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

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Histology of Vascular-Myocardial Wall of Left Atrial Body after Pulmonary Venous Incorporation

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Abstract

During embryonic development the common pulmonary vein (PV) becomes incorporated into the left atrium (LA), giving rise to separate PV ostia. We describe the consequences of this incorporation process for the histology of the LA and the possible clinical implications. The histology of the LA wall in relation to PV incorporation was studied immunohistochemically in 16 human embryos and fetuses, one neonate and 5 adults. The PV wall, surrounded by extrapericardially differentiated myocardial cells, incorporated into the LA body. After incorporation, the composition of PVs and smooth-walled LA body wall was histologically identical. The LA appendage however, consisted of an endocardial and myocardial layer without a vessel wall component. In 2 adults, the myocardium in the LA posterior wall was absent. At the transition of the LA body and the LA appendage, a smooth-walled myocardial zone lacking the venous wall was observed. This zone was histologically identical to the sinus venarum of the right atrium. In conclusion, the LA body arises by incorporation and growth of the PVs, presenting with a histologically identical structure of vessel wall covered by extrapericardially differentiated myocardium of the PVs. Discontinuity of myocardium may be the substrate for arrhythmias, whereas absence of myocardium in some individuals makes this area potentially vulnerable to damage inflicted by ablation strategies. A borderzone between the LA body and the LA appendage is hypothesized to be the left part of the embryonic sinus venosus.

Introduction

During development, the common PV becomes myocardialized whereafter it is incorporated into the posterior LA wall giving rise to separate PV orifices[1]. The degree of incorporation is variable between individuals and only about 75% to 80% of individuals possess 4 discrete PV orifices[2,3]. Though the development and incorporation of the PVs has been studied and several histological studies of the LA and the PVs have been performed, until now, there are no extensive reports that emphasize the histological result of PV incorporation in the LA in subsequent stages in the human. It seems logical to expect characteristic venous wall tissue, consisting of an intima, media with elastin and smooth muscle cells (SMCs) in the LA after the incorporation process, but no histological veno-atrial border has been described in the literature. In the cardiology cathlab this atriovenous border and the structure of the LA and the PV have gained interest in recent years, in relation to the treatment of atrial fibrillation originating from the PV[4]. Ablation strategies, aimed at electrical isolation of the PV from the LA are often targeted at the left atrial tissue just outside the atriovenous junction to avoid the occurrence of PV stenosis by damaging the vulnerable PV vessel wall[5]. The present study investigates the histological outcome of the incorporation process of the PVs in the LA during subsequent stages of development.

Methods

Normal hearts of 16 human embryos and fetuses, 1 neonate and 5 adults were studied. The embryos and fetuses, obtained by legal or spontaneous abortion, ranged from 7 to 22 weeks (crown-rump lengths from 19 to 170 mm). The studies were approved by the local medical-ethical committee. The neonate had died during delivery as a result of asphyxia. Adult hearts were obtained from human bodies used in anatomy teaching lessons. The cause of death was unknown due to privacy regulations. Preservation of the bodies was performed by injection of embalming fluid in the femoral artery, consisting of 36% formaldehyde with a mixture of ethanol, glycerine,

phenol, K_2SO_4, Na_2SO_4, $NaHCO_3$ and Na_2SO_3. The fetal and neonatal specimens were fixed in a 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.2).

Samples/sectioning of material. Embryonic hearts were embedded in paraffin and serially sectioned transversely (5-10 µm). From the neonatal and adult hearts, cross-sectional samples were taken from all PVs, the posterior and anterior LA wall, and the LA appendage. Samples taken from the posterior and anterior right atrial wall and the right atrial appendage served as reference. Samples were embedded in paraffin and sectioned (5 µm) using a Leica microtome. Sections were mounted onto slides using chicken albumine dissolved in glycerine (1:1) and dried at 37º C for at least 24 hours.

Staining. Standard histological stainings were performed with Haematoxilin-Eosin (HE), Elastic Van Gieson (EvG) or elastic Resorcin-Fuchsin (RF) to detect elastic filaments, and Sirius Red to demonstrate collagen fibers [6]. Furthermore, sections were stained immunohistochemically with the pan-muscle actin antibody HHF35 (1:500, Dako, M0635), to study myocardial structure, 1A4 (1:3000, Sigma Aldrich, Product No. A2547) against alpha-smooth muscle (SM) actin, and an antibody against atrial myosin light chain (MLC2a, 1:2000, a generous gift of Steve Kubalak), specific for atrial myocardium. Staining procedures were performed as described previously[7,8].

Results

Embryonic stage: *Seven to 7.5 weeks of gestation, crown-rump length 19 to 24 mm:* Proximal to the heart, 4 PVs drained via a common PV into the smooth-walled segment of the LA (Fig.1a), also called the LA body, that could already be distinguished from the trabeculated LA appendage. The wall of both the intra- and extrapulmonary parts of the veins consisted of 1-3 layers of alpha-SM actin expressing SMCs around the endothelium (Fig.1b,e). The extrapulmonary part of the PVs being continuous with the dorsal wall of the LA were encircled by an additional layer of MLC-positive myocardial cells (Fig.1c,d). In the wall of the intrapulmonary part of the veins myocardial cells were absent (Fig.1f). The LA wall does not express smooth muscle cell actin at this stage.

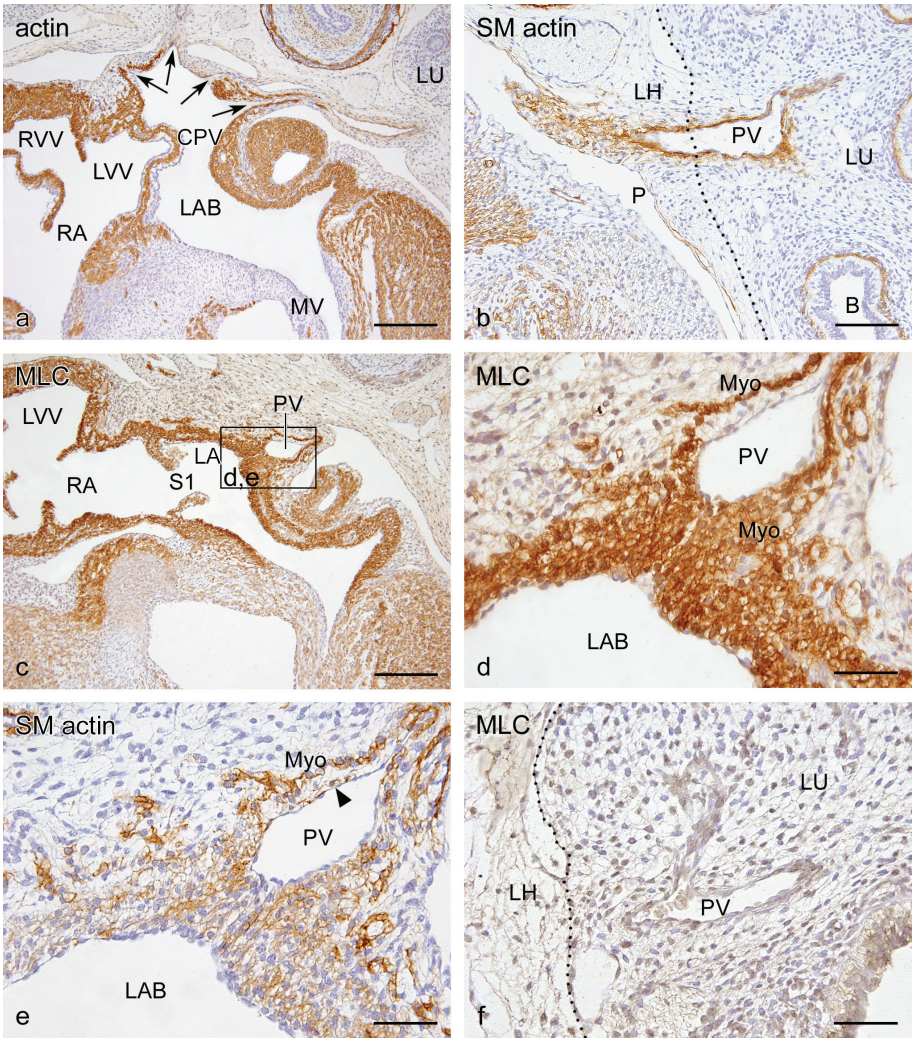
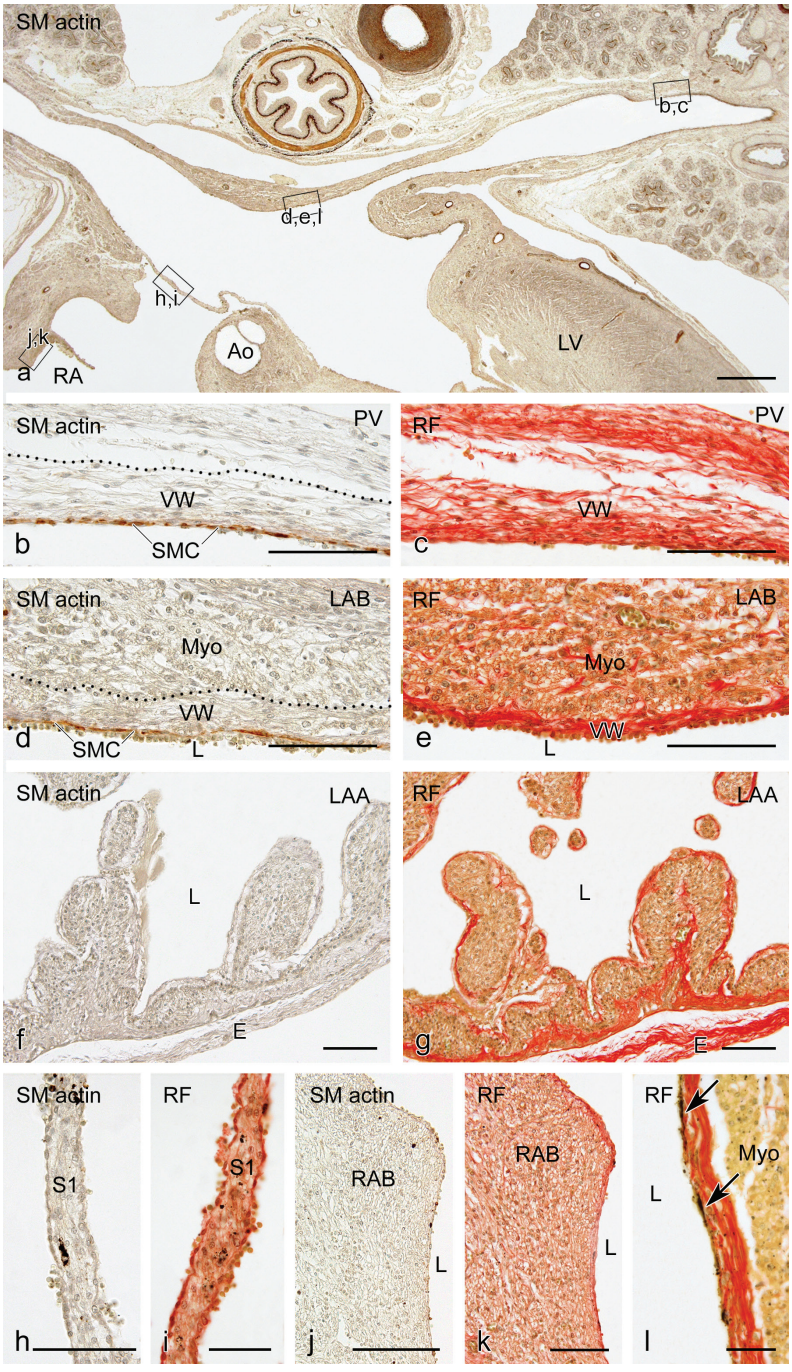


Figure 1a-f

Sections of a 54 days old human embryo stained with HHF35 for muscle actins (a; **actin**), alpha-smooth muscle actin (b,e; **SM actin**), atrial myosin light chain (c,d,f; **MLC**), showing the differentiation of the pulmonary veins (PVs) and their entrance in the left atrial body (**LAB**). a. Four PVs (arrows) drain via one common pulmonary vein (**CPV**) into the LAB. The wall of both intra- and extrapulmonary PVs consists of 1-3 layers of smooth muscle cells (SMC) (b), while an additional layer of actin and MLC positive myocardium (**Myo**) only encircles the extrapulmonary part of the vessel wall (c-f). Consecutive sections in c, d and e demonstrate that part of the MLC-positive myocardial cells surrounding the smooth muscle cells of the PVs (**Myo**) are also 1A4-positive, which is related to early differentiating myocardial cells[21]. Beneath these myocardial cells a real SMC layer develops (e; arrow head). Bronchus (**B**), Intra/extrapulmonary borderline (----), lung (**Lu**), left venous valve (**LVV**), lung hilum (**LH**), mitral valve (**MV**), pericardial cavity (**P**), right atrium (**RA**), right venous valve (**RVV**), septum primum (**S1**). Scale bar = 200 µm in a,c; 100 µm in b,d-f.

Fetal stages: *Ten to 22 weeks' gestation, crown-rump length 42 to 170 mm:* As incorporation of the common PV had progressed, in all but 2 fetuses there were 2 separate right pulmonary venous orifices, while 2 left PVs drained via one common left PV into the LA. At 16 weeks, in 2 fetuses (out of 5) four separate PV ostia were observed. From the lung hilum to the heart, the PVs were completely covered by myocardium. Subendothelially, the wall of the PVs consisted of 1-3 layers of SMCs embedded within a collagenous matrix (Fig.2a-c). This layer extended from the intrapulmonary veins to the smooth-walled LA body (Fig.2d,e). A thin layer of collagen was also found in the LA appendage, atrial septum and in the complete right atrium, but no SMCs were observed (Fig.2f-k). The myocardial layer around the PVs had thickened as well as the subendothelial collagenous vessel wall with SMCs in the PVs and the LA body. The presence of vascular SMCs in the heart was restricted to the LA body. At 21 weeks, the first indication of elastic fiber formation was observed within this layer (Fig.2l).

Figure 2a-l
Sections of a 15.5 week old human embryo. Boxes in Fig.a indicate the position of the enlargements in b-k. RF-sirius red stained sections in c, e, g, i, k and l are adjacent sections of Fig.a, b, d, f, h and j, that are stained for alpha-SM actin. Collagen fibers are red and elastic fibers are black in the RF stained sections.
a-c. Smooth muscle cells (SMCs) have differentiated within the subendothelial collagenous matrix of the pulmonary vessel wall (**VW**). d,e. This differentiating vessel wall extends into the smooth-walled left atrial body (**LAB**). Although the left atrial appendage (**LAA**), the septum primum (**S1**), and the right atrial body (**RAB**) have a thin subendothelial collagen matrix (g, i, k) no SMCs are found (f, h, j). l. At 21 weeks elastin is deposited (arrows) in the differentiating venous vessel wall in the LAB. Aorta (**Ao**), epicardium (**E**), external borderline (------) of the VW, left ventricle (**LV**), lumen (**L**), myocardium (**Myo**). Scale bar = 500 μm in a; 100 μm in b-h,k,l; 50μm in i; 200 μm in j.


SM actin
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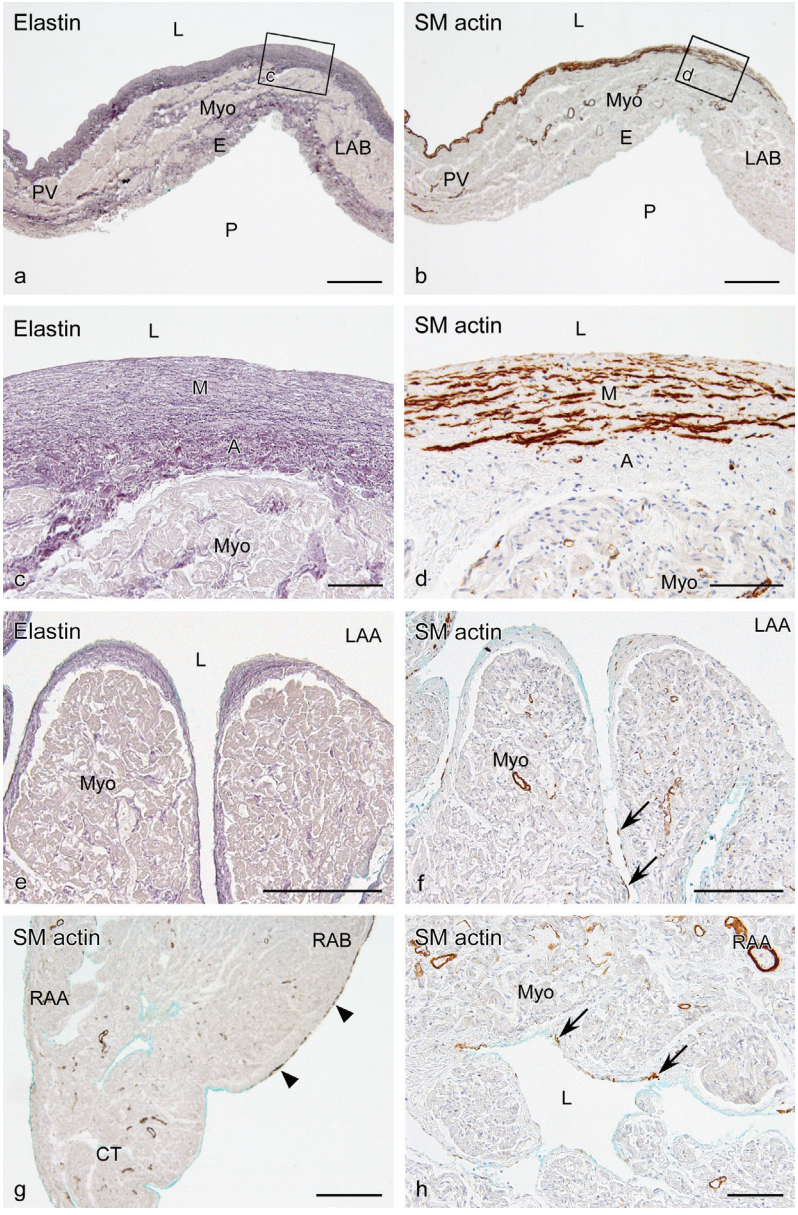
Neonatal stage: *Pulmonary veins and left atrial body:* As in previous stages there were 2 separate right PV ostia and one common left PV ostium. At the transition of the PV ostia and LA body no histological veno-atrial demarcation was found (Fig.3a,b). In both the PVs and the LA body the following layers could be distinguished: an inner venous vessel wall with an endothelium, several medial layers of SMCs with longitudinally oriented elastic fibers, and an adventitial layer with randomly distributed elastic fibers with fibroblasts and vasa vasorum. These vessel wall structures were covered by a myocardial layer and an epicardium on the outside (Fig.3a-d).

Left atrial appendage: In the trabeculated LA appendage the inner surface consisted of an endocardium with a thin subendocardial sheet consisting of well-organized collagen and elastic fibers, but without well-organized layers of alpha-SM actin positive cells. Only a few scattered actin positive cells were found. Characteristic vessel wall layers as seen in the body of the atrium, were lacking (Fig.3e,f). At the outer side, a thick layer of myocardium covered by an epicardium was present.

Right atrium: The right atrial body or sinus venarum consisted of smooth-walled myocardium. The inner side of the wall near the systemic veins consisted of one layer of SMCs. This layer did not represent a proper tunica media as seen in the systemic veins which had a well organized vessel wall with five layers of SMCs. At the junction of the terminal crest with the right atrial body, this smooth muscle layer disappeared (Fig.3g). Within the trabeculated right atrial appendage, only scattered subendocardial SMCs were observed (Fig.3h).

Figure 3a-h
Sections of various segments of a human neonatal heart. a-d At the transition of the PV and LA body no histological veno-atrial demarcation was found. The wall consisted of an endothelium/endocardium, a medial layer (**M**) with smooth muscle cells (SMCs) and elastic fibers, an adventitial layer (**A**) with fibroblasts and elastic fibers, a sheet of myocardium (**Myo**) and an outer epicardium (**E**). e,f In the left atrial appendage (**LAA**) no organized vessel wall was present. Only a subendothelial layer of elastin (e) and isolated SMCs (arrows in f) was present. In the right atrium only one layer of SMCs, not characteristic for a tunica media, could be detected in the right atrial body (arrow heads in g) near the entrance of the caval veins. The histology of the right atrial appendage (**RAA**) is comparable with the LAA with only isolated SMCs (arrows; h). Terminal crest (**CT**), Lumen (**L**), pericardial cavity (**P**). Scale bar = 500 µm in a,b,g; 100 µm in c-f; 200 µm in h.



Adult stage: *Pulmonary veins and left atrial body:* The PVs of 4 adult hearts had discrete PV orifices. In one heart a common left PV orifice was still present. In 2 hearts, an early branching pattern of the right inferior PV was observed. The outer side of

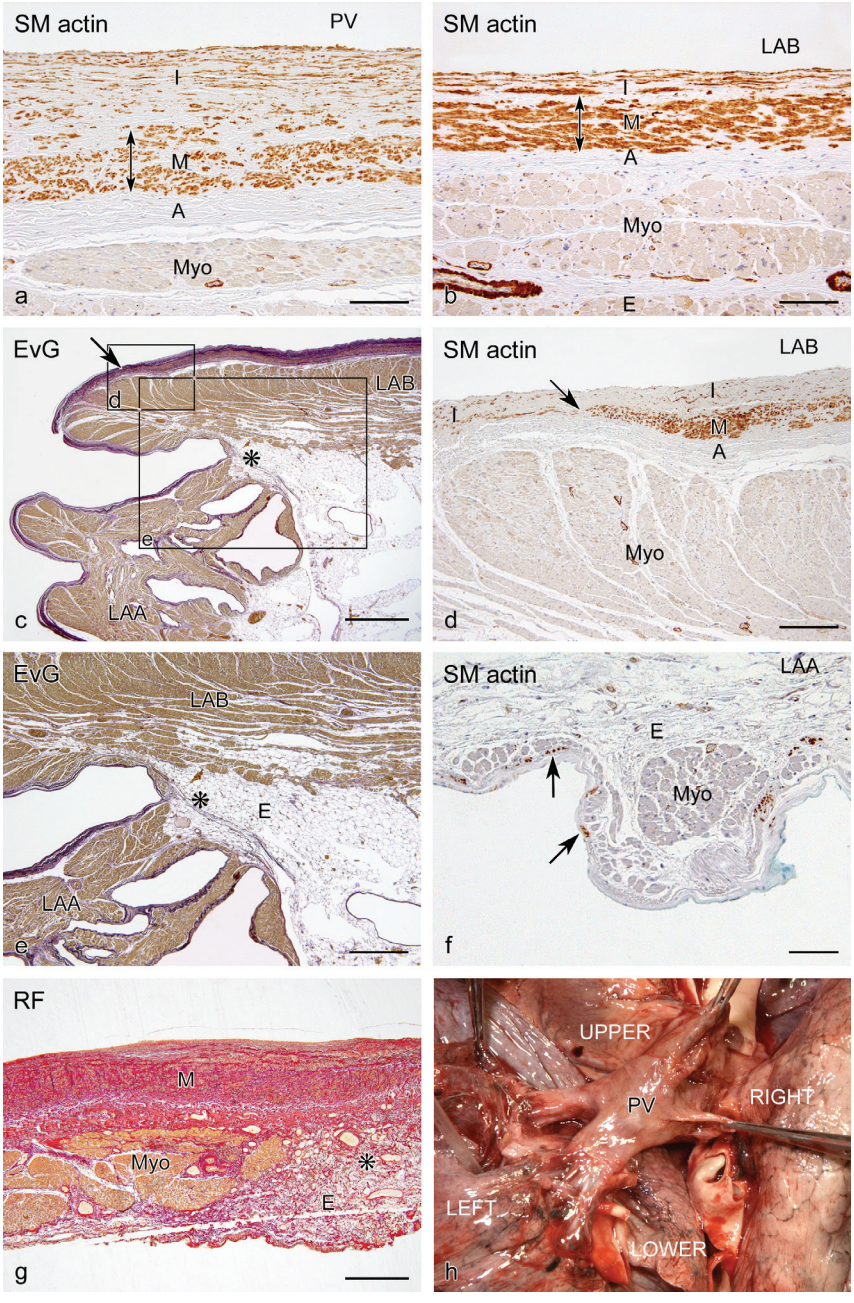
the wall of the LA body consisted of a non-trabeculated myocardial layer. Around the outside of the PVs this myocardium formed a circular or spirally arranged muscular sleeve. The sleeves tended to be thicker and more complete near the junction with the posterior LA body, but tapered more distally. In the PVs, a characteristic vessel wall was found consisting of an intimal layer and a medial layer with SMCs (Fig.4a). In the wall of the entire LA body, in addition to the outer myocardial layer, a prominent vessel wall layer being continuous with the PV vessel wall was present (Fig.4b). In between the media and myocardium an adventitial layer with fibroblasts (not expressing SM actin) was found (Fig.4a,b). In contrast to the neonatal stage, intimal thickening was observed in the PVs and the LA body, which was most profound in the PVs (Fig.4a,b). At the entry of the PVs into the LA, no histological demarcation was found. Interestingly, at the junction of the LA body with the LA appendage, a borderzone with distinctive tissue was identified (Fig.4c-e). The inner surface of this area lacked trabeculation. In contrast to the remaining part of the LA body and the PVs, the characteristic tunica media was lacking in this borderzone (Fig.4d,e). Another finding was that the myocardium at the transition of the LA body and the trabeculated LA appendage was either very thin or absent (Fig.4c,e). Moreover, in 2 of the adult hearts examined, the myocardium covering the LA body in between the confluence of the 4 PVs was discontinuous thus allowing the epicardium to line the vessel wall of the PVs (Fig.4g,h). These areas of discontinuous myocardium were only several millimeters wide. It should, however, be noted that in these areas (partly) isolated bundles of myocytes were observed.

Figure 4a-h

Adult stage sections stained for alpha-smooth muscle actin (a,b,d,f; **SM actin**), Elastic Van Gieson (c,e; **EvG**) and Resorcin-Fuchsin (g; **RF**). (h) is a macroscopical picture of the area in between the confluence of the PVs where discontinuous myocardium is found as shown in (g). Boxes in (c) indicate the position of the enlargements in (d,e). In EvG stained sections collagen fibers are purple and elastic fibers are black, in RF stained sections collagen fibers are red and elastic fibers are black.

a,b. In the pulmonary veins (**PV**) as well as in the left atrial body (**LAB**) an identical and characteristic vessel wall is found consisting of an intimal layer (**I**), a medial layer (**M;** double arrow) with smooth muscle cells (SMCs) and elastic lamellae, and an adventitial layer (**A**) in between the media and myocardium (**Myo**). In contrast to the neonatal stage, a profound intimal thickening is observed, most profound in the PVs. At the junction of the LAB with the left atrial appendage (**LAA**) a borderzone with distinctive tissue is identified (c,d; arrow). The inner surface of this area lacks trabeculation and vessel wall tissue (d). The myocardium at the transition between LAB and LAA is very thin or absent, facing the epicardium (c,e: asterisk). f. On the inner surface of the LAA occasional SMCs are found (arrows) but a vessel wall component is lacking.

g,h. In between the confluence of the four PVs, in two adults, the myocardium (**Myo**) is discontinuous or absent (asterisk in g). Epicardium (**E**). Scale bar = 100 μm in a,b,f; 1mm in c; 200 μm in d; 500 μm in e,g.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Atrial appendages: The wall of the trabeculated LA appendage differed from the PVs and LA body in that it did not contain a vessel wall component. The inner surface consisted of a thin subendocardial collagenous layer with some elastic fibers and an occasional SMC (Fig.4f). The histology of the LA appendage was similar to that of the right atrial appendage (Fig.5a,b).

Right atrial body: The non-trabeculated sinus venarum of the right atrium consisted at the inside of a thin subendocardial collagenous layer covered by myocardium. No characteristics of vessel wall structures were present at this site (Fig.5c,d).

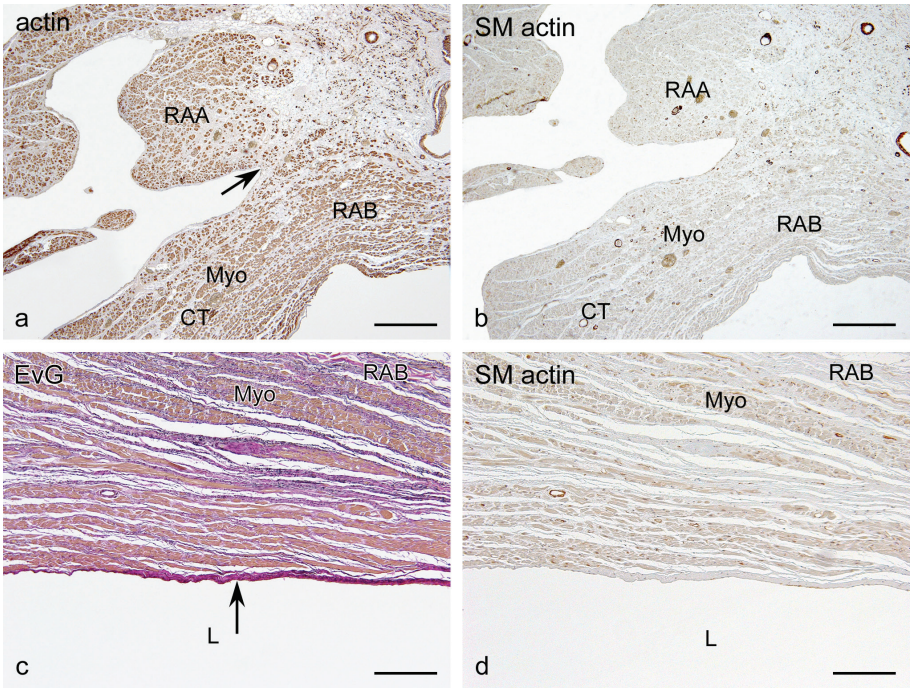

Figure 5a-d
Adult stage sections stained with HHF35 for muscle actins (a; **actin**), alpha-smooth muscle actin (b,d-f; **SM actin**) and Elastic Van Gieson (c; **EvG**). (b) and (d) are adjacent sections of (a) and (c).
a,b. At the transition of the smooth-walled right atrial body (**RAB**) to the trabeculated right atrial appendage (**RAA**), the myocardial layer (**Myo**) is very thin (a; arrow) comparable to the left side. In the RAA, vessel wall tissue is absent (b) which is comparable to the left atrial appendage. c,d. The right atrial body (**RAB**) consists at the inner side of a thin subendocardial layer (c; arrow), covered by myocardium (**Myo**). No characteristics of vessel wall tissue were present at this site (d). Terminal crest (**CT**), lumen (**L**). Scale bar = 500 µm in a,b; 200 µm in c-d.

Discussion

In this study, the histological outcome of the incorporation of the PVs into the LA was described in subsequent developmental stages. Our study demonstrates that, based on histological criteria, 3 different compartments can be distinguished in the LA (schematically demonstrated in Fig.6): the smooth-walled LA body with vessel wall tissue (i.e. incorporated PV tissue); the trabeculated LA appendage, without vessel wall tissue; and a transitional zone in between the LA appendage and the LA body, which is smooth-walled and lacks vessel wall tissue. The right atrial appendage, the body of the right atrium and the atrial septum are not lined by vessel wall tissue. The borderzone between the LA appendage and the LA body histologically resembles the smooth-walled sinus venarum (body) of the right atrium.

The origin of the PVs is an issue of controversy. Based on findings using the marker HNK-1, several authors have described that the myocardium surrounding the primitive PV in the LA is continuous with the sinus venosus in the right atrium[9-11]. However, other authors have disputed this relation[12]. These discrepancies depend largely on different application of definitions (especially how to define the sinu-atrial transition and the sinus venosus), and on different interpretation of possibly similar observations. Regardless of these differences, most authors seem to agree that the myocardium surrounding the primitive PV possesses different characteristics and can be differentiated from the working myocardium of the LA appendage. The aim of the current study was not to provide the answer for the origin of the primitive PV. However, based on the observation of a zone of myocardium at the transition of the LA appendage with the LA body that histologically resembles the sinus venosus in the right atrium, we hypothesize that during incorporation of the PV and the surrounding myocardium into the LA, the contribution of vessel wall to the body of the LA increases, which may reduce the area of sinus venosus myocardium to a small zone encircling the entrance to the LA appendage. An interesting finding was the very thin or even absent myocardium at this junction zone between the LA body and the LA appendage; within this zone slow conduction or conduction block may occur that are prerequisites for the induction of reentry[13].

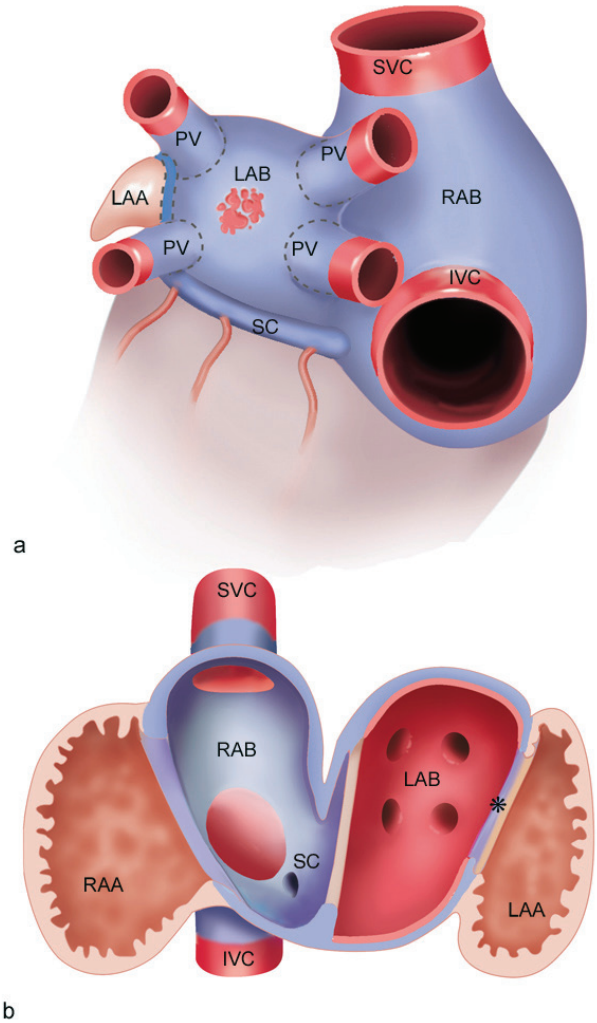

Figure 6a,b

a. Schematic depiction of the outer side of the atrial chambers with the pulmonary veins (**PV**) and systemic veins. The left atrial body (**LAB**) and right atrial body (**RAB**) are covered by myocardium with a smooth-walled inner aspect (**blue**), which stretches out over the extracardiac segments of the PV and over a small peripheral part of the systemic veins (**blue area above and below dotted line**). The left atrial appendage (**LAA**) and right atrial appendage (**RAA**) consist of trabeculated myocardium (**brown**). A zone of smooth-walled myocardium that lacks characteristics of vessel wall tissue is found at the junction of the LAB to the LAA (**blue between dotted lines**). b. Schematic depiction of the tissue types found in the left and right atria seen from the inside of the atria. Vessel wall tissue (**red**), myocardial tissue with a smooth-walled inner aspect (**blue**), primary atrial segment tissue (**brown**), transitional zone (**asterisk**) which is smooth-walled, lacking vessel wall tissue (hypothesized to be left-sided sinus venosus tissue). Coronary sinus (**SC**), inferior caval vein (**IVC**), superior caval vein (**SVC**).

At early stages, the wall of the PVs consisted of a mixture of extrapericardially differentiated myocardial cells and SMCs. These SMCs proliferated and differentiated into an inner venous vessel wall. In the neonate and the adult, due to the incorporation process of the PVs, vessel wall tissue and extrapericardially differentiated myocardium were not only present in the PVs, but also in the LA body. It is currently unclear whether the outer circular or spirally arranged myocardial sleeves are formed due to extracardial mesenchymal triggers as is strongly supported by studies of Kruithof et al.[14,15], or are a direct continuation of the LA myocardium as supposed by Millino in the mouse[16]. Our study demonstrates that the myocardium and venous wall differentiate simultaneously, but does not support either a cardiac or an extracardiac origin.

In the adult PVs and the LA body, beneath the endothelium/endocardium a prominent fibroelastic subendothelial layer was observed, which extended to a lesser extent in the right and left appendages and the atrial septum. Interestingly, in the neonatal stage, only a very thin subendothelial layer was observed, raising the possibility of an aging reaction to different stimuli e.g. continuous shear stress. In congenital heart diseases causing persistent and increased wall tension on the ventricles e.g. aortic stenosis or atresia and hypoplastic left heart, a comparable intimal thickening, so-called fibroelastosis, can be found[17]. However, as all our adult hearts showed this intimal thickening, it is debatable whether these findings represent a true form of fibroelastosis or are part of normal aging. Reactive intimal thickening in the PVs, leading to PV stenosis, can be seen after ablative therapy for arrhythmias, which suggests that the intimal layer is not only susceptible to internal but also to external stimuli[18]. Especially in this context, the results of this study can be of importance since it demonstrates that the LA body contains areas of discontinuous myocardium and areas without myocardium, e.g. in between the PV orifices, that might be the electrophysiological substrate of arrhythmias[13]. An increased susceptibility for arrhythmias may be explained by a difference in the composition of the extracellular matrix, the cellular junctions or the phenotype of these extrapericardially differentiated myocardial cells with regard to the intrapericardially differentiated myocardial cells of the sinus venosus and the atrial segment. These arrhythmias are amenable to catheter ablation but one must be alert that the vessel wall can be easily damaged due to a lack of myocardium. These considerations are specifically

important when performing ablation at the LA dorsal wall, that is in close contact to the esophagus. Recent studies have emphasized the risk of atrio-esophageal fistula due to the application of radiofrequency current at this site[19,20]. Lower dosage of radiofrequency current is therefore recommended when ablating in this area.

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