen in the mesenchyme and epithelium of the coelomic cavity and epicardium (Figure 1d). In the mutants overall expression of RhoA was downregulated (Figure 1h).

**The sinus venosus myocardium**

To evaluate the extent of sinus venosus myocardium we studied the expression of HCN4 in the wildtype and *podoplanin* knockout embryos between 9.5-14.5 dpc. The HCN4 positive region of the sinus venosus area (Figure 2i, k and Figure 3d, f, m, o) overlaps the Nkx2.5 negative (Figure 2g, j and Figure 3h-k) and MLC-2a positive sinus venosus region (Figure 2c-f and Figure 3c, e, l, n). Podoplanin is also expressed in these regions of the sinus venosus myocardium, almost completely overlapping with the Nkx2.5 negative and HCN4 positive regions.
**Sinoatrial node and venous valves**

In the sinoatrial node the MLC-2a positive (Figure 2c-f) and Nkx2.5 negative (Figure 2g, j) region were identical to the HCN4 (Figure 2i, k) and podoplanin (Figure 2h) positive areas. In contrast to the remaining sinus venosus myocardial structures, in the sinoatrial node HCN4 remained positive while Nkx2.5 negative staining was maintained. In the knockout mice the sinoatrial node was hypoplastic (Figure 2a, b, d, f) and the venous valves were shorter and thinner (Figure 2c, e), however, the expression pattern of MLC2a, Nkx2.5 HCN4 and Cx43 (not shown), was not changed compared to the wildtype (Figure 2c-k). Comparable to 10.5 dpc in wildtype embryos, RhoA expression was seen in coelomic mesenchyme and epithelium as well as in sinoatrial node and epicardium (Figure 2l, m). In the mutants, RhoA expression was downregulated in sinoatrial node as well as in coelomic epithelium and epicardium (Figure 2n, o).

**Primary atrial septum and dorsal atrial wall**

In both wildtype and *podoplanin* knockout embryos MLC-2a expression was present in the sinus venosus myocardium and the myocardium of the atrial and ventricular wall (Figure 2a-f and Figure 3a-c, e, l, n). Similar to the sinoatrial node and venous valves, the expression pattern of the mentioned markers was unchanged in the mutants compared to the wildtype embryos. In the mutants, myocardial hypoplasia was seen of the dorsal atrial wall (Figure 2c-f). The atrial septum was thin (Figure 3c-f) and deficient (Figure 2c, e) with a large secondary foramen (Figure 2c, e). The myocardialization process at the base of the atrial septum was almost absent (Figure 2c, e). Additionally in the knockout embryos, the atrioventricular cushion was not fused properly to the top of the ventricular septum resulting into a persisting interventricular communication (Figure 2c, e). There is marked dilatation of the atria in the mutant embryos compared to the wildtype (Figure 2c, e).

---

**Figure 3. Cranio-dorsal view of 3D reconstructions (a and b) and transverse sections (c-o) showing the myocardial hypoplasia of the common pulmonary vein (PV), atrial septum (asterisk) and the wall of the cardinal veins of 12.5 dpc wildtype (WT) and *podoplanin* knockout (*podoplanin*−/−) embryonic mouse hearts.** The intersection lines c, l, e and n refer to the sections (c), (l), (e) and (n) respectively. In the MLC-2a sections (c, e) the hypoplasia of the myocardium around the PV in the *podoplanin*−/− is clearly visible. Moreover, parts of myocardium around the PV, which are also positive for HCN4 (d) and *podoplanin* (g) are missing in the *podoplanin*−/− (compare c, d with e, f). This lack of myocardium around the PV is also seen in the sections stained with Nkx2.5 (h-k) where the Nkx2.5 mosaic myocardium of the PV is missing in the *podoplanin*−/− (k, j) compared with the WT (h, i). The hypoplasia of the atrial septum (asterisk) is seen in the *podoplanin*−/− (c) compared to the WT (c). Sections (l) and (n) are stained with MLC-2a and sections (m) and (o) show HCN4 expression of the same region demonstrating the hypoplasia and the perforations (arrows in n) of the myocardium of the cardinal veins in the *podoplanin*−/−. LA indicates left atrium; LCV, left cardinal vein; LV, left ventricle; RA, right atrium; RCV, right cardinal vein; RV, right ventricle. In the 3D reconstructions: atrial myocardium, light brown; cardiac veins lumen, transparent blue; PV lumen, pink; sinus venosus myocardium, purple; ventricular myocardium, brown. Scale bars: (c), (e), (l), (n) = 60μm; (d), (f-h), (k) = 30μm; (i), (j) = 20μm; (m), (o) = 200μm.
Pulmonary and cardinal veins

In wildtype embryos the wall of the pulmonary and cardinal veins showed MLC-2a (Figure 3c, l) and HCN4 (Figure 3a, d, m) expression while the Nkx2.5 expression pattern was mosaic in the myocardium lining the pulmonary vein (Figure 3h, i) and negative in the wall of the cardinal veins (Figure 3h). The staining for HCN4 was diminished at 15.5 dpc compared to earlier stages whereas the initially Nkx2.5 negative myocardium of the cardinal veins and Nkx2.5 mosaic myocardium of the pulmonary vein wall became positive.

In the knockout embryos, myocardium of the pulmonary vein wall was hypoplastic and almost absent compared to the wildtype (Figure 3c, e). The extent of hypoplasia and lack of myocardium of the pulmonary vein corresponded with the regions normally expressing HCN4.

Figure 4. Sinus venosus (SV, a) and sinoatrial node (SAN, b) myocardial volume estimation of 12 wildtype (WT) mouse hearts of 11.5 dpc (n=3), 12.5 dpc (n=3), 13.5 dpc (n=3) and 14.5 dpc (n=3) and 12 podoplanin knockout (KO) mouse hearts 11.5 dpc (n=3), 12.5 dpc (n=3), 13.5 dpc (n=3) and 14.5 dpc (n=3). Podoplanin knockout embryos have a significant smaller SV and SAN myocardial volume (*P<0.05) compared to WT embryos.
Podoplanin (Figure 3d, f) and podoplanin (Figure 3g). Comparable to the MLC-2a stained myocardium around the pulmonary vein as described above, the Nkx2.5 mosaic area was hypoplastic and almost absent in the knockout mice (compare Figure 3h, i with j, k).

The myocardium of the cardinal veins was also hypoplastic and showed several fenestrations (Figure 3l-o). Both the atrial lumen and the lumen of the cardinal veins were dilated. The sinus venosus (Figure 4a) and separately the sinoatrial node (Figure 4b) myocardial volume, which was estimated by myocardial morphometry, showed a significant ($P<0.05$) decrease of myocardial volume in the knockout embryos compared to the wildtype embryos.

**DISCUSSION**

This study was conducted to elucidate the role of *podoplanin* in the development of the sinus venosus myocardium derived from the specific area of the second heart field which we have named PHF.\(^{13,22}\) Previous studies have shown that mesodermal progenitor cells from the second heart field contribute to the formation and addition of myocardium both at the arterial and the venous pole of the developing heart.\(^{10,13,14,40,41}\) Our observations are based on the study of the *podoplanin* gene and its protein expression in different embryonic stages during cardiac development. We have generated *podoplanin* knockout mouse embryos and used several immunohistochemical markers to study the mutants and wildtypes. We observed that the mutant embryos present severe cardiac malformations and show hypoplasia of the sinus venosus myocardium. These findings have consequences for the development and contribution of the PHF to the sinus venosus myocardium.

**Sinoatrial node and venous valves**

The sinoatrial node is a complex structure that plays a fundamental role in cardiac pacemaker activity.\(^{28}\) Despite its essential role in cardiac conduction the origin of the sinoatrial node is still not well understood. Recently the formation and differentiation of the sinoatrial node at the venous pole of the heart has been described from the novel Nkx2.5 negative and Tbx18 positive precursor cells,\(^{15}\) which were positive for podoplanin.\(^{13}\) In the current study we report HCN4 expression in the podoplanin positive and Nkx2.5 negative sinoatrial node in accordance with observations in other studies.\(^{15,30,42}\) The specific combination of Nkx2.5 negative and podoplanin and HCN4 positive expression in the sinoatrial node during early heart development is in contrast with the expression of these markers in the primary atrial myocardium. The latter suggests a different precursor for the sinoatrial node and the primary atrial myocardium. In contrast to the primary atrial myocardium, which derives from the primary heart field, the sinus venosus myocardium including the sinoatrial node originates from the second heart field,
as concluded from Isl1, Tbx3 and Tbx18 expression at the venous pole of the heart.\textsuperscript{14,15,30,43} To clarify the functional role of \textit{podoplanin} in the development of the sinus venosus myocardium we have studied \textit{podoplanin} mutants and found a hypoplastic sinus venosus myocardium including the sinoatrial node and the venous valves. Another interesting gene involved in the development of the sinus venosus myocardium is \textit{Shox2}.\textsuperscript{21} \textit{Shox2} mutants showed severe hypoplasia of the sinus venosus myocardium comparable to our observations in the \textit{podoplanin} null mice. Moreover, the hypoplastic sinoatrial node in \textit{Shox2} mutants showed aberrant expression of Cx43 combined with abnormal Nkx2.5 positivity. In contrast to the \textit{Shox2} mutants, Nkx2.5 and Cx43 expression patterns remained unchanged in the \textit{podoplanin} knockout mouse hearts, suggesting a different role for \textit{podoplanin} in sinoatrial node / pacemaking development than \textit{Shox2}. Electrophysiological experiments will be carried out to investigate possible arrhythmias in these mutants to solve the mentioned neonatal death.

\textbf{Primary atrial septum and dorsal atrial wall}

Lineage tracing experiments studying \textit{Isl1},\textsuperscript{14} \textit{Fgf10}\textsuperscript{10} and \textit{Tbx5},\textsuperscript{44,45} \textit{Tbx18}\textsuperscript{15} and \textit{Shox2}\textsuperscript{21} have demonstrated the contribution of the second heart field at the venous pole to the formation of the atrial myocardium, which has a distinct molecular composition compared to the heart tube derived from the primary heart field.\textsuperscript{10,14,46}

\textit{Podoplanin} is expressed in PHF as well and not only stains the proepicardial organ derivatives but also the Nkx2.5 negative myocardium in the dorsal mesocardium.\textsuperscript{13} This myocardium is supposed to form part of the dorsal atrial wall as well as the atrial septum. In the \textit{podoplanin} mutant mouse this myocardium is hypoplastic, probably due to diminished PHF-derived myocardial contribution. Another option, is that abnormal epicardial-myocardial interaction plays a role in development of deficient myocardium as is seen in \textit{SP3} mutant mouse.\textsuperscript{47} We already described the deficient EPDC contribution in the \textit{podoplanin} mutant.\textsuperscript{22,23} Therefore, both the hypoplasia of the atrial septum as well as the dorsal atrial wall observed in the current study, are related to the altered contribution of myocardial and epicardial cells from the PHF.

\textbf{Pulmonary and cardinal veins}

A controversy regarding the development of the venous pole concerns the origin of the pulmonary vein. The pulmonary pit develops either in the dorsal mesocardium at the midline,\textsuperscript{48-50} at the left\textsuperscript{51} or the right\textsuperscript{51-53} side of the embryo as a solitary unpaired structure that arises from the sinus venosus\textsuperscript{51-53} or primitive atrium.\textsuperscript{46,48,54} Recently, Männer and Merkel\textsuperscript{55} have described the pulmonary pit as a bilaterally paired structure. We have described that the early common pulmonary vein is surrounded by Nkx2.5 mosaic cells, which are positive for MLC-2a and podoplanin.\textsuperscript{13} Part of this myocardium has also been reported as mediastinal
myocardium. The Nkx2.5 mosaic area forms a myocardial sleeve around the pulmonary vein extending to the atrial septum and Nkx2.5 negative cardinal veins in contrast to the primary atrial myocardium, which is completely Nkx2.5 positive. These data support the formation of the wall of the pulmonary vein to be from the surrounding mesodermal precursor cells at the dorsal mesocardium, which is postulated to derive from the PHF. Concomitant with the higher proliferation rate of the pulmonary vein myocardium, the Nkx2.5 mosaic area became Nkx2.5 positive in contrast to the sinoatrial node and cardinal vein myocardium that remained Nkx2.5 negative but HCN4 positive. This suggests a distinct differentiation rate of the pulmonary vein myocardium compared to the sinoatrial node and cardinal vein myocardium. At 15.5 dpc the cardinal vein myocardium became gradually positive for Nkx2.5, whereas the HCN4 expression was diminished suggesting the gradual completion of the differentiation process at the venous pole.

In the mutant embryos, the diminished myocardial contribution to the wall of the pulmonary vein and cardinal veins is evident. It is not clear whether MLC-2a and Nkx2.5 are directly regulated by podoplanin or whether this is due to altered addition of secondary myocardium from the PHF region by lack of podoplanin.

The role of podoplanin in EMT of the coelomic epithelium

EMT of the coelomic epithelium plays an important role in the addition of cells to the developing heart. An important feature of EMT is downregulation of cell-to-cell adhesion molecule E-cadherin, which is correlated with podoplanin. We have observed upregulation of E-cadherin in the coelomic cavity epithelium of the podoplanin knockout embryos. Regarding the addition of myocardium from the second heart field, the observed hypoplasia at the sinus venosus region might be caused by upregulated E-cadherin in the podoplanin knockout embryos, which causes abnormal EMT of the coelomic epithelium at specific sites of the sinus venosus myocardium.

Moreover, podoplanin is involved in motility of cells where it colocalizes with the ezrin, radixin and moesin (ERM) protein family. ERM proteins bind to the podoplanin ERM-binding site to activate RhoA, a member of the Rho GTPase protein family controlling a wide variety of cellular processes including proliferation, differentiation, cell morphology and motility. The increased RhoA activity leads to ‘podoplanin-induced’ EMT. With regard to this mechanism, lack of podoplanin has resulted in diminished expression of RhoA protein, which may prevent the ‘podoplanin-induced’ EMT with subsequent myocardial abnormalities of the venous pole.

Taken together, we show severe hypoplasia and myocardial abnormalities of the sinus venosus myocardium by lack of podoplanin. In addition, we postulate not only a common origin of
the sinoatrial node, dorsal atrial wall, atrial septum, pulmonary and cardinal veins deriving from the PHF, but also provide a link between the pulmonary vein and sinus venosus myocardium rather than the pulmonary vein and primary atrial myocardium.

**Clinical implications**

Parts of the cardiac conduction system derive from the second heart field, which may imply a role in the etiology of clinical syndromes. Several transgenic mice present with sick sinus syndrome, occurring in a familial form, are due to the lack of Ca2⁺ and other ion channels as well as gap junctions. The dysfunctions include bradycardia, sinus dysrhythmia and sinus node exit block. In our mutant embryos we have observed a hypoplastic sinoatrial node. While podoplanin is involved in water transport, cationic, anionic and amino acid transport and Ca2⁺ dependent cell adhesiveness, it is relevant to perform functional studies in these hearts in future to show dysfunctions such as bradycardia (sick sinus syndrome) comparable to Shox2 mutants.

Several studies suggested an embryonic background of atrial fibrillation originating from the pulmonary and caval veins, based on expression patterns of molecular and immunohistochemical markers. In the current study we observed HCN4 expression in the sinoatrial node and in the myocardium of the wall of the cardinal veins and the pulmonary vein. In the mutant mice this population of HCN4 positive cells is diminished in the sinus venosus myocardium, which may provide a developmental background of arrhythmias originating from this area. Next to disturbances in cardiac conduction the observed deficient myocardial as well as epicardial contribution results in atrial and ventricular septal defects in addition to the already observed myocardial and coronary vascular abnormalities.

**ACKNOWLEDGMENTS**

We thank Jan Lens for expert technical assistance with the figures.

**REFERENCES**


