Cardiac development : the posterior heart field and atrioventricular reentry tachycardia

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General Discussion

For several decades researchers all over the world, driven by curiosity and in their way to discover new treatment modalities for cardiovascular disease, tried to unravel unexplored processes within cardiovascular development. Although knowledge significantly increased on certain mechanisms underlying this complicated developmental process other questions automatically occurred, creating the basis for future (ongoing) research.

In this thesis we focused on normal and abnormal cardiac development in relation to congenital heart disease and supraventricular arrhythmias. In **Part I** of this thesis we described the development of the posterior heart field (PHF) derived venous pole of the heart specifically in correlation to the role of *Shox2* (**Chapter 2 and 3**)[1] and *podoplanin* (**Chapter 4 and 5**)[2,3] in that particular area. Furthermore, the developmental processes in this region seem to have an important role in the anlage of the cardiac conduction system (CCS) and epicardial lineage development of the heart. In the second part (**Part II**) the aetiology of a specific subtype of supraventricular tachycardias (SVTs) i.e. atrioventricular (AV) re-entry tachycardias (AVRTs) are related to normal heart development in mammals (**Chapter 6 and 7**).[4] Finally, **Chapter 8** demonstrates the late outcome of fetal brady- and tachyarrhythmias.[5]

PART I THE POSTERIOR HEART FIELD

9.1 CARDIAC DEVELOPMENT AND THE HEART FORMING FIELDS

In the early tri-laminar embryonic body the up-regulation of specific genes i.e. *GATA-4*[6] and *Nkx2.5*[7,8] in the splanchnic mesoderm already demarcates the heart forming regions. At the present time it is well accepted that the cardiomyocytes forming to the heart derive from two progenitor areas, the so-called first and second heart fields (or lineages).[9-11] As mentioned in **Chapter 1**, it is still unclear whether the contribution of these progenitor areas / fields occurs in a strictly separate manner or that it may be regarded as a continuum during cardiogenesis. The first heart field gives rise to the primary heart tube by fusion of the two laterally located cardiac mesodermal primordia. This primary heart tube mainly encompasses the AV canal and the future left ventricle, which is connected to the inflow and outflow tract. Lineage tracing and immunohistochemical expression studies indicated that these first heart field derived cardiomyocytes of the primary heart tube express Nkx2.5 and become negative for Isl1 after differentiation.[9,12] Subsequently, at the venous as well as the arterial pole second heart field (SHF) derived cardiomyocytes are added to this primary heart tube.[9,10,13-16] At the arterial pole the contribution of the SHF has been divided into two sub-lineages i.e. the anterior[16,17] and secondary heart fields[15] contributing *Isl1* positive cardiomyocytes to the proximal and distal outflow tract, respectively. Although the cardiac outflow tract, with respect to its formation

and maturation is a highly complicated and an appealing cardiac structure, it lays outside the scope of the current thesis. Here, we specifically studied the contribution of the SHF to the venous pole of the heart i.e. the PHF due to the conserved expression of *Shox2* (**Chapter 2 and 3**)[1] and *podoplanin* (**Chapter 4 and 5**)[2,3] in that particular area.
In contrast to the Nkx2.5 positive myocardium from the first heart field, we observed an addition of Nkx2.5 negative cells that were positive for *Shox2* and *podoplanin* as well as the myocardial marker MLC-2a at the venous pole of the heart.[1,2] This population of cardiomyocytes is considered to be derived from the PHF and highly resembles the SHF derived Isl1 positive cardiomyocytes that are added to both poles of the primary heart tube.[9,13] Moreover, *Isl1* mutant mouse embryos fail to extend their primary heart tube at the arterial as well as venous pole.[9] Similar developmental abnormalities were also observed at the venous pole of *Shox2* and *podoplanin* mutants where there is hypoplasia of the Nkx2.5 negative / MLC-2a positive myocardium, which can be studied in **Chapter 2, 3** and **5**.

9.2 THE PHF IN CARDIO-MORPHOGENESIS WITH RESPECT TO THE ROLE OF *SHOX2* AND *PODOPLANIN*

The contribution of the PHF to the venous pole of the heart is suggested to start around 8.5 days post conception (dpc) of mouse heart development shortly after looping of the heart tube has started. In chick however, addition of PHF derived cardiomyocytes occurs already in the tubular heart stage, since in avian the configuration of a tubular straight heart remains present longer as compared to mammals. The signaling pathways and specific genes involved in the development of the PHF partially do have overlap with the SHF derived cardiomyocytes at the outflow tract of the heart: *Isl1*,[9] *Id2*,[18] *Mesp-1*,[19] *Pitx2*,[20,21] *SP3*,[22] T-box genes 2 and 3 (*Tbx2*, *Tbx3*)[23,24] and the bone morphogenetic genes 2 and 4 (*Bmp2, Bmp4*).[15,25] Furthermore, a subset of transcription factors has been identified to be solely responsible for PHF anlage i.e. Tbx18,[12,26] Tbx5[27] as well as the genes described in this thesis: *Shox2* (**Chapter 2 and 3**)[1,28] and *podoplanin* (**Chapter 4 and 5**).[2,3]
In **Chapter 4 and 5** we demonstrate a common gene expression pattern within the PHF derived cardiomyocytes in the *podoplanin* mouse model. In early mouse heart development the expression of *podoplanin* in the cuboidal-shaped epithelium of the coelomic cavity and the adjacent MLC-2a positive sinus venosus myocardium stimulates the possibility that the dorsal coelomic cavity encompasses the area where the PHF derived cardiomyocytes originate from.[2,3] A cuboidal-shape of the epithelium is related to an active Epithelial-to-Mesenchymal Transformation (EMT) process, which can also be observed in other areas during cardiogenesis, like the developing AV cushions[29,30] and the epicardium.[31,32] Interestingly, for *podoplanin* a role in cell migration and invasion in EMT has been established in several cancer types.[33]

In this thesis we were able to demonstrate that during cardiogenesis a similar *podoplanin* mediated EMT processes in the coelomic epithelium occurs via interactions with E-cadherin and Rho-A (**Chapter 5**).[3] For *Shox2* a functional link with regard to the EMT process has not yet been demonstrated. Moreover, it is not expressed in the cuboidal-shaped coelomic epithelium so that a contributory role in EMT remains to be investigated.

The first impression of the important role of the PHF in heart development became obvious by studying the expression patterns of *podoplanin* and *Shox2* in the developing heart. Both genes showed to have a highly restricted spatio-temporal expression pattern that largely overlaps at the developing venous pole of the heart. These areas include the myocardium in which the sinus horns are embedded, the pulmonary veins, the venous valves and parts of the developing CCS including the sinoatrial node (SAN), AV node (AVN) region, common bundle (CB) and primitive bundle branches.[1,2] Moreover at the venous pole expression of *podoplanin* and *Shox2* was also observed in the pro-epicardial organ (PEO) from which the epicardium derives (**Chapter 9.2.1 *"PHF and epicardial development"***).[1,2,34] The abrogated development of the sinus venosus myocardium and epicardial lineage in *Shox2* as well as *podoplanin* knockout mouse embryos confirmed the important role of the PHF in cardiogenesis. Interestingly, mutations in several other genes that have a critical role in PHF anlage show a comparable abnormal cardiac phenotype including: hypoplasia of the sinus venosus myocardium leading to a diminished incorporation of the sinus horns in the developing heart; hypoplasia of the venous valves, the atrial myocardium, the atrial septum and abnormal development of the dorsal mesenchymal protrusion as was demonstrated recently in the *Pdgfr-α* knockout mouse.[35] Furthermore, abnormal pulmonary vein development has been described in *podoplanin* mutants.[36]

Shox2 not only has a role in the anlage of the sinus venosus but also in differentiation of the PHF derived cardiomyocytes.[1,28,37] The PHF derived sinus venosus myocardium is characterized by expression of MLC-2a and the pacemaker channel HCN4 and absence of Nkx2.5, Cx40, Cx43[1] and Nppa at early stages of development.[28] At later stages (>14.5 dpc) except for the SAN, the sinus venosus myocardium attains an "atrial differentiation program" becoming positive for Nkx2.5, Cx40, Cx43 and Nppa and loosing expression of HCN4.[38] As from that moment expression of *Shox2* becomes limited to the SAN region. Therefore, *Shox2* is postulated to have an essential role in differentiation of PHF derived myocardium. This is further substantiated by the fact that the hypoplastic SAN and sinus venosus myocardium in *Shox2* mutants shows an aberrant expression pattern resembling to that seen in normal atrial working myocardium.[1,28] The exact signaling pathways via which *Shox2*, most likely in combination with other genes, regulates differentiation of the sinus venosus myocardium remain to be elucidated. However, some strong candidates are *Tbx3*,[39] *Tbx5*,[37] *Pdgfr-α*[35] and *Pitx2c*,[40] which all show a specific spatio-temporal expression pattern in the PHF derived sinus venosus myocardium during cardiogenesis.

9.2.1 PHF and epicardial development

The epicardium covering the outer layer of the heart derives from the PEO, a cauli-flower like structure located near the sinus venosus area of the developing heart. From the PEO, epicardial cells start to migrate to cover the complete surface of the heart.[41] The epicardium, covering the outflow tract of the heart derives from a separate region.[42] After spreading of the epicardium subsequent EMT of these cells leads to formation of the EPDCs, which enter the heart to fulfill several roles within cardiac development.[41] The significant role of the epicardium in cardiogenesis is largely demonstrated in avian hearts with mechanical / chemical inhibition of the epicardial outgrow from the PEO, leading to several cardiac malformations, including: myocardial non-compaction, abnormal development of the coronary arteries,[43] AV valves,[44] Purkinje fibers[45] and a disturbed anlage of the annulus fibrosus resulting in accessory pathways (APs) causing ventricular pre-excitation.[44,46] Furthermore, abrogated epicardial lineage development may also result in an aberrant cardiac looping process as observed in *Sp3* mutant mouse embryos.[22]

Several genes including, *Shox2* (**Chapter 3**), *podoplanin*,[34] *Pdgfr-α*,[35] *Tbx5*[27,47] and *Tbx18*[48] involved in sinus venosus / PHF anlage also seem to have an imperative role in epicardial lineage development. A postulated common pool of progenitor cells from which both the PHF and the PEO derive may explain why abnormal PHF and epicardial lineage development frequently coincide. Interestingly, recent studies demonstrated a specific region in the dorsal mesocardium characterized by *Tbx18* positive and *Nkx2.5* negative cells, which contributes to both the sinus venosus and PEO in developing hearts.[12,49] Although in these studies this specific area located in the dorsal mesocardium was not referred to as the PHF-region, we truly consider that with respect to the role of this specific area in heart development, it perfectly matches with the definition of the PHF.

In **Chapter 3** the disturbed epicardial lineage development in *Shox2* mutants was demonstrated. The spreading and migration of the epicardium in these mice seemed not to be as abnormal as observed in *podoplanin* and *Pdgfr-α* knockout mouse embryos, which also showed severe epicardial blebbing and multiple myocardial perforations in the ventricles.[34,35] These phenotypic differences may be related to the observation that unlike *Shox2*, *podoplanin* and *Pdgfr-α* are expressed in the complete epicardium covering the heart where both are suggested to be involved in local EMT processes. Moreover, *Pdgfr-α* has recently been demonstrated to be involved in regulation of *Wt1*,[35] which has a major role in regulation of snail and E-cadherin, representing two key factors in the EMT process.[50] Similarly, *podoplanin* (as was demonstrated in **Chapter 5**)[3] also contributes in the regulation of EMT mediators. Consequently, we postulate that especially the local involvement of factors like *podoplanin* and *Pdgfr-α* in EMT leads to a more severely abrogated epicardial lineage development as compared to those who are only expressed in the PEO during cardiogenesis, like *Shox2* (**Chapter 3**).

9.2.2 The PHF in relation to development of the CCS

Two theories have been put forward regarding the development and origin of the cardiomyocytes contributing to the CCS (**Chapter 1.4.1 *"The origin and development of the CCS"***). In short, the *"specification and ballooning theory"* state that the CCS derives from pre-specified cardiomyocytes in the primary heart tube. During cardiogenesis, these cardiomyocytes will retain their primitive phenotype by expression of specific genes / transcription factors that will prevent these cardiomyocytes to attain a working myocardium phenotype.[51-53] The second theory, the "*recruitment theory*" states that the CCS derives from a pool of multi-potent undifferentiated cardiomyogenic cells.[54-56]

As discussed in **Chapter 4**,[2] the second theory is more in line with the contribution of the PHF to the formation of the CCS, since it does not exclude other cell lineages to be involved i.e. the NCCs[57,58] and the PHF (including the epicardium). Moreover, with respect to our observations in the *Shox2* (**Chapter 2 and 3**)[1] as well as *podoplanin* (**Chapter 5**)[3] mouse model, we conclude that the CCS is not a completely primary heart tube derived structure, which argues to the first theory. However, in our opinion the concept of the heart fields including the PHF, demonstrated that overlap exists between the two main theories. Recently, Christoffels and Moorman[59] also did an attempt to link both theories. They suggested that cardiomyocytes that are recruited according to the "*recruitment theory*" can be considered as the precursor cells, which via the "*specification and ballooning theory*" will contribute to the definitive structures of the CCS.[59] Unfortunately, the place of second heart field derived structures was not specifically included in these considerations.

Our data indicated that newly added PHF derived cardiomyocytes to the venous pole, which can be recognized by their unique expression pattern (i.e. positive for MLC-2a, HCN4 and negative for Nkx2.5, Cx40 and Cx43), contribute to major parts of the CCS (**Chapter 2, 3 and 5**).[1,3] In the embryonic heart areas similar in expression pattern and location have also been described in the "*specification and ballooning theory*" that contribute to the mature CCS and are referred to as primitive myocardium.[51-53] Interestingly, as mentioned in **Chapter 1.4.1 *"The origin and development of the CCS"***, the areas of primitive myocardium also show overlap with the transitional zones i.e. the sinu-atrial transition, AV transition, primary ring and ventriculo-arterial transition (**Chapter 1 - Figure 5c**).[60] These transitional zones can be discriminated based on the expression patterns of several markers involved in CCS development.[61-64] Therefore, we may speculate that the primitive myocardium at the venous pole, which largely resembles to the sinu-atrial transitional zone, is PHF derived. The spatio-temporal expression of specific transcription factors / genes like: *Tbx2*, *Tbx5*[59] and *Shox2* (**Chapter 2 and 3**)[1] may regulate the differentiation of the PHF derived primitive myocardium so that it partially forms the CCS, whereas others parts will attain a working myocardial differentiation program. Most likely, comparable differentiation processes occur at other embryonic regions harboring the primitive myocardium, which corresponds to other transitional

zones. Finally, with respect to the role of the heart fields in CCS development, it can not be excluded that some parts of CCS might have a contribution from the first (i.e. primary heart tube) as well as the second heart field.

In this thesis we mainly focused on development of the SAN a structure that can easily be discriminated in early stages of mouse heart development.[65] In *Shox2* and *podoplanin* knockout embryos the SAN is severely hypoplastic. Although it is now well established that the SAN, like the complete sinus venosus myocardium, is a PHF derived structure the exact signaling pathways involved in differentiation of this highly complex region remains largely unknown. Moreover, contemporary studies demonstrated that different regions within the SAN i.e. solid head (or node) and tail along the terminal crest, show distinct expression patterns of genes,[39] which has been related to the even more complex SAN electrophysiological properties.[66]

However, we were able to unravel a small part of this complicated issue, by showing aberrant differentiation of SAN (and sinus venosus) cardiomyocytes in *Shox2* mutants.[1,28] In the SAN an up-regulation of Nkx2.5, Cx40, Cx43 and Nppa that normally encode for an atrial differentiation program was observed. This of course suggests an abnormal pacemaking function in *Shox2* mutant hearts. Most strikingly, the *Shox2* morpholino injections in Zebra fish embryos in **Chapter 2** as well as the electrophysiological recordings in embryonic *Shox2* mutant mouse hearts in **Chapter 3**, indeed confirmed a decreased / bradycardic heart rate.[1] The lowered heart rate in *Shox2* mutants has been related to a decreased expression or absence of the pacemaker channel HCN4 due to up-regulation of Nkx2.5.[28] This of course seems to be a reasonable explanation since bradycardia has been reported in patients with mutations in the *HCN4* gene.[67] Remarkably, in our studies we did not find absence of HCN4 in *Shox2* mutant embryos. Moreover, the intensity of HCN4 expression in the hypoplastic SAN was comparable to that observed in embryonic wildtype hearts. So, what then could be the underlying mechanism of the lowered heart rate in mutants?

In our opinion the hypoplasia of the SAN itself might explain its deteriorated function, since electrophysiological mapping studies in human[68] and animal[69-71] largely demonstrated that pacemaking occurs in a widely distributed area in the SAN creating a pacemaking complex. Under physiological circumstances, as cycle length decreases, the leading pacemaker area shifts towards the upper region in the SAN (head / solid node).[71] On the other hand, an increase in cycle length leads to an inferior shift of the leading pacemaker area. In line with that, it seems reasonable to speculate that in case of hampered SAN anlage the physiological shift of the leading pacemaker-site through the pacemaking complex fails. Consequently, this might underlie the observed bradycardic heart rate in *Shox2* mutant embryos.

Besides the important role of the PHF in development of the SAN, the contribution of the PHF to others parts of the CCS was not studied in detail in this thesis. However, in embryonic hearts the expression of *Shox2*[1] and *podoplanin*[2] highly overlap with other components of the developing CCS i.e. AVN, CB and primitive left and right bundle branches. Furthermore,

expression was observed in the myocardium of the atria that comprise the location of the internodal pathways. Notably, Espinoza-Lewis *et al*. did not find *Shox2* expression in the CB and primitive bundle branches, so that the exact role of *Shox2* in that particular part of the heart remains doubtful.[28] Nevertheless, a more extensive role of the PHF in CCS development is strengthened by the observation that expression patterns of *Shox2* and *podoplanin* highly resembles to that of other markers, which have been used to study CCS development like: CCS-*LacZ*,[62] *MinK-LacZ*,[64] *Hnk-1*,[61,72] *Leu7*[73,74] and *PSA-NCAM*.[75] In the near future lineage tracing studies most probably will have an essential role in unraveling the exact contribution of the PHF in development of the more peripheral regions of the CCS.

9.2.3 The PHF in congenital heart malformations and clinical implications

In **Part I** of this thesis, we demonstrated that the PHF has a major role in heart development, as indirectly can also be appreciated by the high mortalities rates of *Shox2* (**Chapter 2 and 3**)[1] and *podoplanin* (**Chapter 5**)[3] mutant mouse embryos. Similar mortality rates have been reported in other mouse models for PHF development.[35]

The PHF is involved in formation and differentiation of the sinus venosus myocardium but also has an important role in CCS and epicardial lineage development. Consequently, abnormal PHF anlage may encompasses a broad variety of cardiac malformations ranging from structural heart defects to certain types of cardiac arrhythmias, or combination of events like: the association between SAN dysfunction and atrial septal defects (ASD) in patients with mutations in the *Tbx5* gene causing Holt-Oram syndrome;[76] or mutations in *Nkx2.5* that leads to disturbed AV conduction and ASD and / or ventricular septal defects (VSD).[77,78]

With respect to cardiac arrhythmias related to abnormal PHF development, we postulate that they may either occur secondarily to the structural cardiac malformations, or be due to abnormal myocardial differentiation of the PHF derived myocardium i.e. by the maintenance or re-expression of the characteristic expression pattern (positive for MLC-2a, HCN4 and negative for Nkx2.5, Cx40 and Cx43) pre- or postnatally. Therefore, it seems tempting to speculate that the preferential location of arrhythmias like atrial ectopic tachycardia (AET) that are occasionally observed at fetal stages of development, is rendered by areas of abnormal differentiated PHF derived myocardium. However, to our knowledge a clear link has not yet been demonstrated.

Table 1 summarizes the cardiac abnormalities, which are postulated to be related to abnormal PHF anlage.

Abnormal Posterior Heart Field development	
epicardial lineage	**sinus venosus / atrium**
Structural abnormalities * Ventricular septal defects[22, 34] * Myocardial abnormalities[this thesis, 22, 34, 35] - fenestrations - non-compaction * Abnormal cardiac looping[22] * Disturbed annulus fibrosis formation[44, 46] - accessory AV pathways (APs) * Mitral / Tricuspid valve malformation[22, 44] - Ebstein abnormality * Abnormal Purkinje fiber network[45] * Coronary artery defects[34, 43] * Outflow tract abnormalities[22, 34] **Arrhythmia** * Supraventricular tachycardia [44, 46] - AVRT (including WPW)	**Structural abnormalities** * CCS malformations[this thesis, 1, 3, 34, 35] - hypoplasia sinoatrial node * Atrial septal defects[this thesis, 35, 36] * Myocardial abnormalities[this thesis, 1, 3, 34 - 36] - hypoplasia SV myocardium - thin atrial myocardium * Venous valve abnormalities[this thesis, 1, 35] * Abnormal SV incorporation[this thesis, 3, 36] **Arrhythmia** * Sinoatrial node dysfunction [this thesis] - sick sinus syndrome * Supraventricular tachycardia[this thesis] - atrial ectopic tachycardia - atrial fibrillation * AV conduction axis abnormalities - AVNRT, AVB

Table 1. Schematic representation of congenital heart defects that can be related to abnormal development of the Posterior Heart Field (PHF) derived myocardium. The PHF contributes cells to the sinus venosus / atrium and pro-epicardial organ from which the epicardial lineage develops, the congenital heart defects are separated in two columns correspondingly. Whether the PHF also contributes to the atrioventricular (**AV**) conduction axis remains uncertain, therefore this text is indicated in grey. The numbers in this table refer to the individual references that can be obtained from the reference list of this chapter. **AVB** indicates AV block; **AVRT**, AV reentry tachycardia; **AVNRT**, AV nodal reentry tachycardia; **CCS**, cardiac conduction system; **SV**, sinus venosus; **WPW**, Wolff-Parkinson-White.

PART II NORMAL HEART DEVELOPMENT IN RELATION TO AVRT

The development of the mature impulse propagation through the heart not only involves formation of the CCS, proper isolation of specific areas within the heart is extremely important as well. "Leakage" of the cardiac impulse might occur via so-called accessory AV myocardial pathways (APs) at locations where the isolating layer between the atria and ventricles, the annulus fibrosus, is absent. APs are involved in AVRTs, a sub-group of SVTs, which represent the largest group of tachy-arrhythmias within the pediatric age group.[79] Strikingly, almost two-thirds of fetuses diagnosed with SVT based on AP-pathology remains free of arrhythmia before the age of one year without further medical treatment.[80,81]
In **Part II** of this thesis we have related the process of normal heart development, including that of the CCS, AV junction and annulus fibrosus, to spontaneous disappearance of AVRTs at fetal and neonatal stages of development.

9.3 DEVELOPMENT OF THE AV JUNCTION IN RELATION TO TEMPORARY PRESENCE OF APs

9.3.1 Development of the AV junction - Morphology

The AV junction is a complex region in the heart that harbors several important cardiac structures. Imagine that you are facing towards a mature heart from a superior view, from which the atria are cut-off in a transverse plane at the level of the AV junction. Then, two separate orifices including their valvular-apparatus can be discriminated; The mitral valve orifice at the left and at the tricuspid valve orifice at the right side. The fibrous tissue of the mitral and tricuspid valves is in continuity with the annulus fibrosus, surrounding both orifices (**Chapter 1- Figure 7**). In the septal region a single myocardial continuity i.e. the AV conduction axis can be discriminated that penetrates through the annulus fibrosus, to interconnect the atrial with the ventricular (septal) myocardium. This part of the CCS connects superiorly (atrial level) to the AVN and inferiorly (ventricular level) to the bundle branches.
It is noteworthy, with respect to the developmental process underlying the AV junction, that at the very early embryonic stages the heart only consists of a single heart tube. This primary heart tube solely comprises the myocardium contributing to the future left ventricle and AV canal (first heart field derived) and directly connects to the cardiac in- and outflow tract. So, at these stages there is neither presence of an AV junction with two separate AV orifices nor a specialized AV conduction axis.[82] Due to the addition of SHF-derived cardiomyocytes (**Part I**) and complex remodeling processes of the heart, including cardiac looping and septation, the mitral and the tricuspid valve orifice will appear. The formation of the two

independent AV orifices has been demonstrated by our group in a study on the development of the right ventricular inflow tract in CCS-*LacZ* transgenic mice.[83] Shortly after looping of the heart tube, the contours of the primitive left and right ventricles can be discriminated, which are interconnected via the primary interventricular foramen. At these stages (mouse 10.5 dpc; human ≈ E28), the atrial part of the heart still connects to the ventricular part via a single orifice, the primitive AV canal, which by then is mainly positioned above the future left ventricle (**Chapter 1 - Figure 5**). Subsequently, due to outgrowth of the right ventricle, a remodeling of the primary fold will occur, that will eventually form the right ventricular inflow tract.[83] Eventually, by completion of ventricular septation and remodeling of the endocardial located AV cushions, which mainly contribute to formation of the mitral and tricuspid valves, the two individual AV orifices including their valvular apparatuses will appear.

More or less subsequent to formation of the mitral and tricuspid valve orifice, the separation of the AV myocardial continuity in the AV canal is initiated. This process of annulus fibrosus formation, as was already mentioned in **Chapter 1.5.1** ***"Annulus fibrosus: The isolating system"***, involves interaction between epicardially located AV sulcus tissue and endocardial located AV cushion tissue.[84-88] In **Chapter 6** and **7**,[4] we demonstrated that formation of the annulus fibrosus in mouse as well as in human already starts at pre-septation stages of development, by formation of fibrous tissue near the primitive AV canal. Around the twelfth week of human development, as was also demonstrated by others,[84] the complete atrial and ventricular myocardium are separated by the annulus fibrosus through which the AV conduction axis forms the only myocardial continuity between the atria and ventricles.

In this thesis we mainly focused on the role of periostin, primarily known as osteoblast-specific factor 2,[89] in the formation of the annulus fibrosus. Periostin was postulated to have an important role within this particular process because of its proven ability to directly regulate collagen-I fibrillogenesis.[90] In human (**Chapter 7**),[4] mouse (**Chapter 6**) and quail[44,46,91] hearts we did observe high expression of periostin in the developing annulus fibrosus especially at locations where isolation is mandatory, all the way through cardiac development. Intriguingly, state-of-the-art stem cell studies demonstrated that cardiomyocytes-like cells can be newly generated by reprogramming fibroblasts.[92] With respect to that, it is thrilling to speculate whether a sort of natural differentiation mechanism occurs in an opposite direction near the AV junction so that cardiomyocytes become isolating fibroblasts that contribute to the annulus fibrosus. In this thesis, we did however not find a link that substantiates that hypothesis. Our periostin expression data in mouse showed that in the developing AV junction periostin is never co-expressed in cardiomyocytes (**Chapter 6**). For now we may only put forward that periostin is in someway involved in fibrous tissue formation given its proven role in collagen-I fibrillogenesis.[90] Whether periostin is the key factor in AV isolation remains doubtful, since recent studies in *periostin*

mutant mice showed that *periostin* null mutants are largely normal and do not show an abnormal cardiac phenotype.[93]

Other known factors involved in formation of the annulus fibrosus are bone-morphogenetic proteins (BMP) signaling[94,95] and epicardial cells.[44,46,96] The contributory role of BMPs and the epicardium is substantiated by the fact that disturbed BMP signaling in *Alk3* knockout mice[94] as well as hampered epicardial outgrowth in quail[44,46] leads to defects in annulus fibrosus formation with multiple abnormal myocardial AV connections (i.e. APs) between the atria and ventricles that are electrophysiological functional.[44,46] The role of the epicardium / EPDCs in annulus fibrosus formation is substantiated by the observation that *in vitro* cell cultures of human and quail EPDCs express periostin so that they subsequently may have an important role in collagen-I fibrillogenesis.[44] The contribution of especially the epicardium insinuates that the PHF (**Part I**) also has a role in annulus fibrosus development (**Table 1**).

Nevertheless, there are still numerous unidentified signaling pathways involved in the anlage of the annulus fibrosus. Furthermore, it remains highly fascinating why formation of AV isolation especially does not occur in the AV conduction axis leading to complete AV block (AVB).

9.3.2 Development of the AV junction - Electrophysiology

The main purpose of annulus fibrosus development is to prevent "leakage" of the cardiac impulse from the atria to the ventricles (or the other way around) via APs that bypass the AV conduction axis including the AVN and His-Purkinje-system (HPS). Consequently, APs can only be identified once the AV conduction axis has become functional at a specific moment in cardiac development. Before the presence of this axis, the complete primitive AV canal myocardium itself renders the area for normal AV conduction. Fascinatingly, in a looped heart tube the primitive AV canal myocardium was already demonstrated to have slow conducting properties in chick.[97] These conducting properties might be explained by a restricted expression pattern of certain gap junction proteins in that specific area.[98,99] We for instance demonstrated that during development Cx43, involved in rapid impulse conduction through the atria and ventricles, is never expressed in the primitive AV canal / AV junction.[99] (**Chapter 6**) In someway, these slow conducting properties of the primitive AV canal myocardium at the early stages of development resemble the decremental properties of the mature AVN that will reside in this specific area. Therefore, it is tempting to postulate that the AVN, at least partially, derives from this slow conducting primitive AV canal myocardium.

During cardiac development the transition of the ventricular activation pattern from base-to-apex into the mature apex-to-base activation coincides with a functional AV conduction axis mainly with respect to maturation of the HPS. Furthermore, studies in chick and quail hearts demonstrated that this process highly correlates with completion of ventricular septation.[91,100,101](**Chapter 1.4.2** ***"Action potential generation and propagation in the***

developing heart"). However, as demonstrated in **Chapter 6**, the majority of normal developing embryonic mouse hearts already show a mature apex-to-base activation pattern far before completion of ventricular septation. This observation is substantiated by several optical mapping studies performed in pre-septated embryonic mouse hearts, which also demonstrated a functional AV conduction axis at these stages of development.[63,102] These observations might indicate that: (1) the transition to a mature apex-to-base ventricular activation patterns in mouse hearts is not related to completion of ventricular septation as is suggested to be the case in avian hearts; (2) pre-septated embryonic mouse hearts may already have a functional and / or preferential pathways for fast AV impulse propagation, which primarily activates the apex before the base in the developing ventricles.

9.3.3 APs during normal heart development

The incidental presence of APs during normal heart development has already been reported in human,[103-105] mouse[83] and chick.[106] Our group was the first that studied the presence and course of APs during subsequent stages of normal heart development. The initial studies performed in quail demonstrated the presence of electrophysiological functional APs, which appeared to be remnants of the early embryonic primitive AV canal myocardium. These APs were located around both the developing mitral and tricuspid valve orifice and decreased in number and size at subsequent stages of development.[91] This thesis for the first time describes the presence and course of APs during normal mammalian heart development. In both mouse (**Chapter 6**) and human (**Chapter 7**)[4] APs, more or less similar to those observed in quail, could be observed until late stages of fetal life, which gradually decreased in number and size.

Between species, some differences were observed in the preferential location of APs at the annulus fibrosus. At late stages of human heart development most APs were observed sub-endocardially at the lateral aspect of the tricuspid valve orifice.[4] Fetal quail hearts also demonstrated the majority of APs around that orifice, however in quail the APs were positioned to a more septal position in the AV junction.[91] On the other hand, in fetal mouse hearts most APs were located at an antero-lateral position near the mitral valve orifice (**Chapter 6**).

The persistence of especially right sided APs at later stages of development, as observed in human and quail, might be related to the process that is involved in the formation of the right ventricular inflow tract. In contrast to the left ventricular inflow tract, the right inflow tract is a newly formed orifice which originates from a groove in the primary fold.[83] In addition, the concept of the *"transitional zones"* (**Chapter 1.4.1. *"The origin and development of the CCS"***), which describes the presence of four transitional zones in the looped embryonic heart tube based on the expression of CCS developmental markers, contributes to this idea. Especially the dorsal side of the right AV junction may be formed by a myocardium of three transitional zones: the sinu-atrial transition, the AV transition and the primary fold, as can also be

appreciated in **Figure 5d** in **Chapter 1**.[60] The left AV junction however, only receives a contribution from the sinu-atrial transition and the AV transition. Consequently, we postulate that both the physiological delay and a far more complicated developmental process of the right AV junction explains the higher incidence of persistent right sided APs at late fetal stages of development. Furthermore, we may comment that the preferential location of persistent APs (in human) is not related to the location where annulus fibrosus formation initiates during heart development, since **Chapter 7** describes that formation of this isolating structure starts near the right AV junction / future tricuspid valve orifice.[4,84]

As was demonstrated in **Chapter 6**, the majority of APs during fetal stages of mouse heart development are located near the mitral valve orifice. The mean AP-width also appeared larger at the left AV junction. Remarkably, unlike in avian and human,[4,91] in all fetal mouse hearts a broad AP could be observed at an antero-lateral position near the mitral valve orifice. The presence of more or less identical pathways at this specific location in the developing AV junction has been reported before. Rentschler *et al*. demonstrated in their study on CCS development by means of *LacZ* expression several APs that interconnected the left atrium and ventricle along the free wall. These *LacZ* expressing APs, which were not part of the mature CCS, were observed at subsequent stages of development including neonatal mouse hearts and have been related to WPW-syndrome.[63] Thus far, it remains unclear what exactly causes the high incidence of especially left sided APs in mouse fetuses, at least an interspecies differences can not be excluded.

9.3.4 APs - specialized tissue or ordinary working myocardium?

The APs studied in normal heart development in human (**Chapter 7**),[4] mouse (**Chapter 6**) and avian[91] are all supposed to be remnants of the early primitive AV canal myocardium. However, from electrophysiological studies we do know that not all APs share similar electrophysiological characteristics. In Mahaim tachycardia the APs have decremental properties resembling that of the AVN,[107] which might indicate that some APs are composed of specialized conducting tissue. Furthermore, it is well known that APs involved in Mahaim tachycardia have a morphological-relation with the specialized CCS and interconnect the AVN or His bundle to the fascicles or ventricular muscle.[108] Nevertheless, the question remains whether the presence of APs with decremental properties can also be related to the normal process of cardiac development.

Contemporary studies by Jongbloed *et al.* related right ventricular inflow tract development to be one of the underlying mechanisms for atrio-fasciculair tracts. In that study functional APs were observed at the right AV junction, which connected to the right bundle branch via the moderator band.[83] Interestingly, in **Chapter 7** we demonstrated multiple APs between the developing AVN and the ventricular septal myocardium in human fetal hearts. These APs appeared to have a similar cellular morphology as compared to that of AVN cells, which might

indicate that they have specialized conducting properties. Furthermore, we observed that the majority of APs at the tricuspid valve orifice are related to the right AV ring bundle (RAVRB). This ring of specialized cells located around the developing tricuspid valve orifice is believed to be a remnant of the more extensive embryonic CCS that deteriorates during fetal life. The RAVRB can be detected by expression of various markers for the developing CCS, like: *HNK-1*,[61,109] *Leu7*,[73] CCS-*LacZ*.[62,83] In several species a similar structure can also be recognized around the developing mitral valve orifice, which has been referred to as the left AV ring bundle (LAVRB).[2,110,111] In young animals these ring bundles have also been found and tend to express a specific subset of markers, which also demarcate the CCS.[110] The fact that these ring bundles make contact with the AVN via the AV nodal extensions might suggest a role in normal AV conduction, however at present there are no electrophysiological studies which support to this idea.[110]

It seems tempting to postulate that APs with decremental properties (i.e. in Mahaim tachycardia) are those that are related to the embryonic CCS i.e. RAVRB or LAVRB located at the AV junction. The exact mechanism by which these ring bundles deteriorate remains unknown. However, we may speculate that ongoing myocardial differentiation as observed in other parts of the developing CCS has an important role so that these ring bundles (and if present the related APs) loose their specialized (decremental) properties and become ordinary working myocardium.

9.4 AVRT IN THE FETUS AND THE YOUNG – CLINICAL IMPLICATIONS

The AVRTs represent the largest group of tachy-arrhythmias pre- and postnatally and can be potentially life-threatening.[80,81] From fetal life up to adolescence, the APs underlying AVRT can present at the tricuspid as well as mitral valve orifice. The incidence of APs tends to be higher at the left / mitral valve orifice.[112-114] The etiology of the APs underlying this specific type of arrhythmia is largely unknown. In a minority of cases genetic mutations are involved, like *PRKAG-2* (in familiar WPW-syndrome)[115,116] and *ALK3* (in mice).[94] Furthermore, high numbers of APs are found in combination with other forms of congenital heart disease i.e. Ebstein anomally[117,118] and congenital corrected Transposition of the Great Arteries (ccTGA).[119] In rare cases, an intra-cardiac tumor has been reported to serve as substrate for AVRT.[120,121] Intriguingly, the majority of otherwise healthy fetuses and neonates that initially suffer from AVRT remain free of symptoms before the age of one year without additional anti-arrhythmic therapy (**Chapter 8**).[5,79,81] A prolonged cardiac maturation during the first year of life might cause APs to spontaneously disappear, so that AVRT automatically resolves. This is supported by the observation that especially the development of the fibrous structures of the heart that includes the annulus fibrosus, extends into post-natal life.[122,123]

This thesis fully supports the theory that APs are remnants of the early primitive AV canal and that prolonged cardiac maturation leads to disappearance of AVRT during the first year of life. This theory is best substantiated by the observation that: (1) Functional accessory AV myocardial connections are present during normal mouse heart development that might serve as APs (**Chapter 6**);[4] (2) These APs, which can be found around the tricuspid as well as mitral valve orifice, decrease in number and size at subsequent stage of heart development and accordingly might explain the self limiting character of AVRT (**Chapter 6 and 7**);[4] (3) In human hearts many APs were related to the RAVRB that encompasses a part of the embryonic CCS that deteriorates later on, which might explain why some APs have specialized properties (**Chapter 7**).
The ongoing maturation of the annulus fibrosus offers a good explanation for spontaneous AVRT disappearance. However, it remains uncertain why approximately one-third of patients have a recurrent episode of AVRT later in life.[124] In line with that, it is still unknown why the incidence of certain forms of tachy-arrhythmia like AVNRT increases with age.[81,125] At the moment there are no studies giving answers to those specific questions. It seems however interesting to state in general that cardiac development is not a process that is completed at the time of birth. Especially, for cardiac arrhythmias in the paediatric age group it might be speculated that postnatal cardio-morphogenesis has an important role.

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