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

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Pulmonary Vein and Atrial Wall Pathology in Human Total Anomalous Pulmonary Venous Connection

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Abstract

Background. Normally, the inside of the left atrial (LA) body and pulmonary veins (PVs) is lined by vessel wall tissue covered by myocardium. In total anomalous pulmonary venous connection (TAPVC), no connection of the PVs with the LA body exists. These veins have an increased incidence of PV stenosis. We describe the consequences of the absent connection for the histopathology of the wall of the LA body and the PVs, and hypothesize on a mechanism predisposing to PV stenosis.

Methods and results. In 10 human neonates with TAPVC, the wall of the LA body and PVs were studied using histological and immunohistochemical techniques. As controls, 2 normal neonatal and adult hearts and 5 neonatal hearts with partial anomalous venous connection (PAPVC) or situs inversus were studied. In hearts with TAPVC, no vessel wall tissue was found in the LA body and its myocardial layer was hypoplastic. No myocardial sleeve was found around the abnormally draining PVs. In hearts with PAPVC, only the non-LA draining PV lacked myocardial covering, whereas in situs inversus, PVs connecting to the right-sided LA, were normally myocardialized.

Conclusion. An open connection of the PVs with the morphological LA is necessary for the presence of vessel wall tissue in the LA and myocardialization of the PVs. Absence of myocardium covering the PVs is hypothesized to enhance susceptibility to PV stenosis and prevent onset of PV originating arrhythmias. The embryonic posterior heart field may be responsible for the abnormal myocardialization and smooth muscle cell formation in TAPVC.

Introduction

In human cardiac development, at the end of the fifth week, in the dorsal mesocardium the common pulmonary vein (PV) lumenizes, thus forming an open connection between the pulmonary vascular network and the left atrium (LA)[1-4]. Therefore, connections to the systemic circulation[3,5] have become non-functional and regress. Thereafter, a myocardial sleeve, which is continuous with the LA myocardium, is formed around the common PV. The origin of this myocardial layer lining the PVs is a topic of debate. Either LA cardiomyocytes extend into the extracardiac mesenchyme and cover the PVs[6-8], or non-cardiac cells are recruited from the splanchnic mesenchyme and differentiate into myocardial cells which line the PVs[9-11]. Recently, in mice a biphasic process of formation of PV myocardium was described, favouring both points of view[12].

From the tenth week onwards, the PVs grow and are incorporated into the also expanding dorsal wall of the LA. This process results in a smooth-walled LA body consisting of vessel wall tissue on the inner side covered by myocardium on the outside[13]. In hearts with total anomalous pulmonary venous connection (TAPVC), the common PV forms no open connection between the splanchnic plexus and the LA, thus resulting in persistence of (one of) the cardinal veins draining the pulmonary venous blood to the systemic venous circulation[3] which can be divided in a supracardiac, a cardiac and a infracardiac type of drainage. At presentation and especially after repair of anomalous pulmonary venous connections, increased rates of PV stenosis are reported, suggesting susceptibility of these veins to PV stenosis[14-16]. A remarkably low incidence of significant arrhythmias in these patients is reported in literature[17].

Our previous findings[13] have shown that the common PV connects to and incorporates into the expanding LA dorsal wall resulting in vessel wall tissue lining the smooth wall of the LA and a covering of the PVs by a myocardial layer. We hypothesize that, if this process does not take place, as in hearts with TAPVC, there will be an abnormal histology of the structures involved, which may also have clinical consequences. We also postulate on the basis of mouse model studies[12] that PV myocardialization is a genetically left-sided regulated process.

Methods

We studied 5 neonatal heart-lung specimens from the Leiden collection with supracardiac (n=4) or infracardiac (n=1) TAPVC as well as biopsy material of another 5 neonates, taken intra-operatively prior to surgical correction, of either the supracardiac (n=3) or infracardiac (n=2) type of TAPVC. These patients were operated in the University Medical Center of Groningen or Rotterdam. The local Medical Ethical Committee approved the studies. The age of the neonates ranged from 1 day to 2 years. Hearts with atrial isomerism were excluded.

Controls.

Normal neonatal (n=2) and adult hearts (n=2) were used as controls. Three neonatal heart-lung specimens from the Leiden collection with cardiac (n=1) and supracardiac (n=2) partial abnormal pulmonary venous connection (PAPVC) were used to study the myocardialization process of PVs when normal and abnormal pulmonary venous drainage coexist. We additionally examined two neonatal heart-lung specimens with situs inversus to study the influence of laterality, in this case of a right-sided position of the LA, on the myocardialization of the PVs.

The adult hearts were obtained from human bodies used in anatomy teaching lessons. The cause of death was unknown due to privacy regulations.

Samples/sectioning of material:

Study material. From the neonatal heart-lung specimens with TAPVC, tissue blocks were taken from the dorsal wall of the smooth-walled LA body, the LA appendage and the PVs. The surgical biopsies obtained from patients of the University Medical Center of Groningen and Erasmus Medical Center Rotterdam were derived from the LA dorsal wall and the pulmonary venous confluence (exactly taken at the site of the incisions necessary for the correction) and, in some cases, the vertical vein.

Controls.

Normal neonatal and adult hearts. Tissue blocks were taken from the LA dorsal wall and the PVs. In adults, tissue blocks were also taken from the smooth-walled

posterior and anterior right atrial (RA) body, the RA appendage, both caval veins, the smooth-walled RA body above the tricuspid valve and the coronary sinus to compare the presence of vessel wall in the LA and RA.
*Neonatal hearts with abnormalities***.** From the specimens with PAPVC, samples of the normally as well as abnormally draining PVs were taken. In hearts with situs inversus, tissue blocks were taken from the PV-LA transition.

The samples were embedded in paraffin and sectioned (5 µm) using a Leica microtome. The sections were mounted onto slides using chicken albumin dissolved in glycerine (1:1) and dried at 37 °C for ≥ 24 h.

Staining: Standard histological staining procedures were performed with Hematoxylin-Eosin, Verhoeff-Van Gieson or elastic Resorcin-Fuchsin to detect elastic filaments. Additional sections were stained immunohistochemically with a mouse monoclonal against alpha smooth muscle (SM) actin (1A4, 1:10.000, Sigma Aldrich, Product No. A2547, USA), indicating the presence of a vascular wall, and a rabbit polyclonal antibody against atrial myosin light chain (MLC2a, 1:2000, a generous gift of Steve Kubalak), specific for atrial myocardium. After incubation with the primary antibodies overnight, antibody binding for 1A4 was demonstrated with rabbit anti-mouse peroxidase (RAM-PO, 1:250, Dako, Product No. P0260, Denmark) and for MLC2a with goat anti-rabbit biotin (GAR-biotin, 1:200, Vector labs, Product No. BA-1000, USA). The latter procedure was followed by a rabbit biotinylated reagent (ABC, 1:100, Vector Labs, Product No. PK-6100). After sections were rinsed with phosphate buffered saline and tris/maleate (pH 7.6), 3-3' diaminobenzidine tetrahydrochloride (DAB) was used as chromogen and counterstaining was performed with Mayer's haematoxylin. The remaining part of the staining procedures was performed as described previously[1].

Results

Normal neonatal and adult hearts.

In the PVs, a characteristic venous vessel wall was found, consisting of an intimal layer, a medial layer with smooth muscle cells, embedded in a matrix of collagen and elastic fibers and an adventitial layer with fibroblasts and vasa vasorum. On the outside, the PVs were covered by a myocardial sleeve which tapered off towards the lung hilum. In the entire smooth-walled LA body, a prominent vessel wall layer, continuous with that of the PVs, was found, covered on the outside by myocardium. At the transition of the PV ostia and the LA body no histologic veno-atrial demarcation was found. In contrast to the neonatal hearts, in the PVs and LA body of adult hearts intimal thickening was observed, most profound in the PVs (Fig.1a-f).

The RA dorsal wall of the adult hearts was smooth-walled and consisted of myocardium. The inner side of the RA body wall near the superior caval vein consisted of a layer of endocardium overlying a discontinuous layer of elastic fibers, generally indicated as the lamina elastica interna. Between these layers and the myocardium, a vascular tunica media was observed, continuous with that of the superior caval vein (Fig.2a,b). This smooth muscle cell layer was also seen in the region of the orifice of the inferior caval vein. In the circumference of the orifice of the coronary sinus no smooth muscle cells were found. The coronary sinus itself contained a proper tunica media as seen in the superior caval vein, covered on the outside by a myocardial layer (Fig.2c,e). In the remaining part of the smooth-walled RA body no vessel wall tissue was observed (Fig.2c,d).

The RA and LA appendages were trabeculated and lined by endocardium with a subendocardial presence of elastin and collagen fibers and an occasional smooth muscle cell, and a myocardial layer on the outside. No vessel wall layer was present (Fig.2f,g).

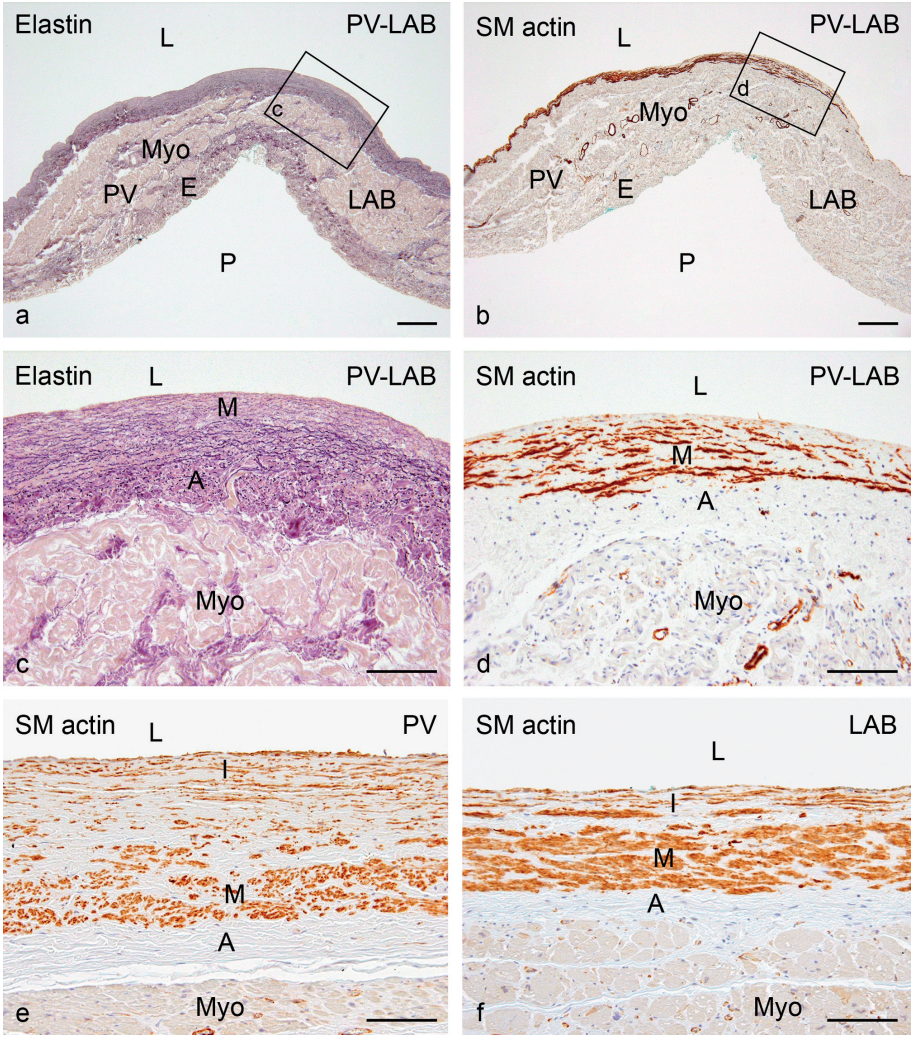

Figure 1a-f

Sections of a normal neonatal heart (a-d**)** and adult heart (e,f**)** stained with elastic Resorcin-Fuchsin (a,c; **Elastin**) and alpha smooth muscle actin (b,d-f; **SM actin**), showing the histological structure of pulmonary venous and left atrial wall. Boxes in a,b indicate the positions of the enlargements in c,d.

a-d. At the anatomical transition of pulmonary veins (**PV**) and LA body (**LAB**), no histologic veno-atrial demarcation is found. The wall consists of endothelium/endocardium, a medial layer (**M**), with smooth muscle cells and elastic fibers, an adventitial layer (**A**), myocardium (**Myo**), and an outer epicardium (**E**).

e,f**.** In the PV as well as in the LAB, conforming to the sections in a-d, an identical and characteristic vessel wall is found. In contrast to the neonatal stage profound intimal thickening (**I**) is observed most obviously in the PV. Lumen (**L),** pericardial cavity (**P**). Scale bar= 300µm in a,b; 100µm in c-f.

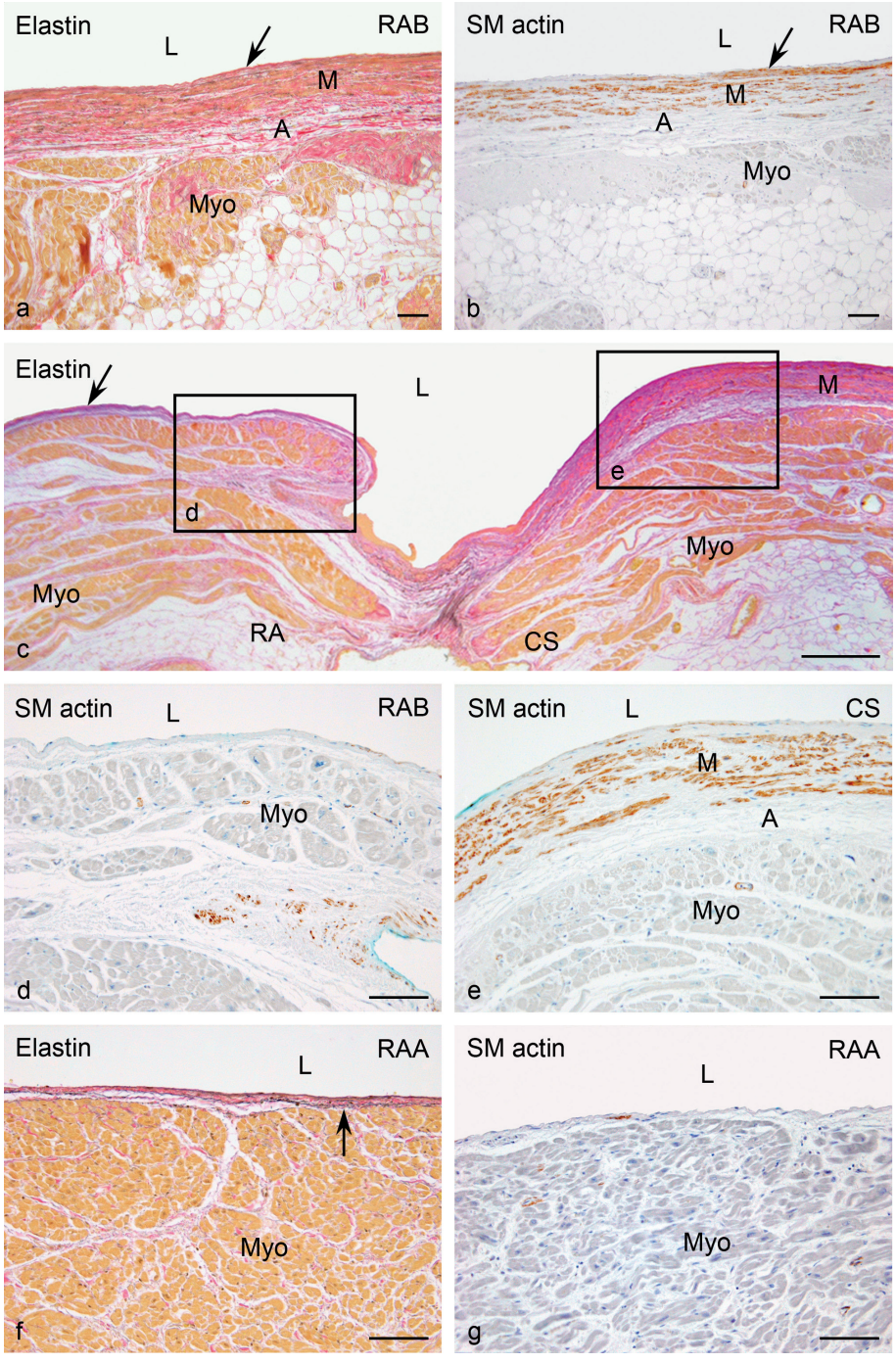
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Figure 2a-g
Sections of the right atrial body of normal adult hearts, stained with Verhoeff-Van Gieson (a,c,f; **Elastin**) and alpha smooth muscle actin (b,d,e,g; **SM actin**). Boxes in c indicate the positions of the enlargements in d,e.
a,b**.** Right atrial body (**RAB**) near the superior caval vein showing organized vessel wall tissue with multiple layers of smooth muscle cells. Intima (arrow**).**
c-e. In the region next to the coronary sinus (**CS**), no vessel wall tissue is found, which is representative for the major part of the smooth-walled RAB. The CS is covered by myocardium. Endothelium (arrow).
f,g. Trabeculated RA appendage (**RAA**). On the inner surface a thin endocardium (arrow) is present and a subendocardial layer with elastic fibers, collagen and occasional smooth muscle cells. There is no proper tunica media. These thin layers are covered by myocardium.
adventitia (**A**)**,** Lumen (**L**), media (**M**), myocardium (**Myo**). Scale bar = 100 µm in a,b,d-g; 300µm in c.

Heart-lung specimens with abnormal PV connections.

Macroscopic findings. Two of the four hearts with the supracardiac type of TAPVC had a situs solitus of the atria, with concordant atrioventricular and ventriculo-arterial connections, a small mitral valve and a hypoplastic left ventricle. Both had a secundum type of atrial septal defect (ASD), one of which had been surgically closed. The PV confluence originally drained via a vertical vein into a systemic vein, in this case the brachiocephalic vein. In the surgically corrected heart, the vertical vein was ligated and the PV confluence was connected to the roof of the LA body, thus establishing a continuous blood flow from the PVs to the LA. One of the other two hearts had a discordant ventriculo-arterial connection, with pulmonary atresia. The other heart had a double outlet right ventricle with infundibular pulmonary stenosis. Both of these hearts had a secundum type of ASD as well as an atrioventricular septal defect (AVSD).

The heart with the infracardiac type of TAPVC also presented with a situs solitus of the atria. There was a concordant atrioventricular connection with mitral atresia and a discordant ventriculo-arterial connection. The LA body and the enlarged RA body were smooth-walled. A secundum type of ASD was present. The PV confluence connected to a vertical vein, which descended through the diaphragm to a systemic vein, in this case the right gastro-epiploic vein that drained into the portal vein.

The three neonatal heart-lung specimens with PAPVC had a situs solitus, with concordant atrioventricular connections. In two specimens, the ventriculo-arterial connections were discordant. One of the four PVs drained either directly or via the brachiocephalic vein into the right superior caval vein in two of the three specimens, and in one the right PVs originally drained directly into the RA which was operatively corrected. Besides a ventricular septal defect (VSD) in two, there were no other serious cardiac abnormalities.

The hearts with situs inversus both had concordant atrioventricular and ventriculo-arterial connections with a right-sided aortic arch. In one a subaortic stenosis, dysplastic aortic valve, secundum type of ASD and an AVSD were present, the other had a perimembranous subaortic VSD.

Microscopic findings. In both the supra- and infracardiac type of TAPVC, the wall of the PV confluence had an intimal, medial and an adventitial layer. The intima consisted of an endothelial layer, a subendothelial layer of collagen and a clear lamina elastica interna with well-oriented elastic fibers. The media consisted of smooth muscle cells embedded in collagen and some elastic fibers. The adventitial layer had randomly distributed collagen fibers, some elastic fibers and scattered smooth muscle cells. Interestingly, in contrast to normally connecting PVs, no myocardial layer was observed around the anomalous connecting PVs (Fig.3a,b).

The posterior wall of the smooth-walled LA body consisted of a slightly thickened subendocardial layer of collagen, elastic fibers and sporadic smooth muscle cells, covered by myocardium. In contrast to the normal LA body, no distinct vessel wall tissue was observed (Fig.3c,d). Moreover, compared to normal, the size of the LA body was significantly diminished and its myocardial layer was translucent and hypoplastic (Fig.4a-d). In one case, even spongious (non-compact) LA myocardium was seen (not shown). Similar to the RA appendage in normal hearts (Fig.2f,g), the LA appendage was trabeculated and consisted of an endocardial layer covered by myocardium.

In the heart-lung specimens with either cardiac or supracardiac PAPVC, the PVs that connected to the LA were normally myocardialized (not shown), whereas the abnormally draining veins (i.e. the veins that did not connect to the LA) had no myocardial covering (Fig.5a-d). Moreover, similar to normal hearts, in cardiac PAPVC no vessel wall tissue was found in the RA body (not shown). The heart-lung specimens with situs inversus showed normally myocardialized PVs connecting to the right-sided morphological LA (Fig.5e,f).

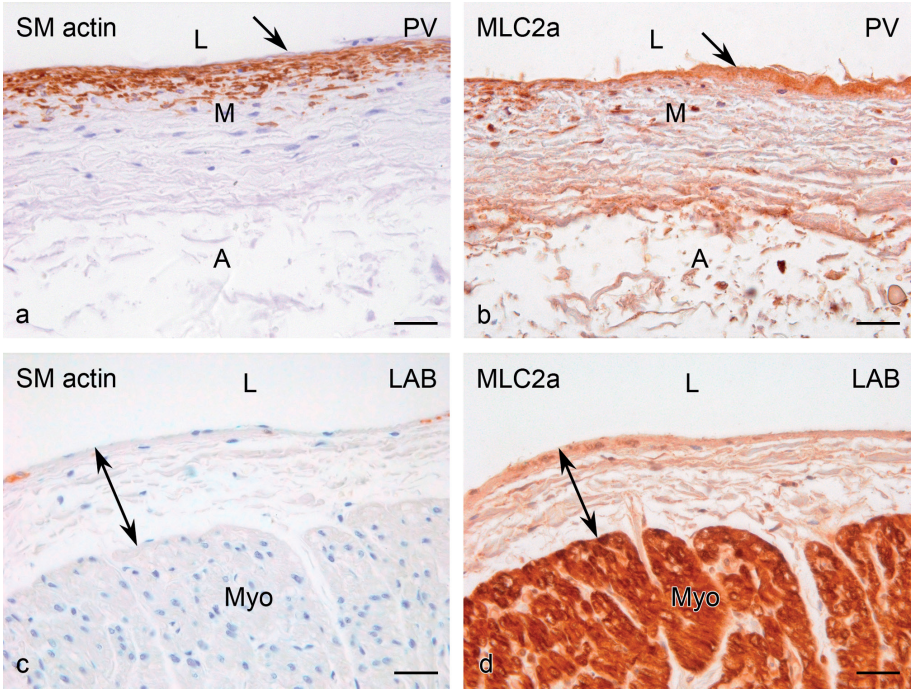


Figure 3a-d
Sections of neonatal heart-lung specimens stained with 1A4 against alpha smooth muscle actin (a,c; **SM actin**) and atrial myosin light chain (b,d; **MLC2a**), showing the histological structure of pulmonary venous and left atrial wall in hearts with total anomalous pulmonary venous connection (TAPVC).
a,b. Pulmonary venous (**PV**) confluence representative for any kind of TAPVC showing vessel wall consisting of a tunica intima (arrow), media (**M**) and a tunica adventitia (**A**) without myocardial covering.
c,d. Smooth-walled left atrial body (**LAB**) with a slightly thickened (sub)endocardial layer (arrow, double head) of collagen, elastic fibers and occasional smooth muscle cells covered by myocardium (**Myo**). No vessel wall tissue was found.
Lumen (**L**). Scale bar = 30µm in a-d.

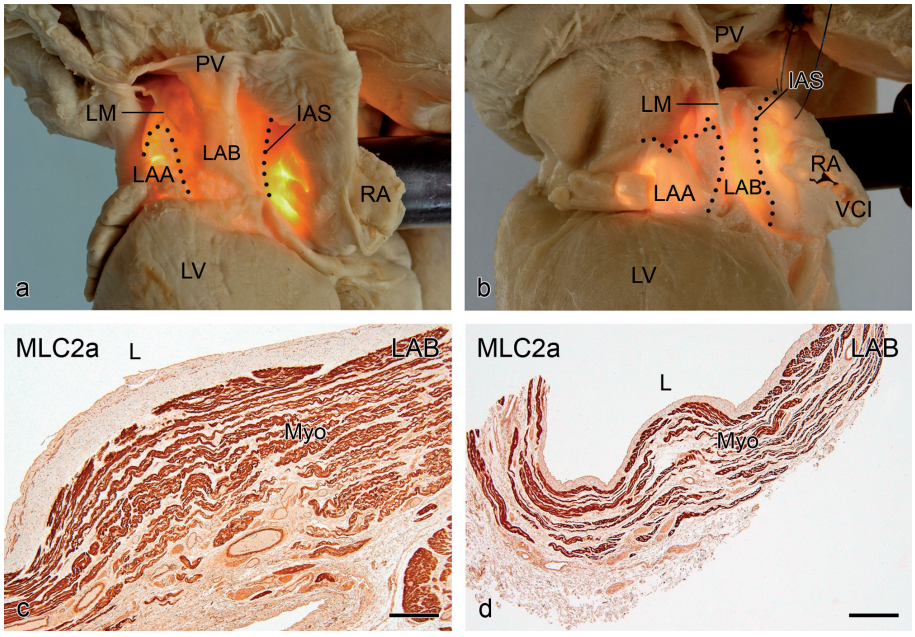


Figure 4a-d

Pictures of a normal neonatal heart (a) and a neonatal heart with the supracardiac type of TAPVC (b) correlated to sections of their LA dorsal wall stained with atrial myosin light chain (c,d; **MLC2a**).

a. Normal heart. The left atrial body (**LAB,** between dotted lines) receives the PVs and is demarcated by the ligament of Marshall (**LM**), at the base of the left atrial appendage (**LAA**), and the atrial septum (**IAS**).

b**.** Supracardiac type of TAPVC. The pulmonary veins (**PV**) are not connected to the LAB but drain via a confluence into the systemic venous circulation. In this case, the LM connects the PV confluence to the left atrium. Note that in TAPVC, the size of the LAB (between dotted lines) is remarkably diminished and that there is more translucency compared to the normal heart.

c. Normal heart. The myocardium (**Myo**) of the LAB has a normal, compact structure.

d. TAPVC. Compared to the normal heart, the myocardium is hypoplastic, causing translucency.

Lumen (**L**), left ventricle (**LV**), right atrium (**RA**), inferior caval vein (**VCI**). Scale bar = 300 µm in c,d.

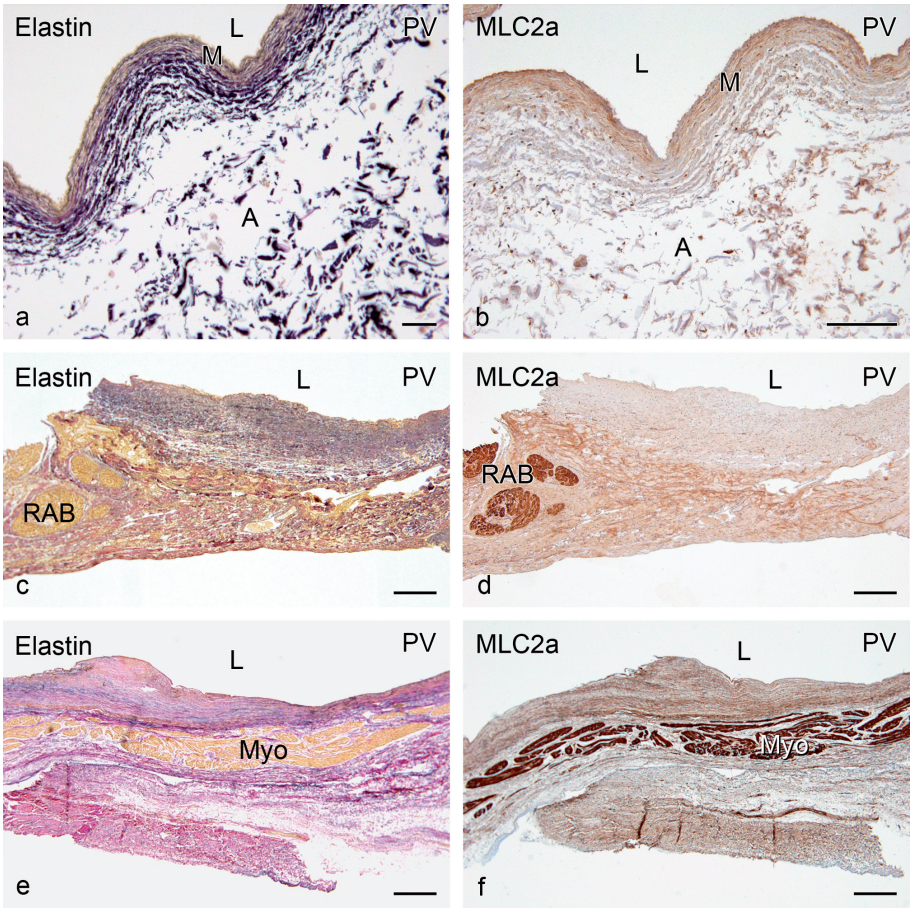


Figure 5a-f
Sections of neonatal heart-lung specimens stained with Verhoeff-Van Gieson for elastic filaments (a,c,e; **Elastin**) and atrial myosin light chain (b,d,f; **MLC2a**), showing the histological structure of the abnormally draining pulmonary vein (**PV**) in partial anomalous pulmonary venous connection (PAPVC; a-d) as well as the histological structure of the PV in case of situs inversus (e,f).
a,b**.** Extracardiac PAPVC. Section of the abnormally draining PV showing a normal vessel wall structure (a), and absence of myocardium (b).
c,d. Cardiac PAPVC. The right atrial body (**RAB**) receives one of the PVs. This PV is not myocardialized.
e,f**.** Situs inversus. The PVs, though right-sided, do have a myocardial layer.
Adventitia (**A**). lumen (**L**), media (**M**). Scale bar = 100µm in a,b and 300 µm in c-f.

Biopsies of patients during surgical correction for TAPVC

Macroscopic findings. In all hearts with the supracardiac type, the PV confluence drained by means of a vertical vein into the brachiocephalic vein. Naturally, in hearts with the infracardiac type, only a vertical vein descending downwards could be identified during operation. In none of these 5 hearts other cardiac anomalies were found.

Microscopic findings. Within the samples taken from the smooth-walled LA body, no vessel wall tissue was detected. The smooth-walled LA body consisted of endocardium and a subendocardial layer, the latter consisting of randomly scattered collagen and elastic fibers, covered by myocardium (Fig.6a-c). The PV confluence consisted of a well organized subendothelial layer of collagen and elastic fibers. The medial layer resembled a proper tunica media and consisted of multiple layers of smooth muscle cells embedded in collagen and some elastic fibers (Fig.6d,e). On the outside of the confluence a proper tunica adventitia was observed. Again, in these veins, no myocardial layer surrounding the vein was present (Fig.6f). Samples of the vertical vein could be taken in two hearts with the supracardiac type and in two hearts with the infracardiac type. In both types the intimal layer was locally thickened (not shown). As in the sections of the anomalous connecting PVs, no myocardial layer was observed (Fig.6g-i).

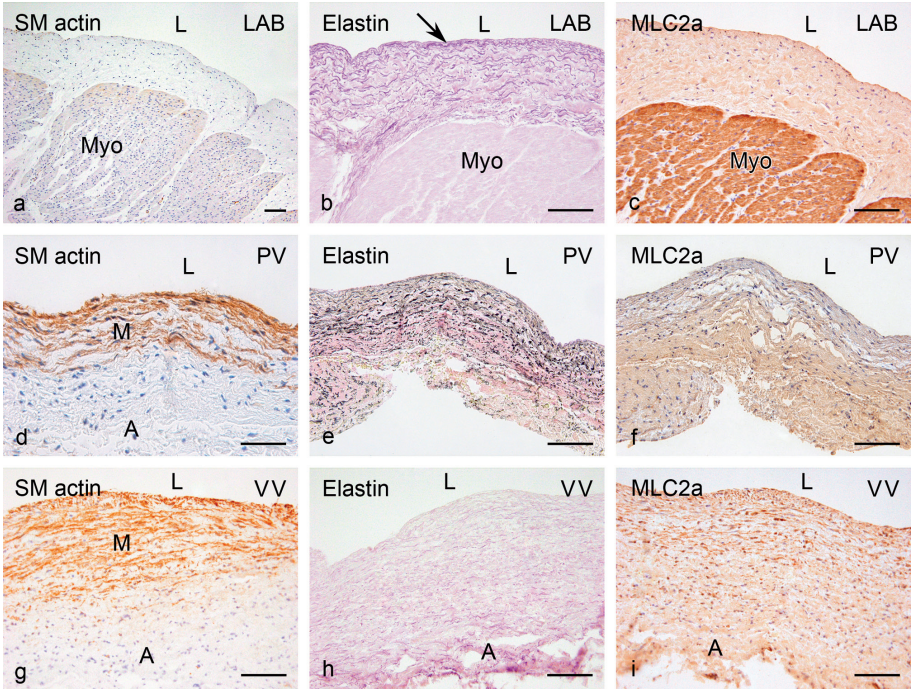
Figure 6a-i

Sections of peroperatively taken samples of left atrial body (**LAB**), pulmonary veins (**PV**), and the vertical vein (**VV**) from neonates operated on for total anomalous pulmonary venous connection. Staining with elastic Resorcin-Fuchsin (b,h; **Elastin**) and Verhoeff-Van Gieson (e; **Elastin**) for elastic filaments; alpha smooth muscle actin (a,d,g; **SM actin**), and atrial myosin light chain (c,f,i; **MLC2a**).

a-c. Smooth-walled LAB, showing absence of vessel wall tissue (a). The thin endocardium (b,arrow**)** and subendocardial layer with collagen and elastic fibers (b), is covered by myocardium (c, **Myo**).

d-f. PV confluence consisting of proper vessel wall components (d,e) but lacking a myocardial covering (f).

g-i**.** Vertical vein histologically resembling the PV confluence with absence of myocardial covering (i**).** Adventitia (**A**), lumen (**L**), media (**M**). Scale bar = 100μm in a-c,f-i; 50μm in d,e.



Discussion

In this study we examined the morphology and tissue characteristics of hearts from patients with anomalous pulmonary venous connections, and compared the results with findings in normal controls. Key findings of our study in patients with TAPVC are: 1. a significantly diminished size of the LA body and reduced thickness of the myocardium; 2. the lack of a vessel wall in the LA body; and 3. the lack of formation of a myocardial muscular sleeve surrounding the PVs.

The origin of the myocardium surrounding the common PV in normal hearts remains an issue of debate. On the basis of morphology and expression patterns of the marker HNK-1 in avian[18], rat[19] and human[20] embryos and Nkx2.5 expression in human embryos[9], our group showed that the myocardium around the common PV belongs to the sinus venosus segment. Based on morphology[4,21] and expression patterns of connexin40 and atrial natriuretic peptide[22], the myocardium around the primitive

PV can be discriminated from the myocardium of the primary heart tube. There are different theories about the (secondary) addition of myocardium lining the common PV.

Recent research using the novel coelomic and myocardial marker podoplanin, has shown that the development of sinus venosus myocardium, derived by recruitment of progenitor cells at the venous pole (the so-called posterior heart field), and surrounding the common PV, is demarcated by podoplanin expression and the absence of Nkx2.5 expression.

Compared to the rest of the sinus venosus myocardium, the PV myocardium undergoes a rapid differentiation and proliferation phase[12], during which the Nkx2.5 expression changes from a mosaic to positive expression pattern[9]. Diminished Nkx2.5 expression is accompanied by expression of the sinus venosus marker HCN4 in the PV myocardium[12], suggesting early transient expression of HCN4 in the PVs during the mosaic Nkx2.5 expression phase. Eventually, HCN4 becomes confined to the sino-atrial node[12]. The myocardium surrounding the common PV is linked to the development of parts of the cardiac conduction system[9,12]. These findings are supported by the (transient) expression of several molecular markers in the myocardial sleeve surrounding the PV, including HNK-1 and CCS-lacZ[20,23]. Therefore, second heart field derived myocardial cells surrounding the PVs may have conduction properties responsible, together with areas of myocardial discontinuity, for the increased susceptibility for arrhythmias arising from the PV area and the LA dorsal wall[10,13,24]. We postulate that the lack of a myocardial sleeve surrounding the PVs in patients with TAPVC, may explain the low incidence of arrhythmias observed in this patient group[17].

Since in TAPVC hearts addition of myocardium at the venous pole is impaired, also sino-atrial node dysfunction can be expected. This hypothesis is supported by several clinical studies[17,25] as well as by the observation that in podoplanin knockout mice, the sino-atrial node is hypoplastic[26].

The findings of our current study that in human hearts with supra- or infracardiac TAPVC, myocardium around the PVs is absent, the LA myocardium is hypoplastic and no vessel wall tissue is found in the LA body, suggest that impaired formation and differentiation of posterior heart field myocardial and smooth muscle cells contributes to the development of TAPVC.

As we have shown, in hearts with TAPVC, as a consequence of absent PV connection and incorporation, the complete LA body is small and smooth-walled, and the myocardium is hypoplastic. Additionally, reduced fetal hemodynamic flow may contribute to the observed small size of the LA in these hearts. Therefore, it is not surprising that surgeons have tried to optimize results of TAPVC correction by augmentation of the LA[15,27].

In this study, in one heart with TAPVC, non-compaction of LA myocardium was seen. Since the posterior heart field seems to contribute to TAPVC and the proepicardial organ is derived from the posterior heart field, an explanation for the spongious LA myocardium can be impaired formation of epicardium derived cells (EPDC's) and altered epicardial-myocardial interaction, which are known to be responsible for abnormal atrial and ventricular myocardium[28,29].

In our study in 60% of TAPVC heart-lung specimens also outflow tract abnormalities were found. Because the posterior heart field is part of the second heart field which contributes to myocardial addition at the arterial as well as the venous pole[30], co-existence of outflow tract and inflow tract abnormalities can be explained.

We hypothesize that an open connection of the common PV into the morphological LA is necessary to trigger recruitment of mediastinal mesenchymal cells, that subsequently differentiate into cardiomyocytes and smooth muscle cells. Based on our findings in hearts with (extra)cardiac PAPVC and in situs inversus, we now know that only PVs connecting to the LA body are myocardialized (Fig.7a-h). This is an important finding which confirms that for PVs, addition of myocardial cells recruited from the posterior heart field, mainly takes place from the morphological left side[9].

In this context, an interesting finding in hearts with cardiac TAPVC and isomeric left atrial appendages was that myocardialization was only found in one "LA", irrespective of the side of PV incorporation. This myocardialization is probably based on left-right patterning as was shown in studies using the left-sided transcription factor Pitx2c[12,31,32]. Pitx2c deficient mice exhibit right atrial isomerism and lack the development of a myocardial sleeve surrounding the PVs[12].

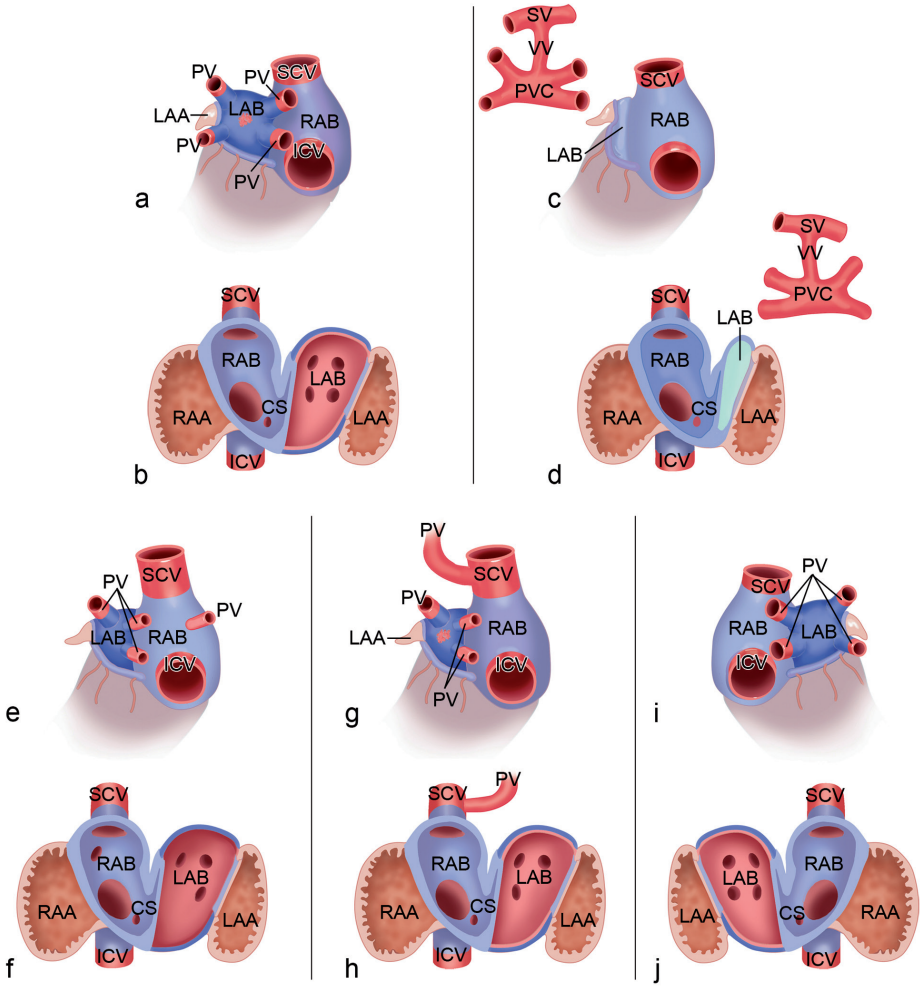

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Figure 7a-j
Schematic depiction of outer (a,c,e,g,i) and inner (b,d,f,h,j) side of atrial chambers and pulmonary veins (**PV**) in normal hearts (a,b), hearts with total anomalous pulmonary venous connection (TAPVC;c,d**),** hearts with (extra)cardiac partial anomalous pulmonary venous connection (PAPVC; e-h) and hearts with situs inversus (i,j).
a,b**.** Normal heart. a. Left atrial body (**LAB**) and pulmonary veins (**PV)** covered by myocardium derived from the posterior heart field (***dark blue***). In the area between the PVs the myocardium can be discontinuous or absent. Right atrial body (**RAB**) covered by sinus venosus myocardium (***light blue***). b. The inner aspect of both LAB and RAB is smooth-walled. In the LAB vessel wall tissue (***red***) is found parallel to PV incorporation. The left and right atrial appendages (**LAA, RAA**) consist of trabeculated myocardium (***brown***).
c,d. TAPVC, extracardiac type. The PVs drain via a vertical vein (**VV)** into a systemic vein (**SV**) and are not covered by myocardium. The LAB consists of smooth-walled hypoplastic myocardium, derived from the posterior heart field. Because of the absence of PV connection and incorporation into the LA, the LAB is small. Moreover the LAB does not contain vessel wall tissue, probably because of impaired differentiation of smooth muscle cells.
e,f. PAPVC, cardiac type. The abnormally connected PV drains directly into the RAB, but no vessel wall tissue is found in the RAB. In contrast to the PVs that are connected to the LAB, the abnormal PV is not covered by myocardium.
g,h. PAPVC, extracardiac type. The abnormally connected PV drains (in)directly into the superior caval vein and is, similar to the cardiac type of PAPVC, not covered by myocardium. The LAB and its incorporated PVs are myocardialized and comparable to the normal heart, vessel wall tissue is present in the LAB.
i,j. Situs inversus. The LAB is located on the right side of the heart and receives the four PVs, which are covered by myocardium. In the LAB vessel wall is found parallel to normal PV incorporation.
Coronary sinus (**CS**), inferior caval vein (**IVC**), pulmonary venous confluence (**PVC),** superior caval vein (**SCV**).

After surgical correction of TAPVC, the pulmonary venous confluence is connected with the LA body. Because of an increased incidence of PV stenosis at the site of the anastomosis, so-called sutureless techniques have been developed and successfully applied[33,34]. We hypothesize that this PV stenosis might be enhanced in TAPVC by differences in wall thickness and contractile properties of cells at the transition of PVs to LA, where vessel wall is directly sutured to myocardium, without an intermediate layer of myocardium protecting the PV. Also, primary PV stenosis is observed frequently in this patient group[35]. With respect to this, absence of the protective layer of myocardium surrounding the PVs may be responsible for the enhanced susceptibility of the PV wall in response to internal or environmental influences, thus making it prone to stenosis. The interaction between the lack of PV myocardial layer and the development of PV stenosis requires further investigation.

In conclusion, in hearts with TAPVC no vessel wall was found in the smooth-walled LA body and its myocardial layer was structurally abnormal, demonstrating marked hypoplasia. No myocardial layer was formed around the PVs. Absence of this

myocardium may prevent the onset of arrhythmias in the PVs but may enhance PV stenosis. Based on our findings in PAPVC and situs inversus, we hypothesize that recruitment of cells, for addition of myocardium and smooth muscle cells at the venous pole, mainly takes place from the morphological left side. An open connection to the LA is mandatory for proper development of the LA vascular wall and myocardium and the pulmonary venous muscular sleeve. The posterior heart field is suggested to be responsible for the abnormal myocardialization and smooth muscle cell formation of the LA dorsal wall and PVs in TAPVC hearts. This knowledge about the PV and atrial morphology in TAPVC hearts may help to understand the mechanisms related to the high occurrence of primary PV stenosis and PV stenosis after surgery for TAPVC.

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