Formation of the Atrioventricular Septal Structures in the Normal Mouse

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Abstract—It is sometimes thought that formation of the atrioventricular septum is equated with fusion of the endocardial cushions and that failure of fusion can explain all deficiencies of atrioventricular septation. Clearly, this is simplistic, but the exact contribution of different primordia to atrioventricular septation is not well understood. To clarify this, we studied normal mouse embryos (days 10 to 15 of gestation), which were serially sectioned and examined by light microscopy. Another group of embryos was examined by scanning electron microscopy after microdissection. Our results show that development of the atrioventricular septal area is highly complex. Proper formation requires the following: remodeling of the inner heart curvature, rotation of the horns of the systemic venous sinus around the pulmonary portal, expansion of the right atrioventricular junction, formation of the muscular atrial and ventricular septa, bridging by the dextrodorsal outflow ridge and the superior endocardial cushion, fusion with the inferior margins of the venous valves, and formation of the mouth of the coronary sinus from the cranial muscular wall of the left sinus horn. Multiple primordia contribute to a central mesenchymal mass (the “septum intermedium”), including the mesenchyme on the leading edge of the primary atrial septum, the atrioventricular endocardial cushions, and the cap of mesenchyme on the spina vestibuli. Fusion of these components closes the ostium primum, completing atrial and atrioventricular septation. Additionally, the spina vestibuli has a mesodermal core, which contributes to the muscularization of the lower margin of the oval fossa. This contrasts with the formation of the upper rim, which occurs as a result of an infolding of the atrial wall itself. (Circ Res. 1998;82:645-656.)

Key Words: mouse • atrioventricular septation • morphogenesis • endocardial cushion

The endocardial cushions have been considered the fundamental “glue” for normal septation in the heart.1,2 In addition, as part of this same process, they are thought to contribute to the membranous ventricular and atrioventricular septal structures,3,4 to the fibrous skeleton of the heart,5 and also to the fabric of the atrioventricular valvar leaflets.6 Despite this, the fate of the endocardial cushions during the process of atrioventricular septation is not well understood. The purpose of the present study, therefore, was to chart the development of the atrioventricular endocardial cushions and establish their contribution to the formation of the atrioventricular junctions and to the atrioventricular septal structures. In addition, we sought to identify the role of any other developmental primordia in producing the definitive atrioventricular septal structures. The present study was based on morphological observation of serial sections of the mouse heart, augmented with scanning electron microscopy.

Materials and Methods
A series of normal embryos was collected to encompass the 10th through the 15th day of gestation. These were either normal embryos from an Rb(11.16)2H/Rb(16.17)7Bnr × C57BL/6J or C57BL/6J × CBA cross. No differences in cardiac structure were observed between the different crosses. Mice were mated overnight. The presence of a copulation plug the next morning was taken as evidence of successful mating, and this day was designated day 1 of gestation. Embryos up to and including 11 days of gestation were staged by the number of somite pairs. Beyond that age, days of gestation are used. To convert to embryonic age (E), subtract 0.5 from the day of gestation. For example, noon on the 12th day of gestation is E 11.5. Pregnant dams were killed by cervical dislocation, the conceptuses were explanted, and extraembryonic membranes were removed. All embryos were examined under a stereomicroscope to establish gross morphology; somite pairs were counted to determine the stage of embryos of <12-day gestation. Embryos collected on days 10 to 12 of gestation were immediately fixed by immersion in 2% glutaraldehyde and 1% formaldehyde and buffered with 0.05 mol/L sodium cacodylate at pH 7.4 (adjusted to 330 mOsm with sodium chloride) for a minimum of 2 hours at room temperature. Embryos of 13 to 15 days of gestation were fixed by perfusion,7,8 followed by overnight immersion/fixation in the same fixative.

Scanning Electron Microscopy
We selected 24 embryos for examination by scanning electron microscopy. After fixation, microdissection was performed by hand under a stereomicroscope by use of iridectomy scissors. All samples were postfixed in 1% osmium tetroxide, dehydrated through a graded alcohol series, critical point–dried using liquid carbon dioxide, mounted on stubs, and then sputter-coated with gold. Samples were viewed on a Zeiss SM940 scanning electron microscope.

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Serial Sections
A series of 41 embryos, at least 6 from each day of gestation, were prepared for serial sectioning. After fixation, embryos were rinsed, dehydrated, embedded in paraffin wax, and serially sectioned at a thickness of 5 μm. The smallest embryos (<50 somites) were embedded in agarose (to facilitate orientation) before processing. Once cut and mounted, sections were dewaxed, rehydrated, stained with Masson’s trichrome stain, dehydrated, and coverslipped using DPX mounting medium. Micrographs of the sections were taken using a Zeiss D-7082 transmitted-light stereo photomicroscope.

Results

10th and 11th Days of Gestation (20 to 50 Somites)
On the 10th day of gestation, the mouse heart is unseptated and tubular. The endocardial cushions are already visible within the atrioventricular canal, which, at this stage, has considerable length compared with its breadth. The heart has already looped to the right, and this, coupled with the length of the atrioventricular canal, produces an extensive inner heart curvature. The atrioventricular canal is situated to the left of the midline of the embryo, and the endocardial cushions fill its lumen. The interventricular foramen, marked externally by the interventricular groove, leads from the well-developed presumptive LV into the presumptive RV, which is small compared with its partner. Most of the right portion of the ventricular loop represents the outflow tract, which at this stage is unseptated. The muscular ventricular septum has yet to form, but by 26 somites, ventricular trabeculations are evident within the apical components of the presumptive RV and LV (not shown).

By the 11th day, at the stage of 38 somites, there has been considerable growth of the muscular ventricular septum so that the RV and LV are now separated apically (not shown). The atrioventricular canal is still committed almost exclu-
sively to the developing LV, but the atrioventricular junction has started to expand to the right (Fig 1A and 1B). Two grooves, or lateral channels, are visible within the substance of the atrioventricular endocardial cushions, although the cushions are not yet fused. At slightly earlier stages, the lateral channels are both perpendicular to the opposing faces of the SC and IC (not shown). The LCH remains symmetrical (Fig 1A and 1B), whereas on the right side, the SC extends over the lateral channel, becoming continuous with mesenchyme on the right parietal wall of the atrioventricular canal (arrow in Fig 1A). This extension of the SC is the first sign of the rightward expansion of the atrioventricular junction. The mural mesenchyme has sometimes been referred to as a “lateral cushion.” This term, to our eyes, implies that the mesenchyme is more extensive than it really is.

At this stage, the LSCV opens into the systemic venous sinus of the RA (Figs 1C, 1D, and 2), to the right of an elevation (denoted by an X in the figures), which can be traced into continuity with the right margin of the orifice of the developing PV. We believe it was this elevation that was described by His9 as the “spina vestibuli.” At \( \approx 20 \) somites (not shown), the pulmonary venous orifice is bound by two symmetrical ridges in the dorsal atrium, which have been described as the RPR and LPR.10,11 For the purposes of this description, the RPR and the spina vestibuli become synonymous. By 38 somites, the extension of the right ridge is, in turn, related to the rightward margin of the IC (Fig 1C through 1G). A dense core can be seen within the elevation on this ridge, which is discrete from the densely stained mesenchyme on the crest of the developing PAS (Fig 1D). The spina vestibuli interposes between the PAS and the right side of the IC (Fig 1F). The dense whorl of cells that forms the “core” of the spina vestibuli can be traced back into the somatic mesoderm of the body of the embryo.

By this stage, the boundaries of the systemic venous sinus (the sinus venosus) within the RA are marked by the venous valves (the sinoatrial valves). There are two such structures, traditionally described as the RVV and LVV (Fig 2). The LVV is seen to be continuous with the RPR (Figs 2 [white arrow], 1G, and 3H), with the LSCV being recognized as a discrete channel running to the left of the midline within the atrioventricular junction (Fig 1G). The PV is now visible as a canalizing channel within the somatic mesoderm. Its orifice opens into the atrium between the pulmonary ridges (solid black arrows in Fig 1G and 1H). The leading edge of the PAS, which is relatively thick at this stage, extends toward the endocardial cushions, but no secondary foramen (OS) is visible within its substance.

Shortly thereafter, at the stage of 40 somites, the PAS has become thinned in its cranial margin, and the secondary
foramen is now seen in its dorsocranial portion (Fig 3A). The leading edge of the PAS has a MC, which is in continuity with the SC (Fig 3B), where it forms one of the boundaries of the still patent OP (Fig 3C). The base of the atrial septum is now markedly thickened in the region of the RPR, and its tissue is continuous with the somatic mesoderm in the area of the foregut (Fig 3E). This ridge also projects toward the atrioventricular canal and abuts the IC (Fig 3F and 3G). The endocardial cushions still remain discrete and separate structures (Fig 3E and 3F). The orifice of the PV within the developing LA is now continuous with the channel developing in the somatic mesoderm, which can be traced into the body of the embryo to its bifurcation near the developing LBs (Fig 3G). The left sinus horn (caval vein) remains a discrete channel within the left atrioventricular groove (Fig 3I).

At the stage of 45 somites, the PAS remains separated from the atrioventricular endocardial cushions by the triangular OP (Fig 4B through 4E). The ostium is bounded by the mesenchyme on the leading edge of the PAS together with the mesenchyme of the cushions (Fig 4B through 4F). These sections taken in the sagittal plane (Fig 4B through 4E) show the extensive nature of the secondary foramen (OS). The LSCV remains a discrete venous channel running behind the LA (Fig 4A), extending within the atrioventricular groove (Fig 4B through 4F), to open between the venous valves in the RA (Fig 4G and 4H). Continuity has also been established at this stage between the mesenchymal mass on the leading edge of the PAS, the SC, and the DDC of the outflow tract (double arrowheads in Figs 4F and 5B). This continuity now roofs the right atrioventricular channel, with the RA being in continuity with the developing RV (Fig 5B through 5E). The bulk of the SC is still positioned to the left of the developing IVS, filling the width of the atrioventricular canal and forming the dorsoinferior margin of the developing subaortic outflow tract. The OP is still patent (Fig 5C), and the atrioventricular endocardial cushions have still to fuse (Fig 5D). The spina vestibuli has its own extensive MC, which shows signs of fusion with the IC (Fig 5G and 5H). The RPR, incorporating the spina vestibuli, is now appreciably larger than the LPR. By this stage, the rightward ventricular margin of the IC is continuous with the crest of the muscular ventricular septum (arrowhead in Fig 5F). When the junction is seen in its entirety, as shown by scanning electron microscopy, the IC is draped across the muscular ventricular crest, dipping markedly toward the right atrioventricular junction (Fig 6A). By 48 somites, the IC projects across the ventricular crest, forming the rightward ventricular tubercle (arrowhead in Fig 6B). The plane of alignment of the developing atrial and ventricular septal structures, revealed by scanning electron microscopy and sectioning, is well to the right of the overall atrioventricular canal (Figs 5B, 6A, and 6B).

Shortly thereafter, by the stage of 49 somites (Fig 7), which is at the end of the 11th day of gestation, all the elements are in place for completion of septation. The cushions within the outflow tract have started to fuse, and the mesenchymal swellings that will become the arterial valvar leaflets are visible. Those within the AO are at a distance from those within the PT (Fig 7A and 7B), and the putative valvar orifices are at right angles to each other. The outflow ridges have fused with each other (Fig 7C), and an extension from the dextrodorsal ridge is in continuity with the ST (arrowhead in Fig 7D) to roof the secondary interventricular foramen. The MC on the RPR (spina vestibuli) has now fused with the IC, which in turn has fused with the SC (Fig 7E and 7F). The IC remains draped across the right side of the muscular ventricular septum (Fig 7F through 7H). Its junction with the SC anchors the dorsoinferior wall of the developing subaortic outflow tract (arrow in Fig 7F) to the crest of the muscular ventricular septum. Within the atrioventricular area, the wall of the LSCV is separated from the derivatives of the right sinus horn by the SS (Fig 7G). The LSCV itself continues to extend as a discrete channel within the left atrioventricular groove, emptying into the RA within the confines of the venous valves (Fig 7G and 7H). The RVV is itself continuous with the IC (Fig 7H; also see Fig 6B).

12th Day of Gestation
By this stage, the proximal DDC and SVC have fused to septate the ventricular outflow tract, although the wall between the newly formed subaortic and subpulmonary outlets remains mesenchymal (Fig 8A and asterisk in 8B). The cushions that will form the developing aortic valvar leaflets remain at right angles and proximal to those that will develop in the PT (Fig 8C and 8D). The ventricular tubercles of the atrioventricular endocardial cushions now form the septal margin of the secondary interven-
tricular foramen (Fig 8D). The SS, which separates the right and left venous horns, is in continuity, ventrally, with the midpoint of the RVV (Fig 8F through 8H). Dorsally, the SS is in continuity with the MC of the spina vestibuli. The mesenchyme on the leading edge of the PAS, which is also in continuity with the MC of the spina vestibuli, has fused with the atrioventricular endocardial cushions to constitute a single mesenchymal mass. This mesenchymal mass has closed the OP, dividing the atrioventricular junction (Fig 8F through 8I). The line of fusion between the endocardial cushions is clearly seen within this developing atrioventricular septal area (arrowhead in Fig 8F). The secondary interatrial foramen (OS) is visible between the upper and lower margins of the PAS (Fig 8H), but as yet, there is no formation of the infolded rim of the oval fossa (Fig 8I).

13th Day of Gestation
The prospective pulmonary infundibulum is positioned cranial to the RV, but its posterior aspect has yet to become muscular (Fig 9A and 9B). The inferior margin of the SVC has now fused with the tubercles of the atrioventricular endocardial cushions, forming the primordium of the membranous septum (Fig 9C). The boundaries of the SC and IC can still be distinguished, with the IC draped across the right side of the muscular ventricular septal crest but with the SC forming the bulk of the dorsoinferior margin of the subaortic outflow tract (Fig 9D). The muscular components of the tension apparatus for the mitral valve are beginning to delaminate from the trabeculated portion of the left lateral wall of the LV (shown by arrowhead in Fig 9D). Dorsal to this area, the right and left atrioventricular orifices are separated by the prominent central mesenchymal mass (asterisk in Fig 9E). Within this mass can be traced contributions from the valves of the systemic venous sinus, the SS, the mesenchyme covering the RPR, the mesenchyme on the leading edge of the PAS, and the atrioventricular endocardial cushions (Fig 9E through 9G). The infolding of the cranial atrial wall, which will form the cranial margin of the oval fossa, is now marked (Fig 9E and open arrow in Fig 9F).

14th Day of Gestation
By the 14th day of gestation, the walls of the subpulmonary infundibulum have largely become muscular, but a fibrous raphe is seen at the site of fusion of the outflow ridges (Fig 10A and arrow in Fig 10B). The cushions forming the leaflets of the aortic valve are directly adherent to the muscular crest of the ventricular septum (Fig 10D), being continuous with the developing anterosuperior part of the membranous septum. These structures form the boundaries of the secondary interventricular foramen (Fig 10E), which has now closed. A muscular band (arrowheads in Fig 10F) can be traced through the front of the right atrioventricular junction, continuing behind the AO, and marks the site of the inner heart curve.
The IC, which will form the septal leaflet of the tricuspid valve, is still draped extensively along the right-hand side of the muscular ventricular septum (Fig 10F, 10G [white arrow], and 10H). As yet, however, there is no delamination of the septal leaflet. The dorsal part of the cushion is by now incorporated within the central mesenchymal mass (asterisk in Fig 10H), which forms an extensive atrioventricular septal area. Its atrial component is becoming muscularized as the bulbus anchorage for the flap valve (PAS) of the oval foramen (open arrow in Fig 10I).

15th Day of Gestation

The subaortic segment of the outflow tract has continued to shorten, so that the aortic valve is now in the same plane as the developing tricuspid and mitral valves (Fig 11A through 11D). It is the newly developed muscular subpulmonary infundibulum, which now forms the roof of the RV (Fig 11A). The line of closure of the secondary interventricular foramen is seen at the junction of the aortic root with the crest of the muscular IVS (Fig 11B and arrow in Fig 11C). Within the central mesenchymal mass, it can be seen that certain areas are becoming fibrous, but the larger part of the mass is becoming muscularized to form the inferior margin of the oval fossa. The developing TT (Fig 11F) can be traced from the muscular base of the PAS in this inferior margin backward into the SS (Fig 11G). The RVV retains its continuity with the IC (Fig 11F). Immediately posterior to this area, the mesenchymal mass has become muscularized and incorporates the anterior wall of the mouth of the superior caval vein (Fig 11G and 11H). As yet, there is still no formation of the septal leaflet of the tricuspid valve (Fig 11D). The aortic leaflet of the mitral valve, however, is seen to be derived exclusively from the SC (Fig 11D), with the zone of fusion with the IC forming its hinge from the muscular ventricular septum (Fig 11E). The mural leaflet of the mitral valve has delaminated from the posterior ventricular wall (Fig 11D), although its tension apparatus remains rudimentary.
Separation of the early cardiac tube into the chambers of the formed heart is achieved by a remarkable diversity of mechanisms and by two fundamentally different processes. On the one hand, there is formation of a conventional septum, i.e., a structure that separates adjacent chambers and can be removed without destroying the walls of the heart. The atrioventricular cushions and the muscular PAS are archetypal septa. In contrast, partitioning can also be attained by folding of the heart walls. For example, the upper rim of the oval fossa develops by an infolding of the atrial wall so that, eventually, this contributes to the separation of the cavities of LA and RA. In this case, the parietal walls become converted into an apparent “septal” structure by the process of folding (even though the process of folding incorporates a wedge of extracardiac space and adipose tissue and thus is not a true septum). Interestingly, the counterpart to this process, with an initially septal structure becoming a parietal wall, occurs in the developing outflow tracts. Here, ridges comparable histologically to the atrioventricular endocardial cushions separate the developing subaortic and subpulmonary outflow tracts. Different parts of the ridges form the primordia of the arterial valvar leaflets, and with subsequent development, the tissues between the sites of formation of the arterial valves become converted into the freestanding subpulmonary muscular infundibulum. It might be thought that atrioventricular septation is relatively simple because the endocardial cushions form a classic septum and retain their position throughout development, but nothing could be further from the truth.

The situation is complicated in the developing atrioventricular septal area because multiple developmental primordia are involved in production of the central mesenchymal mass, which, eventually, divides the developing junctions. These include, in addition to the atrioventricular endocardial cushions themselves, the MCs on the PAS and the RPR, the mesodermal core of the RPR (which can be traced back into the somatic mesoderm), and the valves of the embryonic systemic venous sinus. Furthermore, many other steps in development must occur in correct sequence if these components are to reach their appropriate sites within the developing junctions. These include rotation of the horns of the systemic venous sinus around the developing pulmonary portal, the correct expansion of the right atrioventricular junction, the appropriate formation of the muscular atrial and ventricular septal components, the proper division of the outflow tract, and the correct transfer of the AO to the LV. Only when all these concomitant events have occurred harmoniously can the mesenchymal components fuse and develop in appropriate fashion to finalize septation. Division of the normal atrioventricular junctions, therefore, is remark-
ably complex. It follows that any, or all, of these events can go awry so as to lead to inappropriate division, with production of deficient atrioventricular septation. We will discuss the various events in turn.

Rotation of the Systemic Venous Sinus
Within the stages of development we have charted, the systemic venous sinus has already become demarcated clearly by the venous valves. These valves can be likened to the lips of the mouth, opening into the developing RA with two corners. The upper corner is the septum spurium, whereas the lower corner is directly continuous with the IC. The lips themselves represent the RVV and LVV (the sinoatrial valves). Within the systemic venous sinus thus enclosed, there is an apparently septal component, the SS, which is no more than the adjacent walls of its two tributaries, the right and left sinus horns. Initially, in development, the two horns are symmetrical, and the SS is a midline structure relative to the developing atrium. With growth, the horns of the venous sinus rotate on the site of anchorage of the heart tube to the body provided by the developing pulmonary venous portal. Subsequent to this rotation, and concomitant with formation of the PAS, the systemic venous sinus is incorporated exclusively into the right side of the developing RA. Correct rotation of the systemic venous sinus, therefore, is an essential prerequisite for septation of the atrial component of the heart tube and also for formation of the atrioventricular septum. The TT runs through the cranial part of the RVV, continues through the SS (between the coronary sinus and the orifices of the right superior and right inferior caval veins), and terminates within the spina vestibuli. It is the fusion of the MC on the spina vestibuli with the atrioventricular endocardial cushions that incorporates the TT and the inferior margins of the venous valves within the central mesenchymal mass, which provides the scaffold of the atrioventricular septum.

During the same process, the cranial muscular wall of the left sinus horn, forming the mouth of the coronary sinus, is also incorporated within the atrioventricular area. The development of the venous valves as seen in the mouse, therefore, is directly comparable to that described in man.

“Expansion” of the Right Atrioventricular Junction
For appropriate division of the atrioventricular junctions, it is also necessary for the right atrioventricular orifice to attain an exclusive connection to the RV. In the mouse, on the 10th day of gestation, the atrioventricular canal is situated to the left of the midline, with the atrioventricular endocardial cushions dominating the lumen. Situated to either side of the endocardial cushions are two parallel slitlike channels. These form an H shape, described by Tandler as two T shapes placed base to base.

So, how is exclusive connection of the right atrioventricular orifice to the RV achieved when the atrioventricular canal is initially an exclusive left-sided structure in the tubular heart? Of crucial importance is the position of the crest of the developing muscular ventricular septum relative to the RCH. During this period of development, the heart tube is progressively remodeled. The growth of the RV, with concomitant remodeling of the inner heart curvature, has the effect, when viewed externally, of moving the outflow tract leftward, bringing it to a more ventral position with respect to the atrioventricular canal. The remodeling of this area also brings the RCH of the atrioventricular canal to the right of the crest of the developing muscular ventricular septum—the so-called “expansion” of the right atrioventricular junction. The flow of blood through this RCH, therefore, enters the

Figure 9. Frontal sections of a 13-day embryo. The arrowhead in panel D marks the delamination from the trabeculated portion of the left lateral wall of the LV, forming the tension apparatus for the mitral valve. The asterisk in panel E indicates the prominent central mesenchymal mass separating the right and left atrioventricular orifices. The open arrow in panel F shows the infolding of the cranial atrial wall, which will form the upper margin of the oval fossa. In panel H, the directional arrows are as follows: S, superior; I, inferior; R, right; and L, left. Bar=200 μm.
ventricle to the right of the crest of the ventricular septum. It is this process that establishes the flow pattern necessary for normal septation to proceed, occurring concomitant with the expansion of the RV and producing a clockwise rotation (looking downstream) of the outflow tract. At the same time, the right atrioventricular channel is bridged by the continuity of the dextrodorsal outflow ridge with the SC, and the LCH maintains its dorsoventral orientation. The fusion of the tubercles of the atrioventricular cushions with the proximal ends of the outflow ridges forms the mesenchymal borders of the secondary interventricular foramen, which is to the right of, and at a slightly oblique angle to, the crest of the ventricular septum. The progressive closure of the secondary interventricular foramen then confines the developing tricuspid valvar orifice to the RV and confines the AO to the LV. Subsequent muscularization of the outflow ridges forms the supraventricular crest of the RV and the margins of the freestanding subpulmonary infundibulum.

Alignment of the Ventricular and Atrial Septal Structures

The expansion of the right atrioventricular junction, with concomitant remodeling of the inner heart curvature, brings the muscular ventricular septum to a position where it can partition the ventricular aspect of the atrioventricular junction. The same process also brings the ventricular septum into alignment with the developing PAS. This alignment, when viewed ventrally, is to the right-hand side of the atrioventricular canal but to the left of the developing tricuspid orifice. This configuration leaves the bulk of the SC to the left of the ventricular septum. It is then this SC that contributes predominantly to the aortic leaflet of the mitral valve, with the IC providing its hinge from the crest of the muscular ventricular septum. The remainder of the IC is draped obliquely across the ventricular septal crest. As the muscular ventricular septum grows, the bulk of the IC becomes associated with the right side of the septum. This tissue will, in time, provide the anlagen of the septal leaflet of the tricuspid valve, but this does not delaminate until after term in the mouse heart.

Atrial Septation

The first sign of atrial septation is the growth of its primary component from the roof of the atrial component of the heart tube. Many accounts of this initial development describe the primordium as sickle-shaped. In the mouse, as seen in our material, the ridge that forms the PAS has a straight edge, capped by a prominent mesenchymal mass. This configuration, seen relative to the endocardial cushions, produces a triangular OP, as described by Dalgleish. The primary foramen is closed ventrally by fusion of the atrial extension of the SC with the mesenchyme on the leading edge of the PAS. More caudally, the IC, also bordering the foramen, extends into the floor of the developing RA, fusing with the mesenchyme covering the spina

Figure 10. Frontal sections of a 14-day embryo. In panel A, the directional arrows are as follows: S, superior; I, inferior; R, right; and L, left. The arrow in panel B shows the fibrous raphe at the site of fusion of the outflow ridges, in the wall of the subpulmonary infundibulum, which is otherwise muscular. The arrowheads in panel F indicate a muscular band that runs through the front of the right atrioventricular junction, continuing behind the AO. The white arrow in panel G marks the IC, which will form the septal leaflet of the tricuspid valve, draped along the right-hand side of the muscular ventricular septum. The asterisk in panel H marks the central mesenchymal mass. The open arrow in panel I indicates the flap valve (PAS) of the oval foramen. Bar=200 μm.
vestibuli. This, in turn, is in continuity more cranially with the mesenchyme on the leading edge of the PAS (Fig 12). The pattern of fusion, therefore, is analogous to a process of “zippering,” with fusion first occurring at the caudal and cranial margins of the foramen and proceeding toward a hypothetical point in its center. In this manner, the OP is gradually closed while maintaining its triangular shape. It has been said that closure of the OP takes place before the fusion of the endocardial cushions.20–22 The precise timing of closure is difficult to establish, but our findings indicate that fusion of the atrioventricular cushions themselves certainly plays a part in that closure.

The origin of the mesenchyme on the leading edge of the PAS is contentious. Many authors23–26 equate this tissue with the spina vestibuli of His.9 Puerta Fonollá and Orts Llorca24 support the account of López Rodriguez,23 whom they translate thus: “two areas are always distinguished: one membranous dorsal and the other ventral having an appearance of a clubbing which conserves a structure similar to that of the endocardial cushions and which His named spina vestibuli. The spina vestibuli has the appearance, taken as a whole, of a half-moon, whose concavity forms the free edge of the septum primum, limiting the foramen subseptale dorsally.” They argue that the OP is closed by the fusion of the atrioventricular endocardial cushions with the septum primum but state that this is via the substance of the spina vestibuli. Unequivocally, therefore, they equate the spina vestibuli described by His9 with the entirety of the MC that covers the leading edge of the PAS. This interpretation is in conflict not only with our own observations, but also with His’s illustrations of the spina vestibuli (reproduced in Fig 13). His’s drawings show that it was the elevation on the RPR that was designated as the spina vestibuli. More recently, it seems to be the interpretation of López Rodriguez that has become perpetuated through the literature, being accepted, for example, by Asami and Koizumi25 and by Markwald et al.26

Although the interpretations of Puerta Fonollá and Orts Llorca24 concerning the origin of the mesenchyme on the crest of the PAS are at variance with our own, their data are not. Their serial sections of a stage 13 human embryo show continuity of the RPR with the mediastinal mesoderm of the body stalk. More cranially, in the area of the PAS (which will form the muscular flap valve), free space is seen between it and the dorsal wall of the atrium. This is in concordance with our present findings in...
the mouse. The situation is also comparable in the rat. Igarashi, in a study using scanning electron microscopy, reported that “a small circular lump developed where the dorsal limb of the septum primum connected to the left venous valve” on the 12th embryonic day in the rat. This projection was appropriately described as being in the same location as the spina vestibuli of His. Twelve hours later, it had been absorbed into the dorsal limb of the PAS. We have confirmed that the tissue is, indeed, incorporated within the central mesenchymal mass and contributes to the inferior border of the definitive oval fossa.

What, Then, Is the Spina Vestibuli?
Two of His’s original diagrams (Fig 13, reproduced from Reference 9) clearly show the spina vestibuli as an elevated projection caudal to, and to the right of, the developing PAS (the septum superius). His described the right side of the spine as merging with the eustachian valve (the RVV) and also building the floor of the atrium over the saccus reuniens (a term that has no modern equivalent). The tissue derived from the spina vestibuli was described as forming the cranial margin of the coronary sinus as it empties into the RA. Our findings endorse totally these observations. In contrast, Markwald et al describe the spina vestibuli as a wedge of mesenchyme that extends between the LBs and the two horns of the sinus venosus. They postulate that this mesenchyme then penetrates the posterior atrial wall and migrates to contact the fusing atrioventricular cushions. They seem to be correlating, as one and the same structure, the MC on the primary septum and the mediastinal mesoderm that enters the wall of the atrium through the RPR. In our opinion, it is not helpful to broaden the definition of the spina vestibuli in this fashion. It is the RPR that increases in size and is equated with the elevation that His described as the spina vestibuli. This structure plays a pivotal role not only in atrial septation but also in the formation of the atrioventricular septum. It contains a core of extracardiac tissue that can be traced back into the mediastinal mesoderm in the area ventral to the foregut. These extracardiac mesodermal cells that become incorporated into the heart have a markedly different appearance from both the mesenchyme on the crest of the PAS and the mesenchyme of the atrioventricular endocardial cushions. The elevation on the right ridge, nonetheless, also has its own MC, separate from its “core” of extracardiac mesoderm (Fig 12). The cap may well result from a local epithelial-mesenchymal transformation. We have found no morphological evidence that the core of the spine, continuous with the extracardiac somatic mesoderm, contributes to the luminal mesenchyme on the leading edge of the atrial septum. This septal mesenchyme, as we have stressed, becomes evident concomitant with the formation of the PAS, possibly also as a result of local epithelial-mesenchymal transformation. Thus, the temporal sequence of development suggests that the mesenchyme on the leading edge of the PAS arises in much the same manner as do the atrioventricular endocardial cushions within the atrioventricular canal. This interpretation is supported by the findings of Arrechedera et al, who showed that the endocardial cells covering the PAS in the chick behaved as did those of the endocardial cushions when the latter transformed into mesenchyme, and, more recently, by the study of Gerety and Watanabe. In addition, the homeobox gene Msx-1, which is expressed in atrioventricular endothelium at the time it undergoes an epithelial-mesenchymal transformation, is also expressed in the developing PAS.

Formation of the Secondary Atrial Septum
We have already discussed formation of the PAS and its MC. It is well accepted that the primary septum itself breaks down at its site of origin from the atrial roof to form the secondary interatrial foramen. Our findings support this classical interpretation. The remnant of the primary septum then forms the flap of the oval fossa, with the base of the primary septum being incorporated into the atrioventricular septal area. There is some disagreement regarding the origin of the rim of the oval fossa. Most current textbooks of cardiac embryology show all parts of the rim, which is usually described as the “septum secundum,” as originating from a single sickle-shaped muscular sheet, which is shown forming in much the same manner as the primary septum, and to its right side. In contrast, we have shown that there are two distinct origins for the rim. The upper margin of the oval foramen is formed by an infolding of the muscular atrial walls between the orifices of the superior caval and right PVs. This is completely different from the formation of the lower margin, where the expanded base of the flap valve of the oval fossa is made from several structures, including the spina vestibuli and the venous valves. Morrill described this component as a spurlike thickening that projected into the cavity of the RA between the floor and the PAS and that also had continuity with the LVV: “It appears to be nothing more than a projection, towards the right, of the thickening of septum I and is continuous through the latter with the endocardial cushions of the atrial canal. This structure which is the anlage of septum II, is undoubtedly what His called the spina vestibuli and, taken together with the endocardial cushions, would constitute his septum intermedium.” Although the anatomic description by Morrill is admirably accurate, calling the structure “the anlage of septum II” has probably contributed to the misconception that the secondary septum has a single origin.

Formation of the Atrioventricular Septal Area
The present study has shown that the spina vestibuli, as described by His, contributes not only to the lower border of the oval foramen in the mouse but also to the central fibrous body of the heart. As such, it is but one of several structures that make up the central mesenchymal mass (the so-called septum intermedium). This tissue, after delamination of the septal leaflet of the tricuspid valve, becomes the atrioventricular component of the membranous septum. Although, without specific tissue markers, it is difficult to distinguish the individual mesenchymal components after fusion, the intense staining patterns seen at early stages enable the individual components to be traced. The ventral elevation of the spina vestibuli inserts between, and fuses with, the atrial extensions of the atrioventricular endocardial cushions. Cranially, the MC of the spina vestibuli has continuity with the mesenchyme on the leading edge of the PAS, which, in fusing with the atrial extension of the SC, inserts the “drumstick” of the muscular portion of the PAS into the central mesenchymal mass. The fused structures, having combined to form the central mesenchymal mass, then become the major part of the fibrous skeleton of the heart. There is also an area posterior to the fibrous septum, however, which occupies an atrioventricular location.
This is the area between the offset hinges of the tricuspid and mitral valves to either side of the ventricular septum. In the human, this area is described as the muscular atrioventricular septum. We have pointed out that in the mouse, the area is reduced in size because of the persistence of the LSCV, which drains into the RA through the coronary sinus. It consists of a fibrofatty tissue plane that extends anterosuperiorly from the inferior atrioventricular groove and interposes between the atrial walls and the crest of the muscular ventricular septum, forming an atrioventricular muscular sandwich. We have been unable to clarify the formation of this area in our current material because, in the mouse, the septal leaflet of the tricuspid valve has still to be delaminated at the conclusion of gestation. Further studies are required to elucidate this process as well as to clarify the role of formation of the atrioventricular groove.

Conclusions

The formation of the atrioventricular septal area is remarkably complex. The tissues and processes that permit successful septation are manifold. It is simplistic to imagine that this reflects only appropriate fusion of the atrioventricular endocardial cushions. Similarly, it is equally simplistic to believe that failure of fusion of these cushions can explain all the morphological problems resulting from deficient atrioventricular septation as it occurs in the setting of a common atrioventricular junction. One of the purposes of this investigation was to provide insight into the processes of deficient septation, particularly in the mouse with trisomy 16, thought to be a model of humans with Down’s syndrome. Studies in progress show that the processes are just as complicated in the abnormal as in the normal heart. It is only by paying attention to all the developmental processes presently identified that we will solve the mechanisms of abnormal atrioventricular septation.

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