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chapter 2

Three-Dimensional and Molecular Analysis of the Venous Pole of the Developing Human Heart

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Abstract

Background: Various congenital malformations, as well as many abnormal rhythms, originate from the venous pole of the heart. Due to rapid changes during morphogenesis, lack of molecular and lineage data, along with difficulties in presenting complex morphogenetic changes in the developing heart in a clear fashion, the development of this region in human has been difficult to grasp.

Methods and Results: To gain insight into the development of the different types of myocardium forming the venous pole of the human heart, we performed an immunohistochemical and three-dimensional analysis on serial sections of human embryos ranging from 22 through 40 days of development. Three-dimensional models were prepared in a novel interactive portable format providing crucial spatial information and facilitating interpretation. As in the mouse, the systemic venous myocardium expresses the transcription factor TBX18, while the pulmonary venous myocardium expresses NKX2-5. In contrast to the mouse, a systemic venous sinus is identified upstream from the atrial chambers, albeit initially with non-myocardial walls. From the outset, as in the mouse, the pulmonary vein empties to a chamber with atrial, rather than systemic venous, characteristics. When compared to the mouse, the vestibular spine is a more prominent structure.

Conclusions: The similarities in gene expression in the distinctive types of myocardium surrounding the systemic and pulmonary venous tributaries in man and mouse permit extrapolation of the conclusions drawn from transgenic and lineage studies in the mouse to the human, showing that the systemic and pulmonary venous myocardial sleeves are derived from distinct developmental lineages.
Introduction
The venous pole of the human postnatal heart has a complex morphology, reflecting its development from several components.\textsuperscript{1,2} It comprises the union of the caval veins with the right atrium (sinus venarum), and the connection of the pulmonary veins to the developing left atrium.\textsuperscript{3,4} Various congenital malformations, such as the sinus venosus interatrial communications, anomalous connection of the pulmonary veins, division of the atrial chambers,\textsuperscript{5} and many abnormal rhythms,\textsuperscript{6,7} are known to originate from the venous pole.

Descriptions of the development of the venous pole remained controversial,\textsuperscript{2,8-11} not only due to difficulties in describing the rapid temporal changes in morphology, but also due to inconsistent use of terminology, with the same structures described in different fashion by different investigators.\textsuperscript{4} This holds particularly true for studies on human development, for which data on gene expression are virtually non-existent. In experimental animals, analysis of lineage and gene expression allowed distinction of the components of the atrial chambers, and provided accuracy in defining the boundaries between the bodies of the atrial chambers, their appendages and vestibules, and the systemic and pulmonary venous tributaries.\textsuperscript{1,2,12,13} Connexin40, which forms high conductance gap-junctions, specifically marks the differentiation of the chamber myocardium, distinguishing it from the primary myocardium.\textsuperscript{2} Lineage studies have demonstrated distinct origins of the myocardial sleeves clothing the systemic and pulmonary venous returns, having distinct transcriptional profiles. The sinus muscle expresses transcription factor Tbx18, but not Nkx2-5 as opposed to the myocardium surrounding the pulmonary veins, which expresses from the outset Nkx2-5, but not Tbx18.\textsuperscript{12,13} These different signatures of transcription factors control, in turn, both regions differently. To date, these findings have been extrapolated to development of the human heart. In the absence of reference molecular data for human cardiac morphogenesis it remained uncertain whether this extrapolation is justified.

In this study, therefore, we have delineated the molecular phenotypes of the component parts of the venous pole in the early stages of human cardiac development. So as to facilitate the appreciation of the complex relations between different structures at the venous pole, we have presented our results in a novel three-dimensional interactive fashion, permitting the reader to form his or her independent opinion of our findings (see online Data Supplement, accessible at web-site http://circ.ahajournals.org).
Material and Methods

Collection, Staging and Tissue Processing of Embryos

Human embryos were collected from medically induced abortions performed for social reasons at the Gynaecology Department of the Tartu University Hospital, Estonia. Collection and use of the human embryonic material for research presented here were approved by the Medical Ethics Committees of the Universities of Tartu, Estonia, and Amsterdam, the Netherlands. As soon as was feasible, the aborted tissues were fixed in 4% paraformaldehyde solution. Embryos were then examined under a stereomicroscope, investigated for gross anomalies, and photographed. We graded them in the Carnegie series of developmental stages based on external landmarks, such as limbs, retinal pigment, and the general size of the embryo. After further fixation overnight, embryos were dehydrated in graded ethanol series, immersed in butanol, and embedded in Paraplast. We included only embryos considered normal.

Immunofluorescent Staining and Reconstruction

Standard procedures were used to visualise antibody binding, as detailed in the supplement, which also outlines the use of Amira software (Visage Imaging) to make the reconstructions and the export of the three-dimensional objects into interactive portable document format. The colour-codes for the structures shown in the 3D reconstructions, along with our chosen abbreviations, are listed in Figure 1. The domain of connexin40 expression, labelled in pink, is shown only in the atria, and not in the ventricles.

<table>
<thead>
<tr>
<th>colour-code for the structures</th>
<th>abbreviations</th>
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<tbody>
<tr>
<td>myocardial tissues (all Figures)</td>
<td>aip - anterior intestinal portal</td>
</tr>
<tr>
<td>Cx40-positive myocardium (Fig.4-6)</td>
<td>ao - (dorsal) aorta</td>
</tr>
<tr>
<td>coelomic wall (Fig.3&amp;4)</td>
<td>AVC - atriocentral canal</td>
</tr>
<tr>
<td>foregut &amp; endoderm (Fig.3&amp;4)</td>
<td>cs - coronary sinus</td>
</tr>
<tr>
<td>jelly (Fig.3&amp;4) / intracardiac mesenchyme (Fig.5&amp;6)</td>
<td>cc - coelomic cavity</td>
</tr>
<tr>
<td>transverse septum (Fig.3) / dorsal mesenchyme (Fig.5&amp;6)</td>
<td>Cu - (atriocentral) cushion</td>
</tr>
<tr>
<td>lumen &amp; borders of the systemic veins (all Figures)</td>
<td>fg - foregut</td>
</tr>
<tr>
<td>lumen of the heart tube, dorsal aortae and arteries (all Figures)</td>
<td>ht - heart tube</td>
</tr>
<tr>
<td>lumen of the pulmonary veins (Fig.5&amp;6)</td>
<td>icv - inferior caval vein</td>
</tr>
<tr>
<td>primitive tracheo-bronchial tree (Fig.5&amp;6)</td>
<td>lb - lung bud(s)</td>
</tr>
<tr>
<td>neuro-ectoderm (Fig.3)</td>
<td>ne - neuro-ectoderm</td>
</tr>
<tr>
<td>V/C/UV - vitelline/cardinal/umbilical vein</td>
<td></td>
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<tr>
<td>OFT - outflow tract</td>
<td></td>
</tr>
<tr>
<td>R/LA - right/left atrium</td>
<td></td>
</tr>
<tr>
<td>rlsrv - right/left superior caval vein</td>
<td></td>
</tr>
<tr>
<td>R/LV - right/left ventricle</td>
<td></td>
</tr>
<tr>
<td>pas - primary atrial septum</td>
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<tr>
<td>pr.f - primary foramen</td>
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<tr>
<td>pv - pulmonary vein</td>
<td></td>
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<tr>
<td>sec.f - secondary (atrial) foramen</td>
<td></td>
</tr>
<tr>
<td>st - transverse septum</td>
<td></td>
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<tr>
<td>SV - sinus venosus</td>
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</table>

Figure 1. Colour-code and abbreviations used in the figures and reconstructions.
Technical Limitations

Our study has a number of limitations that derive from the use of human embryonic material, such as the small number of available specimens, and the variable time preceding fixation of the material. The limited number of available embryos did not permit complete optimization of the staining protocol, nor assessment of biological variation between specimens at similar developmental stages. Staining proved to be reproducible, albeit with variations in intensity between embryos for some antibodies.

Results

The reader is encouraged to read the results along with the interactive 3D-PDF file in the online Data Supplement (accessible at web-site http://circ.ahajournals.org).

Myocardial Markers and General Histology

In all but three embryos, we used troponin I as myocardial marker. This antibody recognizes both cardiac (TNNI3) and skeletal isoforms of troponin I. The slow skeletal isoform (TNNI1) is the predominant isoform in the human fetal heart. Since sarcoplasmic reticulum calcium ATPase2 (SERCA2a) is one of the earliest markers of cardiomyocyte differentiation, we verified whether anti-troponin I identified all myocardium that was recognized by the SERCA2a antibody. In three embryos at Carnegie stages 10-12, we stained consecutive sections for SERCA2a, troponin I, and cardiac troponin I. At the venous pole, the domain revealed by SERCA2a and cardiac troponin I was minimally broader when compared to the domain of troponin I (Figure 2A). At the arterial pole, expression of SERCA2a was much weaker than the other myocardial markers (Figure 2B), as has been reported for the rodent embryonic heart.

The antibody against cardiac troponin I was raised in rabbits, preventing its use in combination with other rabbit antibodies. Because of this, we chose troponin I as a reliable marker for delineation of the myocardial tissues. Subsequent to the immuno-staining, sections were stained with hematoxylin-azophloxin, facilitating histological analysis (Figure 2C).

Stage 10 - The Prototypic Linear Heart Tube

The two youngest embryos studied were equivalent to Carnegie stage 10, which corresponds to approximately 22-23 days of development, and embryonic day (E) 8.5 in the mouse. Folding had just been initiated, with the caudal two thirds of the embryos still being flat. The neural tube was not yet closed, there was a wide anterior intestinal portal and a broad coelomic cavity, and the relatively short heart tube was beginning to loop. In the youngest specimen, the heart tube was little more than a myocardialized part of the splanchnic mesodermal layer folded into the coelomic cavity, consisting of a narrow lumen lined by a single layer of endothelial cells, a thick
acelluar layer of cardiac jelly separating the endothelium from the outer myocardial layer (Figures 2C, 3A,D). The dorsal mesocardium had not yet ruptured, albeit the myocardium surrounded the middle portion of the tube in its entirety. At the arterial and venous poles, the tube was not yet closed, and the myocardial wall was contiguous with the non-myocardial coelomic wall (Figure 3C). Caudally, the cardiac jelly extended outside the heart proper over the proximal unfused parts of the cardiac inflow, and was bounded by the endoderm, the coelomic ducts, and the transverse septum (Figure 3A,B,D). Caudally, the vitelline veins were joined to
the umbilical veins, and also received small vessels from the venous plexus of the yolk sac. Cranially, the border of the pericardial cavity was recognized as the reflection between coelomic wall and myocardial heart tube, but caudally the pericardial cavity was continuous with the bilateral coelomic ducts (Figure 3B,C). The coelomic wall covering the venous tributaries, except its most lateral extremes, was already myocardial (Figure 3E, E*). All myocardial cells present at this stage, including those at the venous pole of the heart and adjacent cells of the dorsal coelomic wall, as well as the ventral wall of the foregut, expressed NKX2-5, a core cardiac network transcription factor essential for cardiogenesis (Figure 3D**,E**). Connexin40
was weakly expressed in the endothelial cells of the heart tube, but could not be detected in the endothelium of the veins and aortas, and was not detectable in the cardiomyocytes (not shown).

**Stage 12 - Appearance of the Systemic Venous Sinus and the Pulmonary Pit**

We analyzed three human embryos at Carnegie stage 12, which spans the time from 26 to 30 days after conception, and is equivalent to mouse E9.5. In all embryos the heart tube had looped, and evidence was found of ballooning of the atrial appendages and the apical ventricular trabecular components. An extensive and discrete atrioventricular canal was positioned to the left of the midline (Figure 4B,E). The pericardial cavity remained in communication with the peritoneal cavity through the bilateral pericardio-peritoneal canals, and no epicardial layer was seen on the surface of the heart.

By now, the wall of the common atrial chamber was positive for connexin40, a marker for differentiating working myocardium in the mouse embryo at comparable stages2 (Figure 4A). A small but discrete systemic venous sinus was present upstream relative to the atrial chamber, being more obvious in the older specimens (Figure 4E,F) than in the younger one (Figure 4B,G). The right- and left-sided venous tributaries joined into bilateral common channels, which emptied into the venous sinus. The lumens of these common veins were bordered ventrally by the mesenchyme of the transverse septum, and dorsally by the mesothelial lining of the pericardio-peritoneal canals (Figure 4H). The two common veins themselves were separated by mesenchymal tissue, forming the so-called sinus septum.18

The left- and right-sided appendages had already ballooned from the common atrium, but as yet there was no overt sign of formation of a primary atrial septum. The junction of the venous sinus with the atrium, the sinu-atrial foramen, already located at the right side of the common atrial chamber. The ventral side of this junction was marked by the so-called sinu-atrial fold (dashed lines in Figure 4B,E,F). At the dorsal aspect, there was no distinct morphological landmark marking the junction between the venous sinus and the atrial chamber (Figure 4C,D). At this stage, all myocardium was still NKX2-5 positive (Figure 4H,J). The larger part of the walls of the venous sinus was negative for myocardial markers, with only the most proximal part adjacent to the sinu-atrial foramen acquiring a primary, connexin40-negative, myocardial phenotype (Figure 4K). TBX18, a marker for the tissues making up the systemic venous sinus in the mouse embryo,12 was observed in the mesenchymal tissues bordering the venous sinus and in the pro- pericardium, but was absent from myocardium and tissues bordering the pulmonary pit (Figure 4L).

Dorsally, and in the midline, the reflections between the atrial and coelomic walls formed obvious ridges, which enclosed the pulmonary pit (Figure 4G,H). The space between the pulmonary ridges was filled with a jelly-like substance, which was lined ventrally by endo-
Figure 4. Reconstructions and sections of a stage 12 embryonic heart and related structures. Panels D through F are from a slightly older embryo, for which no connexin40 expression data were available, whereas other panels are from a younger specimen. Dashed lines in B, E and F represent the sino-atrial fold, and dotted lines in panels C and D indicate the pulmonary ridges surrounding the pulmonary pit (arrowheads). Panels H and I show expression of PECAM1 in the endothelium and TnI in myocardium both in pink owing to the use of the same fluorochrome. To allow visualization of the weaker stained endothelial cells, the myocardial staining was overexposed. The sections shown in H, J, K and L were incubated with antibodies as indicated and approximately correspond to the cross-section of the 3D model shown in panel G. Panel I shows the region from a more cranial section corresponding to the boxed area in panel H. The asterisk in G and H points to bigger right pulmonary ridge, arrows in H point to veins joining the venous sinus, the arrowheads in L point to mesenchymal cells expressing TBX18. See text for further description. Scale bars: 100 μm. For abbreviations see Figure 1.
cardium, and dorsally by the loose mesenchyme surrounding the foregut. The primordium of the pulmonary vein was already identifiable in the pulmonary pit as a blind endocardial evagination into the jelly located ventral to the foregut (arrowhead in Figure 4H), the ventral surface of the foregut itself being covered by loose mesenchyme containing capillaries, the so-called mid-pharyngeal endothelial strands (Figure 4I). As in the working myocardium of the atrial chambers, NKX2-5 was strongly expressed in the mesenchyme and myocardium of the ridges, less so in the more prominent right ridge, but not in the mesenchymal wall of the venous sinus (Figure 4J). The myocardium of the ridges was also positive for connexin40 (Figure 4K). The enlarged right ridge contained a mesenchymal mass, which appeared to shift the primordium of the pulmonary vein slightly to the left (Figure 4H).

**Stage 14 - Shift of the Systemic Venous Sinus and Formation of the Pulmonary Vein**

We examined 3 embryos at stage 14, which represents the period of 31 to 35 days after conception, and is equivalent to mouse E10.5. By this stage, the atrial appendages and ventricular apical components were well formed, and the primary atrial and ventricular septal structures were evident. Connexin40 was expressed in the ventricular trabeculations and walls of the atrial chambers, but was absent from the myocardium of the atroventricular canal (Figure 5G). The primary atrial septum was sickle-shaped, with a mesenchymal cap on its leading edge (Figure 5B). Dorso-caudally in between the atriums it was now possible to see the protrusion of mesenchymal tissue into the atrial lumen (Figure 5C-E), producing the so-called spina vestibuli or dorsal mesenchymal protrusion. It already contained small groups of cardiomyocytes, and was contiguous outside the heart with the mediastinal mesenchyme around the forming lung buds, and internally with the inferior atrioventricular cushion and the mesenchymal cap of the primary atrial septum (Figure 5B,C).

By now, the pulmonary vein had a narrow lumen, which drained the peripheral pulmonary vascular plexus around the lung buds to the lower aspect of the left atrium, entering near the atroventricular canal (Figure 5E) to the left of the vestibular spine, which separated the pulmonary venous orifice from the sinu-atrial foramen (Figure 5A,C). The walls of the venous sinus, which had now entirely shifted to the right, were still largely non-myocardial, except the part contiguous to the atrium, which was thickened and porous. In contrast to the myocardium surrounding the systemic venous tributaries, that surrounding the pulmonary orifice was positive for NKX2-5 and connexin40, but negative for TBX18 (Figure 5F-H). A prominent right, and a smaller left, venous valves were now evident guarding the sinu-atrial foramen, and fused cranially to form the so-called spurious septum. A single-cell layer of epicardium, positive for TBX18, now clothed the entirety of the outer surface of the heart. Now also at the venous pole pericardial reflections could be recognized.
Figure 5. Reconstructions and sections of the heart and related structures at stage 14. The asterisk indicates the vestibular spine. Panels A and D show how the newly formed pulmonary vein joins the left atrium running within the dorsal mesenchyme. Panel B shows the systemic venous sinus, the union of the three major veins, right superior caval vein, inferior caval vein and coronary sinus, a continuation of the left superior caval vein; the double-headed arrow points to the primary foramen. In panel C the relationship between the sinu-atrial foramen (black arrowhead) guarded by the venous valves, the vestibular spine, the primary atrial septum, and the orifice of the pulmonary vein (white arrow) is shown. Sections from panels E through H were incubated with antibodies as indicated and correspond to the cross-section of the 3D model shown in panel D. Arrowheads in H point to the cells of the systemic venous sinus and left superior caval vein, which expresses TBX18. See text for further description. Scale bars: 200 μm. For abbreviations see Figure 1.
Both the venous confluence forming the systemic venous sinus, and the left sinus horn or coronary sinus, occupying the left atrioventricular groove (Figure 5A,B), were mostly within the pericardial cavity. The walls of the coronary sinus were partly myocardial, with the myocytes being negative for NKX2-5 (Figure 5F), but expressing TBX18 (Figure 5H) and weakly connexin40 (not shown).

**Stage 16 - Incorporation of the Systemic Venous Sinus into the Right Atrium and**

Myocardialization of the Venous Tributaries

We studied 3 embryos at Carnegie stage 16, approximately 37 to 42 days after conception, equivalent to mouse E11.5. By this stage, the atrial appendages were obvious (Figure 6A), with pectinate muscles now recognizable on their luminal surfaces. The septal structures had also developed further, and a secondary foramen was present in the primary atrial septum (Figure 6C,D). The cushions in the atrioventricular canal had grown together, separating the canal into right and left channels. At the venous pole, in contrast, there had been relatively small morphological changes. When compared to the size of the right atrial cavity, there had been a relative decline in size of the systemic venous sinus, reflecting the initiation of its incorporation into the morphologically right atrium (Figure 6C). As at stage 14, its myocardial walls remained thick and porous (not shown). The coronary sinus was now completely myocardial (Figure 6B), expressed connexin40 and TBX18, but was still devoid of NKX2-5 (Figure 6F-H). It opened between the venous valves into the right atrium, adjacent to the atrioventricular junction (Figure 6C), but its continuation as the left superior caval vein had become much narrower (arrowhead in Figure 6B).

The pulmonary venous plexus continued to drain into the morphologically left atrium through a solitary channel, which joined the heart still adjacent to the atrioventricular junction, but had now acquired a myocardial sleeve (Figure 6C,E). The myocardial surrounds of the orifice were positive for connexin40 and NKX2-5, and negative for TBX18 (Figure 6F-H). The vestibular spine, now forming the base of the primary atrial septum, interposed between the orifice of the pulmonary vein and that of the systemic venous sinus. The diameter of the individual pulmonary veins had increased, so that they could be traced from the bronchopulmonary buds, which were surrounded by an extensive PECAM1-positive capillary network (Figure 6E). The mediastinal mesenchymal tissues remained contiguous with the dorsal cardiac mesenchyme, which fused in turn with the inferior atrioventricular cushion (Figure 6D).
Figure 6. Reconstructions and sections of the heart and related structures in a stage 16 embryo. Panel A shows morphologically distinct atrial appendages on either side of the outflow tract. Panel B shows the pulmonary vein joining left atrium and surrounded by connexin40-positive myocardium. Panel C shows how the mesenchymal tissues at the base of primary atrial septum separate the orifice of the pulmonary vein (white arrowhead) and the mouth of the coronary sinus (black arrowhead). In panels C and D double-headed arrows show the still open primary foramen. Sections from panels E through H were incubated with antibodies as indicated and are slightly caudal to the level shown in panel C. See text for further description. Scale bars: 200 μm. For abbreviations see Figure 1.
Discussion

The recent expansion of molecular technology has revealed functions of the genetic networks driving the development of the vertebrate heart.\textsuperscript{17,22-24} Although the findings in model organisms have been extrapolated to human development, in the absence of reference molecular data for human cardiac morphogenesis it is unclear whether such extrapolation is justified. Furthermore, the scarcity of molecular data, along with the uncertainty regarding the morphological landmarks in the early stages of development of the human heart, has hampered the full understanding of this complex region. We have now used triple immuno-fluorescently labelled serial sections to investigate, in the human embryo, the patterns of expression of multiple genes known to be essential for mouse cardiac morphogenesis and function. This has permitted us, as in the mouse, to delineate different types of myocardium, and to construct accurate morphological models of the developing human heart. We have supplemented these findings with data regarding the expression of relevant genes. The 3D models are presented also in an interactive format (see online Data Supplement), which allows the observer to form an independent opinion concerning the topic under discussion. Our results show that, as in the mouse, the systemic and pulmonary tributaries of the human embryonic heart are surrounded by distinct types of myocardium, supporting the notion that they are derived from distinct lineages. In contrast to the situation in the mouse, we have been able to recognise the presence of a discrete systemic venous sinus at early stages, which subsequently becomes incorporated into the morphologically right atrium.

Systemic Venous Return

In the mouse, evidence from gene expression and lineage analyses, along with morphological studies, has shown that the systemic tributaries connect directly to the atrial chamber at early stages without formation of a discrete systemic venous sinus.\textsuperscript{2} In contrast to the mouse, we observed, first at stage 12 (mouse E9.5), thus before the formation of the venous valves, a discrete systemic venous sinus, which probably is excavated from the body wall and transverse septum into the pericardial cavity earlier in human than in mouse.

The systemic venous tributaries in mouse acquire myocardial sleeves, having a molecular phenotype different from that of the atrial chambers and pulmonary vein.\textsuperscript{12,13} Our current data show that, as in the mouse, the myocardium of the human systemic venous tributaries does not express NKX2-5, but does express TBX18, thus distinguishing it from the pulmonary venous myocardium (see below).

The myocardium of the systemic venous sinus contains the primordium of the sinus node,\textsuperscript{25} responsible for normal pacemaking. We noted a thickened porous structure at the junction of the right superior caval vein and right atrium in embryos at stage 14 and onwards (Figure 5E...
and not shown). This cuff-like structure represents the primordium of the sinus node, which we are currently analyzing in detail.

Two additional structures, the venous valves and the pro-epicardium, deserve additional attention. The morphogenesis of the venous valves, demarcating the sinu-atrial junction, has been well described in human embryos.18 Of the two layers of the embryonic right valve, which remains in the postnatal heart as the Eustachian valve, the layer facing the lumen of the systemic venous sinus had the same molecular make-up as the sinus muscle, whereas the other layer had the atrial phenotype (Figures 5G,6G). TBX18 identified the sinus myocardium and adjacent mesenchyme, including the pro-epicardium. This mesenchymal precursor pool has been shown in chicken to separate early into the pericardial and myocardial lineages.26

**Pulmonary Venous Return**

The principal debate remaining in the development of the cardiac venous pole concerns the origin of the pulmonary orifice, and its relation to the systemic venous sinus.9-11,27,28 When assessing this ongoing debate, we need to distinguish between the development of the venous lumen and the formation of the myocardial sleeve around the lumenized vein. The lumen of the pulmonary vein forms within the endothelial strand, which at the early stages of cardiac development is part of a common splanchnic capillary plexus facing the ventral aspect of the foregut.9,19 The venous primordium acquires its lumen only subsequent to its leftward shift, concomitant with both the formation of lung buds in the mediastinal mesenchyme, and the division of the common atrial chamber into right and left components by the growth of the primary atrial septum.28-30

At the stage of formation of a discrete systemic venous sinus, there is no evidence of lung buds, and hence there can be no pulmonary venous return to the heart. At this stage, nonetheless, the primordium of the orifice of the pulmonary vein is already recognizable as a blind evagination of the endocardium into the jelly between the pulmonary ridges, this being the so-called pulmonary pit (Figure 4H). As in the mouse,2,11 this primordium was located cranially relative to the opening of the systemic venous sinus, and was surrounded by atrial working myocardium. We also found that, in subsequent stages of human development, the right pulmonary ridge enlarged significantly, accumulating more mesenchymal cells than the left. We correlate this mesenchymal accumulation with the development of the vestibular spine, or the spina vestibuli as originally described by His.20 Unlike the situation in the mouse,29 the spine in the human protrudes into the atrial lumen, thus facilitating the entry of the common pulmonary vein to the left atrium. Only after acquiring its lumen does the wall of the pulmonary vein become myocardialized through differentiation of the surrounding mesenchyme.13
Previous studies, largely morphological, have become polarised, arguing that the pulmonary vein was initially connected to either the atrium, or the systemic venous sinus. These variations in opinions may represent differences in interpretation rather than observation. At stage 12 of human development, a capillary plexus is present within the mesenchyme ventral to the foregut, connected through non-lumenized endothelial strands with both the caudally expanding systemic venous sinus and the more cranially located pulmonary pit. At stage 14, the pulmonary venous primordium acquires its lumen, and becomes connected to the morphologically left atrium, being separated from the systemic venous sinus by the vestibular spine. Only subsequent to this connection do the walls of the pulmonary vein become myocardial.

It has been suggested, based on expression of the HNK-1 antigen and the CCS-lacZ transgene in the myocardium of both the systemic venous sinus and the pulmonary vein, that the myocardial sleeves around the pulmonary veins originate from the sinus venosus. This presumption has then been used to explain why cardiac arrhythmias originate so frequently from the pulmonary myocardium. Gene expression and lineage analyses in the mouse, however, unambiguously demonstrated that the musculature of the systemic venous sinus and the pulmonary myocardium have distinct lineages and molecular signatures. As we now show, the genetic profiles of the systemic and pulmonary venous myocardial walls in the developing human heart are also fundamentally different from the onset of their development. As in the mouse, the musculature of the systemic venous sinus expresses TBX18 and lacks NKX2-5, whereas the pulmonary myocardium expresses NKX2-5, but does not express TBX18. The similarity of these transcriptional signatures in mouse and man suggests evolutionary conservation of the genetic program for the formation of these compartments at the venous pole, lending no support to the notion that the pulmonary myocardium originates from the systemic venous sinus.

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Disclosures

None

Reference List


Supplemental Methods

**Immunofluorescence Staining**

Paraffine-embedded embryos were sectioned at 7 μm and sections mounted onto silane-coated slides. Sections were deparaffinised in xylene, rehydrated in graded ethanol series and washed in phosphate-buffered saline (PBS, pH 7.4). Subsequent to treatment with Antigen Unmasking Solution (Vector Laboratories) in a pressure-cooker for 3 min and cooling, sections were incubated in TNB (100 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.5% blocking powder, Perkins & Palmer) for 30-45 min at room temperature (RT) to prevent non-specific binding of antibodies. Then consecutive sections were incubated overnight at RT with triple sets of primary antibodies, raised in 3 different species (goat, rabbit and mouse), to permit subsequent simultaneous detection. Antibodies were diluted 1:250 in Tris-buffered saline (TBS, 100 mM Tris, pH 7.4, 150 mM NaCl). Different mixes of primary antibodies were used on consecutive sections in repetition through the whole series of sections. Here we report the results of staining with the following primary antibodies: goat polyclonal for PECAM1, goat polyclonal for connexin40, rabbit polyclonal for NKX2-5, and goat polyclonal for TBX18 (all from Santa Cruz Biotechnology). Every combination contained troponin I antibody (Chemicon) or, for some embryos, SERCa2a (Abcam), cardiac troponin I (Hytest Ltd.) antibodies as myocardial marker (see Results for further description). The next morning sections were washed 3 times in TNT solution (100 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% Tween-20, Sigma-Aldrich) and incubated at RT for 1.5 - 2 hr in the dark with a mix of fluorochrome-coupled secondary antibodies containing anti-goat Alexa568, anti-rabbit Alexa488 and anti-mouse Alexa680 (Molecular Probes, diluted 1:250 in TBS). For TBX18 biotinylated anti-goat antibody (Jackson ImmunoResearch, 1:250) and Tyramide Signal Amplification kit (Perkins & Palmer) were used according to the manufacturer’s instructions. Then sections were washed again 3 times in TNT solution and covered with 50% glycerol in PBS.

**Staining for general histology**

Subsequent to immunofluorescent staining and photography sections were washed in PBS and stained with hematoxylin and azophloxin according to a standard protocol. Then sections were dehydrated in graded ethanol, washed in xylene and covered with Entellan (Merck). Sections were photographed through a Leica DM3000 light microscope.

**Three-Dimensional Reconstructions**

Stained sections were serially photographed with a fluorescence microscope Leica DM6000, driven by ImagePro Plus 6.2 software (Media Cybernetics, www.mediacy.com) using filters corresponding to each individual fluorochrome, yielding three images per section, representing the patterns of expression of 3 different proteins. Images with myocardial staining were used to reconstruct the entire heart and related structures. Images were first enhanced to produce homogenous bright specific signal and some background for easy identification of the non-stained structures. Amira 4.1 software (Visage Imaging, www.amiravis.com) was used to perform three-dimensional (3D) reconstruction, as described previously. Briefly, alignment of the images was done automatically using a least-squares algorithm with subsequent manual correction of misaligned images. The set of aligned images was used as a template for threshold-determined (myocardium) and manual labelling of the structures. After completing the labels on the original images (XY axis) artefacts due to unequal stretching of sections, imperfect alignment and some damaged sections were corrected manually in XZ and YZ axes. Then the label of the expression domain of connexin40 at the venous pole and atriums was added manually based on the original images of the connexin40 staining. After resampling the label set to reduce the number of voxels, labels were converted into a 3D surface. The created surface was simplified by reducing the number of polygons till 100,000, and further smoothed. Then the 3D data from the Amira Viewer were exported into the Adobe Acrobat 3D (Adobe Systems Inc., www.adobe.com). The protocol to export a 3D object into a PDF file will be published separately.
Supplemental Reference