Normal Development of the Outflow Tract in the Rat

Jing Ya, Maurice J.B. van den Hoff, Piet A.J. de Boer, Sabina Tesink-Taekema, Diego Franco, Antoon F.M. Moorman, Wouter H. Lamers

Abstract—The outflow tract (OFT) provides the structural components forming the ventriculoarterial connection. The prevailing concept that this junction “rotates” to acquire its definitive topography also requires a concept of “counterrotation” and is difficult to reconcile with cell-marking studies. Rats between 10 embryonic days (EDs) and 2 postnatal days were stained immunohistochemically and by in situ hybridization. DNA replication was determined by incorporation of bromodeoxyuridine and apoptosis by the annexin V binding and terminal deoxynucleotidyl transferase–mediated dUTP-X nick end labeling (TUNEL) assays. Starting at ED12, cardiomyocytes in the distal (truncal) part of the OFT begin to shed their myocardial phenotype without proceeding into apoptosis, suggesting transdifferentiation. Myocardial regression is most pronounced on the dextroposterior side and continues until after birth, as revealed by the disappearance of the myocardial cuff surrounding the coronary roots and semilunar sinuses and by the establishment of fibrous continuity between mitral and aortic semilunar valves. Fusion of the endocardial ridges of the truncus on late ED13 is accompanied by the organization of α-smooth muscle actin—and nonmuscle myosin heavy chain–positive myofibroblasts into a central whorl and the appearance of the semilunar valve anlagen at their definitive topographical position within the proximal portion of the truncus. After fusion of the proximal (conal) portion of the endocardial ridges, many of the resident myofibroblasts undergo apoptosis and are replaced by cardiomyocytes. The distal myocardial boundary of the OFT is not a stable landmark but moves proximally over the spiraling course of the aortic and pulmonary routes, so that the semilunar valves develop at their definitive topographic position. After septation, the distal boundary of the OFT continues to regress, particularly in its subaortic portion. The myocardializing conus septum, on the other hand, becomes largely incorporated into the right ventricle. These opposite developments account for the pronounced asymmetry of the subaortic and subpulmonary outlets in the formed heart. (Circ Res. 1998;82:464-472.)

Key Words: development ■ outflow tract ■ semilunar valves ■ rat ■ transdifferentiation
topographic position. We will argue that transdifferentiation, rather than differential growth or apoptosis, is primarily responsible for the proximal movement of the distal myocardial boundary during septation. After septation, the distal boundary of the OFT continues to regress, particularly in its subaortic portion. The conus septum, on the other hand, becomes a well-developed mainly right ventricular structure as a result of ingrowth of myocytes from the surrounding part of the OFT myocardium into the conus septum ("myocardialization"). These opposite developments account for the pronounced asymmetry of the subaortic and supravalvular outlets in the formed heart. Finally, we show that α-SMA–positive neural crest–derived myofibroblasts that populate the walls of the branchial arches and the endocardial ridges, differ from α-SMA–positive cardiac myocytes by their exclusive expression of nonmuscle MHCs.

Materials and Methods

Animals

Wistar rats, obtained from Broekman, Someren, the Netherlands, were kept in a controlled dark-light cycle (light, 11:00 AM to 11:00 PM). Females in estrus were identified and mated. The time of mating was regarded as the beginning of gestation (ED0). Rats were decapitated under CO2 anesthesia, and embryos were harvested. To identify proliferating cells, pregnant animals were equipped with an intraperitoneal Alzet miniosmotic pump (model 2001D) supplying 80 μg BrdU/h, primed with 50 mg/kg BrdU (Sigma Chemical Co) intra-peritoneally, and killed 24 hours later. To identify early apoptotic cells, embryos by ED14 and ED16 were isolated without disturbing the fetal membrane and placental circulation, injected with 2.75 μg (50 μL) recombinant human fluorescein-labeled annexin V, and killed 15 minutes later. Animal welfare was in accordance with institutional guidelines of the University of Amsterdam.

Embryos were fixed overnight in ice-cold methanol/acetic acid/water (2:2:1 vol/vol) for immunohistochemistry in 4% (wt/vol) formaldehyde in PBS (150 mmol/L NaCl and 10 mmol/L sodium phosphate, pH 7.4) for in situ hybridization or in 4% (wt/vol) formaldehyde, 1 mmol/L MgCl2, and 1 mmol/L CaCl2 for 4 hours at 4°C for apoptosis studies. Embedding, storage, and staining procedures were as detailed elsewhere.

A total of 50 immunohistochemically stained embryos (ED10, 3 embryos; ED11, 5 embryos; ED12, 4 embryos; ED13, 9 embryos; ED14, 8 embryos; ED15, 3 embryos; ED16, 7 embryos; ED17, 1 embryo; ED18, 2 embryos; ED19, 3 embryos; ED20, 3 embryos; and postnatal day 2, 2 embryos), 20 BrdU-stained embryos (4 each for ED13 to ED17), 8 TUNEL-stained embryos (2 each for ED14 to ED17), and 16 annexin V–stained embryos (4 each for ED14 to ED17) were investigated. In addition, our hematoxylin-azophloxine–stained reference series of rat embryos (45 specimens between ED10 and birth) was examined when appropriate.

Immunohistochemistry

Serial sections were incubated overnight with appropriately diluted monoclonal antibodies against either α-MHC (1:10, code 249 to 5-A-4), β-MHC (1:10, 219–1D1), α-SMA (1:1000, Sigma IMM-2), desmin (1:50, Monosan, Mon 3001, clone 33), a cytoplasmic antigen of macrophages (1:200, Instruchemie, IC25–896, clone ED1), BrdU (1:50, Becton Dickinson), or a polyclonal antibody against fibronectin (1:6000, AB 1942, Brunschwig Chemie). To retrieve the BrdU epitope, sections were submerged in boiling 10 mmol/L sodium citrate buffer for 1 minute and quenched in ice-cold PBS before being exposed to antibody. Early apoptotic cells were identified by their binding of annexin V, whereas late apoptotic cells were identified by the TUNEL assay according to the manufacturer’s instructions. Annexin V binding and labeled DNA nicks were demonstrated with a peroxidase-coupled monoclonal antibody against fluorescein (converter-PD, 1:15, Boehringer-Mannheim). Antibody binding was visualized with diaminobenzidine and H2O2, as described.

In Situ Hybridization

In situ hybridization was performed as described in detail previously. Antisense RNA probes were made from human NMMHC A cDNA (934 bp), human NMMHC B cDNA (4650 bp), mouse NMMHC cDNA (350 bp), and rat MLC2A cDNA (550 bp). NMMHC A and NMMHC B RNAs were labeled with two nucleotides; the others, with one.

Three-Dimensional Reconstruction

Selected hearts of immunostained serial sections of rat embryos were reconstructed three-dimensionally as described and rendered with the help of a medical artist.

Nomenclature

The OFT is defined as that part of the embryonic heart that connects the embryonic right ventricle with the aortic sac. The aortic sac, in turn, is the mesenchymal structure that connects the OFT with the branchial arch arteries. Our denarcamation of the boundaries of the OFT follows that provided by Pexieder. Its distal arterial boundary is taken to coincide with the distal boundary of the myocardium. Externally, its proximal ventricular boundary can be identified by a furrow on the myocardial surface, whereas its internal boundary corresponds with that of the endocardial ridges and with the boundary between the developing trabeculae of the right ventricle and the nontrabeculated wall of the OFT. The septal endocardial ridge takes origin on the ventricular septum and covers the left lateral and posterior wall of the OFT, whereas the parietal ridge takes origin on the right lateral part of the junction between the OFT and right ventricle and covers the right lateral and anterior wall of the OFT (see Reference 39). A characteristic bend halfway down the OFT corresponds internally with a fairly acute change in direction of the endocardial ridges; in accordance with Pexieder, we will designate the portion of the OFT that is proximal (upstream) to this bend as the conus and the portion distal (downstream) to it as the truncus.

Results

Changes in the Configuration of the Distal Myocardial Boundary

Soon after the heart tube becomes identifiable at Thelier’s stage 12 (ED9), the OFT develops into a prominent feature of the ascending loop of the heart tube. As demonstrated by the staining pattern of α- and β-MHC, the distal boundary of the myocardium of the OFT initially does not reach the aortic sac. In the specimen shown in Fig 1A, the myocardium reaches up to the developing bend between the conal and the truncal part of the
OFT. The myocardial expansion toward the arterial pole is completed in the course of ED10 (Theiler’s stages 13 and 14). During ED11 (Theiler’s stages 15 and 16), the distal myocardial rim remained circular in configuration and apposed to the aortic sac (Fig 1B). However, at ED12 (Theiler’s stage 17), we observed that the staining intensity of both α-MHC (not shown) and β-MHC (Fig 1C to 1F) started to decline on the right side of the distal boundary. Although the cells in the area showing this heterogeneous weak MHC staining property on ED12 no longer expressed MHCs on early ED13 (Theiler’s stage 18), the process was seen to have spread to adjacent myocardial cells (Figs 2 and 3). Meanwhile, a similar process had also started on the left side of the OFT (Fig 3), so that the originally flat distal myocardial boundary of the OFT had transformed into a saddle-shaped structure by late ED13 (Theiler’s stage 19). At this stage, the “seat” of the saddle was deeper on the right than on the left side and extended halfway through the truncus (Fig 3F and 3G). Meanwhile, growth of the right ventricle caused the bend in the OFT to begin straightening. In the course of ED14, MHC-positive cells continued to shed their myocardial phenotype, but now the anterior and posterior “tongues” had also become involved. As a result, the MHC-positive dextroposterior boundary of the myocardium was located more proximal than the rest of the distal rim of the myocardium (Figs 3F, 3G, and 4).

We measured the length of the myocardial OFT, ie, the distance between the distal rim and the ventricular base of the endocardial ridges, between ED13 and ED18. The distance declined ≈60% between ED13 and ED14 to remain virtually constant thereafter until ED18. This regression could not be accounted for by local apoptosis, which was absent from the heart at this stage (Fig 5E). Furthermore, the OFT myocardium had a very low DNA-synthesizing activity, as demonstrated by a very low incorporation of BrdU between ED12 and ED15 (Fig 5A and 5C). In fact, the difference in BrdU incorporation between OFT and ventricular myocardium was so pronounced in this period that it could have been used to demarcate both cardiac segments. In sharp contrast, however, the areas that were in the process of shedding their myocardial

**Figure 1.** Right lateral views of reconstructions of the OFT in ED10, ED11, and ED12 rat embryos (panels A, B, and C, respectively) and sections of the ED12 embryo (panel C) showing the expression of β-MHC (panel D), α-SMA (panel E), and desmin (panel F) in the heart. Panels A to C show the distal boundary of the OFT as identified by the expression of β-MHC (interrupted line), the myocardium losing its myocardial phenotype (panel C, asterisk in shaded area), and the reflection of the epicardium to the pericardium (arrow pointing at stippled line). Note the development of the bend between the truncal and the conal portions of the OFT. Note also that the area showing regression of β-MHC staining (panel D, arrows) cannot yet be recognized in the α-SMA-stained and desmin-stained sections (panels E and F, respectively). AS indicates aortic sac; 4th, part of aortic sac giving access to fourth branchial arch arteries; 6th and 314th, sixth and combined third and fourth branchial arteries, respectively; and RV, right ventricle. Bar=50 μm.

**Figure 2.** Fate of the distal boundary of the OFT myocardium in early (panels A to D) and mid-ED13 (panels E to J) rat embryos. The embryo in panels A to D was sectioned transversely, and that in panels E to J was sectioned frontally. Panels A, B, H, and I were stained for the presence of β-MHC; panels C and E to G, for the presence of α-SMA; and panels D and J, for the presence of desmin. Panel B is a higher magnification of the box appearing in panel A. Panels F, I, and J are serial sections, and panels E and H are neighboring sections. Note the regressive staining for β-MHC (panels A, B, H, and I), leaving the anterior and posterior “tongues” of remaining myocardium (panel H). Panels C and E to G show the population of endocardial ridges by α-SMA-positive myofibroblasts; the aorticopulmonary septum (APS, panel E) is laterally continuous with the septal and parietal ridges (S and P, respectively; panels F and G). The intercalated ridges (I) contain many desmin-positive cells (panel J) but virtually no α-SMA-positive cells (panel F). A indicates aorta; PT, pulmonary trunk; RV, right ventricle; and 6th, 6th arch arteries. Bar=100 μm (panels A, C, and D) and 50 μm (panels B and E to J).
phenotype were characterized by a rather high level of incorporation of BrdU on both ED12 (not shown) and ED13 (Fig 5A and 5C). The loss of myofibrillar staining therefore occurs in healthy nonreplicating cells and is followed by a mitotic wave. From ED15, DNA synthetic activity gradually increased in the remaining myocardium of the OFT, so that from ED17 onward, it could no longer be distinguished by this criterion from the ventricular myocardium (not shown). The coronary arteries were found to be useful structures for following the subsequent fate of the distal boundary of the OFT myocardium. Coronary arteries could first be identified unambiguously near the distal rim of the OFT at ED16 (Fig 6A), although suggestive structures were already seen at ED15 (Fig 4G to 4I). More important, Fig 6A clearly shows that the aortic root of the coronary arteries was localized within the confines of the myocardium of the OFT. This cuff of OFT myocardium still supported the semilunar valves and, hence, the fragmented staining pattern of $\beta$-MHC suggested regressive changes. Indeed, 1 week later, the myocardial cuff was no longer present, and a fibrous continuity had developed between the mitral valve and the semilunar valves of the aorta, whereas the semilunar valves of the pulmonary trunk were supported by a free-standing pulmonary infundibulum (not shown; see Reference 24).

Remodeling of the Endocardial Tissue of the OFT

Up to ED12, the endocardial tissue of the OFT lined the inside of the myocardial tube as an almost uniformly thick cuff. $\alpha$-SMA–positive cells could be seen to populate the endocardial ridge tissue as a result of their conjugation with the myocardial cuff. The endocardial tissue of the OFT was, however, not homogenous in this regard. By ED12, the endocardial tissue of the OFT consisted of a rather thick layer that tapered off toward the aortic root (Fig 3). More anteriorly, the endocardial tissue continued in the atrioventricular septum as a rather thin layer (Fig 3). From ED12 onward, the endocardial tissue of the OFT became progressively thinner in the region adjacent to the aortic root and thicker in the region adjacent to the pulmonary trunk (Fig 4). The transition between the endocardium and the myocardium was marked by a layer of endocardial cells that became progressively thicker toward the aortic root (Fig 4). The endocardial tissue of the OFT was, therefore, not a homogeneous structure but consisted of two distinct layers: an outer layer that was continuous with the myocardium and an inner layer that was continuous with the intima of the coronary arteries. The outer layer of the endocardial tissue of the OFT was composed of cells that were intensely desmin-positive and $\alpha$-SMA-positive (Fig 3). The inner layer of the endocardial tissue of the OFT was composed of cells that were intensely $\alpha$-SMA-positive and $\beta$-MHC-positive (Fig 3). The transition between the endocardium and the myocardium was marked by a layer of endocardial cells that became progressively thicker toward the aortic root (Fig 4). The endocardial tissue of the OFT was, therefore, not a homogeneous structure but consisted of two distinct layers: an outer layer that was continuous with the myocardium and an inner layer that was continuous with the intima of the coronary arteries.

Figure 3. Fate of the distal boundary of the OFT myocardium in late ED13 embryos (panels A to E), dextroanterior view of OFT on early ED13 revealing area with regressing myocardial phenotype (asterisk in shaded area in panel F; reconstruction is based on the embryo shown in panels A to D of Fig 2), and right lateral view of OFT late on ED13 (panel G; reconstruction is based on the embryo shown in panels A to E). Panels A to E show transverse sections of the embryo stained for the presence of $\beta$-MHC (panels A and C), $\alpha$-SMA (panels B and E), and desmin (panel D). The sections show that the distal boundary of the OFT is further distal on the left than on the right side, that the portion of the wall of the OFT that has lost its $\beta$-MHC–positive staining has become intensely desmin-positive (see panels C and D), and that the $\alpha$-SMA–positive myofibroblasts in the aortocaval septum (APS, panel E) are anteriorly and posteriorly (panel B) continuous with the same cells in the septal and parietal endocardial ridges (S and P, respectively). Note the regression of the bend between truncal and conal portions of the OFT (panels F and G) and the anterior course of the ascending aortic trunk above the OFT compared with the straight ascending course of the pulmonary trunk (PT, panel G). A indicates aorta; RV, right ventricle; and 6th, 6th arch arteries. Bar=100 µm.

Figure 4. Fate of the distal boundary of the OFT myocardium in ED14 (panels A to F) and ED15 (panels G to I) embryos and right lateral view of the OFT of the ED14 embryo (panel J) showing that fusion is complete in the truncus portion and is progressing in the conus portion. The panels show frontal sections stained for the presence $\alpha$-SMA (panels A, D, and G), $\beta$-MHC (panels B, E, and H), and desmin (panel I). Panels C and F are stained with hematoxylin and azoflochsin to show the transition of endothelium (thick layer) into endocardium (thin layer) at the level of the valve swellings. Panels B, E, H, and J show that the myocardial rim supporting the aortic semilunar valve occupies a more proximal position than that supporting the pulmonary valve. Panels A, D, and G show that the accumulation of the $\alpha$-SMA–positive myofibroblasts into a central whorl-shaped structure leaves the developing valve swellings in the septal and parietal ridges virtually free of $\alpha$-SMA–positive cells. The valve swelling of the intercalated ridge does not express $\alpha$-SMA either but is positive for desmin (panels G and I). Panels G and H show a structure that is suggestive for a developing coronary artery (arrows). A indicates aorta; APS, aortocaval septum; and PT, pulmonary trunk. Dotted lines in panels D, G, and I indicate luminal boundary of endocardial tissue. The interrupted line in panel J indicates the distal rim of myocardium. Bar=100 µm.
main ridges because they contained very few ridges. These structures could be easily distinguished from the parietal and septal ridge persisted to form the so-called intercalated beyond the rightward bend, the endocardial tissue between the proximal part of the OFT, the endocardial tissue became concentrated entirely within both ridges, but in its distal portion, ie, the myocardium overlying the intercalated ridges from which the “seat of the saddle” forms (Fig 3). Indeed, these parts of the intercalated ridges incorporated far less BrdU than did the parietal and septal ridges (Fig 5C). Coincident with the loss of MHC staining in the overlying myocardium, the prevalence of desmin-positive cells in the intercalated ridges increased (Figs 2J and 4I). These phenotypic characteristics of the intercalated ridges (above the bend) had increased considerably more in size than their proximal counterparts and touched each other at the midpoint of the truncus, squeezing the lumen into narrow left- and right-sided passages that featured the characteristic star shape of the semilunar valve (Figs 2E to 2J and 3A to 3D). Proximal to the developing valves, the lumen of the OFT was hourglass-shaped with wider anterior and posterior passages because of the locally more lateral position of both ridges. The left-sided narrow passage was continuous with the anterior (future subpulmonary) route, whereas the right-sided narrow passage was continuous with the posterior (future subaortic) route. Just distal to the developing valves, where the wall of the OFT had lost its myocardial phenotype, the ascending aorta occupied a dextroposterior position, and the pulmonary trunk occupied a sinistroanterior position. These parts of the ascending aorta and the pulmonary trunk were being separated by the descending, intensely α-SMA–positive aorticopulmonary septum (Fig 3A to 3D). The aortic route changed direction from posterior in the conal part of the OFT, via the right side of the original truncal part of the OFT, to sharply anterior in the ascending aortic trunk (Fig 3G), whereas the pulmonary route changed direction only from anterior in the conal part of the OFT to the left side of the original truncal part of the OFT before proceeding straight into the sixth arch arteries. This configuration persisted during subsequent development.

The α-SMA–positive cells in the endocardial ridges appear to play an important role in initiating and/or enforcing fusion of the OFT myocardium (see panels C and D; IR is delineated by dotted line in panel C). However, in the areas showing a regressing myocardial phenotype, DNA replication is strong (panel A, arrow). Cells at the distal boundary of the OFT do not go into apoptosis (see panels E and F). Soon after fusion of the conal portion of the ridges, extensive apoptotic activity develops (panels G to I; panel H is a magnification of the boxed area in panel G). A indicates aorta; APS, aorticopulmonary septum; P, parietal ridge; PT, pulmonary trunk; RV, right ventricle; and S, septal ridge. Bar=100 μm (panels A to F, H, and I) and 50 μm (panel G).

**Figure 5.** DNA replication (panels A to D) and apoptosis (panels E to I) in the embryonic OFT. The sections in panels A and B were from ED13 hearts; in panels C to F, from ED14 hearts; and in panels G to I, from ED16 hearts. All panels show frontal sections, except panel I, which is a sagittal section. Panels A and C show BrdU incorporation; panels E and I, the TUNEL assay; panels G and H, the annexin V binding assay; and panels B, D, and F, MHC distribution for reference. DNA replication is very low in the OFT myocardium (see panels A and B) and in the intercalated ridges (IRs) (see panels C and D; IR is delineated by dotted line in panel C). One of our objectives was to delineate the portion of the endocardial ridges from which the semilunar valves develop. In early ED13 embryos (Theiler’s stage 18), separate parietal and septal ridges could be distinguished, but the lumen of the OFT was still undivided. In these embryos, both fourth arch arteries were connected with the anterior part of the OFT via a short median ascending trunk, whereas both sixth arch arteries had their origin directly over the posterior part of the OFT (Fig 3F). Half a day later (Theiler’s stage 19), the profile of the ridges had changed profoundly. The distal portions of the ridges (above the bend) had increased considerably more in size than their proximal counterparts and touched each other at the midway point of the truncus, squeezing the lumen into narrow left- and right-sided passages that featured the characteristic star shape of the semilunar valve (Figs 2E to 2J and 3A to 3D). One of our objectives was to delineate the portion of the endocardial ridges from which the semilunar valves develop.
of the ridges. In late ED13 hearts, the α-SMA–positive cells could be seen to align longitudinally between the attachment of the septal and parietal ridges on the lateral wall of the OFT on the one hand and their place of origin in the mesenchyme surrounding the aortic sac on the other hand (Fig 3A to 3D). Fusion of the ridges is accompanied by accumulation of the α-SMA–positive cells into a highly characteristic whorl-like structure at the site of fusion (Fig 4), depleting the more peripheral parts of the newly formed septum of α-SMA–positive cells. These α-SMA–depleted parts of the septum subsequently formed, together with the α-SMA–sparse intercalated ridges, the leaflets of the semilunar valves (Fig 7).

Below the valvular swellings, the laterally positioned ridges were still separate at ED14, even though their volume had markedly increased (Fig 4). On their fusion on ED15 (Theiler’s stage 21), the parietal and septal endocardial ridges form the conal (or infundibular) septum between the subpulmonary and subaortic outlets of the ventricles. Soon after fusion, many of the nonmyocardial cells of the septum were seen to undergo apoptosis (Fig 5G to 5I). Apoptotic cells were identified both by binding of annexin V to their membranes (early apoptotic cells) and by terminal-transferase labeling of nicked nuclear DNA (late apoptotic cells). Annexin V binding was first seen on ED15, whereas both parameters were positive on ED16 and ED17. Apoptotic involution did not include the surrounding myocardium. The apoptotic cells were found to attract fetal macrophages to execute their removal (Fig 8B and 8C).

Meanwhile, myocardial cells at the margin of the fused ridges started to migrate into the ridges to replace the regressing nonmyocardial cells (Fig 8A). Active migration is suggested by the slender shape of the myocytes and by the presence of apoptotic cells and macrophages at the myocardial front line (Fig 8B and 8C). The frequency of BrdU accumulation in these migratory myocardial cells was not higher than in the adjacent OFT myocardium (not shown), suggesting that migratory cells were not primarily recruited by cell multiplication. Myocardialization was completed first at the ventricular end of the conal septum on ED16 and last at the distal end (Figs 7 and 8). On myocardialization, the conal septum forms the supraventricular crest, which separates the right ventricular inlet from the outlet, and the outlet portion of the muscular ventricular septum, which separates the subaortic from the subpulmonary outlet. As a result of the ongoing regression in the myocardium in the subaortic outlet (see previous section), the distal portion of the outlet septum becomes the posterior part of the freestanding subpulmonary infundibulum.

**MHCs of the SMA-Positive Cells in the Ridges**

The α-SMA–positive cells in the endocardial ridges of the OFT appear to have an important morphogenetic function. To establish their contractile properties, we determined the type of MHC with which the actin was associated (Fig 9). Staining for MLC2A was used to delineate the myocardium (not shown). In situ hybridization analysis showed that cells in the endocardial ridges were characterized by a strong expression of NMMHCb and to a much lesser extent by the expression of NMMHca, whereas no expression of SMMHC was seen. Further distal, the walls of the branchial arch arteries stained very intensely for NMMHcb and, to a lesser extent, NMMHca and weakly for SMMHC. The differential staining of the periesophageal tissue and the pharyngeal pouch confirmed that the NMMHca signal did not result from cross hybridization with NMMHCB. The intensity of the SMMHC signal could be related to the very strong signal that was seen in the smooth muscle cells in the developing lung.

**Discussion**

The OFT provides the structural components to form the definitive architecture of the ventriculoarterial connection. Its normal development has been the subject of numerous studies. The formation of the OFT portion of the myocardial tube follows a proximodistal direction and is completed at ED12 in the rat (Theiler’s stage 17, Carnegie stage 14 in human embryos [authors’ unpublished data, 1997], and Hamburger and Hamilton stage 22 in the chick). Subsequently, its relative size declines. The present study addresses this second phase in OFT development and shows...
that the partition of the OFT into a conal and a truncal portion (see Reference 38 and references therein) is useful in view of their very different developmental fates.

**Fate of the Truncal Portion of the OFT Myocardium**

In the formed heart, the truncal portion of the OFT has become restricted to the myocardium supporting the semilunar valves. In addition to confirming the role of a low mitotic activity in its decline in relative size (see also References 50 and 51), we present evidence that the distal portion of the OFT loses its myocardial phenotype and becomes the proximal part of the ascending aorta and pulmonary trunk (the distal intrapericardial portion of these vessels develops from the part of the aortic sac that becomes surrounded by pericardial cavity). We observed two phases in this process, namely, an early phase, which starts at ED12 in the rat and ends when the semilunar valves have become delineated at ED14, and a more protracted second phase, which lasts until the end of the first postnatal week. Since the loss of myocardial phenotype seen during the first phase is associated with activation of DNA replication in previously silent cells but not with apoptosis, this process appears to qualify as a local transdifferentiation of cardiomyocytes into cells of the wall of the great arteries. This hypothesis is supported by earlier electron microscopical observations by Arguello et al suggesting that a similar process occurred in the Hamburger and Hamilton stage 27 chicken embryo. Furthermore, in the tattooing experiments of Thompson et al in the same species, approximately half of the labeled cells from the distal portion of the OFT ended up in the adjacent mesenchyme. We do not know at present why the transdifferentiation process starts and advances most on the dextroposterior side, but the coincident acceleration of the growth of the right ventricle and the ensuing straightening of the OFT may well be involved. Nevertheless, the dextroposterior predominance of the process accounts for the different levels of the aortic and pulmonary semilunar valves in the formed heart.

Also after the emergence of the semilunar valves in the proximal portion of the truncus, the surrounding myocardium continues to regress. This could be unambiguously demonstrated by showing that the myocardial cuff that initially supports all sinuses of the semilunar valves and surrounds the coronary arteries at their origin from the aorta disappears toward the end of the first postnatal week. Furthermore, in the chicken embryo, the wall of the aortic sinuses and of the coronary arteries does not consist of neural crest–derived cells, in contrast to the more distal parts of the great arteries. In the subaortic but not in the subpulmonary portion of the OFT, myocardial regression continues and leads to the formation of the fibrous continuity between aortic and mitral valves, as was also noted by Jackson et al. However, in contrast to the transdifferentiation process discussed in the previous paragraph, the disappearance of OFT myocardium in the fetal and neonatal hearts had characteristics of regressive changes, such as fragmentation of the MHC staining pattern.

**Fate of the Conal Portion of the OFT Myocardium**

The conal portion of the OFT becomes largely incorporated into the right ventricle as its infundibular portion. The developmental importance of the conus stems from the development and fate of the conal septum. As we have also shown in human hearts, the conal septum becomes myocardialized. In the human embryo, the migratory cardiomyocytes could be distinguished from their resident counterparts by their small size and slender shape, as well as by their irregular arrangement of myofibrils. In the rat embryo, the phenotypic differences are not so pronounced, but the presence of apoptotic cells and macrophages behind the front line of the myocytes in the septum supports migration. The pronounced apoptotic activity in the conal septum (see also References 48, 49, and 54 to 56) and the associated recruitment of macrophages (see Reference 31) may well produce the migratory signals, as a similar process was not seen at the level of the developing valves. The origin of the apoptotic cells remains to be established, with the neural crest–derived cells as leading candidates.

The conal septum separates not only the subaortic and subpulmonary outlets but also the tricuspid inlet to the right ventricle from its infundibular outlet. In 8-week-old human embryos, we could trace the lower boundary of the conal septum to the supraventricular crest, the medial (conal) papillary muscle, and the lower boundary of the smooth-walled...
conus of the right ventricle. The distal end of the conal septum may not be myocardialized and persist as the ligament of the conus, at the base of the aortic and pulmonary roots. As a result of the ongoing regression of the myocardium in the subaortic outlet, the distal portion of the myocardialized outlet septum becomes the posterior part of the freestanding subpulmonary infundibulum.

Myofibroblasts of the Endocardial Tissue

The very strong expression of α-SMA in neural crest-derived cells that populate the walls of the branchial arches and the endocardial ridges of the OFT is well known in both mammals and birds. Although several studies have shown that the smooth muscles in the wall of the aorta and pulmonary arteries of embryonic and fetal mammals express the SM1 and SMemb isoforms of SMMHC and the B form of nonmuscle MHC (NMMHC), data on the differential distribution of SMMHC and nonmuscle MHC in the walls of arteries of neural crest and mesodermal origin were not yet available. We show that the expression of NMMHCB and, to a lesser extent, NMMHCα are confined to the branchial arch arteries, ie, arteries with a contribution from the neural crest. This association of NMMHCB with the neural crest is also strengthened by its presence in neural cells. On the other hand, the descending aorta, which does not receive a contribution of the neural crest, expresses only SMMHC. Inside the endocardial tissues of the OFT, SMMHC was not expressed (see also Reference 35), whereas its expression in the ascending aorta and pulmonary trunk was initially weak.

Although we studied only phenotypic markers, the coexpression of α-SMA and nonmuscle MHCs by a subpopulation of cells in the endocardial ridges and in the wall of the branchial arch arteries certainly suggests that this phenotype identifies cells with a neural crest origin. In this respect, it is remarkable that such cells were virtually absent in the intercalated ridges and subsequently seemed to migrate away from the part of the parietal and septal ridges that form the semilunar valve leaflets to generate a very dense "whorl" at the site of fusion of the ridges. This description implies that the neural crest does not play a prominent role in semilunar valve development. This hypothesis is supported by the finding that mice deficient for the transcription factors Sox4, Pax3, or retinoic acid receptors all suffer from a common trunk, but only a deficient function of the endocardial ridge tissue (Sox4 deficiency) is associated with a deficient morphogenesis of the semilunar valves.

Conclusion

A striking feature of the aortic and pulmonary blood channels is that major changes in the direction of both routes are realized at different locations. The aortic route is characterized by a pronounced and long forward bend in its extracardiac trajectory to connect the posterior subaortic outlet with the fourth arch arteries (aortic arc). The pulmonary route, on the other hand, takes a forward turn inside the right ventricle, winds around the supraventricular crest and the muscular outlet septum, but, from there on, ascends straight into the pulmonary trunk. The layout of this pattern becomes visible and is completed in the rat embryo in a single day (ED13, Carnegie stage 16 in the human embryo) and basically does not change thereafter. It coincides temporally with the appearance of the semilunar valve anlagen. Although it is generally accepted that the spiraling course of the septal and parietal ridges plays a major role in realizing these connections between ventricles and arteries, the transformation of these ridges into structures (the semilunar valves and the outlet septum) without such a spiraling topographical relation has remained an issue. We have now shown that this issue can be resolved if the transfer of the cells of the distal portion of the OFT to the root of the great arteries is taken into account. Nevertheless, the fate of these cells will need to be established more definitely by lineage marking.

Acknowledgments

We thank I.E.M. Oosterling for skillfully preparing the drawings of the three-dimensional reconstructions and C. Gravemeijer for his photographs.

References

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Jing Ya, Maurice J. B. van den Hoff, Piet A. J. de Boer, Sabina Tesink-Taekema, Diego Franco, Antoon F. M. Moorman and Wouter H. Lamers

Circ Res. 1998;82:464-472
doi: 10.1161/01.RES.82.4.464

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/82/4/464

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