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Qayyum, S.R.; Webb, S.; Anderson, R.H.; Verbeek, F.J.; Brown, N.A.; Richardson, M.K.

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SONIA R. QAYYUM,[1] **SANDRA WEBB,**[1] **ROBERT H. ANDERSON,**[2]
FONS J. VERBEEK,[3] **NIGEL A. BROWN,**[1] AND **MICHAEL K. RICHARDSON**[1]*
[1]Department of Anatomy and Developmental Biology, St. George's Hospital Medical
School, London SW17 ORE, United Kingdom
[2]Cardiac Unit, Institute of Child Health, University College London,
London WC1N 1EH, United Kingdom
[3]Imaging & BioInformatics, Hubrecht Laboratory, Netherlands Institute for
Developmental Biology, 3584 CT Utrecht, The Netherlands

ABSTRACT

There is no agreement, in the chick, about the number of the endocardial cushions within the outflow tract or their pattern of fusion. Also, little is known of their relative contributions to the formation of the arterial valves, the subpulmonary infundibulum, and the arterial valvar sinuses. As the chick heart is an important model for studying septation of the outflow tract, our objective was to clarify these issues. Normal septation of the outflow tract was studied in a series of 60 staged chick hearts, by using stained whole-mount preparations, serial sections, and scanning electron microscopy. A further six hearts were examined subsequent to hatching. At stage 21, two pairs of endocardial cushions were seen within the developing outflow tract. One pair was positioned proximally, with the other pair located distally. By stage 25, a third distal cushion had developed. This finding was before the appearance of two further, intercalated, endocardial cushions, also distally positioned, which were first seen at stage 29. In the arterial segment, the aortic and pulmonary channels were separated by the structure known as the aortopulmonary septum. The dorsal limb of this septum penetrated the distal dorsal cushion, whereas the ventral limb grew between the remaining two distal cushions, both of which were positioned ventrally. The three distal endocardial cushions, and the two intercalated endocardial cushions, contributed to the formation of the leaflets and sinuses of the arterial roots. The two proximal cushions gave rise to a transient septum, which later became transformed into the muscular component of the subpulmonary infundibulum. Concomitant with these changes, an extracardiac tissue plane was formed which separated this newly formed structure from the sinuses of the aortic root. Our study confirms that three endocardial cushions are positioned distally, and two proximally, within the developing outflow tract of the chick. The pattern of the distal cushions, and the position of the ventral limb of the aortopulmonary septum, differs significantly from that seen in mammals. Anat Rec 264:273–283, 2001.   © 2001 Wiley-Liss, Inc.

Key words: heart; chick; developmental biology; scanning electron microscopy; embryology; morphogenesis

There have been numerous descriptions concerning septation of the outflow tract of the heart in different vertebrate species. It remains difficult to correlate the findings between different species, and reports regarding septation within the same species are frequently conflicting. To an extent, these difficulties reflect the formidable task of interpreting three-dimensional relationships in a structure as complex as the developing heart, but some do represent real morphologic differences between species. A firm understanding of these differences remains important, both because of the clinical significance of cardiac malformations (Anderson and Becker, 1992), and the use of animal models to establish the mechanisms of normal and abnormal development. Regardless of the species being studied, there is also considerable confusion regarding the nomenclature of the segments within the outflow tract, and their corresponding cushions.

The outflow tract of the heart connects the developing right ventricle with the arterial segment, and hence the

arteries in the pharyngeal arches. As part of the original embryonic heart tube, it is surrounded in part by a myocardial sleeve (Thompson and Fitzharris, 1984) which, during development, undergoes an apparent retraction (Thompson and Fitzharris, 1979; Thompson et al., 1983). In addition, the outflow tract itself undergoes a complex process of septation and valvar formation to produce the ventricular outlets, whereas the arterial segment gives rise to the systemic and pulmonary arterial channels of the adult. The fate of the endocardial cushions (Davies, 1927; Kramer, 1942; Patten et al., 1948; Markwald, 1984) found within the outflow tract is crucial to these processes. In the chick, there is disagreement about the number of these cushions. Some workers have described only two cushions (de la Cruz et al., 1977; Pexeider, 1978; Icardo, 1990), whereas others have described three (Tonge, 1869; Langer, 1895; Greil, 1903; Hochstetter, 1906; Shaner, 1962; Laane, 1978; Waldo et al., 1998). Of those who recognise three cushions distally, some state that two of them fuse cranially to form a common cushion, but remain unfused caudally (Laane, 1978; Waldo et al., 1998). Yet others have simply described two longitudinal cushions occupying the entire length of the developing outflow tract (Los, 1978). This finding is in contrast to the situation in mammals, where, to the best of our knowledge, a third distal endocardial cushion has never been reported. In mammals, previous reports have either described four endocardial cushions within the outflow tract, with two positioned distally and two proximally (Kramer, 1942; Van Mierop et al., 1963; Anderson et al., 1974; Ya et al., 1998; Yasui et al., 1999), or else have described two opposing cushions which extend through the length of the outflow tract (Goor et al., 1972; Nakajima et al., 1996).

There is further disagreement concerning the pattern of fusion of the cushions. The consensus is that the distal cushions and the aortopulmonary septum contribute together to a distal septum, whereas the proximal cushions fuse to form a proximal septum and contribute to closure of the interventricular foramen. In an interesting, but neglected, early study, Tonge (1869) argued that, in the chick, the distal cushions, of which he described three, functioned largely in formation of the arterial valves and the supporting walls of their sinuses. The only structures he recognised as septating the outflow tract were the aortopulmonary septum and the proximal cushions. The aortopulmonary septum itself is formed by cells that migrate from the neural crest, entering the arterial segment between the fourth and sixth pairs of arteries supplying the pharyngeal arches (Waldo et al., 1998). At its proximal extent, when viewed on histologic sections, the aortopulmonary septum is continuous with two columns of compact cells, the dorsal and ventral limbs (Laane, 1978; Los, 1978; Thompson et al., 1983; Waldo et al., 1998). Some authors state that each of these limbs splits a distal cushion in half to create a total of four valvar primordia (Kramer, 1942; Icardo, 1990). Tonge (1869), in contrast, stated that the ventral limb, in the chick, passed between the two ventral cushions. Unfortunately, Tonge's model, although endorsed at the turn of the century, has been ignored by more recent authors. If true, it represents a significant difference between the chick and mammals.

In mammals, it is generally agreed that there are only two endocardial cushions distally. Each of these is held to contribute two leaflets to the arterial valves, one to the aortic valve and one to the valve of the pulmonary trunk. The third leaflet of each valve is said to be derived from an intercalated endocardial cushion (Kramer, 1942). The six endocardial valvar primordia, thus created are then said to undergo excavation on their distal face, becoming converted into the fibrous leaflets of the aortic and pulmonary valves (Hurle, 1979).

We have attempted to resolve all these various issues by re-examining the development of the chick outflow tract. We have used scanning electron microscopy and stained whole-mount preparations, combined with serial histologic sectioning and reconstruction, to ascertain the number of cushions present, their pattern of fusion, and their relationship to the retracting myocardial cuff. We have also clarified the position of the limbs of the aortopulmonary septum in relation to the three distal ridges and explained the formation of the separate aortic and pulmonary roots.

MATERIALS AND METHODS

We examined 36 normal chicken embryos, either as whole-mounts or after serial sectioning, and 24 by scanning electron microscopy. We also examined two 1-day and one 15-month posthatching chick hearts by histologic sectioning, and three 15-month-old chick hearts by gross dissection. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

Collection and Fixation of Embryos

Fertilised chicken eggs of the *Hi-Sex* strain were obtained commercially (Poyndon Farm, Herts, UK). They were incubated at $38 \pm 1°C$ on stationary shelves. Embryos were removed from the egg into saline and staged according to Hamburger and Hamilton (1951). Some were perfused-fixed by injecting Bouin's fluid into the ventricle through a glass capillary needle. The whole embryo was then further immersed in Bouin's fluid for at least 4 hr.

Histology

Embryos were dehydrated through a graded series of alcohols to 100%, cleared with methyl salicylate, and then embedded in paraffin wax (three changes, 30 min, 60°C, under vacuum). Sections were cut on a rotary microtome at a nominal thickness of 3–7 microns. Sections were triple-stained with haematoxylin, eosin, and Alcian blue.

Whole-mounts

After fixation, embryos were stained overnight in 0.03% Alcian blue, dehydrated to 100% ethanol, and cleared with methyl salicylate. The hearts were then dissected. Each heart was placed in methyl salicylate and rotated in various axes by using forceps. Images were captured through a colour video camera (JVC) connected to a stereo dissecting microscope. Single-frame playback was used to study the heart in different planes of view.

Scanning Electron Microscopy

Embryos were harvested as described above and perfuse-fixed overnight with half-strength Karnofsky's fixative. Subsequent to fixation, specimens were rinsed in buffer. After post-fixing for 2 hr with osmium tetroxide, specimens were rinsed twice in a buffer and the passed through a graded series of 35%, 70%, 90%, and two

TABLE 1. The stage of appearance of selected developmental events in the outflow tract of the chick[a]

Character	Stage
Endocardial cushions of the outflow tract	
Proximal right	21[b]
Proximal left	21[b]
Distal dorsal	21[b]
Distal right-ventral	21[b]
Distal left-ventral	25
Pulmonary intercalated	29
Aortic intercalated	29
Aortopulmonary septum	
Limbs of septum enter distal segment of outflow tract	25
Limbs of septum fuse with each other	30
Proximal outflow ridges	
Proximal right and left cushions	
beginning to fuse	29–30
Myocardialisation beginning	31
Completely fused	34
Arterial valvar leaflets	
Cavitation beginning in distal end of distal cushions	30–31

[a]The stages indicated here are when the named structure was distinct. For example, the pulmonary intercalated cushion was faintly indicated stage 28 but was not prominent until stage 29.
[b]Examination of earlier embryos showed that these conditions or structures were already present prior to stage 21 (data not shown).

changes of 100% ethanol for dehydration. Specimens were then left overnight and dried by using the critical point method. They were mounted on aluminium stubs and sputter-coated with gold.

Three-Dimensional Reconstructions

A three-dimensional model, showing the relative positions of the outflow cushions, was made from a serially sectioned embryo at HH stage 28. The reconstructions were derived from an aligned stack of images (Verbeek et al., 1995), followed by conversion to a volume model (Verbeek and Huijsmans, 1998), which was used to generate rendered images.

RESULTS

The staging of the key developmental events is listed in Table 1. In this account, "distal" means toward the arterial end and "proximal" toward the ventricular origin of the outflow tract. So as to avoid some of the previous problems with nomenclature, we have used descriptive terms to account for the developing outlets. Thus, the portion of the heart tube that is surrounded by a myocardial sleeve and that runs from the distal part of the ventricular loop will be considered to represent the outflow segment. It will be described as having proximal and distal components, which are analogous to the so-called "conotruncus." The segment that then extends from the distal end of the myocardial tube to the pharyngeal arches will be described as the arterial segment.

Stages 21–24

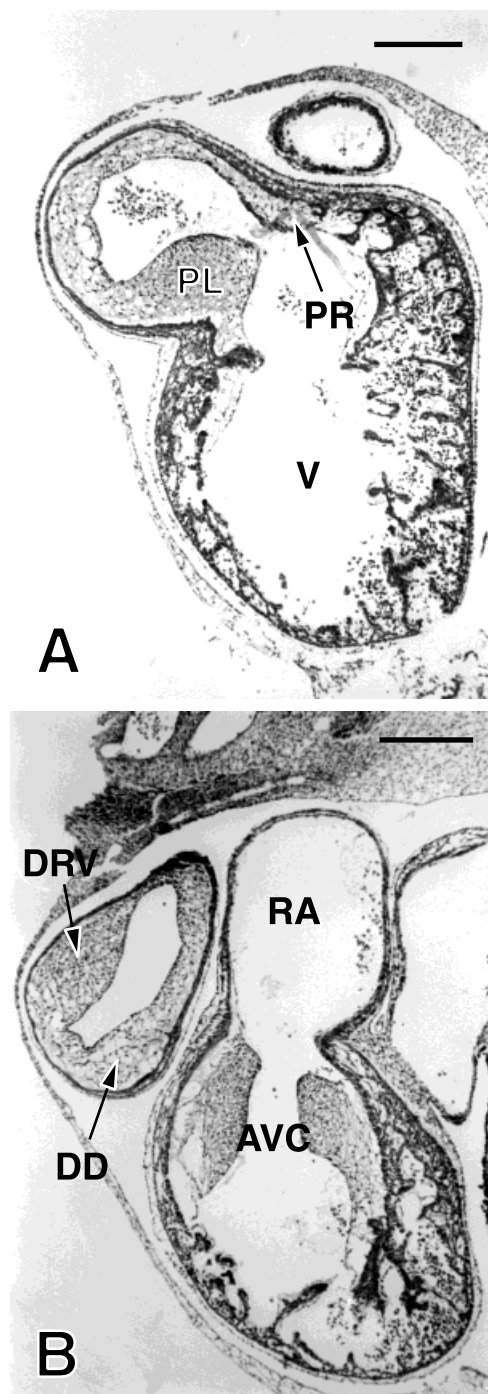Both the whole-mount preparations and the serial sections showed two proximal endocardial cushions posi-

Fig. 1. The two members of each pair are from the same chick embryo. **A:** The proximal outflow tract is seen in sagittal section at stage 23. The proximal cushions are widely separated, and positioned to the left (PL) and the right (PR). **B:** Only two cushions are seen in the distal outflow tract located ventrally (DRV) and dorsally (DD). For other abbreviations, see list. Scale bars = 200 μm in A,B.

tioned within the lumen of the proximal part of the outflow tract (Fig. 1A). One was positioned to the left and, hence, is called the proximal left cushion. The other was positioned to the right and so is described as the proximal right cushion. A second pair of endocardial cushions were
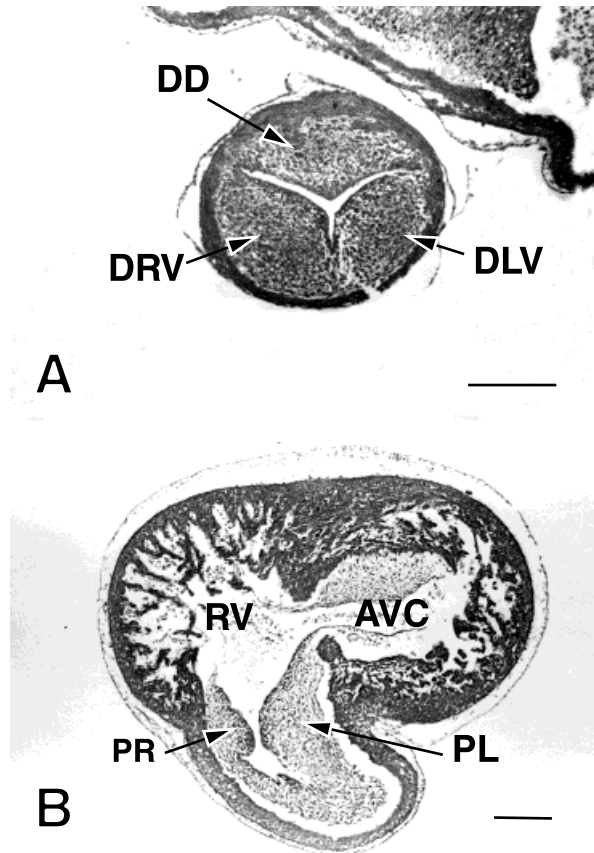
Fig. 2. These sections, from an embryo at stage 25, are cut in transverse section. **A:** The distal left ventral (DLV) cushion has now appeared in the distal outflow tract. **B:** Paired cushions (PR, PL) remain in the proximal outflow tract. In all the transverse sections, ventral is shown to the bottom of the picture so as to correspond with the diagrams in Figures 7–9. For other abbreviations, see list. Scale bars = 200 μm in A,B.

positioned within the distal outflow tract (Fig. 1B), with an area of flat subendocardial tissue in the gap between them in the ventral aspect of the lumen. One distal cushion was positioned dorsally, and is, therefore, the distal dorsal cushion. The other was positioned ventrally, and we will call it the distal right ventral cushion. We have used the term distal *right* ventral cushion for this structure, because the gap between the two distal cushions
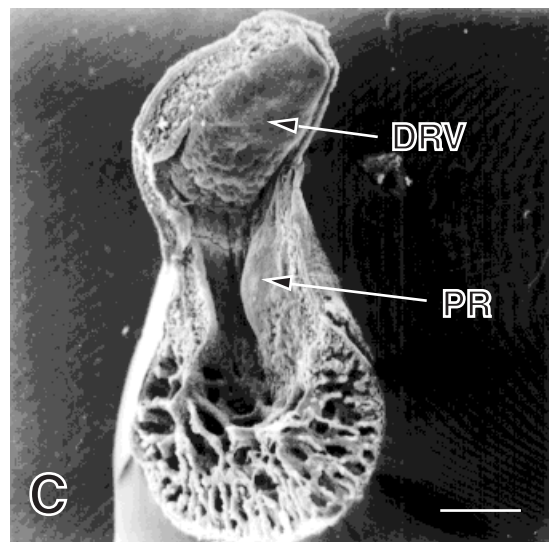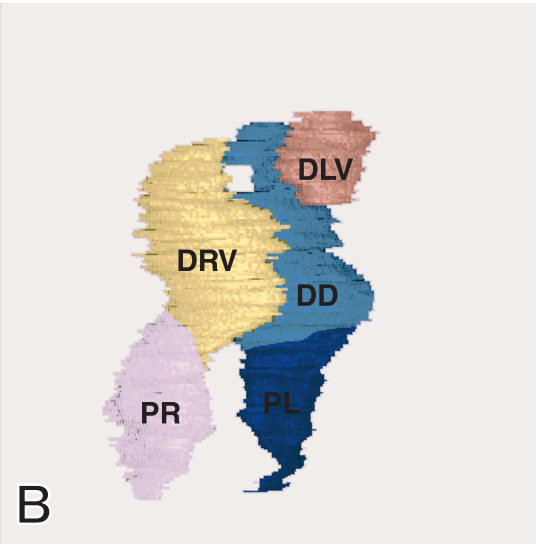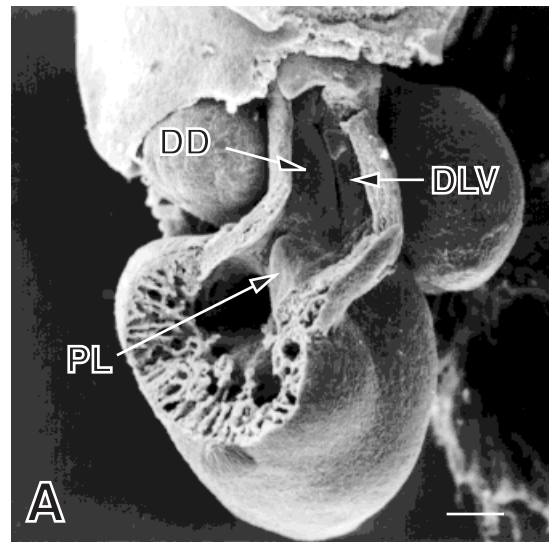

Fig. 3. **A:** Scanning electron photomicrograph from chick embryo at stage 26, right/lateral view, with the right wall of the outflow tract removed by microdissection. The dorsal (DD) and the left ventral (DLV) cushions can be seen in the distal segment. The proximal left cushion (PL) can also be seen in the proximal segment. Note the appearance of a marked groove separating the two distal cushions. **B:** A reconstruction of the serially sectioned heart from a chick embryo at stage 28 shows the relative positions of the outflow cushions. This reconstruction is oriented in a manner similar to the heart above. The reconstruction confirms the presence of three cushions distally. **C:** The counterface of A. The distal right ventral cushion (DRV) can be seen as a large structure within the distal segment, and the proximal right cushion (PR) is within the proximal segment. Note the lack of continuity between the right proximal and the distal cushions. For abbreviations, see list. Scale bars = 200 μm in A,C.
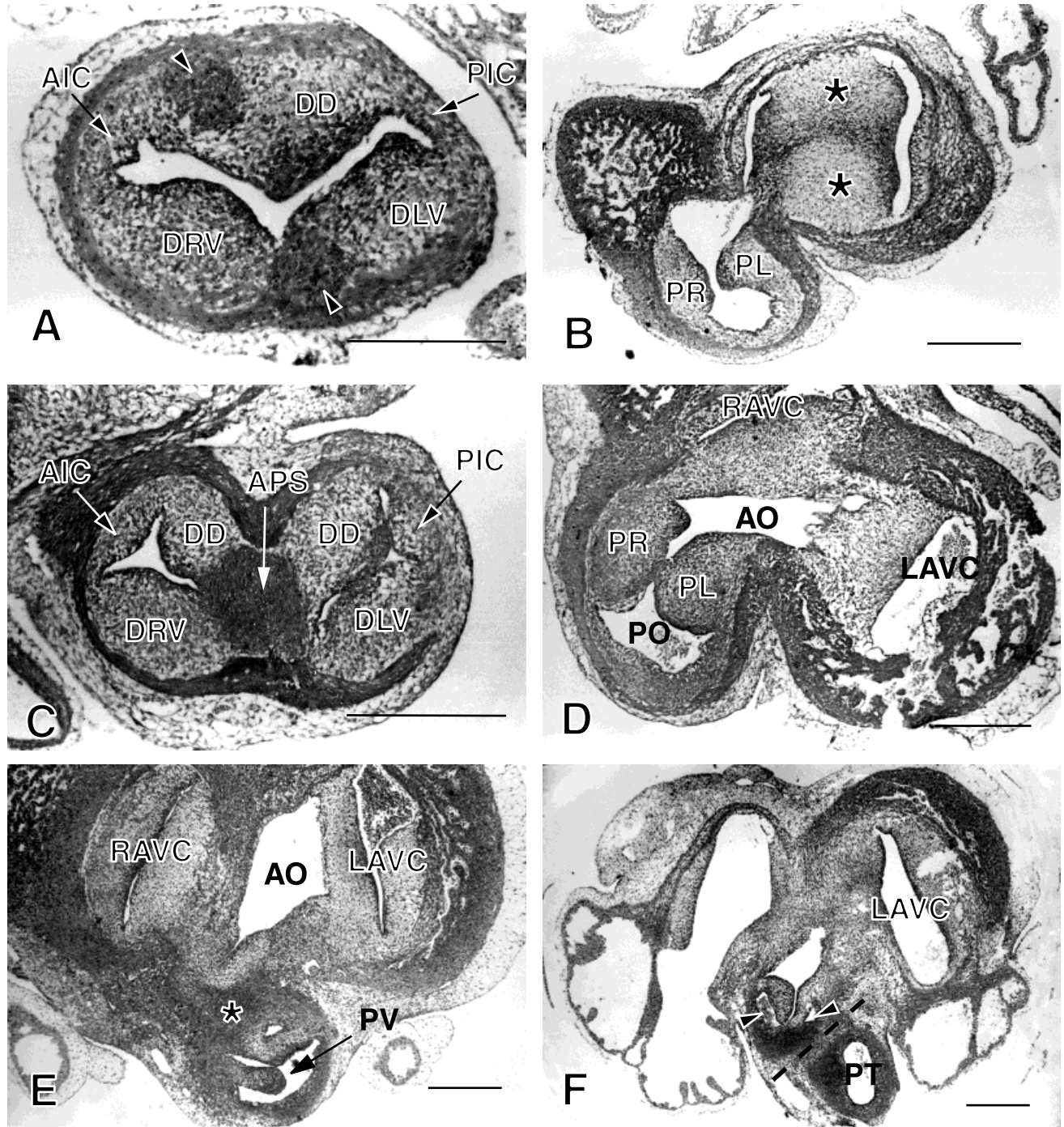
Fig. 4. The two members of each pair come from the same chick embryo. **A:** Distal outflow tract, transverse section (stage 29). Note the two limbs of the aortopulmonary septum (arrowheads), with the ventral limb passing in the furrow between the two ventral cushions (DRV, DLV), but with the dorsal limb embedded within the distal dorsal cushion (DD). Note also that the intercalated cushions (AIC, PIC) have now appeared. **B:** Proximal outflow tract, transverse section (as A). It shows the unfused proximal cushions (PR, PL). The asterisks show the fused atrioventricular cushions. **C:** Distal outflow tract, transverse section (stage 30). Note the splitting of the dorsal cushion (DD) by the aortopulmonary septum (APS) to form two dorsal entities. The aortopulmonary septum itself now forms the partition between the primordia of the arterial valves. The intercalated cushions are now clearly seen (AIC, PIC). **D:** Proximal outflow tract, transverse section (as C). Note the close proximity of the proximal ridges (PL, PR). The developing aortic outlet (AO), separating from the pulmonary outflow (PO), is being displaced to a central position by remodeling of the atrioventricular cushions (RAVC, LAVC). The right atrioventricular cushion is fusing in places with the proximal right outflow cushion. This encircles the aortic outlet with endocardial cushion tissue. **E:** Pulmonary valve (PV), oblique/coronal section (stage 31). Note that the pulmonary arterial sinuses are beginning to develop. The proximal ridges have fused at their caudal aspect to form a septum proximally, which is beginning to undergo myocardialisation (asterisk). The subaortic outlet (AO) is wedged between the atrioventricular orifices (RAVC, LAVC). **F:** Aortic valve, oblique/coronal section (as E). Note the development of the aortic arterial sinuses (arrowheads) and the plane of extracardiac fibrous tissue (dotted line) which is appearing between them and the pulmonary trunk (PT). For other abbreviations, see list. Scale bars = 300 μm in A–F.
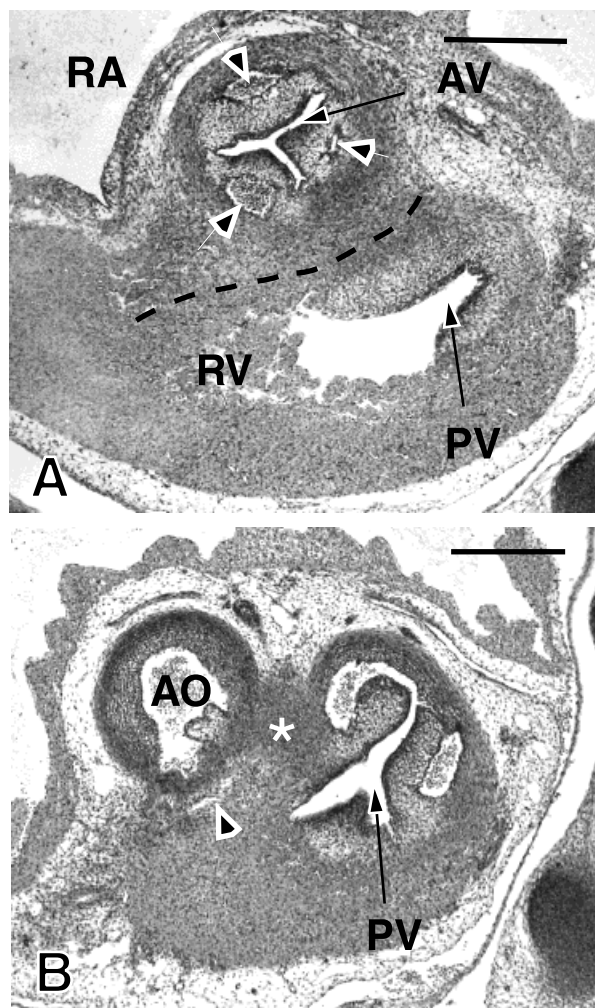
Fig. 5. A chick heart at stage 34, seen in transverse plane. **A:** This proximal section shows the developing sinuses of the aortic valve (arrowheads). Note that the aortic root remains surrounded fully by myocardium and that myocardialising cushions, at this stage, separate the aortic root from the developing subpulmonary infundibulum (dotted line). **B:** A section taken more distally shows the ligament of the conus (asterisk) formed from the remnant of the aortopulmonary septum at the sinutubular junction. The subpulmonary infundibulum is visible adjacent to the aortic outlet, which shows only the most distal part of the aortic valvar leaflets (AV). The valves are now in different orientations, and at different levels, within the outflow tract. Note the beginning of appearance of the tissue plane, which will separate the infundibulum from the aortic root (arrow). For other abbreviations, see list. Scale bars = 300 μm in A,B.

The junction between the distal dorsal and proximal left cushions was marked by a shallow indentation. The proximal left cushion, in turn, was also continuous, along the inner heart curvature, with the superior endocardial cushion of the atrioventricular canal.

The transition from outflow tract to arterial segment marked the upper limit of the distal endocardial cushions, and this coincided with the distal extent of the myocardial sleeve. A dark-staining mesenchymal wedge, the so-called aortopulmonary septum, was seen between the arteries supplying the fourth and sixth arches (not shown).

Stages 25–28

At stage 25, a new endocardial cushion had developed in the distal segment of the outflow tract. This third distal cushion, which as explained we termed the distal left ventral cushion, was to the left of the distal right ventral cushion (Fig. 2A). Two cushions remained proximally, with the left cushion continuous with the superior atrioventricular cushion (Fig. 2B). The findings were confirmed by electron microscopy and by reconstruction of serial histologic sections (Fig. 3). Two limbs could now be traced from the aortopulmonary septum into the distal outflow tract. The dorsal limb entered the distal dorsal cushion, whereas the ventral limb lay between the two distal ventral cushions.

Stage 29

By this stage, intercalated endocardial cushions were becoming evident, one each in the developing aortic and pulmonary halves of the distal segment (Fig. 4A). They lay at the same level as the upper limit of the distal cushions. The intercalated swelling in the pulmonary component lay between the dorsal and left ventral cushions, whereas the aortic swelling was between the distal dorsal and the distal right ventral cushions. The proximal cushions were now approaching each other at the centre of the lumen, albeit still as separate structures (Fig. 4B).

Stage 30

The arterial segment was appreciably longer at this stage when compared with the length of the outflow tract. This gave the appearance of shortening and proximal displacement of the distal cushions. The two limbs that could be traced from the aortopulmonary septum had fused with each other, splitting the distal dorsal cushion in half, and separating the developing aortic and pulmonary channels. Each outflow tract now contained three valvar primordia (Fig. 4C); one from the intercalated cushion, one from half of the distal dorsal cushion, and one from either the distal left ventral or the distal right ventral cushions At the level of the distal segment, the arterial valves and the supporting sinus walls were beginning to develop through excavation of the distal cushions. More proximally, the cushions had begun to fuse in a proximal-distal direction (Fig. 4D), thus, forming a septum between the developing subpulmonary and subaortic outlets, which were still encased in a common myocardial sleeve.

Continuity between the proximal right cushions and the right free edge of the central cushion mass in the atrioventricular canal had become more prominent. This zone of fusion now formed the medial wall of the right atrioventricular canal, and the right wall of the aortic outlet. Therefore, the outlet from the developing left ventricle had become encircled by endocardial cushion tissue (Fig. 4D).

becomes occupied later by a third distal endocardial moiety, which is not an intercalated cushion. As this third cushion was also positioned ventrally, it is described as the distal *left* ventral cushion. The distal right ventral cushion could be traced along a somewhat oblique course on the ventral wall of the outflow tract and was in alignment with the proximal right cushion, although it was almost completely separated from it by a deep indentation. The distal dorsal cushion, in contrast, ran a straight course on the dorsal wall of the outflow tract (Fig. 1B). When it approached the proximal segment, it turned leftward, becoming aligned with the proximal left cushion.
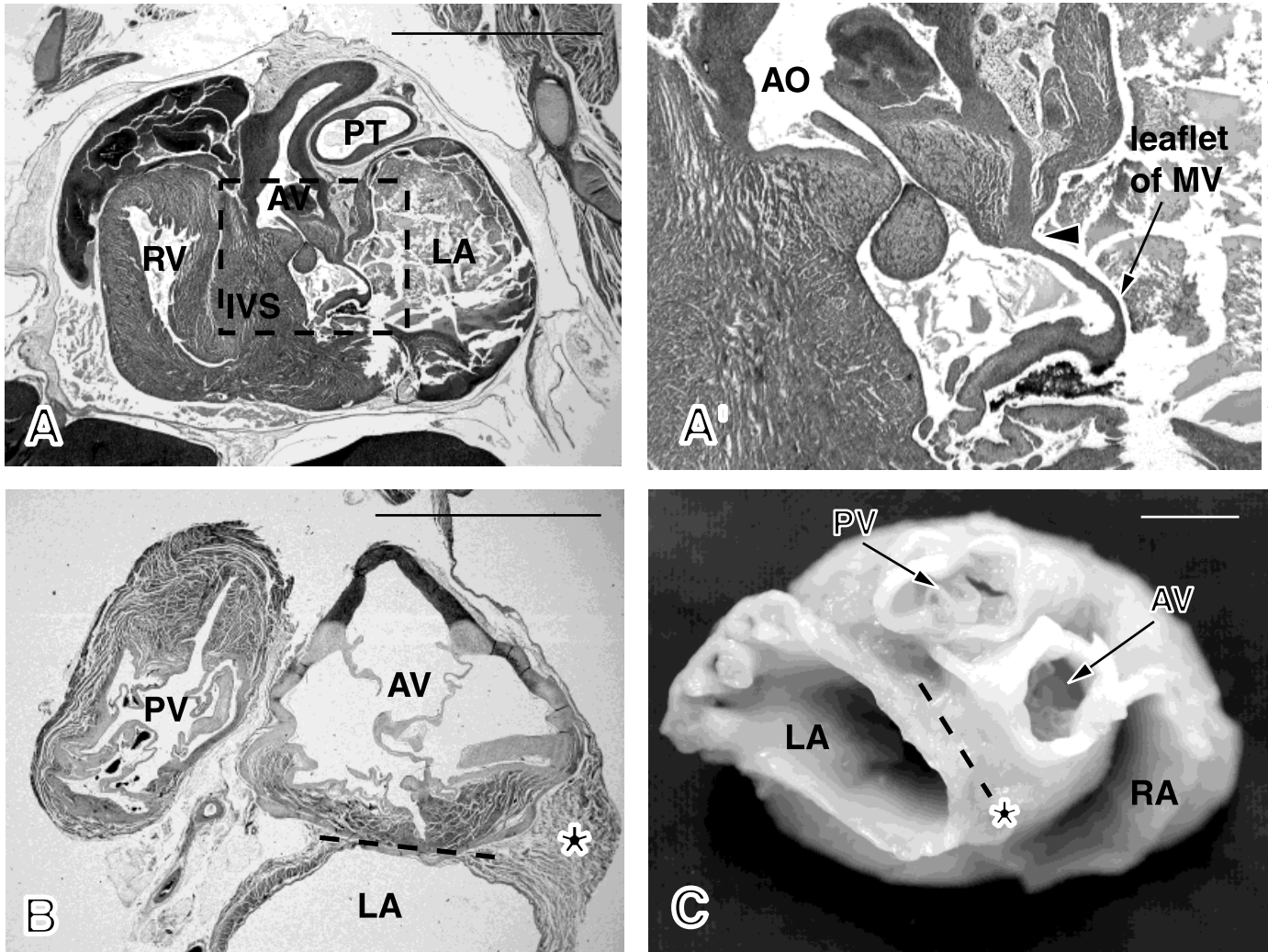
Fig. 6. **A:** Coronal section through the heart from a 1-day-old chick. The boxed area is shown, enlarged, in **A′**. There is muscular separation between the attachments of the aortic valvar leaflets and the hinge of the aortic leaflet of the mitral valve (arrowhead) in the region of the inner heart curve. **B:** The short axis section through a normal chick heart of 15 months of age confirms the presence of this muscle within the inner heart curvature (dotted line). **C:** The gross structure of the heart from which B was prepared is shown. It shows the definitive relations of the pulmonary and aortic valves (PV, AV) relative to the left atrium (LA). The asterisk shows the base of the ventricular mass. Note that the heart in B is rotated counterclockwise relative to the heart C. For other abbreviations, see list. Scale bars = 4 mm in A–C.

Stage 31

In all specimens examined, excavation of the distal cushions had produced valvar sinuses, but still within a common outflow segment surrounded by myocardium. The aortic outlet now occupied a central position between the right and left atrioventricular orifices (Fig. 4E). The developing leaflets of the aortic and pulmonary valves no longer lay in the same plane, but were becoming offset and angled relative to each other (compare Fig. 4E with 4F). The proximal extent of the fusing cushions, now attached to the crest of the muscular interventricular septum, was now seen to contain myocardial cells. This myocardialisation contributed ventrally to the dorsal wall of the developing subpulmonary infundibulum, and dorsally to the transiently myocardial ventral wall of the aortic root (Fig. 4F). At this stage, nonetheless, the fused cushions still formed a septal structure within the common outflow tract (Fig. 4E).

Stage 32–34

The caudal ends of the proximal cushions were still unfused. However, the myocardialised component of the fused portion of the proximal cushions no longer occupied a septal location. This finding was because a sleeve of free-standing muscular subpulmonary infundibulum had formed from its ventral aspect, with an extracardiac tissue plane appearing between it and two of the sinuses of the aortic root (Fig. 5A). The structure that initially separated the two channels within the arterial segment had disappeared. A raphe persisted at the level of the sinutubular junctions, where it formed the ligament of the conus (Fig. 5B). More proximally, the newly formed myocardium of the subpulmonary infundibulum no longer interposed between the cavities of the ventricles and no longer represented a "true" septum. The myocardium on the dorsal aspect, in contrast, continued to encircle the walls of the developing arterial sinuses up to the level of the sinutu-
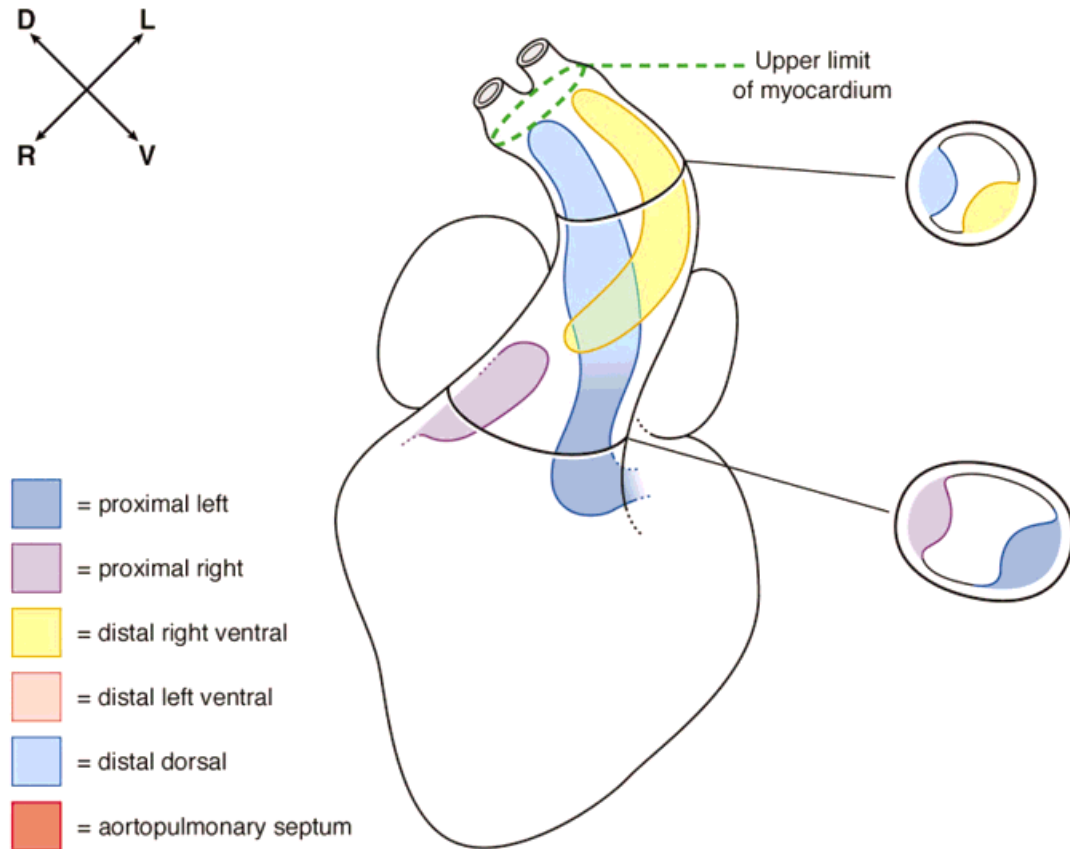
Fig. 7.   A schematic diagram summarising our findings on the relationships and terminology of endocardial cushions developing within the outflow segment and the aortopulmonary septum. This diagram, illustrating the situation at stage 24, is shown as viewed from the front in oblique right lateral projection. Only two cushions are present proximally and distally at this stage. D, dorsal; L, left; V, ventral; R, right.

bular junction (Fig. 5B). The mural tissue of the distal cushions was now forming the walls of these arterial sinuses, whereas the luminal tissues were differentiating into the arterial valvar leaflets (Fig. 5A). The aortic and pulmonary sinuses themselves, by now, had become separated from each other by an extracardiac tissue plane. Thus, the area between them, extending from the ventricular outlets to the sinutubular junctions, was also beginning to lose its initial septal nature.

Other Late Events

At stage 34, the external walls of the valvar sinuses had remained encircled by myocardium (Fig. 5). In the postnatal heart, in contrast, the boundary between the myocardium and the more distal fibroelastic tissue lay not at the sinutubular junction, but two-thirds of the way down the length of the valvar sinus. Up to stage 30, the arterial valves had been located at the same level, and in the same plane. Beyond this stage, the valves adopted markedly different configurations. By stage 34, the pulmonary infundibulum swept across from the right, then assumed a vertical position just proximal to the pulmonary valve. Therefore, the valve itself came to lie in an almost horizontal plane and was parallel to the plane of the atrioventricular junction. The aortic valve lay almost at a right angle to this, so that blood flowed not parallel to the long

axis of the heart, as it does through the pulmonary valve, but obliquely, in a right-superior direction. At this stage, therefore, the pulmonary valve lay ventral and to the left relative to the aortic valve, a configuration that persists in the formed heart (Fig. 6C). None of the specimens examined showed any evidence of fibrous continuity between the leaflets of the aortic and mitral valves (Fig. 6A,B).

DISCUSSION

Our study has shown that, in the chick, there are three endocardial cushions within the distal segment of the outflow tract. These distal cushions, together with two intercalated cushions, give rise to the leaflets of the arterial valves, and to the walls of their supporting sinuses of Valsalva. They do not seem to form any septal structure in the formed heart. Thus, although both the distal cushions and the aortopulmonary septum function initially as a true septum, dividing the aortic and pulmonary components of the arterial segment and the distal outflow tract, subsequently both these structures lose their septal nature. In the fully formed heart, an extracardiac tissue plane separates not only the proximal parts of the arterial trunks, but also the valvar sinuses of the aortic and pulmonary roots.

In a similar manner, the proximal cushions also fuse initially to form an intracardiac septum. But, as the cush-
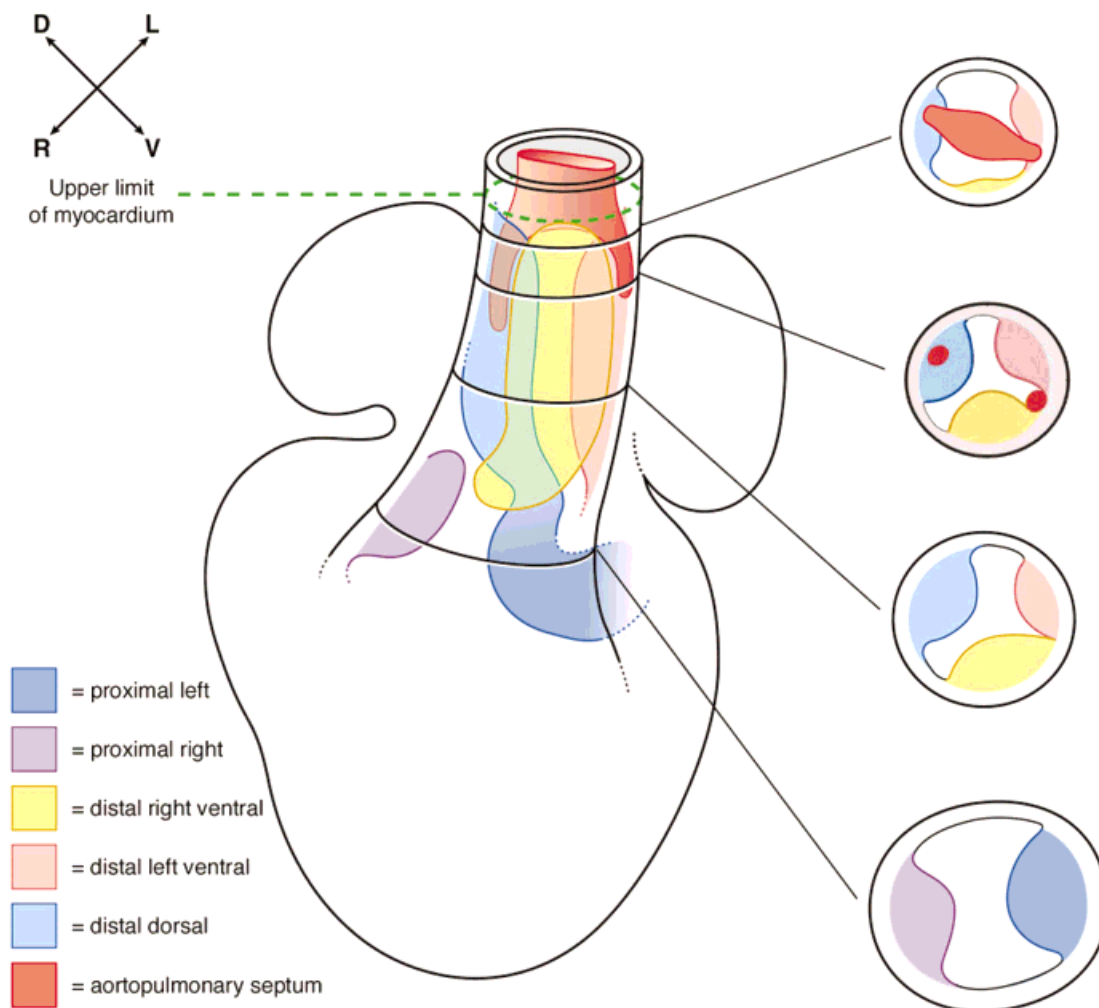
Fig. 8.   This schematic diagram is also shown viewed from the front in the oblique right lateral projection and represents the arrangement at stage 26/27. The overlapping of the two proximal ridges in this angle of view has not been shown. The third distal cushion has now appeared. Note that the upper cut-away passes through the main body of the aortopulmonary septum. D, dorsal; L, left; V, ventral; R, right.

ions become muscularised within the outflow tract, they too lose their septal location. The component that initially functioned as a septum becomes converted in part into the dorsal wall of the free-standing muscular subpulmonary infundibulum. Thus, the entire complex that initially separated the developing aortic and pulmonary channels within the developing arterial segment and the distal embryonic outlet loses its septal status in the definitive heart. This finding means that it is not possible to identify a discrete muscular outlet septum in the normal heart of the chick, a fact that is also true of mammals. In the chick, the entire process under discussion is best described as separation of the outflow tracts, with septation being only the initial step in this process.

Our findings as described above differ in several respects from some previous reports. These differences may, in part, arise because inferences made from knowledge of the mammalian pattern of development are imposed on the developing avian heart. In fact, there are significant differences between mammalian and avian hearts. Thus, in mammals, the morphologically tricuspid valve is a fi-

brous structure with three leaflets. In birds, the right atrioventricular valve is a unifolate muscular structure. In mammals, a discrete membranous septum interposes between the subaortic outflow tract and the right-sided chambers. Such a structure is lacking in the avian heart. Indeed, as pointed out by Shaner (1962), in many respects the avian heart is more comparable in its development to that of sauropsids, including the alligator. These differences are seen both in embryonic and adult features.

In terms of detail, we found that the third cushion within the distal outflow segment appeared later than the other two, and before the appearance of the intercalated cushions. Importantly, it arose as a new structure and was not formed by the aortopulmonary septum splitting one of the existing distal cushions. Our findings are shown diagrammatically in Figures 7–9. They endorse earlier descriptions that accounted for three ridges in the distal outflow tract of the chick but are in contrast to the more recent descriptions of two cushions seen proximally and distally (de la Cruz et al., 1977; Pexeider, 1978; Icardo,
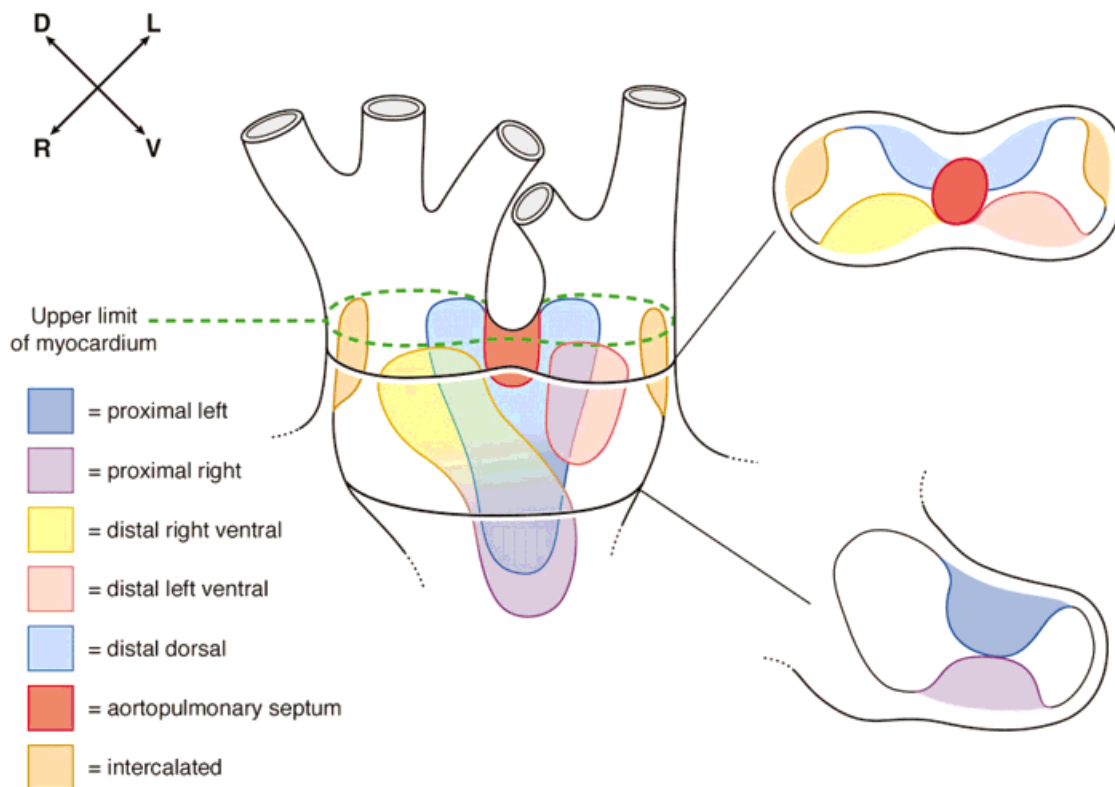
Fig. 9. This schematic diagram illustrates the arrangement of the outflow cushions at stage 30. The intercalated cushions have now appeared, but the myocardium still extends to the distal margins of the cushions. Note that the proximal cushions have now almost fused. D, dorsal; L, left; V, ventral; R, right.

1990). The latter pattern, although characteristic for mammals, does not exist in the chick.

A common model for development of the outflow tract envisages a "conotruncal" septum. This septum, believed to be derived from the fused proximal cushions, together with a tissue mass comprising the distal cushions and aortopulmonary septum, is held to divide the presumptive aortic and pulmonary channels. In our specimens, we were unable to find any definitive distal septum derived from endocardial cushion tissue. Instead, we saw the cushions producing primordia of six valvar leaflets, with the central mass of the aortopulmonary septum serving initially to divide the two channels more distally. It is possible, as suggested previously by Tonge (1869), that the sole fate of the distal cushions is to provide the substance for formation of the arterial valvar leaflets and the walls of their supporting sinuses of Valsalva.

After separation of the developing arterial roots by the aortopulmonary septum, the latter structure, which was initially septal in nature, no longer remains visible. Instead, the separated aortic and pulmonary roots, the latter supported by its muscular infundibulum, are found in its place. This arrangement is comparable to the situation seen in the formed human heart (Merrick et al., 2000). The presence of the extracardiac space that appears between these definitive structures is, perhaps, explained by studies which show that the fate of cells derived from the neural crest is to die through a process of apoptosis (Poelmann et al., 1998).

Neural crest cells have been demonstrated below the level of the sinutubular junctions by both Poelmann et al.

(1998) and Jiang and his colleagues (2000). If these cells do undergo apoptosis, then their death and subsequent disappearance would explain well the absence of any septal structures below the level of the sinutubular junction in the mature chick heart. This, in turn, eliminates the need to explain the formation of a "truncal-conal," or muscular outlet septum. As explained, such a structure does not exist in the mature heart. Our findings, nonetheless, are in agreement with previous work showing that the fused proximal cushions initially constitute a mesenchymal outlet septum (van den Hoff et al., 1999). Like Van den Hoff and his colleagues, we have also observed the invasion of this septum by cardiomyocytes in a process of myocardialisation. However, our findings indicate that that the proximal septum after myocardialisation becomes, in its larger part, the posterior wall of the freestanding subpulmonary infundibulum, losing in this process its initially septal nature.

The leaflets and sinuses of the arterial valves develop from the distal cushions of the outflow tract just proximal to the initial level of transition between the myocardial cuff and the arterial wall. The subsequent change in the level of the ventriculoarterial boundary in the walls of the maturing valvar sinuses suggests either that the original myocardium has continued physically to retract, or else that it has transdifferentiated into fibroelastic tissue. Further studies are required to elucidate these events and to determine the mechanisms of formation of the valvar leaflets as opposed to the arterial sinuses and the steps involved in the separation of the aortic and pulmonary components.

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