The development of the venous pole of the heart
Mommersteeg, M.T.M.

Citation for published version (APA):
Mommersteeg, M. T. M. (2009). The development of the venous pole of the heart

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 3

The sinus venosus progenitors separate and diversify from the first and second heart fields early in development

Mathilda T.M. Mommersteeg, Jorge N. Domínguez, Cornelia Wiese, Corrie de Gier-de Vries, John B.E. Burch, Andreas Kispert, Nigel. A. Brown, Antoon F.M. Moorman, Vincent M. Christoffels

Submitted for publication
Abstract

Aims: The venous pole is a complex part of the heart, often involved in congenital heart defects. During development, it receives contributions from second heart field and from \(Tbx18^{+}\) progenitors. To elucidate these distinct contributions, we investigated the origin of the \(Tbx18^{+}\) sinus venosus progenitor population in the cardiogenic mesoderm and its spatial and temporal relation to the second heart field during heart development.

Methods and results: \(GFP^{+}\) mesenchyme derived from \(Tbx18^{+/GFP}\) embryos was cultured to demonstrate its potential to form Nkx2-5- sinus venosus myocardium. Dil-labeling and short term lineage analysis using a mutG5/hsp68/lacZ line indicated that the sinus venosus progenitor population is localized lateral and caudal in the splanchnic mesoderm, opposite from the medially located second heart field. The early splanchnic mesoderm initially broadly expressed Nkx2-5 and second heart field marker Isl1, but subsequently became partitioned into an Nkx2-5+ Isl1- myocardial subpopulation (cardiac crescent), an Isl1+ second heart field subpopulation, and a lateral Nkx2-5- Isl1- subpopulation, corresponding to the sinus venosus progenitors. 3D reconstructions of the myocardium, the \(Isl1^{+}\) domain and the \(Tbx18^{+}\) domain of hearts between E8.5-10.5 showed the relocation of the sinus venosus progenitor population and the second heart field during folding of the embryo. The two populations remained separate, except for a small overlapping \(Isl1^{+} Tbx18^{+}\) area at the right lateral side of the inflow tract, which indicates the location of initiation of sinus node development.

Conclusion(s): Our data indicate that in addition to the first and second heart fields, the cardiac mesoderm contains an additional progenitor population, which contributes to the sinus venosus myocardium. After patterning of the mesoderm, this sinus venosus progenitor population remains spatially separated from the second heart field population during development.
Introduction

The initial vertebrate embryonic heart tube is formed when the two bilateral cardio-
genic mesodermal regions in the splanchnic mesoderm, called the first heart field, differentiate into myocardium and fuse at the midline. After initial heart tube formation, the heart tube elongates by addition of Isl1+ Nkx2-5+ second heart field cells not only to the anterior pole and dorsal mesocardium, but also to the venous pole. Only recently, the posterior (caudal) region of the murine and chicken second heart field that contributes to the atria and inflow tract has been mapped. However, although Isl1+ Nkx2-5+ second heart field cells provide a large contribution to the myocardium of the inflow tract, this myocardium is taken up into the atrium by embryonic day (E) 9.5. From E9.5 onwards, the sinus venosus myocardium differentiates at the inflow tract that is marked by the expression of T-box transcription factor Tbx18 and the absence of Nkx2-5 expression. This sinus venosus myocardium differentiates around the connection of the cardinal veins to the atrium, and will form the right and left myocardial sinus horn, including the venous part of the venous valves, during fetal life. In adults, most of the right sinus horn myocardium is incorporated into the right atrium. In humans, the left sinus horn will lose its connection to the body and form the coronary sinus, whereas in mouse it will persist as the myocardium surrounding the left superior caval vein. The sinus venosus is an important part of the heart as it constitutes the sinus node and its derivatives are a common focus for atrial arrhythmias. Furthermore, the sinus venosus myocardium is often involved in congenital malformations.

To gain insight into the molecular and developmental processes involved in sinus venosus myocardium formation, we identified the location of the sinus venosus progenitors and their relation with the second heart field, from the onset of their formation in the lateral plate mesoderm until the development of the sinus venosus myocardium.

Methods

Mice

For the Tbx18m2Akis (Tbx18GFP) transgenic mouse line, the IRES.lacZ knock-in construct was modified to harbor an EGFP cassette in the start codon. The mutG5/hsp68/lacZ transgenic line was made as follows. Nucleotides 295-300 of the 868-bp GATA5 F-fragment were replaced with a KpnI site (i.e. TTAACA was mutated to GGTACC) and this fragment was inserted upstream of the promoter in the
hsp68/lacZ plasmid. The isolated mutG5/hsp68/lacZ reporter cassette was used to make a transgenic line. Embryonic age was determined according to the vaginal plug, with noon of the day on which the plug was first observed being taken as embryonic day (E) 0.5. Embryos and fetuses were dissected in PBS, fixed in 4% paraformaldehyde overnight and embedded in paraffin. Amnion or tail biopsy genomic DNA was used for PCR assays to detect the lacZ or Gfp transgenes. Animal experiments were performed in agreement with national and institutional guidelines.

**Dil-labeling**
Embryos ranging from the 4 to 7-somite stages were injected with Dil and DiR (molecular Probes) and cultured for 24 to 48 hours as described.

**Embryonic explant cultures**
For the explant cultures lateral parts of the Gfp+ mesenchyme and control ventricle of E9.5 Tbx18+/GFP heterozygous embryos were micro-dissected. Immediately after micro-dissection was checked if the explant was beating. Beating Gfp+ tissue and not beating ventricles were excluded from the experiment. Immediately 4% paraformaldehyde fixed control tissue was washed in PBS and checked for the presence of myocardium by fluorescent immunohistochemistry. Explants were cultured as previously described. After incubation for 96 hours, each sample was washed in PBS, followed by fixation in 4% paraformaldehyde in PBS, and three PBS rinses, before staining by fluorescent immunohistochemistry.

**Non-radioactive in situ hybridization**
Non-radioactive in situ hybridization of 12 μm embryo sections was performed as described. The probe for Isl1 was kindly provided by S. Evans (Skaggs School of Pharmacy, University of California, San Diego). Other probes have been described previously.

**Fluorescent immunohistochemistry**
Paraffin sections of 7 μm were pressure cooked for 3 minutes in Antigen unmasking solution (H-3300, Vector Laboratories Inc) after deparaffination and rehydration. After cooling down, the sections were processed according to the TSA tetramethylrhodamine system protocol (NEL702001KT, Perkin Elmer LAS). For double staining with two primary antibodies from different species, a fluorescent secondary antibody was added during the biotinylated secondary antibody or streptavidine-HRP step of the TSA protocol. The following primary antibodies were used: goat polyclonal against Tbx18 (1:250, C-20 Santa Cruz), Tbx3 (1:250, E-20 Santa Cruz), Isl1 (1:250, neuronics), rabbit polyclonal antibodies against Nkx2-5 (1:250, H-114 Santa Cruz), cTnl...
(1:1000, Hytest Ltd), Hcn4 (1:250, Chemicon), and monoclonal antibodies against MF20 (1:50, Hybridoma bank, Iowa City, IA, USA). Fluorescent secondary antibodies used were Alexa 488 goat anti-rabbit and goat anti-mouse (1:250, Molecular Probes). Nuclei were counterstained with TOPRO, SYTOX green or SYTOX orange nucleic acid stain (Molecular Probes).

3D-reconstructions
Three-dimensional visualization and geometry reconstruction of patterns of gene expression determined by in situ hybridization or immunohistochemistry were carried out as described previously. Files with reconstructions are available upon request.

Results

*Tbx18-positive mesenchymal explants can form sinus venosus myocardium.*
The sinus venosus myocardium forms from a *Tbx18*+ mesenchymal population ventral caudal of the heart tube. To provide evidence that the *Tbx18*+ mesenchyme is able to form the sinus venosus myocardium, we made explant cultures of the GFP+ mesenchyme of *Tbx18+/GFP* embryos. GFP+ cells were isolated from the lateral side of the inflow tract of E9.5 embryos, thereby excluding the pro-epicardium (Figure 1A). The left ventricle was taken as control tissue. Immunohistochemistry on freshly isolated GFP+ tissue showed that 77% of the isolations (n=31) were free from contamination with myocardium (Figure 1B). 23% showed contamination of sparse myocardial cells at the borders. After 96 hours of culturing, 93% of the GFP+ explants (n=31) showed expression of myocardial marker MF20 (Figure 1C), whereas 50% of the explants was beating. Both the right and left side showed myocardial potential. Furthermore, 85% of the newly formed myocardium was negative for Nkx2-5 (n=13) (Figure 1C), which is the typical feature of sinus venosus myocardium. Therefore, we conclude that the *Tbx18*+ mesenchyme is a cardiac progenitor population that has the potential to differentiate into sinus venosus myocardium.

*The sinus venosus originates in the caudal lateral part of the cardiac crescent.*
The late onset of *Tbx18* expression limits its usefulness as a marker to answer the question where the progenitors of the sinus venosus are located at earlier stages prior to heart tube formation. We used a transgenic mouse line in which a mutated Gata5 enhancer drives expression of LacZ that is active specifically at the ventral side of the inflow tract, where the sinus venosus progenitors reside, and in the first proepicardial cells at E9.0 (Figure 2A). Tracing its expression back in development
to E7.5, β-galactosidase expression was visible in the lateral plate mesoderm (Figure 2B). Interestingly, cross-sections through the LacZ+ area at E7.5 showed that the expression of LacZ was restricted to the mesodermal cells at the lateral-most edge of the lateral plate mesoderm, where it borders with the yolk sac (Figure 2C). Although these data do not provide direct lineage proof, due to the relative stability of β-galactosidase, these results indicate a relation between the lateral-most side of the lateral plate mesoderm and the ventral part of the inflow tract.

To confirm the lineage relationship between the cells lateral of the cardiac crescent and the ventral side of the inflow tract, we performed fluorescent cell tracing experiments. Labels were placed on the caudal lateral rims of the cardiac mesoderm in embryos ranging from E7.5-8.0, on both the right (DiR) and left (DiI) side (Figure 2D). These lateral rims are the location of initiation of Tbx18 expression (Figure 2D).22 The embryos were then cultured for one to two days. In all properly developed embryos (n=14), labels in the caudal lateral region of the cardiac mesoderm were found in the ventral side of the inflow tract (Figure 2D and see Figure 4 for a better view of the corresponding Tbx18+ region). Labels placed at the right side of the cardiac mesoderm were found to contribute to the right side and labels placed on the left side contributed to the left side of the ventral inflow tract (Figure 2D). These results confirm that the cells from the caudal lateral part of the cardiac crescent contribute to the Tbx18+ ventral side of the inflow tract.

Progressive partitioning of the splanchnic mesoderm into heart fields

To assess the location of the sinus venosus progenitors relative to the heart fields, we examined the early expression patterns of second heart field marker Isl1 and first and second heart field marker Nkx2-5 in the lateral plate mesoderm. At E7.0, before the first cardiomyocytes differentiate from the splanchnic mesoderm, the complete lateral plate mesoderm expressed Isl1 (Figure 3A).25 The expression of Isl1 in the lateral plate mesoderm was found from most cranial to most caudal and further into the allantois (Figure 3A). In the cranial half of the embryo, Isl1 and Nkx2-5 showed overlapping expression in the medial and lateral cells of the lateral plate mesoderm. (Figure 3A). This broad expression pattern indicates that these markers are active in all putative heart fields. At E7.5, the lateral plate mesoderm has formed a splanchnic and somatic layer and the first MF20+ myocardial cells (“first heart field-derived”) could be observed in the splanchnic mesoderm (Figure 3B). Nkx2-5 expression was down-regulated in the somatic mesoderm, but was maintained in the new myocardium and in the splanchnic mesoderm just medial of the myocardium, now recognizable as the second heart field. Isl1 expression, on the other hand, was disappearing from the myocardium, but was maintained in a broader area than Nkx2-5 in the second heart field mesoderm (Figure 3B) and extended into the paraxial
Figure 1. Tbx18+ mesenchyme differentiates into Nkx2-5- myocardium. A, Whole mount E9.5 embryo stained for Tbx18 mRNA. The dotted areas indicate the regions used for explant culture. B, Tbx18+/GFP mesenchyme and control ventricle scanned at day 0 for Gfp and stained for MF20 and SYTOX orange, showing that the Tbx18+/GFP mesenchyme is negative for MF20. B, Tbx18+/GFP myocardium and control ventricle after 96 hours of culturing, stained for Nkx2-5, MF20 and SYTOX orange. The Tbx18+/GFP mesenchyme has differentiated into Nkx2-5- myocardium. A, atrium; EV, embryonic ventricle; OFT, outflow tract; PE, proepicardium; mes, mesenchyme; myo, myocardium.
Figure 2. Lineage relationship between the cells of the lateral caudal rim of the cardiogenic mesoderm and the ventral caudal side of the heart tube

A, transversal section of an E9.0 mutG5/hsp68/LacZ embryo stained for β-galactosidase. β-galactosidase staining is observed at the ventral side of the venous pole, including the proepicardium. B, E7.5 mutG5/hsp68/LacZ embryos stained for β-galactosidase, showing staining in the lateral plate mesoderm. C, transverse section of embryo in B, revealing that the staining observed in B is located at the most lateral side of the splanchnic mesoderm (arrowhead). D, three embryos labeled with DiR (right) and DiI (left) at E7.5-E8.0 and after culturing at E9.5. Arrowheads indicate the location of the labels. Labeling of the lateral side of the caudal cardiac crescent can be found at the ventral side of the venous pole at E9.5. I/OFT, in/outflow tract L/RV, left/right ventricle; NF, neural fold; SPM, splanchnic mesoderm.
mesoderm shown to contribute to the head musculature. At the lateral border of the myocardium, where we mapped the sinus venosus progenitors, the mesodermal cells now lacked expression of both Nkx2-5 and Isl1 (Figure 3B). Between E7.5 and E8.5 the embryo folds, and the cranial Isl1 expressing area became relocated dorsal of the forming heart tube (Figure 3C). Caudal of the heart tube, in the ‘legs’ of the splanchnic mesoderm, the pattern as seen at E7.5, with an Nkx2-5+ Isl1+ expression domain medial and Nkx2-5- Isl1- mesoderm on the lateral border, was maintained (Figure 3C). In conclusion, after the initial co-expression of Isl1 and Nkx2-5 in most of the cranial lateral plate mesoderm, the splanchnic mesoderm becomes from medial to lateral partitioned into Nkx2-5- Isl1+, Nkx2-5+ Isl1+ and Nkx2-5- Isl1- subpopulations. The Nkx2-5+ Isl1+ subpopulation, the first heart field, is the first subdomain to differentiate into myocardium and to down-regulate Isl1 (Figure 3D, region 1). The medial of the first heart field located second heart field, remains completely positive for Isl1 until differentiation into myocardium. This region differentiates into myocardium only after the differentiation of the first heart field and starts to express Nkx2-5 only just prior to its differentiation (Figure 3D, region 2). The lateral-most Nkx2-5- Isl1- subpopulation correlates to the late differentiating sinus venosus progenitor population (Figure 3D, region 3).

**Separation of the Isl1+ and Tbx18+ populations is maintained during development.**

To gain further insight into the properties of the sinus venosus progenitor population during cardiac morphogenesis, we made three-dimensional reconstructions of embryonic mouse hearts ranging from E8.5, just after initiation of Tbx18 expression and before formation of the sinus venosus, to E10.5, when the sinus venosus has started to form (Figure 4). At E8.5, when the heart has started to loop, the myocardium (grey) is completely surrounded by Isl1+ mesenchyme (yellow). Strong Tbx18 expression (blue), which is initiated around E8.25,22 can be observed at E8.5 at the ventral and lateral side of the inflow tract (Figure 4B). A rim of Isl1 expressing cells is located between the myocardium of the ventral side of the inflow tract and the Tbx18+ mesenchymal population.

One day later, at E9.5, the heart has looped and the chambers have differentiated (Figure 4A,B). The Isl1+ population is completely lining the myocardial border at the medial and dorsal side of the heart, in continuity with the outflow tract, the dorsal mesocardium including the dorsal mesenchymal protrusion and the developing pulmonary vein, and the atria. However, at the ventral side of the inflow tract the Tbx18+ population now lines the myocardium in a horseshoe-like shape, with the Isl1+ population located in the centre of the horseshoe (Figure 4B). At the ventral side of the horseshoe, Tbx18 is expressed in the pro-epicardium, whereas at the lateral sides...
the \textit{Tbx18}+ population surrounds the common cardinal veins at their entrance to the heart (Figure 4A). Until this stage, expression of \textit{Tbx18} is confined to the mesenchyme, as the sinus venosus myocardium has not yet developed. At E10.5, the formation of the sinus venosus myocardium has been initiated and \textit{Tbx18} is now expressed in both the mesenchyme (blue) and the sinus venosus myocardium (green) (Figure 4B). A stripe of \textit{Tbx18}+ mesenchyme can be found lining the coelomic cavity, medial of the forming urogenital ridge (Figure 4B). In conclusion, the sinus venosus progenitors become located ventral caudal of the heart tube after folding of the embryo. Visualization of the sinus venosus and second heart field expression domains shows that these populations remain separated during heart formation.

\textbf{Sinus node development is initiated in the presence of both \textit{Tbx18} and \textit{Isl1}.}

Although the \textit{Tbx18}+ and \textit{Isl1}+ populations are physically separated and represent two distinct pools of progenitor cells, we found a small area of overlapping expression (Figure 4B). At the lateral-most sides of the inflow tract, \textit{Tbx18}+ cells have a small overlap (purple) with the \textit{Isl1}+ cells at E8.5 (Figure 4B). After E8.5, the \textit{Tbx18} and \textit{Isl1} co-expressing area becomes restricted to the right lateral side. Immunohistochemistry showed that as soon as the sinus venosus myocardium starts to develop around E9.5, a small \textit{Isl1}+ area is visible in this myocardium (Figure 5A). One day later the \textit{Tbx18}/\textit{Isl1} co-expressing area correlates with the just-developing \textit{Tbx3+/Nkx2-5-} sinus node (Figure 5B,C,D), which was previously shown to express \textit{Isl1}.\textsuperscript{27} Further differentiation of the sinus horn myocardium occurs in absence of \textit{Isl1}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Progressive partitioning of the splanchnic mesoderm into heart fields. A-C, schematic representations of the cardiac mesoderm and the myocardium of E7.0 (A), E7.5 (B) and E8.0 (C) embryos. The level of sectioning is shown, apostrophes indicate the corresponding sections. The transverse sections are stained with immunohistochemistry for Nkx2-5, Isl1, MF20 and TOPRO. A, at E7.0 Nkx2-5 and Isl1 are expressed in both the splanchnic and somatic mesoderm (white arrowheads). Red arrowheads indicate the slightly more medial Isl1 expression, compared to Nkx2-5 expression. Grey arrowheads indicate Isl1 expression in the endoderm. 3D reconstructions of the Isl1 and Nkx2-5 expression area show that Isl1 is expressed in the complete lateral plate mesoderm continuing into the allantois. Nkx2-5 is expressed in a cranial subset. B, at E7.5 MF20+ myocardium has formed. Medial of the Nkx2-5+ myocardium the Isl1+ Nkx2-5+ second heart field is located. The lateral-most mesoderm is Nkx2-5- Isl1- (white arrowhead). Red arrowhead indicates the more medial Isl1 expression compared with Nkx2-5 expression. C, at E8.5 Isl1 becomes located dorsal of the heart tube cranially (red arrowheads). Caudally Isl1 expression remains located medial, and the Nkx2-5- Isl1- mesoderm lateral (white arrowheads). Grey arrowheads indicate Isl1 expression in the endoderm. D, schematic representation of the location of the distinct progenitor populations in the splanchnic mesoderm and the expression in time of Isl1, Nkx2-5 and MF20 in these populations. AL, allantois; END, endoderm; ECT, ectoderm.}
\end{figure}
The development of the venous pole of the heart
Figure 4. The Tbx18+ progenitor population is located in a horseshoe-like shape around the Isl1+ second heart field population. A, three dimensional reconstruction of a complete E9.5 embryo and of its heart with only the Tbx18+ domain (upper reconstruction) or the Isl1+ domain (lower reconstruction). B, reconstructions of the hearts of E8.5, E9.5 and E10.5 embryos shown from the left and caudal side, indicating that the Tbx18+ and Isl1+ domains remain separated during development, except for a small overlapping area. EV, embryonic ventricle; CCV, common cardinal vein; LA, left atrium; L/RV, left/right ventricle; OFT, outflow tract; RV, right ventricle.
Thus, the Tbx18+ and Isl1+ cell populations contribute to two spatially separated parts of the heart, except for the lateral-most border of these domains, which show overlapping expression. The right-sided domain will form the sinus node of the heart.

Discussion

In this study we identified and mapped the sinus venosus progenitor population and provide insight into the distinct contributions of progenitor cells to the venous pole of the heart. Our data indicate that the sinus venosus myocardium forms from a cardiac progenitor population located lateral and caudal in the cardiac mesoderm (Figure 6). This population is spatially distinct from the second heart field progenitor population, which is located medial in the cardiogenic mesoderm. With the folding of the embryo the sinus venosus progenitor population is translocated to a position ventral and caudal of the heart tube, where it will form the myocardium of the sinus venosus (Figure 6). 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.

The lateral-most region of the caudal cardiac splanchnic mesoderm contributes to the sinus venosus

The splanchnic mesoderm contains all the progenitors of the heart. The current view is that the first mesoderm to differentiate into myocardium is the “first heart field”, which forms the cardiac crescent and subsequently the primary heart tube. The mesodermal cells medial to these cells are called the “second heart field”, which will contribute cells to the arterial and venous pole and dorsal mesocardium of the initial heart tube.3,28 Recent proliferation analysis has indicated that a single, bilateral growth center in the Isl1+ domain contributes cells to both poles of the heart during the period in which both the first and the second heart fields contribute myocardium to the heart, indicating the existence of a single cardiac progenitor pool that contributes to the complete heart.9 Initially, Isl1 and Nkx2-5 are co-expressed in the cardiac mesodermal cells (Figure 3).7,25,29 Only at subsequent stages, the splanchnic mesoderm becomes partitioned into subpopulations, corresponding to the first heart field, second heart field, and sinus venosus progenitor populations. These subpopulations can be divided further into subdomains when taking into account other gene expression domains. The division into a cranial and caudal part, for example, is thought to be established by reduction of Fgf8 expression due to retinoic acid signaling.30,31 Taken together,
Figure 5. The sinus node develops in the presence of both Tbx18 and Isl1. Sagittal serial sections of an E9.5 (A), E10.5 (B), E11.5 (E) and transverse serial sections of an E11.5 (D) embryo stained for Tbx18, Isl1, Nkx2-5, Tbx3 and cTnI protein. A, at E9.5 a small area of Isl1 expression is visible in the first Nkx2-5- myocardial cells (dotted region). Note that the pulmonary vein develops in the Tbx18- Nkx2-5- Isl1+ Tbx3+ pulmonary mesenchyme. B, at E10.5 the Nkx2-5- sinus venosus myocardium is positive for Isl1 and Tbx3 at the right side. C, 3D reconstruction of the myocardium and Tbx18+ and Isl1+ domains of an E10.5 embryo, showing the overlapping area of Tbx18 and Isl1 expression on the right side. D, transverse serial sections an E11.5 indicating that the Tbx18+ Isl1+ cTnI+ area overlaps with the Tbx3+ sinus node. E, at E11.5 the sinus venousus continues to differentiate from Isl1+ mesenchyme (arrowhead). (R)A, (right) atrium; L, liver; OFT, outflow tract; PE, proepicardium; PV, pulmonary vein; RCV, right caval vein; SAN, sinus node; SV, sinus venosus.
these data indicate that the complete heart is formed from a single progenitor population in the splanchnic mesoderm that becomes partitioned into distinguishable subdomains that contribute to distinct parts of the heart.

Early lineage tracing experiments in chick already indicated the relation of the caudal lateral part of the splanchnic mesoderm with the ventral caudal side of the heart tube. Also Recent Dil labeling in mouse and chicken has shown that the atrial progenitors are located most caudal in the second heart field. However, these studies did not map the progenitors of the sinus venosus, because the sinus venosus will only form after the stages analyzed in these studies. Using both short term lineage analysis and Dil-labeling we now show that also the sinus venosus has its origin in the most lateral rim of the caudal cardiac mesoderm. Although the mutG5/hsp68/LacZ embryos show LacZ expression in the lateral rim of the complete cardiac crescent, the cranial part of this rim is thought to be the region of fusion of the cardiac mesoderm to form the ventral heart tube. The mutG5/hsp68/LacZ results also indicate that the sinus venosus shares its origin with the proepicardium, which will give rise to the epicardium, coronary vessels and fibroblasts. The proepicardium, in turn, is thought to share its origin with the septum transversum, which will eventually form the diaphragm located immediately caudal to the heart. Our findings are consistent with tracing experiments and expression analysis that indicated lineage relation between the lateral rim of the splanchnic mesoderm shortly after gastrulation and the septum transversum after folding of the embryo.

The sinus venosus precursors are distinguished from the first and second heart fields by their early loss of Isl1 and Nkx2-5 expression

Previous lineage data suggested that the sinus venosus myocardium is the sole part of the myocardium derived from Nkx2-5-negative cells. However, the initial expression of Isl1 and Nkx2-5 in both the splanchnic and somatic mesoderm (Figure 5) suggests that also the sinus venosus myocardium has a history positive for Nkx2-5 and Isl1. A stronger reporter line, like the Gata4 line, would probably show the positive history for Nkx2-5 and Isl1 expression of the sinus venosus myocardium. These results suggest that early in development the heart fields are indistinguishable using these markers, suggesting that patterning of the cardiac progenitor population into heart fields occurs later. Further, these data indicate the limitations of Isl1 and Nkx2-5 and of the respective Cre lines to distinguish heart field contributions to the heart.

In contrast, the actual expression pattern of these genes does allow to assess the locations of the populations of cells that will contribute to the distinct parts of the heart. We found that Isl1 and Nkx2-5 are expressed in the sinus venosus progenitors.
only transiently very early in development. The sinus venosus progenitors lose Nkx2-5 and Isl1 expression long before their differentiation into the sinus venosus myocardium. This early loss of Nkx2-5 and Isl1 distinguishes the sinus venosus progenitors from the first and second heart field progenitors, that only turn off Isl1 after the initiation of differentiation into myocardium, and that never turn off Nkx2-5. The sinus node, however, maintains Isl1 expression. Furthermore, the sinus node receives a contribution of cells from an area of overlap between the Tbx18+ sinus venosus progenitors and the Isl1+ second heart field progenitors, raising the possibility that this co-expression could be crucial for sinus node development.

The mechanism of sinus venosus formation is highly conserved, as in Drosophila a similar mechanism is described for the formation of the ostia, the equivalent of the murine sinus venosus.\textsuperscript{38} Also the expression pattern of Tbx18 in the sinus venosus is found to be conserved from zebrafish\textsuperscript{39} and Xenopus,\textsuperscript{40} to chick\textsuperscript{41} and mouse.\textsuperscript{22}

\textbf{Insight into the origin of the distinct parts of the venous return may help to understand congenital malformations}

The sinus venosus is involved in congenital heart defects and is an origin for ectopic foci underlying atrial fibrillation.\textsuperscript{12,13} Knowledge of the origin of the distinct cardiac progenitor cells, their spatial relation during development, and their contribution to distinct parts of the heart, will be helpful in understanding how congenital malformations develop. For example, the sinus venosus myocardium is long thought to have a common origin with the myocardium around the pulmonary vein. However, in contrast to the sinus venosus, the pulmonary myocardium develops in the Nkx2-5+ Isl1+ dorsal mesocardium,\textsuperscript{42} which has its origin in the medial cardiac crescent. Therefore, development of defects involving both structures is not likely to have its origin in a common progenitor pool, but in genetic and morphological defects.

Defects in sinus venosus myocardium and sinus node development manifest only after E9.5 and are found in Tbx18 and Shox2 mutants.\textsuperscript{10,43} Tbx18 mutants show a reduced and delayed development of the sinus venosus myocardium,\textsuperscript{10} with loss of the sinus node head.\textsuperscript{16} In absence of Shox2 the sinus venosus myocardium is nearly absent and the sinus venosus gene program is lost, with ectopic expression of Nkx2-5 in the remnant of the sinus venosus.\textsuperscript{43,44} Absence of Shox2 results in bradycardia. The absence of Nkx2-5 from the sinus venosus and sinus node and its presence in the atrial myocardium is likely to be important for normal pacemaker function and the localization of pacemaker activity to the sinus venosus myocardium.\textsuperscript{24,42,44,45}
Figure 6. Fate map of the cardiac mesoderm. Schematic representation showing the fate map of the cardiac mesoderm. The sinus venosus progenitor population becomes relocated from caudal lateral in the splanchnic mesoderm, to ventral caudal of the heart tube. The dotted line indicates the level of sectioning. A, atrium; AIP, anterior intestinal portal; CCV, common cardinal vein; EV, embryonic ventricle; HT, heart tube; OFT, outflow tract; VEN, ventral; DOR, dorsal; CRA, cranial; CAU, caudal; L, left; R, right.
Acknowledgements

We thank Gert van den Berg, Bram van Wijk and Saskia van der Velden for their contributions. This work was supported by grants from the Netherlands Heart Foundation (96.002) to V.M.C. and A.F.M., from NWO VIDI (864.05.006) to V.M.C, from the EU (LSHM-CT-2005-018630) to V.M.C, A.F.M, and from the British Heart Foundation (RG RG-03-012) to N.A.B.
References

4. Snarr BS, O'Neal JL, Chintalapudi MR, Wirrig EE, Phelps AL, Kubalak SW, Wessels A. Isl1 Expression at the Venous Pole Identifies a Novel Role for the Second Heart Field in Cardiac Development. Circ Res. 2007;101:971-974
5. Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell. 2003;5:877-889
8. Galli D, Dominguez JN, Zaffran S, Munk A, Brown NA, Buckingham ME. Atrial myocardium derives from the posterior region of the second heart field, which acquires left-right identity as Pitx2c is expressed. Development. 2008;135:1157-1167


42. Mommersteeg MTM, Brown NA, Prall OWJ, de Gier-de Vries C, Harvey RP, Moorman AFM, Christoffels VM. Pitx2c and Nkx2-5 are required for the formation and identity of the pulmonary myocardium. *Circ Res.* 2007;101:902-909

