The development of the venous pole of the heart
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Summary
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Congenital heart defects are the most commonly found congenital defects accounting for 25% of all birth defects. A large number of congenital heart defects originate from the venous pole of the heart, including sinus venosus defects, atrial septal defects and anomalous connections of the pulmonary veins. The venous pole region is not only a common location of anatomic malformations, but is also frequently involved in cardiac arrhythmias, like atrial fibrillation and sick sinus syndrome.

In this thesis, we focus on the development of the myocardium at the venous pole of the heart. The venous pole of the heart includes the myocardium of the atrial body, the myocardium clothing the systemic caval veins, also known as the sinus venosus myocardium, and the myocardium clothing the pulmonary veins. We provide novel insights into how the sinus venosus and pulmonary myocardium develop, from the nature and location of their progenitors in the cardiac crescent to the mechanisms of formation of the actual myocardial structures and what can go wrong during the development of these areas. The introduction, Chapter 2, shortly summarizes the development of the venous pole and discusses the possible underlying causes of abnormal electrophysiological properties in the setting of atrial fibrillation from a developmental point of view. Atrial fibrillation is the most commonly encountered cardiac arrhythmia in clinical practice and affects approximately 1% of the total population, and one-tenth of those surviving to reach the age of 80 years. Both the myocardium surrounding the caval veins and the myocardium surrounding the pulmonary veins are found to be a location of origin of atrial fibrillation, with the great majority being found to originate from the myocardial sleeves clothing the pulmonary veins. Knowledge about the development of the venous pole from the work in this thesis and literature is combined to try to understand why certain regions in the heart are the favoured sites for arrhythmogenesis.

In the next three chapters we concentrate on the development of the sinus venosus myocardium. The embryonic sinus venosus is composed of a right and left myocardial sinus horn. The right horn will form the proximal myocardial parts of the superior and inferior caval veins along with the floor of the systemic venous sinus. The left horn persists as the left-sided superior caval vein in the mouse, but becomes the coronary sinus in human.

In Chapter 3 the origin of the sinus venosus progenitors in the cardiogenic mesoderm in relation to the other cardiac progenitors is studied. The initial embryonic heart tube is formed when two bilateral cardiogenic mesodermal regions in the splanchnic mesoderm, the first heart field, differentiate into myocardium and fuse at the midline. Cells that are located medial in the cardiogenic mesoderm, called 'second heart field’ cells, will contribute to the arterial and venous pole and dorsal mesocardium after initial formation of the heart tube, causing the heart tube to elongate. In this study, using Dil-labeling and short term lineage analysis, we showed that the
sinus venosus progenitor population is localized caudal in the cardiogenic splanchnic mesoderm, lateral of all other cardiac progenitors. Due to folding of the embryo the second heart field progenitors are relocated to dorsal of the heart tube, whereas the sinus venosus progenitors become located ventral of the venous pole. Visualization of the sinus venosus and second heart field expression domains with 3D reconstructions showed that these populations remain separated during heart formation. All cardiac progenitors initially express transcription factors Isl1 and Nkx2-5. The sinus venosus progenitor population can be distinguished from the first and second heart field by the loss of expression of Isl1 and Nkx2-5 more than a day before its differentiation into myocardium.

**Chapter 4** describes the process of myocardium formation at the systemic venous pole of the heart. The myocardium initially forming the venous pole will be taken up into the atrial myocardium. Only after 9.5 days of development, after the initiation of the development of the chambers, sinus venosus progenitors differentiate into myocardium. This sinus venosus myocardium is characterized by the absence of Nkx2-5 expression. Lineage experiments showed that the sinus venosus myocardium, in contrast to the remainder of the heart, develops from Nkx2-5-negative progenitors. The sinus venosus specifically expressed Tbx18, shown to be required for normal sinus venosus development. Mice deficient for Tbx18 have a delayed formation of the sinus venosus myocardium and have defective caval veins.

The role of Nkx2-5 in the development of the sinus venosus is further explored in **Chapter 5**. Here we focus on the development of the sinus node in the sinus venosus myocardium. The initial heart tube is composed of slowly conducting myocytes that possess intrinsic pacemaker activity. Dominant pacemaker activity, however, can always be found at the caudal-most part of the venous pole, causing electrical activity and contraction to originate from the venous to the arterial pole. At the venous pole of the initial heart tube, the domain of Hcn4 expression, which is required for the pacemaker activity in murine embryos, overlaps with that of Nkx2-5. Concomitant with the formation of the Nkx2-5-negative sinus venosus caudal to the heart tube, expression of Hcn4 becomes restricted to this newly formed myocardium. Nkx2-5 knock-out and hypomorphic embryos showed that this factor is essential for the confinement of Hcn4 expression to the Nkx2-5-negative sinus venosus, and the sinus node. The sinus node develops as a thickening on the right side in the sinus venosus after E9.5 and immediately upon its formation expresses Tbx3. Tbx3 was found to suppress chamber differentiation, providing a mechanism by which the Tbx3-positive sinus node is shielded from differentiating into atrial myocardium. Pitx2c suppresses the default program for sinoatrial node formation on the left side, as Pitx2c-deficient fetuses form sinus nodes at both the right and left side.

The sinus venosus myocardium forms at the ventral caudal side of the atria, and will surround the systemic venous entrance to the heart. The other venous en-
trance to the heart, the pulmonary vein, develops a connection on the dorsal side of the atria. This connection is found in the midline of the common atrium. However, due to the formation of the atrial septum from the right pulmonary ridge, the pulmonary vein gains exclusive connection to the left atrium. This connection is clothed by a myocardial sleeve, similar to the myocardial layer surrounding the caval veins at their connection to the right atrium. In both human and mouse the pulmonary vein starts with a single connection. In mouse this single connection is maintained with long myocardial sleeves. In human, the pulmonary vein including its myocardium becomes incorporated into the left atrium. As a consequence, four pulmonary veins with short myocardial sleeves enter the left atrium. Chapter 6 focuses on the development of the myocardium surrounding the pulmonary vein. Our data indicate a biphasic model for the development of the pulmonary myocardium. First, a myocardial population forms by differentiation from the mesenchyme surrounding the proximal part of the pulmonary vein. Secondly, the pulmonary myocardium initiates a phase of rapid proliferation and expansion to form the pulmonary myocardial sleeve. Pitx2c-deficient mice did not develop a pulmonary myocardial sleeve because they seem to fail to form the initial pulmonary myocardial cells. Lineage analysis excluded contribution of atrial cells to the pulmonary myocardium. Furthermore, lineage analysis showed that whereas the sinus venosus derives from Nkx2-5-negative progenitors, the pulmonary myocardium derives from Nkx2-5-expressing progenitors, indicating a distinct origin of the two venous systems. Nkx2-5 and its target gap-junction gene Cx40 are expressed in the atria and in the pulmonary myocardium, but not in the sinus venosus, which expresses the essential pacemaker channel Hcn4. When Nkx2-5 protein levels are lowered in a hypomorphic model, the pulmonary myocardium switches to a Cx40-negative, Hcn4-positive phenotype resembling that of the sinus venosus. This switch to a pacemaker-like gene program indicates a possible role for Nkx2-5 in the development of atrial fibrillation.

Just before the pulmonary myocardium starts to develop, a mesenchymal cell population, called the ‘spina vestibuli’ or dorsal mesenchymal protrusion, develops on the right side of the developing pulmonary vein, from where it will connect to the atrioventricular cushions and contribute to the atrioventricular septal complex. In Chapter 7 we used lineage analysis to show that the spina vestibuli is the only part of the atrioventricular septal complex that does not derive from the endocardium, but from the extracardiac mesoderm. Three dimensional reconstructions visualized that this non-endocardium-derived mesenchyme does not form a visible protrusion into the atrial lumen. The morphogenesis of this area is of great clinical interest, because of the high incidence of atrial and atrioventricular septal defects.