General Introduction
The interest in pulmonary vein development has rapidly increased since the role of the pulmonary venous myocardial sleeve in the foundation of potential atrial arrhythmias\textsuperscript{1,2} and the vulnerability for potential pathogenic processes\textsuperscript{3} has become more and more clear. A more detailed understanding of normal and abnormal pulmonary vein development requires comprehension of general cardiac development, since the so-called heart fields, that play a very important role in the formation of the complete heart, play an essential role in the composition and differentiation of the pulmonary venous wall as well\textsuperscript{4}. Abnormalities in this (vessel) wall composition as well as the presence and re-activation of embryonic cardiac conduction system cells can be responsible for the onset of arrhythmias\textsuperscript{1}. Besides that, abnormal pulmonary vein development, leading to abnormal pulmonary vein anatomy, may be held responsible for a lot of clinical entities.

**Cardiac development**

The primary heart tube develops from two parallel cardiogenic plates located in the anterior splanchnic mesoderm. After fusion of these crescent shaped cardiogenic plates in the midline, a primary myocardial heart tube is formed, cranially connected to the pharyngeal arches and caudally to the omphalomesenteric veins. The myocardial tube is lined on the inside by cardiac jelly (later on replaced by the endocardial cushions) and endocardial cells that are continuous with the endothelium of the embryonic vascular plexus\textsuperscript{5,6}. This primary heart tube has an asymmetric shape as a consequence of predetermined left-right patterning and cardiac looping\textsuperscript{6-8}. Cardiac differentiation into future atrial and ventricular components already takes place in the cardiogenic plate stage, based on expression of cardiac-specific genes like Nkx2.5\textsuperscript{8} and GATA4-6\textsuperscript{8}. In normal development, the heart tube loops to the right, resulting in a position of the outflow tract frontal to the atria and wedging of the aorta in between the atrioventricular orifices. During heart development, cardiac chambers and transitional zones are distinguished, the latter being involved in cardiac septation, valve formation and development of the cardiac conduction system. There has been much debate about which heart segments are discernable in the primitive heart tube, but most data point out that it is initially composed of a small atrial segment,
an atrio-ventricular canal and a primitive left ventricle\textsuperscript{5,6}. The splanchnic mesoderm forming the primary heart tube is referred to as the \textbf{first heart field} or first lineage, and has the potential to generate differentiation into myocardial cells\textsuperscript{10,11}. To this primary myocardium, new myocardium is secondarily contributed (second lineage), recruited from the so-called \textbf{second heart field}, located in the splanchnic mesoderm dorsal to the primary tube (Fig.1)\textsuperscript{12}. Islet 1 (Isl 1), a marker of undifferentiated cardiac progenitor cells, and inhibitor of DNA binding 2 (Id2) have shown that major parts of the arterial as well as the venous pole of the heart are derived from this second heart field\textsuperscript{13,14}. Therefore, to simplify understanding of terms, processes at the arterial pole (outflow tract) and venous pole (inflow tract) will be described separately.

\textbf{Myocardial recruitment at the arterial pole}

Several studies have been performed using different lineage markers such as fibroblast growth factor (Fgf)\textsuperscript{8} and 10\textsuperscript{15}, Islet (Isl)\textsuperscript{13} and Tbx1\textsuperscript{16} to trace these cells into their cardiac destination. As a result of this, we know that undifferentiated mesoderm, recruited from the \textbf{anterior heart field}, is responsible for addition of myocardium of the complete right ventricle and the proximal arterial outflow tract\textsuperscript{12,17}, whereas the \textbf{secondary heart field}, being the distal part of the anterior heart field, forms the distal outflow tract\textsuperscript{18}. Both the anterior heart field and the secondary heart field are part of the second heart field (Fig.1).

An extracardiac cell type contributive to the formation of the definitive heart at the arterial pole is the neural crest cell, derived from the crest of the neural tube\textsuperscript{18,19}. At the arterial pole, these cells have an active role in the formation of the pharyngeal arch arteries and, to a lesser extent, in the septation of the outflow tract (Fig.1), since neural crest ablation has lead to cardiac outflow tract malformations\textsuperscript{19}. Therefore, it is not surprising that a good interaction of both second heart field and neural crest cells is a prerequisite for the normal outflow tract development.

Finally, besides genetic factors being the cause of cardiac anomalies, also epigenetic (environmental) factors can play a role in remodeling of the outflow tract, as seen in diabetes\textsuperscript{20,21}, hyperhomocysteinemia\textsuperscript{22} and shear stress\textsuperscript{23}. 

Figure 1. Schematic representation of the first and second heart field contribution to cardiac development. The primary heart tube, which contains the left ventricle (LV), the atroventricular canal (AVC) and parts of the atria, is derived from the Isl-1 negative precursors of the first heart field and is depicted in brown. The secondary added myocardium, derived from Isl-1 positive precursors of the second heart field, is depicted in yellow. The second heart field is divided into a secondary heart field, that contributes to the distal outflow tract (DOT) and an anterior heart field that contributes to the right ventricle (RV) and the proximal outflow tract (POT) at the arterial pole of the heart. At the venous pole of the heart, myocardium is added from the posterior heart field, which contributes to the posterior wall of the atria and the interatrial septum, the dorsal mesenchymal protrusion (DMP), the sinoatrial node (SAN), the myocardium of the sinus venosus (SV), the pulmonary veins (PV), and the cardinal veins (CV), including the coronary sinus (CS), and probably part of the central conduction system (CCS). The posterior heart field also contributes to the proepicardial organ (PEO), which is the source of the epicardium and epicardium derived cells. Cardiac neural crest cells (dark blue) migrate to the heart and enter the heart both at the arterial and venous pole. Inflow tract (IFT); ganglia (ggl); outflow tract (OFT); pharyngeal arch arteries (PAA). Adapted from Gittenberger-de Groot et al.\textsuperscript{6}. 

General Introduction
Myocardial recruitment at the venous pole

Analogous to the arterial pole, there has been special interest for markers specific for recruitment of myocardium from the second heart field to the venous pole of the heart, such as Pitx2c<sup>24</sup>, Nkx2.5<sup>24,25</sup>, Tbx18<sup>26</sup>, Shox2<sup>27</sup> and podoplanin<sup>25,28</sup>. This region of the second heart field, located posteriorly to the primary heart tube, is involved in the addition of myocardium to the sinus venosus area, including the sino-atrial node, the pulmonary veins, the cardinal veins and the coronary sinus, as well as the main body of the atria, and is referred to as the posterior heart field (Fig.1).

Moreover, at the venous pole, two other extracardiac cell types play a role in the formation of the definitive heart structures. In the first place, neural crest cells that migrate into the inflow tract<sup>29</sup>, are important for the development of the sympathetic and parasympathetic ganglia<sup>30</sup>, and possibly are the indication of the atrioventricular conduction system<sup>31</sup>. Secondly, epicardial cells that are derived from the coelomic epithelial lining (coelomic mesothelium) of the posterior heart field, forming the proepicardial organ (PEO)<sup>32-34</sup> (Fig.1). Cells derived from this PEO grow out over the myocardial heart tube<sup>35</sup> and undergo epithelial-to-mesenchymal transformation (EMT). The so-called epicardium-derived cells (EPDC’s)<sup>36-38</sup> migrate into the atrial and ventricular myocardium and are essential for the formation of compact myocardium<sup>39</sup>, the atrioventricular valves<sup>36</sup>, the main coronary arteries<sup>40,41</sup> and differentiation of the Purkinje network<sup>42</sup>. Moreover, they might play a role in the pathogenesis of endocardial fibroelastosis.
**General pulmonary vein development**

During the fourth week of embryonic development, two primary lung buds can be discriminated at the caudal end of the respiratory diverticulum, that develop in the surrounding splanchnic mesenchyme. Subsequently, these lung buds differentiate into the bronchi and their bifurcations in the future lungs. The cartilaginous plates, the bronchial smooth muscle and connective tissue as well as the pulmonary connective tissue and capillaries are derived from the splanchnic mesenchyme, that forms a plexus of veins, by which the primitive lungs drain to the systemic circulation. This splanchnic plexus is also connected to the endocardium of the primitive heart tube by means of a strand of endothelial cells, situated in the mesocardium behind the heart, which is the anlage of the future common pulmonary vein (Fig.2). When this strand lumenizes, initially, the lungs can drain centrally, to the atrial part of the heart, as well as peripherally, to the systemic veins. After atrial septation, the common pulmonary vein drains to the left atrium and starts to grow. The pulmonary-to-systemic venous connections are not necessary anymore and regress. Abnormal development of the common pulmonary vein might be related to persistence of the primitive pulmonary-to-systemic connections which is the likely substrate for anomalous pulmonary venous connection.
Figure 2. Schematic depiction of pulmonary vein development.

a,b. Lateral (a) and frontal (b) view. The splanchnic vascular network surrounds the two lung buds (LB) and drains to the systemic circulation by means of primitive pulmonary-to-systemic connections to the right and left cardinal veins (RCV/LCV) or the right and left umbilical and vitelline veins (RUVV/LUVV). In the region of the heart, this splanchnic plexus (SP) is connected to the sinus venosus segment of the heart (SV, blue) by means of the midpharyngeal endothelial strand (MPES), which is the remaining part of a strand of endothelial cells that initially runs from the arterial to the venous pole in the dorsal mesocardium.

c. When the endothelial strand, which is the anlage of the future common pulmonary vein (CPV) lumenizes, the splanchnic plexus can drain its blood to the systemic circulation as well as to the heart. Although atrial septation has started, the CPV still drains to the sinus venosus segment that is connected to a common atrium.

d. The CPV grows and dilates, becoming the main route for drainage of pulmonary venous blood. The primitive pulmonary-to-systemic connections are not necessary anymore and regress. After atrial septation has completed (IAS), the part of the sinus venosus containing the CPV is placed to the left, becoming part of the left atrium (LA).

e. The CPV has bifurcated, so that the right and left lung (RL/LL) drain to the heart by means of two left and two right pulmonary veins. The right cardinal vein becomes the superior caval vein (SCV) and the left cardinal vein gives rise to the coronary sinus (CS), both connecting to the sinus venosus part of the right atrium (RA).

f,g. By incorporation of the CPV, initially, one right and one left CPV drain to the LA (f). After incorporation has completed, four separate pulmonary vein (PV) ostia can be identified on the inner side of the LA (g). The extracardiac part of the LCV regresses, becoming the so-called ligament of Marshall.

Development of the cardiac conduction system

In human embryos peristaltic contractions of the heart tube can be observed from the age of 3 weeks of development. After the heart has developed into its definitive segments, the myocardium differentiates either into working myocardium or in conducting myocardium, in which process neural crest cells as wells as EDPC’s play an inductive role. According to the ballooning model, the working myocardium of the atria and the ventricles differentiates from the outer curvatures of the looping heart tube, whereas the conducting myocardium might originate from the inner heart curvature.
The conduction system can be divided into a central conduction system and a peripheral Purkinje network. The central conduction system has a sino-atrial and an atrioventricular component connected by the atrioventricular node. Studies using the antibody against HNK-1 and the transgenic CCS-LacZ mouse strain have described the developing cardiac conduction system in mice and men\textsuperscript{48-50} and show that there are two main transitional zones contributing to the central conduction system: the sinus venosus myocardium including the sino-atrial transition (or ring) and the primary fold (or ring) between the ventricular segments. The sino-atrial ring includes the left and right venous valves, the septum spurium, the myocardium surrounding the coronary sinus and the pulmonary veins and the interatrial Bachmann's bundle\textsuperscript{51}. Three internodal pathways, derived from the sino-atrial ring, connect the sino-atrial (SA) node with the atrioventricular (AV) node (Fig.3). The first pathway, represented by the fused venous valves or septum spurium, runs anterosuperiorly and connects to the transient anterior AV node, located to the anterior side of the right atrioventricular ring (rAVR), posterior to the aortic root. The second pathway runs laterally in the base of the right venous valve (the later crista terminalis of the right atrium) and connects together with the third pathway, running within the left venous valve through the base of the atrial septum, to the posterior AV node. Whether these anterior and posterior AV node anlagen fuse or whether the regular AV node solely consists of the embryonic posterior AV node is not completely clear\textsuperscript{5}, but the functional AV node connects to the His bundle and the bundle branches. The atrioventricular conduction system is mainly derived from the primary ring, which encircles the aortic orifice and the right atrioventricular canal, thus forming the rAVR. (Fig.3). It was shown that the right ventricular moderator band is also continuous with the rAVR\textsuperscript{51}.

In the normal heart, only the SA node, the AV node, the His bundle and both bundle branches remain functional, while the internodal pathways, the embryonic conduction tissue surrounding the coronary sinus and the pulmonary veins and the rAVR disappear\textsuperscript{5}. Therefore, discrimination between the developmental conduction system and the functional conduction system is essential since rhythm disturbances can be explained from the embryonic disposition of the conduction system as a result of dedifferentiation of embryonic cells and reinitiation of conduction system capacities\textsuperscript{51}. 18
Figure 3. Embryonic parts of the conduction system derived from the sinoatrial ring (green) and the primary fold (yellow). The sinoatrial ring includes the right venous valve (RVV), the left venous valve (LVV), the septum spurium (SS), the myocardium surrounding the coronary sinus and the pulmonary veins (PV) and Bachmann’s bundle, being part of the left atrioventricular ring, running posterior to the aorta, in the roof of the left atrium (LA) to the right atrium (RA). The tissue of the primary fold or ring encircles the right atrioventricular canal (forming the right atrioventricular ring), and the aortic orifice. The definitive conduction system (depicted in red) consists of the sinoatrial node (SAN), the atrioventricular node (AVN), the common atrioventricular bundle (CB), the left bundle branch (LBB), and the right bundle branch (RBB). The parts of the conduction system depicted in yellow and green are supposed to disappear. It remains to be investigated if the anterior atrioventricular node (aAVN), which is connected to the SAN by the anterior internodal pathway running in the SS, becomes part of the regular AVN or solely features as an embryonic structure in addition to persisting in some cardiac malformations. Neural crest cells (NCC) can be found in proximity to structures of the cardiac conduction system, both as precursors of the autonomic neuronal network (depicted in dark blue) and after apoptosis as possible inducing cells (depicted in shaded blue).

Aorta (Ao); epicardial derived cell (EPDC); left ventricle (LV); pulmonary trunk (PT); right ventricle (RV).

Modified after Gittenberger-de Groot et al.\(^6\).
Although molecular biology, by the use of transgenic animal models, has attributed to the knowledge of normal and abnormal heart development, one has to realize that congenital heart malformations mostly are the result of highly complex cascades of gene interactions combined with epigenetic factors that cannot be deduced to isolated entities.

**Aim of this thesis**

In this thesis we describe normal and abnormal pulmonary vein development in human and mouse hearts, and focus on the histo(patho)logy of the pulmonary venous and left atrial dorsal wall, in order to elucidate the role of the posterior heart field in the formation and differentiation of the pulmonary venous vessel wall and its possible consequences for the onset of arrhythmias and susceptibility for pulmonary vein stenosis. Another aim of this thesis is that the understanding of normal pulmonary vein development will contribute to the understanding of the development of abnormal pulmonary venous connections and its clinical consequences.

**Chapter outline**

In Chapter 2 the normal development of the pulmonary veins and the left atrial dorsal wall is described in embryonic, neonatal and adult human hearts, with special focus on the histological consequences of the incorporation process of the pulmonary veins into the left atrium. The findings are discussed in relation to the pathogenesis of atrial arrhythmias.

In Chapter 3 the development and differentiation of the myocardium and vascular wall of the pulmonary veins, left atrial dorsal wall and atrial septum in wild type and *podoplanin* knockout mouse embryos of embryonic stages (E) 10.5-E18.5 is described, using 3-D reconstruction and immunohistochemistry. The role of the posterior heart field myocardial marker podoplanin in this developmental process is discussed.
In Chapter 4 the histopathological findings of the pulmonary venous and left atrial dorsal wall in human neonatal hearts with total anomalous pulmonary venous connection are attributed to abnormal posterior heart field contribution to the development of the venous pole of the heart and related to the findings in Chapter three. A clinical link to the origin of pulmonary vein stenosis is made.

Chapter 5 presents a clinical report on unilateral pulmonary vein stenosis with a contralateral pulmonary varix as an example of abnormal pulmonary vein development. A pulmonary varix is a dilated, tortuous pulmonary vein, usually resulting from obstructed pulmonary vein drainage. In this report, we try to link the clinical condition to an embryologic explanatory hypothesis which may or may not be combined with environmental factors (acquired disease).

Chapter 6 is an extended general discussion which reviews normal and abnormal pulmonary vein development, including the content of the chapters two to six. It outlines the importance of the posterior heart field in the etiology of congenital heart disease and emphasizes the need for improvement of knowledge on its (epi) genetic background in the future.
Reference List


General Introduction


