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Published in:
Heart

DOI:
10.1136/heart.89.9.1110

Citation for published version (APA):
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Heart 2003;89;1110-1118
doi:10.1136/heart.89.9.1110

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DEVELOPMENT OF THE HEART: (3) FORMATION OF THE VENTRICULAR OUTFLOW TRACTS, ARTERIAL VALVES, AND INTRAPERICARDIAL ARTERIAL TRUNKS

Robert H Anderson, Sandra Webb, Nigel A Brown, Wouter Lamers, Antoon Moorman

In the first part of our review of cardiac development, we explained the changes occurring during the transformation of the solitary primary heart tube into the primordia of the definitive heart, describing how this involved the processes of looping, and subsequent formation from the primary tube of the components of the atriums and ventricles. In the second part of our review, we then accounted for the steps involved in separation of the atrial and ventricular chambers, emphasising that the processes were more complicated than the simple formation of partitions within the respective atrial and ventricular primordia.

The subject of this, our third review, is the transformation of the initially solitary outflow portion of the heart tube into the intrapericardial parts of the aorta and the pulmonary trunk, their arterial valves and sinuses, and the subarterial ventricular outflow tracts. In our first review, we summarised some of the problems that continue to plague the understanding of the development of these outflow structures. Thus, initially the entirety of the primary heart tube contained within the confines of the pericardial cavity possesses a myocardial phenotype. Yet, in the definitive heart, the walls of the intrapericardial arterial trunks, along with the sinuses of the arterial valves, and small parts of the subarterial ventricular outlets, have an arterial or fibrous phenotype. The steps involved in the changes of the walls from the myocardial to the arterial and fibrous phenotypes have yet to be clarified. And then, cushions, or ridges, of endocardial tissue initially fuse to divide the entirety of the solitary outflow segment into the presumptive systemic and pulmonary outlets. With subsequent development, these cushions lose their septal function, as the arterial valves and trunks, along with the subpulmonary muscular infundibulum, develop as free-standing structures with their own discrete walls within the pericardial cavity. Again, to the best of our knowledge, no satisfactory solution has yet been given to explain the loss of septal function of these cushions. It is also well recognised that migrations of cells from extracardiac sources, such as the neural crest, make significant contributions to the components of the definitive outflow tracts. The routes of migration, nonetheless, and the degree of persistence of the extracardiac cells within the heart, have still to be clarified. Recently, separately and in collaboration, we have made studies and reviews of these processes, using animal models, and comparing the findings with the arrangement in the human heart. It is on the basis of these recent works that we have constructed this review of development of the outflow tract.

INITIAL DEVELOPMENT OF THE SOLITARY OUTFLOW TRACT

In our first review, we explained how cells from the primary cardiac crescent, formed bilaterally within the embryonic disc, migrated into the cervical region of the developing embryo to form the primary heart tube. We also explained how, with further growth, cells from a second cardiogenic area, located posterior to the dorsal wall of the developing pericardial cavity, migrated into the cardiac region. The cells from this secondary heart field populate the outflow tract and the aortic arches (fig 1). There is also evidence that they contribute to the developing right ventricle, in part from the evidence shown in fig 1, and also because markers placed at the cranial end of the initial straight heart tube (fig 2A) are eventually, subsequent to looping, found at the level of the interventricular foramen (fig 2B). We described the subsequent formation of the right ventricle, and its separation from the left ventricle, in our second review. We are concerned here with the changes occurring in the outflow tract. Subsequent to looping of the primary heart tube, this component extends from the distal end of the developing right ventricle to the boundaries of the pericardial cavity (figs 3 and 4).

As is seen in the scanning electron micrographs from the mouse heart (fig 3), subsequent to looping, the outflow tract possesses a characteristic dog-leg bend in its course from the right ventricle to the margins of the pericardial cavity. This bend divides the outflow tract into proximal and distal portions. The arrangement seen in the mouse heart, and reached during the 10th day of development, when the mouse has from between 40–45 somites, occurs in the developing human in the 12th through the 14th Carnegie stages.
PRIMARY SEPTATION OF THE OUTFLOW TRACT

Through the stages of looping, important remodelling has also been occurring within the arteries that run from the distal end of the heart tube through the pharyngeal arches, taking their origin from the area known as the aortic sac. Five pharyngeal arches are found in mammals, and initially arteries are formed within each arch that encircle the developing pharynx, running bilaterally and terminating in the dorsal aorta (fig 4). The arteries from the first two arches are subsequently transformed into small vessels with the skull, while the arteries of the third arch become incorporated into the carotid arteries. It is the arteries running through the fourth and sixth arches that are significant in the development of the outflow tract. By the 16th Carnegie stage, the artery of the left fourth arch has become a major pathway from the solitary outflow tract to the dorsal aorta, but the artery running through the left sixth arch has also become a prominent structure. When the outflow tract is first identified, it has exclusively muscular walls (fig 5) that extend to the borders of the pericardial cavity, where the tract becomes continuous with the aortic sac (fig 5B). At this stage, endocardial jelly clothes the entirety of the lumen of the primary tube (fig 5). Concomitant with the remodelling of the arteries extending through the pharyngeal arteries, the endocardial jelly concentrates itself into pairs of facing cushions that extend through the length of the outflow tract. Using the dog-leg bend as a boundary, these cushions can, like the outflow tract itself, be divided into proximal and distal moieties. Reconstructions, nonetheless, show that the cushions are continuous throughout the outflow tract, spiralling round one another as they run from the distal end of the right ventricle to the aortic sac (fig 6). By the time the cushions have solidified, and begin to approach each other across the lumen of the solitary outflow channel, significant changes have already begun to take place within the walls of the distal segment. Cells from the pharyngeal mesenchyme have begun to invade the parietal parts of the wall of the distal outflow tract (fig 7). The distal cushions themselves are located superiorly and
inferiorly in this part of the outflow tract as they extend towards the aortic sac (fig 8). Within the aortic sac itself, a transverse wedge of tissue is now seen separating the arteries feeding the fourth and sixth arches, respectively (fig 8). By this stage, the arteries that will eventually nourish the lungs have cavitaded within the mediastinal mesenchyme, and have joined the underside of the arteries of the sixth arch (fig 8). The right sided arteries of both the fourth and sixth arches by now have begun to involute (fig 9). The wedge of tissue that separates the fourth and sixth arches in the posterior wall of the aortic sac is the so-called “aorto-pulmonary septum”. In reality, it is no more than the mass of posterior mediastinal mesoderm between the arch arteries (fig 8).

The invasion of the parietal walls of the distal outflow tract, in the areas between those occupied by the cushions, is the first step in the change from a myocardial to an arterial phenotype for the outflow tract. At more or less the same time, however, there is an additional invasion of cells into the outflow tract. These cells enter the distal endocardial cushions themselves, and can be traced as rods of densely staining tissue within the substance of the cushions as far as the dog-leg bend (fig 10), and beyond the bend into the proximal cushions (fig 6). These are the cells derived from the neural crest. As far as we have been able to establish, they enter the cushions from the sides of the pharynx, and not through the so-called “aorto-pulmonary septum”. As already emphasised, our studies in rat, mouse, and human indicate that this “septum” is a relatively insignificant structure.

Be that as it may, the next important step in development is fusion of the endocardial cushions. This starts distally, and progresses proximally. The fusion of the cushions distally divides the distal part of the outflow tract itself into the intrapericardial components of the aorta and the pulmonary trunk (fig 9). Concomitant fusion of the distal ends of the now joined cushions with the wedge shaped mesenchyme of the
posterior wall of the aorta sac (the “aorto-pulmonary septum”) connects the developing aorta with the artery of the left fourth arch. Note the proximal parietal cushion (star) in the outflow tract. The distal outflow tract extends to the margin of the pericardial cavity (arrow) and its parietal wall is becoming infiltrated by a densely stained population of cells from the pharyngeal mesenchyme (triangle), with the phenotype of the wall closest to the aortic sac beginning to adopt an arterial rather than myocardial pattern.

Figure 7 This section, in the sagittal plane, comes from a human embryo at Carnegie stage 15. It shows how the proximal outflow tract is supported exclusively by the developing right ventricle, which at this stage has no direct connection with the developing right atrium (dotted line). Note the proximal parietal cushion (star) in the outflow tract. The distal outflow tract extends to the margin of the pericardial cavity (arrow) and its parietal wall is becoming infiltrated by a densely stained population of cells from the pharyngeal mesenchyme (triangle), with the phenotype of the wall closest to the aortic sac beginning to adopt an arterial rather than myocardial pattern.

Figure 8 This section, from the same human embryo as shown in fig 7, shows the arrangement of the distal outflow tract. The endocardial jelly has now become organised into cushions, positioned superiorly and inferiorly (stars), which approach each other across the lumen. A further population of densely stained cells, this time derived from the neural crest, is beginning to infiltrate the cushions. Note the arteries to the fourth (4) and sixth (6) pharyngeal arches arising from the aortic sac, and the left pulmonary artery running within the mediastinal mesenchyme. The wedge of mesenchyme between the fourth and sixth arch arteries (arrow) is the so-called aorto-pulmonary septum.

The proximal outflow tract (fig 10B), the distal outflow tract has transformed into the separating intrapericardial parts of the aorta and the pulmonary trunk, with no evidence now visible of the fused distal cushions themselves. Instead, both the arterial trunks distal to the dog-leg bend have begun to develop walls with an arterial phenotype (fig 9).

At this stage, therefore, the cushions have fused down to the level of the dog-leg bend (fig 10A). The rods of condensed mesenchyme derived from the neural crest have now themselves fused at the level of the dog-leg bend, where they form a characteristic whorl (fig 10A). Prongs of condensed mesenchyme, again derived from the neural crest, can be traced from the whorl into the proximal parts of the cushions, which as yet have still to fuse to divide the most proximal part of the outflow tract (fig 6 and fig 10B).

**FURTHER SEPARATION OF THE PROXIMAL OUTFLOW TRACT**

By the 18th Carnegie stage, the original dog-leg bend in the outflow tract has become transformed into the developing sinutubular junction. At this stage, just before closure of the embryonic interventricular foramen, the walls of the distal outflow tract have become completely arterialised, and developing fibroadipose tissue now interposes between the separate walls of the intrapericardial components of the aorta and the pulmonary trunk (fig 11, 12, and 13). Proximal to the dog-leg bend, however, it is still possible to recognise the cushions that are septating this part of the outflow tract. The prongs of condensed mesenchyme derived from the neural crest are still prominent, and the entirety of the tract is still encased within an encircling myocardial wall (fig 12). Significant changes are by now taking place in the cushions contained within the myocardial wall. In addition to the cushions that have fused to separate the proximal outflow tract into prospective aortic and pulmonary arteries, the proximal parietal wall of the aorta sac (the “aorto-pulmonary septum”) has fused down to the level of the dog-leg bend (fig 10A). The rods of condensed mesenchyme derived from the neural crest have now themselves fused at the level of the dog-leg bend, where they form a characteristic whorl (fig 10A). Prongs of condensed mesenchyme, again derived from the neural crest, can be traced from the whorl into the proximal parts of the cushions, which as yet have still to fuse to divide the most proximal part of the outflow tract (fig 6 and fig 10B).
pulmonary components, two further intercalated cushions have grown in the opposite quadrants of the common outflow tract (fig 6). Formation of cavities in the fused distal parts of the proximal cushions, along with similar cavitation in the intercalated cushions, now produces the primordia of the arterial valvar leaflets and sinuses. These structures, therefore, are formed in the most distal part of the proximal outflow tract, immediately upstream relative to the developing sinutubular junction (figs 11 and 12). Sections taken in the short axis of the developing sinuses show that the cavitation of the cushions leaves the central luminal part of each cushion to form the arterial valvar leaflets, with the peripheral part arterialisng to form the wall of the supporting valvar sinuses (fig 13). The rightward and inferior of the intercalated cushions, initially positioned anteriorly (fig 6), forms one sinus of the aortic valve, while the opposite leftward and superior intercalated cushion, initially positioned posteriorly (fig 6), forms the non-adjacent sinus of the pulmonary valve. The adjacent sinuses and valvar leaflets, in contrast, are excavated from the fused distal parts of the proximal cushions, with each of the two fused cushions forming one sinus and leaflet of the aortic valve, together with the adjacent sinus and leaflet of the pulmonary valve (fig 13). At this stage, nonetheless, the original myocardial wall of the proximal outflow tract continues to encase both developing valves. The developing coronary arteries have to pierce this myocardial cuff so as to gain access to the adjacent aortic valvar sinuses (fig 13). Between the 18th (figs 11 and 12) and the 20th (fig 13) Carnegie stages, it becomes possible to discern the plane of cleavage that is developing between the aortic and pulmonary roots, this following the track of the prongs of condensed mesenchyme that extended into the proximal outflow tract from the whorl initially formed at the dog-leg bend.

By the 20th stage, the most proximal parts of the cushions have also fused. Within this most proximal part of the outflow tract, however, notably different processes are taking place. Whereas the initial musculature of the walls of the distal outflow tract has disappeared very rapidly as the arterial trunks achieve their arterial phenotype, and while the muscular cuff surrounding the distal part of the proximal outflow tract also disappears, albeit far more gradually, muscular tissue is now being added to the outflow tract in its most proximal portion. This occurs by the process now known as “myocardialisation”. The myocardial cells in the walls of the proximal outflow tract grow into the most proximal parts of the cushions as they fuse, converting the endocardial septum into a muscular partition (fig 14). As shown in fig 10, initially the most proximal parts of the outflow cushions are attached parietally and septally within the roof of the developing right ventricle. Thus, as the cushions fuse and muscularise, they span the outlet from the right ventricle, which initially supports both developing outflow tracts. With continuing...
growth during the 18th through 20th Carnegie stages, the space beneath the free proximal edge of the fused cushions becomes increasingly smaller, until eventually the entire leading edge of the fused outflow cushions becomes attached to the right ventricular surface of the crest of the muscular ventricular septum, thus walling the aorta into the left ventricle (fig 14). At the stage of adherence of the myocardialising outlet partition to the primary muscular ventricular septum, so-called closure of the embryonic interventricular communication, the infero-posterior wall of the developing

Figure 13 These sections are from a human embryo at Carnegie stage 22, after the interventricular communication has closed. They are cut transversely across the developing arterial valves (A), and the intrapericardial arterial trunks (B). Cavities have now developed in the cushions occupying the distal part of the proximal outflow tract, forming two layers that give rise to the valvar leaflets lumina (blue arrows) and the walls of the supporting sinuses on the mural aspect (red arrows). The mural components are beginning to arterialise. The sinuses and their accompanying leaflets derived from the intercalated cushions, shown by the arrows, form one sinus each for the aorta and pulmonary trunk. The walls of both these sinuses have arterialised, the myocardial covering having disappeared. The myocardium still forms a cuff, however, around the sinuses and leaflets excavated from the fused proximal cushions (stars). Each of the two cushions will contribute one sinus and leaflet to the aorta, and a facing sinus and leaflet to the pulmonary trunk. Note that the coronary arteries will need to perforate the persisting myocardial cuff to enter the aortic sinuses. The second panel (B), taken at a superior level, shows that the intrapericardial parts of the aorta (AO) and pulmonary trunk (PT) now have their distinctive arterial walls. A plane of fibroadipose tissue (dotted line) has appeared between them at the site initially occupied by the distal cushions (see fig 9).
The subaortic outflow tract retains its muscular phenotype (fig 12). This wall is part of the original inner heart curvature. Only at a much later stage is this muscular tissue converted into fibrous tissue, producing the area of fibrous continuity between the leaflets of the aortic and mitral valves and, at the same time, severing the electrical continuity at this site between the atrial and ventricular muscle masses.

The fused and muscularised outflow cushions then span the roof of the newly separated right ventricle between the septum and the developing leaflets of the tricuspid valve (fig 14). With extension of the developing fibroadipose tissue plane that already separates the aortic and pulmonary trunks (fig 13B), the muscularised partition becomes converted into the free-standing infundibulum of the pulmonary valve, and part of the supraventricular crest of the right ventricle. The regression of the musculature that initially surrounds the aortic valvar sinuses (fig 14C) also contributes to formation of the plane of tissue that, in the definitive heart, separates the aortic root from the subpulmonary infundibulum (fig 15).

Regression of the muscular wall of the distal part of the proximal outflow tract, not complete until the end of the third month of development, at the same time places the fibrous triangles formed beneath the attachments of the valvar leaflets at the sinutubular junction in potential continuity with the extracardiac space (fig 16), explaining the formation of the interleaflet triangles of the subarterial outflow tracts.

**COMMENT**

The account we have given for formation and separation of the outflow tracts and intrapericardial arterial trunks differs notably from those found in current accounts. We have made no reference to the “truncus” and “conus”, although these can be considered synonymous with the regions we have described as the proximal and distal components of the outflow tract. These parts are separated by the prominent dog-leg bend that, eventually, marks the site of formation of the definitive sinutubular junctions. We have purposely avoided the use of “truncus” and “conus”. This is because most
previous investigators have argued that the separation between these components can be made “at the level of formation of the arterial valves”. But they then described the valves and their supporting sinuses as being formed from the “truncus”, in other words, the distal part of the outflow tract as we have described it. The valves, however, occupy a significant length of the outflow tract. It is not possible accurately to divide the tract according to the “level of formation of the arterial valves”. In fact, the boundary between the two parts of the primordial outflow tract is destined to become the sinu-tubular junctions. The valves and their supporting sinuses, therefore, are developed from the proximal part of the outflow tract, or the “conus” in classical terminology. The initial muscular wall of this distal part of the proximal outflow tract, however, plays no part in the formation of either the valvar leaflets or their supporting arterial sinuses. Instead, these structures are formed by formation of cavities within the cushions, with the central, or luminal, parts of the cushions forming the leaflets, and the peripheral, or mural, parts arterialising to form the sinusial walls. All the cushions are involved in this process, with the adjacent sinuses of the aorta and pulmonary trunk derived from the cushions that initially septated the outflow tract, and the non-adjacent sinuses and leaflets derived from the intercalated cushions. Tonge provided an excellent and accurate account of these processes as long ago as 1856, albeit that this has been somewhat ignored in recent years.

The other significant difference between our account and recent “classical” versions is that we consider the distal cushions of the outflow tract initially to septate the common outflow channel into the intrapericardial portions of the aorta and the pulmonary trunk. We have found no evidence to support the notion that the “aortopulmonary septum” grows down to septate the distal outflow tract. According to our observations, the cushions, having achieved their role in initial septation, disappear by a process yet to be determined as the arterial trunks develop their own walls within the pericardial cavity. At the same time, the walls of the distal outflow tract, again by a process as yet undetermined, rapidly change from a myocardial to an arterial phenotype. Initially, we had thought that this change occurred by transdifferentiation, a mechanism also favoured by Arguello and colleagues. Recent experiments in our laboratory, however, suggest that the cells of the arterial trunks have never possessed a myocardial heritage (unpublished observations). We are currently exploring the mechanisms of change. All we can say at this stage is that they occur with great rapidity.

The change in phenotype of the walls of the outflow tract can be considered as a retreat of the myocardium. This occurs in two distinct phases. The first is the phenotypic change in the wall of the distal outflow as discussed above, which is remarkably rapid. The second phase is much more gradual, and involves the disappearance of the myocardium that initially surrounds the developing arterial sinuses, along with part of the inner curvature of the heart in the roof of the left ventricle. The former process had not received attention before the study of Ya and colleagues. The latter process is well recognised as “absorption of the conus”, eventually producing fibrous continuity between the leaflets of the aortic and mitral valves.

For some time, arguments have raged as to whether this “conal absorption” is necessary for the aorta to become connected to the left ventricle. Further arguments have concerned the fate of the spiralling outflow ridges subsequent to their fusion, and whether this requires “detorsion”. Our current observations provide the resolution to both these debates. The disappearance of the musculature within the inner heart curvature is an event occurring subsequent to the connection of the aorta to the left ventricle. The ventriculo-arterial connections are achieved simply by adhesion of the fused and muscularised proximal outflow cushions to the primary muscular ventricular septum. It is no surprise, therefore, to find that otherwise normal hearts can be encountered with muscular tissue still present between the hinges of the aortic and mitral valves. Concerning the “detorsion” of the outflow ridges, this is no more than an optical illusion. It is well recognised that the pulmonary trunk spirals round the aorta as it exits from the right ventricle and bifurcates within the pericardial cavity. Thus, the initial spiralling course of the outflow cushions (fig 6) is retained in the definitive heart. It has simply been transferred to the arterial trunks because of the change in the phenotype of their walls.

ACKNOWLEDGEMENTS
The work done by Professors Anderson and Brown, and Dr Webb, during the preparation of the concepts set out in this review, was supported generously by the British Heart Foundation. The work of Professors Lamers and Moorman was equally well supported by the Dutch Heart Foundation.

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REFERENCES
   * These references are the first two parts of our trilogy, completed in this review.
   * It was the pioneering work of Le Douarin that first showed the importance of the neural crest in development, and Kirby and her colleagues then extended this work to show the significance of migrations of cells from extracardiac sources to formation of the cardiac outflow tracts and arch arteries.
   * The first work to question the conventional division of the outflow tract into "truncus" and "conus", showing how, during development, there was a retreat of the myocardium initially clothing the entirety of the developing outflow tract.
   * The review published in Journal of Anatomy, along with our manuscript submitted for publication, represent the work drawn upon in the production of this final review concerning development of the outflow tract of the heart.
   * References 8–10 are the recent studies that have revealed the presence of the so-called secondary heart field, that gives rise to the outflow tract, and probably to the greater part of the developing ventricle.
   * This important work summarises the so-called Carnegie stages used to grade the formation of the developing human embryo.
   * This superbly illustrated study, by a group of anatomists from Japan, shows the steps involved in the formation of the system of aortic arches.
   * The important study that showed how myocardium was added to the ventricular outflow tract by invasion of the cushions from the adjacent myocardial walls. Subsequently it has also been shown by the same group that some cells within the cushions also become converted to myocardial cells.
   * In this investigation, we questioned the existence of a “arterial valvar annulus”, emphasising that the semilunar attachment of the valvar leaflets incorporated three triangles of fibrous tissue, adjacent to the sinutubular junctions, at the distal extent of both ventricular outflow tracts.
   * This important study, much neglected of late, described how the leaflets and sinuses of the arterial valves in the chick are formed by cavitation of the cushions within the outflow tract.
   * The authors provided evidence to show that, in the chick, the cells of the distal outflow tract changed during development from a myocardial to an arterial phenotype. There is no evidence this far, however, of such a mechanism occurring in man.
   * An important early review that first described the changes occurring within the developing outflow tracts and applied the findings to congenital cardiac malformations.
   * This study resurrected the role of the proximal outflow tract as the “factory” for formation of lesions such as double outlet right ventricle and discordant ventriculo-arterial connections.
   * This important study showed that, in some otherwise normal hearts, small segments of ventricular myocardium could be found in the inner heart curvature interposed between the leaflets of the aortic and mitral valves.