Normal and abnormal development of the intrapericardial arterial trunks in humans and mice

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Aims
The definitive cardiac outflow channels have three components: the intrapericardial arterial trunks; the arterial roots with valves; and the ventricular outflow tracts (OFTs). We studied the normal and abnormal development of the most distal of these, the arterial trunks, comparing findings in mice and humans.

Methods and results
Using lineage tracing and three-dimensional visualization by episcopic reconstruction and scanning electron microscopy, we studied embryonic day 9.5–12.5 mouse hearts, clarifying the development of the OFTs distal to the primordia of the arterial valves. We characterize a transient aortopulmonary (AP) foramen, located between the leading edge of a protrusion from the dorsal wall of the aortic sac and the distal margins of the two outflow cushions. The foramen is closed by fusion of the protrusion, with its cap of neural crest cells (NCCs), with the NCC-filled cushions; the resulting structure then functioning transiently as an AP septum. Only subsequent to this closure is it possible to recognize, more proximally, the previously described AP septal complex. The adjacent walls of the intrapericardial trunks are derived from the protrusion and distal parts of the outflow cushions, whereas the lateral walls are formed from intrapericardial extensions of the pharyngeal mesenchyme derived from the second heart field.

Conclusions
We provide, for the first time, objective evidence of the mechanisms of closure of an AP foramen that exists distally between the lumens of the developing intrapericardial arterial trunks. Our findings provide insights into the formation of AP windows and the variants of common arterial trunk.

Keywords
Mouse development • Neural crest • Second heart field • Aortopulmonary window

1. Introduction

Over the past decade, studies of animal models have transformed our understanding of cardiac embryology. In particular, the use of genetic cell lineage analysis, along with the ability to assay large numbers of specimens, has permitted the accurate tracking of distinct cellular contributions within the developing mouse heart. These findings have provided a framework for understanding cardiac development in humans, where comparable studies are hampered by limited availability of specimens and the impossibility of following cell lineage. As revealed in a recent review by Okamoto et al.,¹ there are numerous accounts concerning the development of the outflow tract (OFT).

Arbitration between the various theories is complicated by the fact that the definitive intrapericardial pulmonary and aortic outflow channels each possess three segments, namely the intrapericardial arterial trunks distal to the sinutubular junctions, the arterial roots with their valves, and the subvalvar ventricular OFTs, although development is usually explained in terms of only two components, the truncus and the conus.² There is no consensus as to how these two components relate to the three definitive intrapericardial parts of OFTs, nor the exact temporal and morphological changes that separate the distal common lumen into separate intrapericardial aortic and pulmonary trunks. In addition, describing any embryonic structure as an OFT septum is somewhat problematic, given that in the fully formed heart there is no septum between these two arterial components.
It is well established, nonetheless, that during development the aortic and pulmonary channels are separated by mechanisms involving intrapericardial migration of new populations of cells, including those derived from an Isl1-expressing (Is11) progenitor population in the pharyngeal mesoderm termed the second heart field4 and neural crest cells (NCC).4 Using lineage analysis, along with techniques providing three-dimensional visualizations, we have now analysed the contributions made by these populations during separation of the intrapericardial arterial trunks distal to the primordia of the arterial valves. We have not, in this study, assessed the mechanics of separation of the arterial roots, nor the formation of the subpulmonary infundibulum, although our findings are pertinent to previous studies describing how these components are separated by an aortopulmonary (AP) septal complex.4,5 Subsequent application of our approach to mouse mutants, however, has permitted us to provide mechanistic insights with regard to the morphogenesis of AP window and common arterial trunk.

2. Methods

Details and references are provided in Supplementary material online.

2.1 Mouse and human embryos

We used CD1 (Charles River), Parkes, and C57Bl6 mice, employing R26R and R26EYFP reporter lines in combination with the Wnt1-cre and Isl1-cre lines to label permanently NCC and second heart field cells, respectively, as well as Isl1-lacZ mice. Mice were maintained according to the regulations of the UK Home Office, and the studies conformed to Directive 2010/63/EU of the European Parliament. We also studied eight human embryos between Carnegie stages (CS) 13 and 18 obtained from the MRC-Wellcome Human Developmental Biology Resource housed at Newcastle University. Ethical approval for the collection and use of this material was gained from Newcastle University. These studies conformed with the principles outlined in the Declaration of Helsinki.

2.2 Histology and immunohistochemistry

Standard protocols were used for the staining of paraformaldehyde-fixed embryos. An anti-green fluorescent protein antibody, which also recognizes yellow fluorescent protein, was used to identify positive cells in fixed samples from R26EYFP. Xgal staining was used to detect β-galactosidase expression in samples from R26R and Isl1-lacZ mice. Each experiment was repeated a minimum of three times, and included appropriate controls.

2.3 Three-dimensional reconstruction

Three dimensional reconstructions of five serially sectioned staged embryos encompassing embryonic days (E) 9.5–12.5 were carried out according to standard protocols. Sections were stained with MF20 antibody to mark the myocardium, and alcian blue to stain the cushion tissue.

2.4 Scanning electron microscopy

Embryos from E9.5 through 12.5, using at least five embryos for each stage, were cacodylate-fixed, dissected, and processed by standard techniques to show the salient anatomic features.

2.5 High-resolution episcopic microscopy

Embryos were prepared and imaged as described in Supplementary material online. We studied 5 data sets from mice at E10.5, 25 data sets from E11.5, and 12 data sets from E12.5. For comparative purposes, we also studied eight human embryos as already described.

3. Results

3.1 The OFT prior to septation

The cardiac OFT extends from the ventricular mass to the margins of the pericardial cavity. A prominent bend, more acute in humans than in mice (compare Figure 1A and B), initially permits distinction of proximal and distal components. At the margins of the pericardial cavity, the lumen of the OFT is continuous with that of the aortic sac, a manifold embedded within the pharyngeal mesenchyme which gives rise to the pharyngeal arch arteries (Figure 1D and E). At E9.5 (25 somites, equivalent to CS13 in humans), the walls of the OFT are exclusively myocardial (Figure 1C). By early E10.5, the jelly lining the walls is populated by NCCs, which are contiguous extrapericardially with those forming the walls of the aortic sac and arch arteries (Figure 1C).

By late E10.5 (35 somites, CS14), short non-myocardial spurs are seen cranially and caudally, which indent the myocardial margins towards the ventricles and express Isl1 (Figure 1D). Initially at E10.5, a prominent mid-sagittal ridge, populated by NCCs, separates the right and left sides of the aortic sac between the third and fourth arches (Figure 1F and Supplementary material online, Figure S1D). The ridge has attenuated by early E11.5 (not shown), by which time the junction between the OFT and the aortic sac has shifted caudally, so as to lie between the fourth and sixth arches. By this stage, the dorsal wall of the aortic sac has developed a subtle protrusion (arrow in Figure 2A). It is this protrusion, rather than the mid-sagittal ridge, which represents the earliest sign of separation of the cranial aortic and caudal pulmonary channels.

Over the period continuing to late E11.5 (about 50 somites, CS15), the distal non-myocardial tissue, initially seen cranially and caudally, expands and rotates counterclockwise in the ventral view (compare Figure 1D and G; see Supplementary material online, Figure S2A), producing right-sided and left-sided walls to the OFT that are non-myocardial (Supplementary material online, Figure S2C), and producing a fishmouth configuration for the distal myocardial margins. The myocardial components of the wall now overlie the distal extent of the opposing cushions, which extend proximally throughout the proximal parts of the OFT (Supplementary material online, Figure S2B and D), whereas the non-myocardial components overlie the blood-flow channels. The cushion located caudally in the distal part of the OFT terminates proximally on the septal side of the right ventricle (orange in Supplementary material online, Figures S2 and S3 and Movie S1), whereas the second cushion, located cranially in the distal OFT, extends rightwards and parietally, when traced towards the ventricles (yellow in figures).

Up to the early stage of E11.5, the aortic sac and arch arteries are left–right symmetrical, with the sac giving rise cranially to the third and fourth arch arteries, and caudally to the sixth arch arteries (Figure 1G and H; Supplementary material online, Movie S2). By the end of E11.5, and during E12.5, the developing pulmonary arteries have become evident, taking their origin from the mid-ventral portions of the sixth arch arteries (Figure 1K, Supplementary material online, Movie S2). Within the distal OFT, the apposition of the opposing cushions has now created two lateral channels, even prior to the fusion of their facing surfaces (Figure 2, Supplementary material online, Figure S3). When traced proximally, the channels spiral (Supplementary material online, Figure S3A and C and Movie S3), with the left-sided distal channel moving cranially towards the cavity of the right ventricle, and the right-sided channel directed caudally (Supplementary material online, Figures S2 and S3). By this stage, the oblique protrusion from the dorsal wall of the aortic sac (dashed line in Figure 3D; double arrow in Figure 4D) serves to
direct the caudal sixth arch arteries towards the left-sided channel, and the cranial fourth arch arteries towards the right-sided channel. Eventual fusion of the protrusion with the distal ends of the cushions closes the space initially existing between these developing aortic and pulmonary channels, thus forming a transient AP septum. By analogy to the foramen primum, which exists between the leading edge of the septum primum and the atrioventricular cushions, the space itself can be considered to be the AP foramen.

3.2 Separation of the distal intrapericardial channels

At the beginning of E11.5, the extrapericardial aortic sac has a cranial part, giving rise to the fourth arch arteries, and a caudal part supporting the sixth arch arteries (Figure 2G and Supplementary material online, Movies S4 and S5). The dorsal wall, which separates the arterial orifices, is longer in mice compared with humans (compare Figure 3A and B). As discussed earlier, the space between the dorsal wall and the distal margins of the cushions at this stage is an AP foramen (boxed regions in Figure 2A–F and circled in H). Analysis of the 25 data sets from E11.5 embryos shows that the foramen closes rapidly during E11.5 (Figure 2). When viewed externally, the OFT and its adjoining pericardial walls are initially smooth (Figure 4A). Concomitant with the bulging of the sixth arch arteries into the pericardial space caudally, and the appearance of spiralling counterclockwise grooves laterally, the latter showing the locations of the eventual separation of the developing intrapericardial aortic and pulmonary trunks (Figure 4B and C and Supplementary material online, Movie S6), there is a reduction in the size of the foramen. Dissections through the OFT
reveal formation of a narrow waist caudally, close to the pericardial margins (black arrows in Figure 4E). Extrapericardially, excavation of the intermediate part of the aortic sac, particularly on the right side, frees the pharyngeal mesenchyme to form the wall of the extra-pericardial ascending aorta, which is continuous at the borders of the pericardial cavity with the right-sided non-myocardial tissue derived from the second heart field (Supplementary material online, Figure S3D and Movies S7–S9). Similar processes on the left side form the intra- and extrapericardial parts of the pulmonary trunk (Figure 5). By the middle of E11.5, the foramen itself is no more than a pinhole (Figure 3E). The sixth arch arteries, encased within the extra-pericardial mesenchyme (Figure 4D), have also begun to remodel at this stage, with the right sixth arch artery showing marked regression dorsal to the origin of the right pulmonary artery (Supplementary material online, Figure S2B). Eventual disappearance of this right-sided artery is necessary to achieve full separation of the pulmonary and aortic circuits.

### 3.3 Two populations of NCCs close the AP foramen

At E9.5 and 10.5, through to early 11.5, the dorsal wall of the aortic sac is predominantly of NCC origin (Figure 1C and F), overlying a region made up of cells expressing Isl1 (Figure S8–S10). By this stage, the distal cushions are also filled with NCCs (Figure 5A), which can be traced laterally into the pharyngeal mesenchyme (Figure 1C and F). Fusion of the protrusion with the distal ends of the cushions, each initially covered by an endothelial layer, produces a continuum of NCCs (Figure 2F), although with the two populations derived from medial and lateral origins (Figure 5A). This central structure formed within the distal OFT functions transiently as an AP septum. Only subsequent to the fusion of the two populations of NCCs does it become possible to recognize the condensed whorl-like structure present within the tissues now separating the newly formed intrapericardial aortic and pulmonary channels. This condensed tissue can be traced into the unfused cushions more proximally, where it forms the columns of condensed mesenchyme known as the ‘prongs’ (Supplementary material online, Figure S3). The transient septum formed by fusion of the protrusion and the distal cushions subsequently expresses alpha-smooth muscle actin (aSMA; Figure 5E) and, with ongoing development, its surfaces arterialize to form the adjacent walls of the intrapericardial arterial trunks. The most distal adjacent walls are formed from the surfaces of the protrusion, whereas the proximal walls are derived from the distal cushions. The core of the central tissue mass is then replaced by connective tissue (Figure 5F).

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**Figure 2** (A–F) Episcopic images showing the reduction in size and eventual closure of the AP foramen in E11.5 mouse hearts. All are shown from the right side, having removed the parietal wall of the cardiac structures. The insets show the foramen in detail. The arrow in (A) marks the protrusion of the dorsal wall of the aortic sac. (G–I) The remodelling of the junction of the OFT with the aortic sac, comparing early (G), middle (H), and late (I) stages of E11.5. The developing aortic channel is shown in red, along with the third and fourth arch arteries, whereas the pulmonary channel and sixth arch arteries are shown in deep blue, with the pulmonary arteries arrowed. The circle in (H) shows the closing AP foramen.
in contrast, do not express Isl1. As described earlier, being of NCC origin, they are formed distally from the protrusion, and proximally from the distal tips of the cushions (Figure 5A). The dimple marking the closing AP foramen shows the site of union between these two sources of NCCs (Figure 3F).

3.5 Mechanistic insights relating to morphogenesis

If our concept of normal development is correct, malformations related to an abnormal protrusion of the dorsal wall of the aortic sac should be separable from defects caused by failure of the fusion of the OFT cushions. With this in mind, we examined mouse models and human specimens, hoping to provide insights into the morphogenesis of lesions such as AP window and common arterial trunk. Loop-tail (Lp) mice have a mutation in Vangl2. At E11.5, the pharyngeal region of Lp/Lp embryos shows varying numbers of NCCs within the OFT cushions, with an uneven distribution of the NCC in the dorsal wall of the aortic sac (Figure 6A). In some mice, by E12.5, we found that the cushions had fused with each other, forming separate aortic and pulmonary roots, but had failed to fuse with the protrusion, leaving an AP window (Figure 6B and C). Other Lp/Lp mice, in contrast, showed common arterial trunk, with the failure of the fusion of the outflow cushions, but with separation intrapericardially of the aortic and pulmonary channels due to the formation of a dorsal protrusion (Figure 6D).

Splotch 2H (Sp²H) mice carry a mutation in Pax3, which reduces the population of NCCs. At E11.5, there is obvious deficiency of pharyngeal NCCs (compare Figure 6E with 1F and 5A). The dorsal wall of the aortic sac is markedly thinned, with a much reduced population of NCCs, and no dorsal protrusion, since the animals also lack sixth aortic arch arteries. By E12.5, the OFT cushions remain unfused (Figure 6F), and, at later stages, the majority of Sp²H/Sp²H mutants have a common arterial trunk. Deficiency of NCCs in the Sp²H mutant, therefore, is associated with the failure of the fusion of the structures required for the separation of the more proximal parts of the OFT, along with the absence of the sixth arch arteries, and hence no potential for the formation of an AP septum.

The malformations seen in mice are directly comparable with lesions found in humans. The phenotypic feature of human AP windows is the presence of a communication between the intrapericardial arterial trunks, but with normal formation not only of the aortic and pulmonary roots, but also of the proximal walls of the intrapericardial arterial trunks (Figure 6G). This implies the failure of the closure of the AP foramen, but normal fusion of the outflow cushions. In contrast, the phenotypic feature of human common arterial trunk is the presence of a common arterial valve, indicating a lack of cushion fusion. This is typically seen with the pulmonary arteries arising side-by-side at the margins of the pericardial cavity (Figure 6H), with the absence of the sixth arch arteries again meaning that there is no protrusion of the dorsal wall of the aortic sac. Less frequently, there can be separate intrapericardial aortic and pulmonary channels (Figure 6I), along with the presence of a left sixth arch, suggesting relatively normal formation of the protrusion, but again with the overall failure of the fusion of the cushions.

3.4 Different origins of the lateral and facing walls of the intrapericardial arterial trunks

The non-myocardial lateral and distal parts of the developing walls of both the intrapericardial aorta and the pulmonary trunk (Figure 1f) express Isl1 (Figure 5B and C), revealing their origin from the second heart field. The adjacent, or facing, walls of these channels, so that eventually there is no longer any anatomic septum between the intrapericardial arterial channels.

Figure 3 Episcopic images showing the rotation of the boundary of the distal OFT with the pharyngeal mesenchyme. (A) The aortic sac with cranial and caudal components at early E11.5, which gives rise symmetrically to the fourth and sixth arch arteries, respectively. The asterisk shows the intermediate part of the sac, with the dorsal wall separating the systemic and pulmonary parts of the sac. (B) A CS14 human embryo showing a comparable arrangement. (C and D) Views of a mid-E11.5 OFT cut transversely through the closing AP foramen (arrow head in D), close to the aortic sac, as shown in the inset in (C). The oblique orientation of the protruding dorsal wall of the aortic sac is shown as the dashed line in (D). (E) An overview of an E11.5 heart, showing the orientation of sections (A) and (F). (F) A view made by removing the parietal wall of the aorta, as in Figure 2F, just prior to the closure of the AP foramen. The dotted line shows the border between the intra- and extrapericardial aortic components. The upper double-headed arrow shows the walls distal to the foramen, derived from the protrusion, whereas the lower double-headed arrow shows the adjacent wall of the aorta proximal to the foramen, derived from the fused distal cushions.
4. Discussion

Numerous hypotheses have been advanced to explain the transformation of the distal part of the developing OFT from a channel with a common lumen into the aortic and pulmonary trunks. In some studies, these changes have been explained on the basis of the fusion of an AP septum with the distal ends of the outflow cushions. The structure defined as the AP septum is equivalent to the entity we have described as the protrusion. We prefer to use the term 'protrusion' rather than septum for three reasons. First, there is no comparable septal structure in the postnatal heart. Second, it is only the distal half of the embryonic septum between the intrapericardial trunks that is derived from the protrusion, the proximal part being derived from the distal cushions. Third, and perhaps the most important, it is more customary to consider the AP septum as separating the more proximal parts of the OFT, specifically the arterial roots and the ventricular OFTs. We agree with these findings concerning the role of the cushions as septal structures within the more proximal parts of the OFTs, but this has not been the subject of this current investigation. Rather, we have addressed the formation of the intrapericardial trunks distal to the primordia of the arterial valves. The AP septal complex as described by the later investigators does not become evident until after the separation of the intrapericardial arterial trunks. This separation, eventually leading to the formation of discrete adjacent walls of the aorta and pulmonary trunk, also requires marked remodelling and excavation at the junction between the OFT and the aortic sac. This remodelling, not previously described in this regard, is regression of the dorsal part of the right sixth arch.

The changes we see in our mouse hearts are comparable with those observed in the human heart, although also with subtle differences. There is a greater degree of bending of the OFT in humans, more obvious formation of the initial non-myocardial spurs seen cranially and caudally at the margins of the pericardial cavity, and a

Figure 4 Scanning electron micrographs of E10.5 and 11.5 mouse embryos. (A) (E10.5) and (B–F) (E11.5): The junction of the OFT and the mediastinal wall, viewed inferiorly, with the transected foregut and bilateral cardinal veins visible beneath. Note the progressive asymmetric bulging of the left and right sixth aortic arches (labelled 6 in B, C, D, and E) into the pericardial cavity. This, together with excavation at the pericardial border, gives the impression that the OFT is being twisted ant clockwise in this ventral view. In (D), the OFT has been transected in the plane indicated by the white line in (C). This shows that the lumen at the site of the cut is made up of a large right-sided cranial portion, which will be the intrapericardial aorta (ipa), and a small left-sided caudal portion, which will be the pulmonary trunk (ipp). The double-headed white arrow shows the oblique orientation of the AP foramen and the protrusion. (E) An OFT that has been sectioned longitudinally (transverse to the embryo, at the level shown by the white line in D) and is viewed superiorly. Note the narrowness of the junction (arrows) between the OFT and the body. The septal cushion (SC) is visible in the inferior portion of the OFT. (F) A later E11.5 junction of the OFT and the mediastinal wall, viewed superiorly, showing the pronounced remoulding between the ipa and ipp. aa, aortic arch.
shorter dorsal wall of the aortic sac. The comparable findings in humans and mice, nonetheless, permit us to suggest mechanistic insights not only for normal development, but also for the pathogenesis of lesions such as common arterial trunk and AP window. With regard to normal development, our observations support the important role of NCCs in filling the outflow cushions and separating the more proximal parts of the OFT.\textsuperscript{11,12} We also show the importance of a second migration of cells derived from the neural crest, specifically those contained in the protrusion from the dorsal wall of the aortic sac. Thus, we have demonstrated two pathways for the migration of NCCs into the OFT. Cells migrate laterally to populate the cushions, whereas others enter medially forming a cap on the part derived from the second heart field. Our findings also confirm the importance of the intrapericardial migrations from the second heart field;\textsuperscript{3} our lineage tracing showing that the non-myocardial components derived from the second heart field will form the lateral walls of the intrapericardial trunks, endorsing a similar study in the developing avian heart.\textsuperscript{10} The facing walls of the trunks, in contrast, are derived from NCCs within the fused protrusion and cushions, whereas those in the protrusion are supported by a core derived from the second heart field. This core eventually becomes replaced by the connective tissue interposed between the walls of the arterial trunks, and described circumferential seams between these components. We also envisage the presence of seams between the component parts, but our findings indicate that the seams would extend longitudinally from the sinutubular junctions to the distal margins of the pericardial cavity. Waldo et al.\textsuperscript{14} however, studied the chicken heart, whereas our interpretations are based on findings from mice, supported by findings in humans.\textsuperscript{13}

With regard to the pathogenesis of congenital cardiac malformations, we had suggested previously, in a brief review,\textsuperscript{15} that failure to close the embryonic AP foramen would provide a rational...
explanation for the morphogenesis of AP window. Our current images now provide the detailed evidence underscoring this mechanistic insight, which is further supported by analysis of human hearts with AP windows. The separate nature of the arterial trunks proximal to the window is strong circumstantial evidence supporting our interpretation that the adjacent walls are derived distally from the protrusion, but proximally from the cushions. Our current findings also endorse the concept that the failure of the fusion of the outflow cushions themselves is responsible for producing the common arterial trunk. As already discussed, we fully recognize in this regard the importance of contributions from NCCs during normal as opposed to abnormal development, but our findings have shown the duality of these contributions. Both are important in explaining the full spectrum of morphology seen in the setting of common arterial trunk. When seen in humans, common arterial trunks can sometimes show extensive separation of the intrapericardial aortic and pulmonary components. This implies some degree of formation of the intrapericardial protrusion from the aortic sac, a feature also shown in some of our Lpq mice. This arrangement is termed pulmonary dominance in humans, and is found with either interruption of the aortic arch or severe aortic coarctation, and is found with the presence of the arterial duct implying the formation of the left sixth arch artery. Common arterial trunk in humans, however, is found most frequently with a predominantly aortic intrapericardial arterial component, the pulmonary arteries typically arising side-by-side from the dorsal part of the trunk, and with no formation of any distal AP septal structure in the absence of any sixth arch artery. The pathogenesis of malformations involving the intrapericardial arterial trunks, therefore, involves processes that reduce the intrapericardial protrusion of the dorsal wall of the aortic sac and requires failure of the fusion of the outflow cushions, or a combination of these events—these mechanistic insights only being understandable on the basis of normal development as currently described.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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