The handle http://hdl.handle.net/1887/20556 holds various files of this Leiden University dissertation.

Author: Scherptong, Roderick Wiebe Conrad
Title: Characterization of the right ventricle: embryonic development, noninvasive imaging and electrocardiography
Issue Date: 2013-02-26
Chapter 3

Morphogenesis of Outflow Tract Rotation During Cardiac Development: The Pulmonary Push Concept

Roderick WC Scherptong
Monique RM Jongbloed
Lambertus J Wisse
Rebecca Vicente-Steijn
Margot M Bartelings
Robert E. Poelmann
Martin J Schalij
Adriana C Gittenberger-de Groot

ABSTRACT

Understanding of cardiac outflow tract (OFT) remodeling is essential to explain repositioning of the aorta and pulmonary orifice. In wild type embryos (E9.5–14.5) second heart field contribution (SHF) to the OFT was studied using expression patterns of Islet 1, Nkx2.5, MLC-2a, WT-1 and 3D-reconstructions. Abnormal remodeling was studied in VEGF120/120 embryos. In wild type, Islet 1 and Nkx2.5 positive myocardial precursors formed an asymmetric elongated column almost exclusively at the pulmonary side of the OFT up to the pulmonary orifice. In VEGF120/120 embryos the Nkx2.5 positive mesenchymal population was disorganized with a short extension along the pulmonary OFT. We postulate that normally the pulmonary trunk and orifice are pushed in a higher and more frontal position relative to the aortic orifice by asymmetric addition of SHF-myocardium. Deficient or disorganized right ventricular OFT expansion might explain cardiac malformations with abnormal position of the great arteries, such as double outlet right ventricle.
INTRODUCTION

Morphogenesis of the outflow tract (OFT) is a complex and delicately orchestrated process. During proper development of the OFT the great arteries achieve their definitive morphologic relationship, with the aorta situated in a central position right posterior of the pulmonary trunk. Maldevelopment of the OFT results in an abnormal position of the aorta and pulmonary trunk, observed in some forms of congenital heart disease, such as tetralogy of Fallot, transposition of the great arteries and double outlet right ventricle, often characterized by a side-to-side arrangement of the great vessels. The malpositioning of the great arteries and their respective orifices in these instances has major consequences, with early mortality without timely intervention. Knowledge of normal OFT development is mandatory as a first step in comprehension of the background of these malformations, as well as to aid in early (prenatal) diagnosis.

During embryonic development the myocardium of the OFT is derived from the second heart field, also referred to as anterior or secondary heart field. In this paper we refer to this population as the anterior heart field, which includes the coelomic wall covering the pericardial cavity in the OFT region of the heart. This population of cells expresses amongst others Isl1, Tbx1 and Tbx3, GATA4, Nkx2.5 and WT-1. Expression of some of these genes, including Tbx3 and Isl1, is also observed in neural crest derived cells, and a recent study indicates a dual origin (myocardial and neural crest cells) of Isl1 derivatives in the heart. Anterior heart field cells contribute to the vascular wall of the great arteries and differentiate into a myocardial phenotype upon migration to the myocardial OFT. While at the beginning of development the myocardial OFT is short, orchestrated interaction between products of the aforementioned genes results in continuing addition of cells from the anterior heart field to the OFT, which lengthens in response. Although the exact time span of cellular addition from the anterior heart field to the myocardial OFT is unclear, addition until embryonic day (E) 11.5 in mouse was previously described. Using immunofluorescent stainings and deposits of Indian ink in chick an increase of the myocardial part of the OFT up to stage HH24 has been demonstrated, corresponding to E12.5 in mouse.

The future aorta and pulmonary trunk and their respective orifices have a side-by-side position early in cardiac development. It is suggested that, besides lengthening, a rotational motion of the OFT is required for normal positioning of the aorta and pulmonary trunk and their respective orifices. Multiple developmental signaling factors such as Pitx2c and FGF8/10, expressed in the anterior heart field in an asymmetrical fashion, have been reported as potential regulators of this rotational motion. Several mutant mouse models with a phenotype that includes OFT malformations are currently available, including models with alterations in expression in vascular endothelial growth factor (VEGF) signaling. We have previously studied VEGF 120/120 mutant mouse embryos, that solely express the VEGF120 isoform, and found a high susceptibility of these embryos for OFT malformations, including tetralogy of Fallot and double outlet right ventricle. It was postulated that the anterior heart field-derived subpulmonary myocardium is highly sensi-
tive for signaling for VEGF and Notch, which may underlie the observed malpositioning of the OFT vessels in the VEGF 120/120 mutants. However, a direct link with a contribution of the anterior heart field mesenchyme to the OFT has not been studied thus far. In general, studies in which the dynamics of OFT rotation are related to the expression of markers more typical for myocardial precursors in the anterior heart field during consecutive developmental stages, are currently lacking. Hence, it is unknown how and if the anterior heart field contribution to the myocardial OFT could be involved in OFT rotation and whether we are dealing with an already described shortening of the subaortic OFT\textsuperscript{20, 21} or with a marked, relative lengthening of the subpulmonary OFT. The aim of the current study was to assess how the specific architecture of the anterior heart field could result in an asymmetric addition of OFT myocardium during cardiac development. We demonstrate asymmetry in contribution of myocardial precursors within the anterior heart field and postulate a basis for OFT rotation and for subsequent normal positioning of the aortic and pulmonary trunk orifice. Additionally we studied this phenomenon in VEGF120/120 embryos which have been described with double outlet right ventricle.

RESULTS

Early myocardial outflow tract development

At embryonic day (E) 9.5 and E10.5, the OFT was not septated yet and was positioned entirely above the primitive right ventricle. At E9.5, the OFT consisted of a myocardial part lined on the inside by cardiac jelly (in subsequent stages developing to endocardial cushion tissue), and of an aortic sac, connecting to the pharyngeal arch arteries (Figure 1). At this stage, the pro-epicardial organ at the venous pole of the heart could be distinguished and expressed WT-1. No epicardial covering of the primitive heart tube was present yet at this stage, with the exception of a few cells (Figure 1a), while the coelomic wall covering the OFT was also still negative for WT-1. Nkx2.5 expression was observed in the ventral endoderm of the foregut, in mesenchymal cells at the level of the OFT as well as in the coelomic wall being more pronounced at the left side of the OFT (Figure 1b). At stage E10.5, faint expression of MLC-2a was observed in the mesenchyme surrounding the aortic sac at the left side, where lining of the coelomic cavity joins the OFT myocardium (arrow in Figure 1c). Here MLC-2a expression intensified along the myocardial OFT towards the body of the right ventricle, indicating myocardial differentiation (Figure 1c). Expression of Isl1 was marked within the cardiac mesoderm and faded towards the differentiating myocardium of the OFT (Figure 1d). Nkx2.5 was strongly expressed in the anterior heart field, including the coelomic wall, and in the OFT myocardium at E10.5 (Figure 1f). The expression of both Isl1 and Nkx2.5 within the anterior heart field mesenchyme was observed as a central cluster of cells ventral to the foregut, extending preferentially on the left side of the aortic sac and surrounding the left 6th pharyngeal arch artery (Figure 1d, f), forming the region surrounding the pulmonary trunk and the future ductus
Figure 1. Expression patterns of MLC-2a, Nkx2.5 Isl-1 and WT-1 in myocardial and mesenchymal regions of the developing outflow tract. Sections through the outflow tract of a wildtype mouse heart embryonic day (E) 9.5 (a,b) and E10.5 (c-f). 

a. Expression pattern of WT-1. Besides a few WT-1 expressing epicardial cells (arrowhead), epicardial covering of the heart is largely absent at this stage. Expression of WT-1 in the coelomic wall in the region of the outflow tract (OFT) is also still largely absent.

b. Nkx2.5 expression can be observed in the endoderm (End), as well as in the coelomic wall bordering the OFT (arrowhead), more pronounced on the left side as compared to the right.

c. The myocardial marker MLC-2a stains the myocardium of the OFT. The myocardium is lined on the inside by endocardial cushion tissue (EC). A more faint MLC-2a staining is seen in the region of the second heart field mesoderm (arrow) being more prominent on the left side of the aortic sac (AoS).

d. Expression of Isl-1 is prominent in the endoderm (End) and the anterior second heart field mesoderm (asterisk). It extends along the left lateral side of the AoS up to the myocardial OFT. Isl1 expression is also found in some of the myocardial cells (M) and the coelomic wall lining (Coe, arrowhead in d), being also more prominent on the left side as compared to the right side. The faint staining in the right-sided mesenchyme most likely represents vascular smooth muscle cells of the developing arterial wall.

e. Expression pattern of WT-1. Note the expression in the region of the coelomic wall at the right side (arrowhead), as compared to the lack of expression in the coelomic epithelium at the left side (open arrow head).

f. Nkx2.5 expression is markedly asymmetric (asterisk) and found more prominently of the left side of the OFT (future pulmonary trunk) and only sparsely on the right side (future aorta). Note the pronounced Nkx2.5 expression in the cells of the coelomic wall on the left side (arrowhead), as compared to the largely absent expression in the coelomic wall on the right side (open arrowhead). CJ: cardiac jelly, DAo: dorsal aorta, G: foregut, LA: left atrium, LV: left ventricle; RV: right ventricle; Bar: 100μm.
arteriosus. On the right side in the cardiac mesoderm, in the region bordering the aortic wall, expression of Isl1 and Nkx2.5 was absent at this stage. Moreover, the faint MLC-2a staining was missing. A WT-1 positive coelomic wall now covered the Nkx2.5 negative mesoderm on the right (putative aortic) side. Lacking at this stage was a WT-1 positive coelomic wall covering the Nkx2.5 positive population on the left (putative pulmonary) side (Figure 1e). WT-1 positive epicardial cells, derived from the pro-epicardial organ, were found on the atrial and ventricular myocardium.

**Expression of myocardial progenitor markers during septation and before outflow tract rotation**

At E11.5, septation of the aorta and pulmonary trunk and orifice level had initiated by condensed mesenchyme (Figure 2a). A comparable expression pattern of Isl1, Nkx2.5, MLC-2a and WT-1 expression with stage E10.5 was observed. A mesenchymal column of Nkx2.5 positive...
cells extended from the cardiac mesoderm in the anterior heart field to the left side of the OFT beneath the left 6th pharyngeal arch artery, where it connected to the subpulmonary myocardium (Figure 2a-c and f,g). 3D-reconstruction of the OFT myocardium, based on the expression pattern of MLC-2a, demonstrated an anterior and posterior cranial extension of myocardium resulting in a saddle-shaped distal myocardial OFT border (Figure 2d,e and h,i, dotted lines). The condensed mesenchyme of neural crest origin connects to the endocardial cushions at the top of the myocardial extensions. Mesenchymal expression of Nkx2.5 (indicated in yellow in the 3D-reconstructions) and Isl1 could be observed as a central cluster of cells located in the pre-pharyngeal mesoderm dorsal to the level of the OFT (Figure 2i), extending in the indentation between the myocardial extensions on the left side where the pulmonary trunk connects to the subpulmonary myocardium (Figure 2d). This area of the coelomic wall lacked WT-1 (Figure 2j; 3D-reconstructions of epicardial covering are shown in Figure 2 k,l). Nkx2.5 expression was absent in the indentation between the myocardial extensions on the right side in the aortic orifice region (Figure 2e) where the coelomic wall is positive for WT-1 (Figure 2j; Figure 2k,l). Similar to E10.5, Isl1 expression in the cardiac precursor cells showed an asymmetric distribution favouring the pulmonary side (not shown). In the OFT myocardium the expression was lost whereas Nkx2.5 as well as MLC-2a was present in both the subaortic and subpulmonary myocardium. An interactive pdf file of a 3D-reconstruction of stage E11.5 is provided in online Supplemental File 2a).

Positioning of the pulmonary trunk and aorta: The pulmonary push concept

At E12.5, a distinct column of Nkx2.5 expressing mesenchymal cells was found exclusively within the indentation at the orifice level at the pulmonary side but not in the aortic region (Figure 3a-c, Supplemental file 1). From stage E12.5 onwards, the entire heart was covered by WT-1 expressing epicardial cells. In contrast to previous stages, the coelomic epithelium at the left side of the outflow tract now also expressed WT-1 and, similar to previous stages, WT-1 was expressed in the coelomic epithelium at the right side. From E12.5 to E14.5, the orifice of the future pulmonary trunk became progressively positioned in an anterior and rightward direction (Figure 3a, d, black dot and arrow). An interactive pdf file of a 3D-reconstruction of stage E12.5 is provided in online Supplemental File 2b).

At E14.5, the pulmonary trunk and orifice reached their definitive position, which is anterior to the aortic orifice (Figure 3 d-f). The mesenchymal column of Nkx2.5 expressing cells was no longer present at E13.5 and E14.5, indicating that myocardial precursors are not incorporated anymore from the anterior heart field into the OFT. Concurrent with the repositioning of the pulmonary orifice, the atrioventricular canal became positioned below the aortic orifice as it expanded rightward during development (Compare green dot and arrow in Figure 3b and e).
MORPHOGENESIS OF OUTFLOW TRACT ROTATION

Abnormal development: Expression of myocardial progenitor markers in the VEGF120/120 model

To test the hypothesis of the pulmonary push concept, development of the OFT was studied in VEGF120/120 mouse embryos of stage E10.5. For the current study, focus was directed specifically at the Nkx2.5 and Isl-1 positive myocardial precursors at the OFT of the heart,
as compared to results obtained in wild type embryos. In wild type embryos at stage E10.5, a well-organized Nkx2.5 positive, Isl-1 positive cluster of cells was observed in the cardiac mesenchyme (Figure 4a-c, asterisk in Figure 4b), and an elongated column of Nkx2.5 positive cells was observed extending along the left side of the OFT (dotted area in Figure 4b). In the stage E10.5 VEGF120/120 embryos a large cluster of cells was also observed in the cardiac mesenchyme (asterisk in Figure 4e), however, in contrast to wild type, we observed an abnormal

![Figure 4](image.png)

**Figure 4.** Expression of myocardial progenitor markers in the VEGF120/120 model. Results in E10.5 in wild-type (a-c) and VEGF 120/120 (e-g) embryos. a-c: Three subsequent sections, stained for MLC-2a (a), Nkx2.5 (b) and Isl1 (c) are shown. In wild type embryos, a group of Isl1/Nkx2.5 positive cells are located behind in the heart in the pre-pharyngeal mesoderm (asterisk in b). An elongated column of Nkx2.5 (b) Isl1 (c) positive cells can be observed extending along the left side of the outflow tract (OFT) (indicated by the dotted line in b). Upon differentiation, cells increasingly express the myocardial marker Mlc2a (a). d-f: Three subsequent sections in VEGF120/120 embryos, stained for MLC-2a (d), Nkx2.5 (e) and Isl1 (f) are shown. In VEGF 120/120 embryos, the cluster of Isl1 (g) and Nkx2.5 (f) positive precursors (asterisk in f) shows an abnormal organisation. The column of cells extending on the left side of the OFT is only very short (compared length of the dotted lines in b and f). Abbreviations: AoS; aortic sac, End: endoderm, LA: left atrium, G: foregut, 6th: sixth pharyngeal arch artery. Bar: 100μm.
organisation of this Nkx2.5 positive anterior heart field mesoderm in front of the pharynx. The extension of this population along the pulmonary side of the OFT up to the pulmonary myocardium was very short with only a small area facing the coelomic cavity (Figure 4d-f, compare the length of the dotted lines in Figure 4b and 4e).

**DISCUSSION**

OFT remodeling is an essential part of heart development. Normal separation and positioning of the great arteries of the OFT is necessary for the heart to sustain its function as a central pump coordinating the blood flow through the systemic and pulmonary circulations. Congenital OFT malformations may severely compromise normal physiology, resulting in hemodynamic overload, cyanosis and early death. Knowledge of the developmental processes resulting in proper OFT formation and remodeling, as well as the pivotal cells or cell groups involved, is essential to comprehend when and why malformations, resulting in congenital heart disease, occur. In recent years, the relevance of the contribution of cells derived from the second heart field has been attested, although the exact mechanism of OFT remodeling, including the final positioning of the aorta and the pulmonary trunk and their orifices, is unclear.

It has been shown that the anterior part of the second heart field plays a crucial role in normal OFT development together with neural crest and epicardium. During cardiovascular development, cells from the three above-described populations are recruited to the linear heart tube via both the arterial and the venous pole from E9.5 onwards. It has been demonstrated previously that the myocardial part of the OFT comprising both the subaortic and subpulmonary myocardium, is derived from the second heart field. This myocardium has been shown to have a common lineage relationship to the right- and left-sided head muscles, respectively.

We have, based on results in the VEGF120/120 model, previously postulated that the second heart field-derived subpulmonary myocardium may be highly sensitive for signaling of factors like VEGF and Notch, which may underlie the observed malpositioning of the OFT vessels in the VEGF120/120 mutants, but did not show a direct link with a contribution of the second heart field mesenchyme to the OFT in this study.

The pulmonary trunk originates from the posterior left side of the aortic sac, whereas the putative aorta is originally positioned on the right side. After OFT development is completed, however, the pulmonary trunk has obtained a right anterior position. A dynamic process is required during cardiac development resulting in a rotational movement of the pulmonary trunk and orifice around the aorta.

A mechanism of cell death, predominantly present in the subaortic myocardium resulting in a shortening of the subaortic OFT, has been proposed as the mechanism for proper
formation of the ventriculo-arterial connections.\textsuperscript{20, 21, 33} Cell death is a well known mechanism in heart development and has been described to occur at the level of the OFT.\textsuperscript{34-35} However, a mechanism of asymmetric active shortening of the OFT by cell death alone is unlikely to explain the complex positioning of the OFT and does not take into account an concomitant asymmetrical gaining of length of the subpulmonary myocardium, while the subaortic myocardium remains relatively short from the onset.\textsuperscript{36}

It was previously reported that asymmetrical expression of Fgf 8/10, Pitx2c and possibly Tbx1 within the anterior heart field are involved in the induction of asymmetrical OFT growth.\textsuperscript{6, 37} Experimental knock-out of these genes resulted in the association with a large variety of fundamental defects in OFT development.\textsuperscript{38} Especially relevant seems to be the co-expression Tbx1 with Pitx2, an important gene in right-left signaling, in the pharyngeal mesoderm of the OFT. Crossing of heterozygous Tbx1 and Pitx2 mice results in cardiac anomalies including double outlet right ventricle.\textsuperscript{37} Experiments using right-and left sided second heart field labeling experiments in the chick OFT indicate contributions of second heart field precursors to distinct lateralized regions of the OFT.\textsuperscript{39} It was unclear whether other known markers of myocardial precursors within the anterior heart field demonstrate a similar asymmetric expression pattern that could explain the above-described dynamics of OFT remodeling. We have now shown that it is also applicable to Nkx2.5 and Isl1 expression. It is remarkable that the Nkx2.5 and Isl1 positive area of the anterior heart field was the last part of the heart to be covered by a WT-1 expressing coelomic epithelium i.e. the epicardium. On the aortic side, that is supposed to originate from the right sided pre-pharyngeal mesoderm (current study, and\textsuperscript{39-40}), the disappearance of the Nkx2.5 positive population at day 9.5 was followed by the start of expression of WT-1 in the coelomic wall. A possible interaction between Nkx2.5 and WT1 needs further study.

Our studies suggest that lengthening of the subpulmonary myocardium of the OFT is active until day 12.5 which is at least one day longer than proposed in literature\textsuperscript{12, 41} but in line with studies in chick that have indicated contributions to the OFT up to HH24 (correlating with mouse day12.5), respectively.\textsuperscript{13}

The aim of the current study was to assess whether anterior heart field architecture could provide clues relevant for normal positioning of the aorta orifice and pulmonary trunk during mammalian heart development. The main finding of the present study, based on 3D-reconstructions of consecutive developmental stages, was that myocardial precursor cells were asymmetrically positioned in the OFT during development, as they were distinctively observed at the pulmonary side, whereas they were almost absent at the aortic side. The current study shows for the first time that continued addition from the Nkx2.5 expressing mesenchymal myocardial precursors below the left 6th pharyngeal arch artery may push the future pulmonary artery orifice in an anterior and rightward direction.

In addition, we have shown that this addition was disorganized and the extension towards the left side of the OFT shorter than in wildtype in the VEGF120/120 model, that has a phe-
notype in which OFT malformations are predominant.\textsuperscript{19} It was recently indicated that the myocardium at the base of the pulmonary trunk and the myocardium at the base of the aorta originate from distinct developmental portions of second heart field derived cells.\textsuperscript{40} Clonal studies showed a larger number of cell clones in the subpulmonary region, indicative for a higher proliferative rate, whereas in the subaortic region clones were very small.\textsuperscript{40} The subpulmonary myocardium remained in a posterior position in the Pitx2c knock-out mouse that presents with OFT malformations such as transposition of the great arteries and double outlet right ventricle.\textsuperscript{14} We postulate that the dominant left-sided expression of Pitx2c induces the expression of Nkx2.5 within the anterior heart field, which might be disturbed in Pitx2c knock-out mouse embryos. In our study of the VEGF120/120 embryo we could discern the malpositioning of the Nkx2.5 positive mesenchymal cells at the pulmonary side of the OFT in line with Bajolle et al. The disorganization of the Nkx2.5 positive SHF mesenchyme in front of the pharynx is indicative of an abnormal differentiation of the SHF. The small area of Nkx2.5 positive myocardial precursors covering the pulmonary side of the OFT could underlie the observed tetralogy of Fallot and double outlet right ventricle in the VEGF120/120 hearts\textsuperscript{19} in which subpulmonary myocardium is insufficiently added.

In transposition of the great arteries, the arterial orifices are positioned in almost one plane\textsuperscript{36} supporting the fact that addition of myocardium to the right ventricular OFT has either not taken place or was deficient. Similar observations have been made for mouse hearts with double outlet right ventricle with a marked shortening of the right ventricular OFT.\textsuperscript{42, 43} To date specific patterns in the second heart field for Nkx2.5 and Isl1 have not been reported. Results in the VEGF120/120 model in the current study support the postulated mechanism of a deficient and disorganized addition of myocardial precursors to the pulmonary side of the OFT. An asymmetric contribution of anterior heart-field derived myocardium around and specifically below the left 6th pharyngeal arch artery during crucial stages of OFT development, provides an explanation for the dynamic process that results in positioning of the pulmonary artery orifice in its normal left anterior location. It must be questioned whether a true rotational motion, based on spiraling outflow tract ridges, occurs. The latter explanation has dominated the positional remodeling for several decades, and an abnormal torsion was considered the underlying mechanism for OFT tract abnormalities as observed in transposition of the great arteries.\textsuperscript{15–16, 44, 45} This explanation for rotation of the outlet however has also been the subject of controversy over the past decades, and several authors have indicated that no such rotation occurs during normal development.\textsuperscript{5, 46–48} Given the second heart field contributions to OFT remodeling, new concepts for related abnormalities occur. Obviously, proper positioning of the great arteries needs to occur in order to achieve the normal morphological relation of the aorta and pulmonary trunk. Results of our study support the occurrence of rotation. We propose that, rather than a mechanism based on spiraling of the endocardial OFT cushions, an asymmetrical lengthening of the right ventricular OFT
causes the pulmonary trunk to be pushed towards its definitive position in front of the aorta, which is observed as a rotation.

CONCLUSION

We conclude that the addition of Nkx2.5 positive myocardial precursors from the anterior heart field occurs during normal development predominantly below the left branch of the 6th pharyngeal arch artery. We postulate that this results in a movement without spiralization of the OFT in which, due to the continued addition of right-sided myocardium, the pulmonary trunk orifice is pushed in a rightward and anterior direction, which presents a new explanation for the rotation of the pulmonary orifice and trunk. This mechanism is referred to as pulmonary push, which results in a rotation of the pulmonary orifice to an anterior position. As the term rotation has been intrinsically used in many ways and for many OFT structures, we prefer the use of “pulmonary push” to describe the mechanism of OFT positioning. Deficient or disorganized positioning of the Nkx2.5 positive precursors, as was observed in the VEGF120/120 mutant, might explain cardiac malformations with side-by-side position of the great arteries.

EXPERIMENTAL PROCEDURES

Embryonic material and immunohistochemical procedures

The handling of all animals and embryos was according to the Guide for Care and Use of Laboratory Animals, as published by the NIH. Wildtype mouse embryos were obtained from the CLB-Swiss strain. In addition to studies of wildtype embryos and to test the hypothesis of the pulmonary push concept, VEGF120/+ mice were crossed to obtain VEGF120/120 embryos and VEGF+/+ wild type littermates. The day the vaginal plug was detected, was designated embryonic day (E) 0.5. Pregnant female mice were sacrificed on consecutive days from E9.5 onward up until E14.5 for wildtype mice and, per day, three embryos were harvested for the study. For VEGF120/120 mice, stage E10.5 was studied. For further immunohistochemical staining, all embryos were embedded in paraffin after fixation in 4% paraformaldehyde in phosphate buffered saline (0.1 M, pH 7.2) and subsequent dehydration. Embryos were sectioned transversely to the body axis (5μm). Due to the position of the heart in the thorax, the level of the outflow tract was therefore positioned in a more frontal plane. Sections were serially mounted on glass slides. Immunohistochemical staining was performed with antibodies against the myocardial marker MLC-2a (1/6000, kindly provided by S.W. Kubalak, Charleston, SC, United States); Nkx 2.5 (1/4000, Santa Cruz Biotechnology Inc., CA, United
States, SC-8697), expressed in atrial and ventricular and outflow tract myocardium, as well as in the mesenchyme of the second heart field; the second heart field marker Islet 1 (Isl1) (1/400, mouse monoclonal antibody, clone 39.4D5, Developmental Studies Hybridoma Bank) and Wilms Tumor 1 (WT-1) (1/1000, Santa Cruz Biotechnology Inc., CA, United States, SC-192), to show the coelomic wall covering and the pro-epicardial organ derived epicardium. The slides were first incubated for 45 min using ABC-reagent (Vector Laboratories, Burlingame, United States PK 6100), and then with 400 μg/mL 3,3'di-aminobenzidin tetrahydrochloride (DAB, Sigma-Aldrich, St Louis, United States, D5637) dissolved in trismaleate buffer pH 7.6 to which 20 μl H2O2 was added. The latter incubation was done 5 min for MLC-2a and 10 min for Nkx 2.5, Isl1 and WT-1. Furthermore, counterstaining was done using 0.1% hematoxylin (Merck, Darmstadt, Germany) for 5 sec, and the slides were subsequently rinsed with tap water for 10 min. Finally, slides were dehydrated and mounted with Entellan (Merck, Darmstadt, Germany).

Three-dimensional reconstruction

To describe the dynamics of OFT development within a spatial context, three-dimensional reconstructions were made from E9.5 to ED14.5 embryos. Micrographs of serial sections were processed using the AMIRA software package (Template Graphics Software, San Diego, CA) as described previously. First, the myocardium was reconstructed using the expression pattern of MLC-2a, after which the expression of Isl1, WT-1 and Nkx2.5, was superimposed to depict myocardial progenitors within the anterior heart field. Isl-1 expression was used to demarcate the anterior heart field, Nkx2.5 was used to identify precursors of OFT myocardium, and WT-1 to show which parts of the outflow tract were covered with epicardium.

Acknowledgements

We thank Ron Slagter for designing the animation of outflow tract remodelling.
REFERENCES


40. Bajolle F, Zaffran S, et al. Myocardium at the base of the aorta and pulmonary trunk is prefigured in the outflow tract of the heart and in subdomains of the second heart field. Dev Biol 2008; 313:25-34.


48. De la Cruz MV, Da Rocha JP. An ontogenetic theory for the explanation of congenital malformations involving the truncus and conus. Am Heart J 1956;51:782-805.

Supplemental file 1

Animated 3D-reconstruction demonstrating the column of Nkx2.5 expressing cells (bright yellow) within the heart and anterior heart field at ED 12.5. This column is rendered transparent during part of the animation to demonstrate its relation to the aorta (red) and pulmonary trunk (dark blue). The outflow tract and right ventricle are depicted in light-yellow, whereas the left ventricle is depicted in grey. Other colour coding: Light blue: endocardial cushion tissue
Supplemental files 2a, b en c

Interactive pdf files of 3D-reconstructions in WT embryos stage E11.5 (Supplemental file 2a), stage E12.5 (Supplemental file 2b) and E14.5 (Supplemental file 2c) that can be used to optimise insight of the orientation of the Nkx2.5 positive column of cells in relation to other cardiac structures. For viewing these files, Adobe reader version 8.0 or higher is required.

To use the interactive file, one should open the pdf file, and expand the cardiac compartments by clicking on the small “plus: sign in the upper left hand panel, just left of the text “Heart E11.5”, “Heart E12.5” or “Heart E14.5” for supplemental figures 2a, 2b or 2c, respectively. In some versions, the so called “Toggle Model Tree”, that can be found in the superior toolbar (just above the 3D figure) needs to be clicked before the upper and lower left hand panels appear.

The default setting of the 3D-image in the right hand panel is an anterior view of the heart, with all the cardiac compartments depicted. Colour coding is as follows: Grey: myocardium, dark blue: ductus arteriosus/pulmonary trunk/pulmonary arteries. Red: aorta, yellow: Nkx2.5 expressing mesenchymal cells, light blue: endocardial cushion tissue.

These different cardiac compartments and colour codes are also shown in the left hand side of the pdf, after clicking the expansion (“plus”) icon described above. By clicking on the marks
(v's) one can choose to eliminate different compartment from the figure. Also, by right clicking on the text describing the different compartments, one can choose for the option “transparent” in order to make the compartment of choice transparent to visualize the Nkx2.5 positive column of cells more clearly. In the lower left hand panel, other views are depicted, that will appear when clicking on them. These other views include a left lateral view, right lateral view, dorsal view and a superior view. One can also manually choose the preferred view by moving the mouse over the 3D-animation.