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The role of the second heart field in pulmonary vein development : new insights in the origin of clinical abnormalities

Douglas, Y.L.

Citation

Douglas, Y. L. (2010, October 20). *The role of the second heart field in pulmonary vein development : new insights in the origin of clinical abnormalities*. Retrieved from <https://hdl.handle.net/1887/16065>

Version: Corrected Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).

Yvonne L. Douglas^{1,2,#}, Edris A.F. Mahtab^{2,#}, Monique R.M. Jongbloed², Pavel Uhrin³, Jan Zaujec³, Bernd R. Binder³, Martin J. Schalij⁴, Robert E. Poelmann², Marco C. DeRuiter², Adriana C. Gittenberger-de Groot².

Both authors contributed equally

¹Department of Cardio-thoracic Surgery, University Medical Center Groningen, Groningen, 9700 RB, The Netherlands. ²Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, 2300 RC, The Netherlands. ³Department of Vascular Biology and Thrombosis Research, University of Vienna, Vienna, A-1235, Austria. ⁴Department of Cardiology, Leiden University Medical Center, Leiden, 2300 RC, The Netherlands.

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**Pulmonary Vein, Dorsal Atrial Wall
and Atrial Septum Abnormalities
in *Podoplanin* Knockout Mice with
Disturbed Posterior Heart Field
Contribution**

Pediatric Research 2009;65:27-32

Abstract

The developing sinus venosus myocardium, derived from the posterior heart field, contributes to the atrial septum, the posterior atrial wall, the sino-atrial node and myocardium lining the pulmonary and cardinal veins, all expressing podoplanin, a coelomic and myocardial marker. We compared development and differentiation of the myocardium and vascular wall of the pulmonary veins, left atrial dorsal wall and atrial septum in wild type with *podoplanin* knockout mouse embryos (E10.5-E18.5) by 3-D reconstruction and immunohistochemistry. Expression of Nkx2.5 in the pulmonary venous myocardium changes from mosaic to positive during development pointing out a high proliferative rate compared to Nkx2.5 negative myocardium of the sino-atrial node and cardinal veins. In mutants, myocardium of the pulmonary veins, dorsal atrial wall and atrial septum was hypoplastic. The atrial septum and right sided wall of the pulmonary vein almost lacked interposed mesenchyme. Extension of smooth muscle cells into the left atrial body was diminished. We conclude that myocardium of the pulmonary veins, dorsal atrial wall and atrial septum as well as the smooth muscle cells are derived from the posterior heart field regulated by *podoplanin*.

Introduction

The developmental origin and molecular mechanisms controlling the formation of the myocardium of the pulmonary veins (PV) and the smooth muscle cells (SMCs) of the PV media are a matter of debate. The PV myocardial sleeve develops by either migration of myocardial cells from the left atrium (LA)¹ or by recruitment of extracardiac mesenchymal cells which differentiate into myocardial cells². Based on expression patterns of *Nkx2.5*, *Islet1* and *Pitx2c* the LA and PV myocardium was suggested to be formed by the addition of myocardial cells from the second heart field at the venous pole³.

The second heart field, part of the splanchnic mesoderm, is involved in the addition of (myocardial) cells to the primary heart tube at both the arterial (the *secondary* and *anterior* heart field) and the venous pole (the *posterior* heart field)⁴. *Islet1*, a marker of undifferentiated cardiac progenitor cells, is expressed throughout the second heart field⁵. Myocardial cells can differentiate from second heart field derived mesenchymal cells, and SMCs may also differentiate from mesenchyme⁶.

The transmembrane glycoprotein podoplanin, a coelomic and myocardial marker expressed in the posterior heart field⁷, has gained interest for its role in epithelial-to-mesenchymal transformation (EMT) and in formation of the myocardium and coronary SMCs at the venous pole of the heart⁸. It is found in the proepicardial organ, epicardium, sinus venosus myocardium including the sino-atrial node, the PVs and cardinal veins, the dorsal atrial wall and the base of the atrial septum extending into the developing atrial and ventricular cardiac conduction system. *Podoplanin* promotes EMT by binding ezrin, radixin, moesin (ERM) proteins that activate RhoA, and by downregulation of the cell-to-cell adhesion molecule E-cadherin⁹. Lack of *podoplanin* leads to altered EMT and e.g., to abnormal formation of sinus venosus myocardium and decreased numbers of SMCs of the coronary artery media⁸. In extra cardiac tissues *podoplanin* functions as a possible signalling molecule and is involved in the development of osteoblasts¹⁰ intestinal and alveolar epithelium¹¹ podocytes and mesothelium of the visceral peritoneum¹² and lymphatic endothelium¹³. During cardiac development, podoplanin is expressed specifically in the second heart field derived myocardium and mesenchyme at the venous pole of the heart^{7,8}.

Our previous study in humans¹⁴ demonstrated that during incorporation of the PVs in the LA body, the inner lining of the LA body presents vessel wall tissue. The outer layer of the LA is formed by myocardium covered by epicardium. The origin of the SMCs of the LA dorsal wall is still a topic of discussion.

In the present study, we have compared the morphology and development of the wall of the PVs, LA dorsal wall and atrial septum between wild type and *podoplanin* knockout mouse embryos of embryonic stages (E) 10.5-18.5. We hypothesize that the posterior heart field is involved in the formation and differentiation of both myocardial cells and SMCs of the PVs and LA dorsal wall regulated by *podoplanin*.

Material and methods

Generation of the embryonic and neonatal mice

This study was performed at the department of Anatomy & Embryology of the Leiden University Medical Center and was approved by the Animal Research Committee. *Podoplanin* knockout mice were generated by homologous recombination in embryonic stem cells from the 129S/v mouse line as reported previously⁸.

General description

We investigated the morphology and development of the sinus venosus region of the heart, especially the PVs, LA dorsal wall and atrial septum in 32 wild type mouse embryos of E10.5 (n=4), E11.5 (n=3), E12.5 (n=4), E13.5 (n=5), E14.5 (n=4), E15.5 (n=3), E16.5 (n=3), E17.5 (n=3) and E18.5 (n=3), and compared these with 28 *podoplanin* knockout mouse embryos of E10.5 (n=4), E11.5 (n=5), E12.5 (n=5), E13.5 (n=3), E14.5 (n=4), E15.5 (n=4) and E18.5 (n=3).

Immunohistochemistry

Immunohistochemistry was performed with antibodies against alpha-smooth-muscle actin (1A4, 1:2000, Sigma Aldrich, Product No.A2547, USA) to detect differentiated SMCs and developing myocardium, atrial myosin light chain 2 (MLC2a, 1/6000, kindly provided by S.W. Kubalak, Charleston, SC, USA) specific for (atrial) myocardium, NK2

transcription factor related locus 5 (Nkx2.5, 1/4000, Santa Cruz Biotechnology, Inc., CA, USA, SC-8697) as an early marker of undifferentiated cardiac progenitor cells, and podoplanin (clone 8.1.1., 1/500, Hybridomabank, Iowa, USA) as a marker of the posterior heart field myocardium. Fixation, preparation and staining procedures were completed according to standard protocols⁸.

3-D reconstructions

We made 3-D reconstructions, as described previously⁸, of the composition of PV, LA dorsal wall and atrial septum of MLC-2a and 1A4 stained sections of *podoplanin* wild type and knockout embryos (E15.5).

Morphometry

Sinus venosus myocardial volume estimation was performed of 12 wildtype mouse hearts of E11.5 (n=3), E12.5 (n=3), E15.5 (n=3) and E18.5 (n=3) and 12 *podoplanin* knockout hearts of E11.5 (n=3), E12.5 (n=3), E15.5 (n=4) and E18.5 (n=2). Statistical analysis was performed with an independent sample-*t*-test ($P < 0.05$) using SPSS 11.0 software (SPSS Inc, Chicago, Ill) as described previously⁸.

Results

Podoplanin mutants demonstrate marked mesenchymal and myocardial abnormalities, including hypoplasia of the pro-epicardial organ, abnormal epicardium, hypoplastic chamber myocardium, atrial, ventricular and atrioventricular septal defects, and an abnormal coronary arterial vascular wall^{7,8}. These abnormalities are related to diminished EMT which was reported in a previous paper⁸. Below, the morphological development of the wall of the PV, LA and atrial septum will be described in subsequent stages. Abnormal PV connections were not observed. In Table 1a sinus venosus myocardial volumes between wild type and knockout mice are compared. Table 1b provides an overview of qualitative expression of myocardial and SMCs markers used.

Myocardial Morphometry

a

E	WT		<i>Podoplanin</i> ^{-/-}		t-test p value
	Myocardial volume (mm ³)	SD	Myocardial volume (mm ³)	SD	
11.5	0.0094 (n=3)	0.0029	0.0050 (n=3)	0.0017	0.0457
12.5	0.0120 (n=3)	0.0025	0.0071 (n=3)	0.0032	0.0348
15.5	0.0424 (n=3)	0.0052	0.0300 (n=4)	0.0047	0.0108
18.5	0.2871 (n=3)	0.0307	0.1688 (n=2)	0.0287	0.0115

Expression of Markers

b

E	Markers	PV wall		LA dorsal wall		Atrial septum		Media PV	
		WT	Mutant	WT	Mutant	WT	Mutant	WT	Mutant
10.5	MLC2a	++	+	++	+	Not Developed			
	Nkx2.5	++	+	++	+	Developed			
	Actin	++	+	++	+	Yet			
12.5	MLC2a	++	+	+++	++	+++	++	++	++
	Nkx2.5	++	+	++	++	++	++	++	++
	Actin	+	+	+	+	+	+	-	-
15.5	MLC2a	+++	+++	+++	+++	+++	+++	+	+
	Nkx2.5	+++	+++	+++	+++	+++	+++	+	+
	Actin	-	-	-	-	-	-	++	+
18.5	MLC2a	+++	+++	+++	+++	+++	+++	-	-
	Nkx2.5	+++	+++	+++	+++	+++	+++	-	-
	Actin	-	-	-	-	-	-	+++	+

Table 1. a: Sinus venosus myocardial volume estimation of 12 wild type (WT) and 12 *podoplanin*^{-/-} mouse hearts. In all stages, mutants have a significant smaller myocardial volume (P<0.05) compared to WT embryos. E: embryonic day, SD: standard deviation. b: A qualitative overview of the expression of markers at several locations in *podoplanin* WT and knockout (mutant) embryos. At early stages, the expression of all markers is diminished in mutants compared to the WT embryos, whereas in advanced stages these differences are no longer apparent, indicating delayed differentiation in mutants. Left atrium (LA); pulmonary vein (PV). +++strong, ++medium, +weak, -absent expression.

E10.5

In the right part of the common atrium, the right and left venous valves delineated the ostia of the cardinal veins entering the sinus venosus. In both wild type and knockout

embryos, MLC2a was expressed in the sinus venosus and chamber myocardium (Fig.1a,c-e). In the mesenchyme of the dorsal mesocardium in wild type embryos, the lumen of the primitive PV was surrounded by a mainly left sided concentration of MLC2a positive myocardium that stained, however, less markedly for MLC2a compared to the common atrium (Fig.1a,d). In knockouts, this myocardium was thinner with clearly diminished MLC2a expression in a larger part in the dorsal mesocardium and left part of the atrial dorsal wall (compare Fig.1d with e). This diminished expression was seen in the region normally expressing podoplanin (Fig.1b, Table 1b). In wild types the primitive PV myocardium in the region of the dorsal mesocardium had mosaic Nkx2.5 expression, defined as a mixture of Nkx2.5 positive and negative cells. Similar to MLC2a expression, the Nkx2.5 mosaic myocardial cells were mainly situated on the left side of the dorsal mesocardium (Fig.1f). Moreover, in knockouts, this area was hypoplastic showing less Nkx2.5 expressing cells (compare Fig.1f with g).

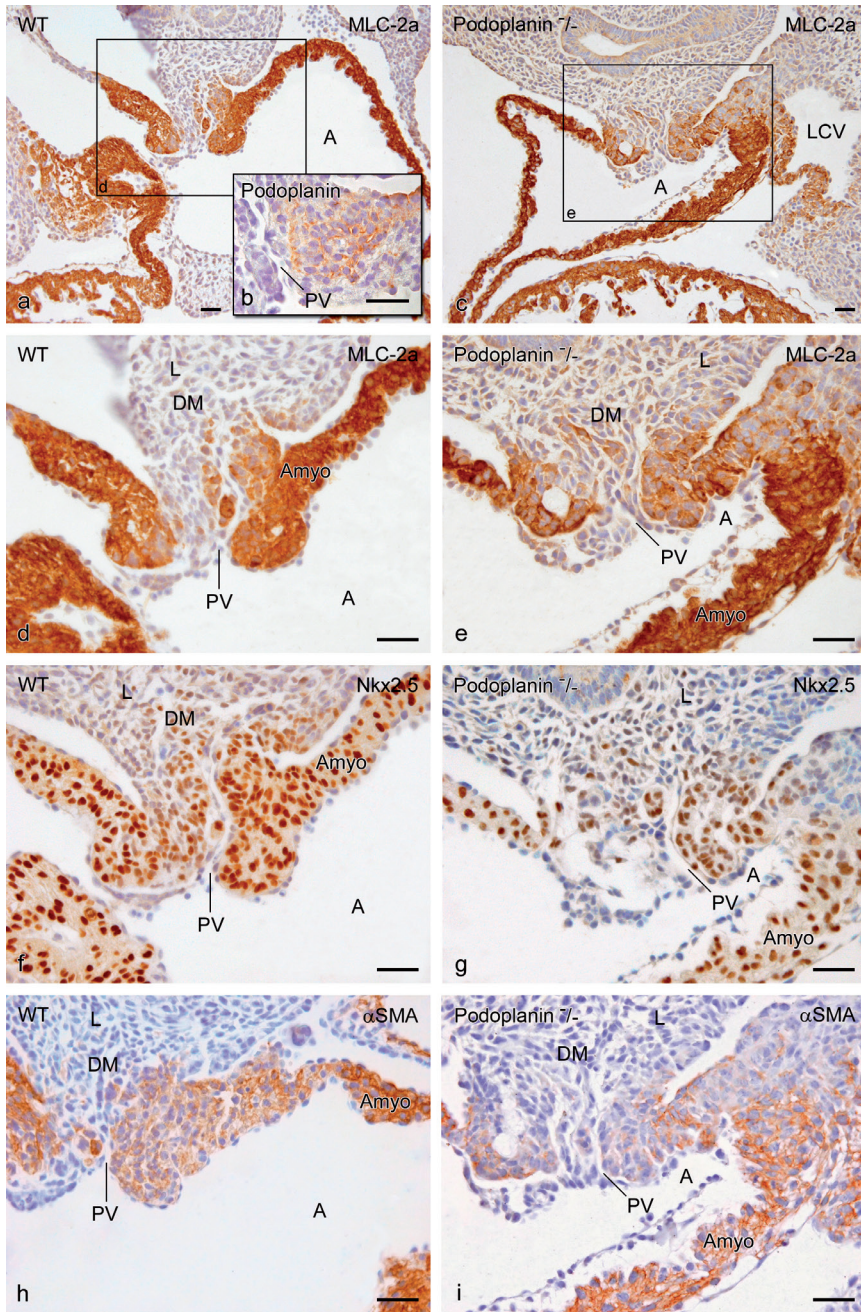
The alpha-smooth-muscle actin antibody 1A4 was co-expressed in the MLC2a-stained myocardium of the common atrium and ventricle of the wild type embryos. Similar to MLC2a, actin expression around the primitive PV and left part of the atrial dorsal wall was weaker (Fig.1h). In mutants, actin expression was diminished in a larger region compared to the wild type (Fig.1i).

E12.5

The common PV bifurcated into tiny left and right PVs, covered by a MLC2a positive myocardial layer which, in contrast to E10.5, could now be observed in equal density on both sides of the PV (Fig.2a,c). MLC2a expression around the PV was weaker than the expression in the myocardium of the atria (Fig.2a,c). In the dorsal mesocardium and around the PV Nkx2.5 expression was mosaic, while in the wall of the cardinal veins Nkx2.5 staining was negative (Fig.2d). In the atrial myocardium of the wild type embryos MLC2a as well as Nkx2.5 were positive, while smooth muscle actin had almost disappeared, particularly in the LA dorsal wall (Fig.2e). In the myocardium of the ventricles (not shown) and the wall of the cardinal veins (Fig.2e) smooth muscle actin was still present. Actin expression in the wall of the PV was more extensive than the MLC2a expression (Fig.2e).

In the *podoplanin* knockout embryos, MLC2a (Fig.2f,g) and actin positive (Fig.2i) as well as Nkx2.5 mosaic (Fig.2h) myocardium around the PV was thin and locally even absent (compare Fig.2a,c with f,g) in a region where podoplanin expression was seen in wild type embryos (Fig.2b). The LA dorsal wall and the atrial septum were thin and the myocardium was hypoplastic (compare Fig.2j,k with l,m). The atrial septum showed a large secondary foramen and myocardialization at the base of the atrial septum was absent (Fig.2k,m). Additionally, the atrioventricular cushion was not fused properly to the top of the ventricular septum resulting in a interventricular communication (Fig.2j,l).

Figure 1. Transverse sections of *podoplanin* wild type (WT,a,b,d,f,h) and knockout (Podoplanin^{-/-},c,e,g,i) mouse embryos of stage (E)10.5 comparing the development of the pulmonary vein (PV). Boxed area in a and c are magnified in d and e. MLC2a expression is seen around the primitive PV on the left side of the dorsal mesocardium (DM) (a,d) where also podoplanin (b) and mosaic Nkx2.5 expression (f) is seen. Compared to the atrial myocardium (Amyo) the MLC2a expression at the DM and atrial (A) dorsal wall is weaker corresponding with the expression pattern of alpha-smooth-muscle actin (α SMA, h), which marks the developing myocardium. In knockouts, a larger region shows less expression of MLC2a (c,e), Nkx2.5 (g) and α SMA (i), corresponding with altered differentiation rate of myocardium at these regions. Left cardinal vein (LCV), Lung (L). Scale bars 30 μ m.



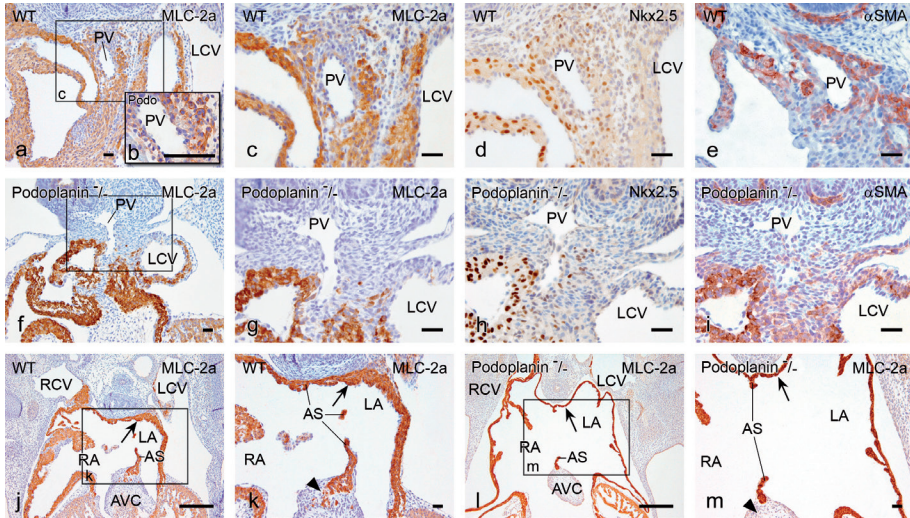


Figure 2. Transverse sections of *podoplanin* wild type (WT, a-e, j, k) and knockout (Podoplanin^{-/-}, f-i, l, m) mouse embryos of stage (E)12.5 comparing the development of the pulmonary vein (PV, a-i), atrial septum (AS, j-m) and left atrial (LA) dorsal wall (j-m). In WT embryos PV is surrounded by myocardium expressing MLC2a (a, c), podoplanin (b) and mosaic Nkx2.5 (d) and completely positive for alpha-smooth-muscle actin (α SMA, e). In mutants the expression of these markers around the PV is almost absent (compare a, c-e with f-i) and myocardium of the LA dorsal wall is hypoplastic (compare arrow in j, k with l, m). The AS in the knockouts is thin and deficient with a large secondary foramen (k, m). Moreover, myocardialization process of the AS with the deficient atrioventricular cushion (AVC, compare j with l) is absent (see arrowhead in k and m), which might cause an atrioventricular septal defect. Boxed area in a, f, j and l are magnified in c, g, k and m. Left cardinal vein (LCV), Right atrium (RA), Right cardinal vein (RCV). Scale bars a-i, k, m 30 μ m, j, l 200 μ m.

E15.5

In both wild types and knockouts, the myocardium of the atria, the atrial septum and of the wall of the cardinal and PVs expressed MLC2a (Fig.3a-f). In mutants, the myocardium of the atrial septum and the PVs was hypoplastic (compare Fig.3c, d with e, f). Moreover, compared to wild type, the PVs in knockout embryos seemed to be dilated (compare Fig.3c with e). In both wild type and knockout embryos, the Nkx2.5 mosaic expression in the wall of the PV became overall positive (Fig.3g, i).

In wild type embryos 1A4 co-staining was specifically observed in the MLC2a positive sub-endothelial layer of the PVs and LA dorsal wall (Fig.3h). In the outer MLC2a positive myocardial layer of the wall of the PVs, cardinal veins, dorsal atrial wall and the entire atrial myocardium (not shown), 1A4 staining had disappeared (Fig.3h). In

knockout embryos, smooth muscle actin was almost absent in the sub-endothelial layer of the PVs and LA dorsal wall, which still showed MLC2a staining (compare Fig.3a,h with b,j). In areas with 1A4 expression, distribution of positive cells was discontinuous compared to the wild type embryos (compare Fig.3h with j).

E18.5

The common part of the PVs was incorporated into the LA. In both wild type and knockout embryos MLC2a expressing myocardium was seen in the atria and the wall of the cardinal and PVs, while the myocardium now extended into the lungs (Fig.4a,b). Similar to previous stages, the myocardium was hypoplastic in knockouts, with dilation of the atria, the cardinal and PVs (compare Fig.4a,b with c,d). In the dorsal mesocardium less mesenchymal cells were observed compared to the wild type (compare asterisk in Fig.4 a with b).

In the PV and LA dorsal wall of the wild type embryos, smooth muscle actin (1A4) was present between endothelium and MLC2a positive myocardium (Fig.4e). Hence, the 1A4 positive actin layer was MLC2a negative in contrast to the previous stages (compare Fig.3d,h with Fig.4c,e). The 1A4 staining at other parts of the heart was absent (not shown), except for the wall of the cardinal veins and ventricular myocardium. Again, hardly any 1A4 staining was seen in the sub-endothelial layer of the PV and LA dorsal wall of the *podoplanin* knockout embryos (compare Fig.4e with f).

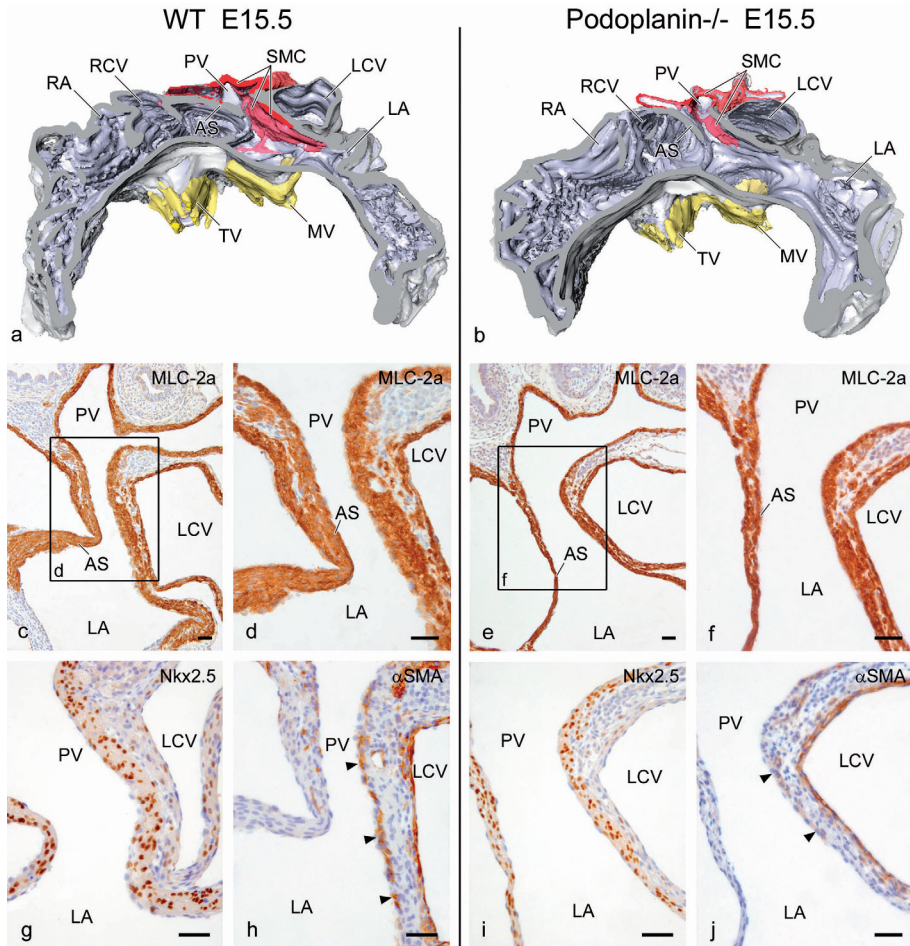


Figure 3. 3-D reconstructions (a-b) and transverse sections (c-j) of *podoplanin* wild type (WT, a, c, d, g, h) and knockout (*Podoplanin*^{-/-}, b, e, f, i, j) mouse embryos of stage (E)15.5 showing hypoplasia of atrial septum (AS) and altered smooth muscle cells (SMC, red) formation in the wall of the common pulmonary vein (PV) and the posterior wall of left atrium (LA) of the mutants. a and b are a cranial view of the PV orifice into the LA showing the diminished amount of SMCs in the PV and LA dorsal wall in knockouts. Boxes in c and e are the positions of the enlargements in d and f. Compared to WT embryos, the MLC2a positive myocardium of the AS, the left cardinal vein (LCV) and the somewhat dilated PVs in knockouts is hypoplastic with interposition of less mesenchymal tissue (compare c, d with e, f). The myocardium of the wall of the PV is differentiated and is seen as a myocardial cuff around the PV, which expresses Nkx2.5 (g, i). In WT embryos SMCs (arrowheads in h) are incorporated into the LA, whereas in knockouts SMCs are almost absent (arrowheads in j). Mitral valve (MV, yellow), Right atrium lumen (RA, light gray), Right cardinal vein lumen (RCV, light gray), tricuspid valve (TV, yellow). Color codes: light gray (lumen), dark gray (MLC2a positive myocardium). Scale bars 30µm.

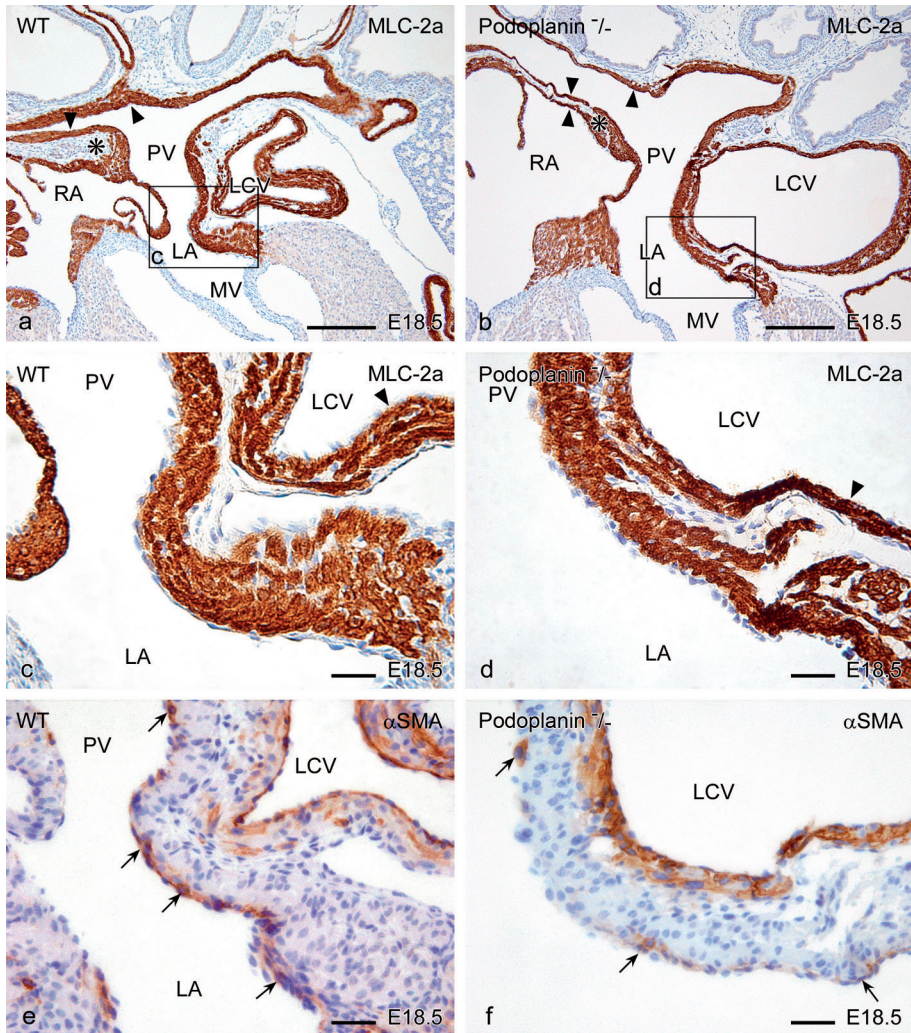


Figure 4. Transverse sections of *podoplanin* wild type (WT, a, c, e) and knockout (Podoplanin^{-/-}, b, d, f) mouse embryos of stage (E) 18.5 comparing the development of pulmonary vein (PV). Sections a-d are stained with MLC2a as myocardial marker and sections e and f are stained with alpha-smooth-muscle cell actin (α SMA). Boxes in a, b are the positions of the enlargements in c, d. a, b. MLC2a expression is seen in WT and mutants in the myocardium of left (LA) and right atrium (RA), left cardinal vein (LCV) and pulmonary veins (PV) (a, b). In knockouts, these structures are dilated and have hypoplastic myocardium (arrowheads in a-d) with interposition of less mesenchymal tissue (a, b; asterisks). Smooth muscle cells (SMC) are seen in the sub-endothelial layer of the PV and LA dorsal wall (arrows in e). In the mutants, SMCs are almost absent, which might be caused by either impaired SMC formation or differentiation due to the lack of *podoplanin* (compare arrows in e with f). Mitral valve (MV). Scale bars a, b 200 μ m; c-f 30 μ m.

Discussion

During human development, a vascular lining comprising SMCs and an adventitia will evolve at the inside of the LA body, that is lined on the outside by atrial myocardium¹⁴. Findings in the current study show a similar phenomenon in the LA of wild type mouse embryos. A difference between mouse and human specimen concerns the extension of the myocardial tissue surrounding the PV into the lung. The merits of our current study are that the posterior heart field plays a role in the addition of not only PV myocardium, but also in the formation and differentiation of SMCs that line the LA body. We demonstrated hypoplasia of the myocardium of the PV, LA dorsal wall and the atrial septum in *podoplanin* knockout mice. The vessel wall of the PVs and its extension into the LA was underdeveloped in knockout embryos.

Myocardial development

It has been reported earlier that the myocardial sleeve around the PV is formed by either migration of existing atrial cardiomyocytes¹, or by recruitment and differentiation of mesenchymal cells of the splanchnic mesoderm². The latter supports earlier advances in the study of cardiac development that underline the relevance of addition of myocardium to the primary heart tube¹⁵. The second heart field⁵ or second lineage^{4,5} concerns an anteroposterior extension of splanchnic mesenchyme from where cells are recruited for addition to both the arterial pole and the venous pole of the heart. Several studies have been performed using different lineage markers such as *fibroblast growth factor (Fgf)8* and *10*¹⁶, *Islet (Isl)1*⁵ and *Tbx1* and *18*^{17,18} to trace these cells into their cardiac destination. Special interest was raised in markers specific for recruitment of myocardium from the second heart field to the venous pole of the heart such as *Pitx2c*³, *Nkx2.5*^{3,7}, *Shox2*¹⁹ and *podoplanin*^{7,8}. *Podoplanin* promotes EMT by binding ERM proteins that activate RhoA and by downregulation of the cell-to-cell adhesion molecule E-cadherin^{8,9}. In *podoplanin* knockout mice E-cadherin is upregulated causing abnormal EMT which may lead to abnormal formation of myocardium at the venous pole of the heart⁸. Thus, the hypoplasia of the myocardium of the wall of the PVs, atrial septum and LA dorsal wall observed in the current study in *podoplanin* knockout mice could be explained

by impaired addition of myocardium from the posterior heart field due to abnormal EMT by lack of *podoplanin*.

Another explanation for the myocardial hypoplasia in the *podoplanin* knockouts could be abnormal epicardial-myocardial interaction. Previously we demonstrated that altered epicardial-myocardial interaction leads to deficient ventricular myocardial formation in *SP3* mutants²⁰ as well as in *podoplanin* mutant embryos⁸. In *SP3* mutants, WT-1 expression, a transcription factor involved in development of epicardium derived cells (EPDCs)²¹, was downregulated and the mutant hearts showed EPDC-related cardiac abnormalities comparable to *podoplanin* mutants. The impaired formation of EPDCs and altered epicardial-myocardial interaction, resulting in hypoplastic atrial and ventricular myocardium in *podoplanin* knockouts, has already been described and related to the deficient contribution of the posterior heart field^{8,22}. Therefore, the hypoplasia of the PVs, atrial septum and LA dorsal wall reported in the current study could be related to the altered contribution of epicardium and myocardium from the posterior heart field.

In early stages, Nkx2.5 and alpha-smooth-muscle actin (1A4) are expressed in undifferentiated myocardium. At these stages, Nkx2.5 is mosaic around the PVs and absent in the wall of the cardinal veins⁷, whereas actin expression is present in the wall of the pulmonary and cardinal veins. The Nkx2.5 mosaic expression in the wall of the PVs rapidly becomes completely positive concomitant with a higher proliferation rate of the PV myocardium compared to the cardinal veins³. Consequently, 1A4 expression from E15.5 on is confined to the medial layer of the vascular wall of the pulmonary and cardinal veins. These findings suggest that the pulmonary and cardinal veins have a common precursor derived from the posterior heart field⁷, but a distinct proliferation rate³ accounting for a distinct differentiation based on Nkx2.5 expression.

Expression of markers in putative PV myocardium starts at the left side of the dorsal mesocardium, indicating that PV myocardium is preferentially added from the left side of the posterior heart field, regulated by progenitor cells that play a role in left-right patterning, as was reported for *Pitx2c*^{3,23,24}. Next to hypoplasia, mutants showed diminished expression, that was predominantly observed in the earlier stages (Table 1a,b). As in later stages these differences were no longer apparent, this suggests a delayed differentiation of the myocardium and SMCs in mutants.

Smooth muscle cell development

The origin of the SMCs at the venous pole is as yet not well understood. SMCs may differentiate from mesenchymal cells⁶. At the arterial pole formation and differentiation of SMCs has been reported from the splanchnic mesoderm or from the neural crest²⁵. The latter process requires cross-talk between the endothelial and the muscular component²⁶. DeRuiter and colleagues have described the formation of the SMCs of the dorsal aorta by transdifferentiation from endothelial cells²⁷.

Research in this field is complicated as alpha-smooth-muscle actin also stains the primitive myocardium. We demonstrated that this staining was less extensive in the developing myocardium of the *podoplanin* mutant mice, supporting delayed or defective myocardial differentiation. At the stage that normally alpha-smooth-muscle actin disappears from the myocardium, only the SMCs retain their expression of this marker. In *podoplanin* knockout mice we observed a diminished extension of SMCs in the LA dorsal wall as compared to normal. This phenomenon may be caused by impaired formation of SMCs from the posterior heart field derived dorsal mesocardium or abnormal or delayed differentiation of the SMCs in the PV and LA body in absence of *podoplanin*. The disturbed EMT in *podoplanin* knockouts probably leads to abnormal formation of SMCs as was shown for the coronary artery SMCs development in mutant mice⁸.

In conclusion, as *podoplanin* is a marker of the myocardial and mesenchymal cells derived from the second heart field at the venous pole of the heart, this study supports evidence that the myocardium of the PVs, LA dorsal wall and atrial septum is derived from the posterior heart field. Moreover, we can state that *podoplanin* not only plays a role in the development of myocardium, but also in the formation of the SMCs.

The clinical relevance of our findings needs further research. In the mutants, complex atrial defects were observed. In the majority, there was fusion of the primary atrial septum with the AV cushions, but the secondary foramen was enlarged and the AV cushions did not fuse properly with the ventricular septum resulting in an AVSD with shunting at the ventricular level. These findings were described in a previous study⁸.

The study of human and mouse models with isomerism, atrial arrhythmias and cases with abnormal pulmonary venous return are on their way. More insight into the variation in myocardial cuffing of the PVs in the human population might enlighten us on the variability of occurrence of ectopic automaticity in the PV myocardial sleeve²⁸, which is suggested by the finding that the length of the PV sleeve corresponds to the frequency of occurrence of ectopic PV beats as observed in electrophysiological studies^{28,29}. In podoplanin knockout embryos, we have observed deficient sinus venosus myocardium with myocardial discontinuities. Areas lacking myocardium can be regarded as low voltage areas (comparable to scar tissue) that may form the substrate of reentry circuits. Electrophysiological testing in mutant mice is necessary to further investigate this hypothesis.

Acknowledgements

We thank Bert Wisse for preparation of the 3D-reconstructions, and Jan Lens for preparation of the figures.

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