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A Spatiotemporal Evaluation of the Contribution of the Dorsal Mesenchymal Protrusion to Cardiac Development

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Abstract

The mesenchymal tissues involved in cardiac septation are derived from different sources. In addition to endocardial-derived mesenchyme, the heart also receives contributions from the neural crest, the proepicardium, and the dorsal mesenchymal protrusion (DMP). Whereas the contributions of the neural crest and proepicardium have been thoroughly studied, the DMP has received little attention. Here, we present the results of a comprehensive spatiotemporal study of the DMP in cardiac development. Using the Tie2-Cre mouse, immunohistochemistry, and AMIRA reconstructions, we show that the DMP, in combination with the mesenchymal cap on the primary atrial septum, fuse with the major atrioventricular cushions to close the primary atrial foramen and to form the atrioventricular mesenchymal complex. In this complex, the DMP constitutes a discrete prominent mesenchymal component, wedged in between the major cushions. This new model for atrioventricular septation may provide novel insights into understanding the etiology of congenital cardiac malformations.

Keywords

septation; atrial septum; atrioventricular cushions; muscularization; dorsal mesocardium; spina vestibuli

INTRODUCTION

Congenital heart defects (CHD) are the most common birth defects and are present in nearly 1% of all newborns (Eisenberg and Markwald, 1995; Hoffman and Kaplan, 2002). With a majority of these defects involving abnormalities in valve and septal formation (Clark, 1987; Pierpont et al., 2000), great attention has been focused upon understanding the developmental processes of valvuloseptal morphogenesis (Odgers, 1939; Wenink and

Gittenberger-de Groot, 1985; Lamers et al., 1995; Webb et al., 1998a; de Lange et al., 2004). An early event that appears to be critical in these events is the mesenchymalization of the endocardial cushions (Markwald et al., 1977). During this process, the cushions are populated by mesenchyme derived from the endocardium, via an epithelial-to-mesenchymal transformation (EMT) (Markwald et al., 1975; Runyan and Markwald, 1983; Gerety and Watanabe, 1997), as well as by cells derived from non-endocardial sources, including neural crest- and epicardial-derived cells (Gittenberger-de Groot et al., 1998; Waldo et al., 1999; Perez-Pomares et al., 2002; Nakamura et al., 2006). In addition, extracardiac mesenchyme associated with the dorsal mesocardium, contributes to the mesenchymal cell-mass at the venous pole of the heart. Historically, this mesenchyme has been referred to with terms such as spina vestibuli (or vestibular spine) (Tasaka et al., 1996; Anderson et al., 1999; Kim et al., 2001; Mommersteeg et al., 2006; His, 1880), endocardial proliferation of the dorsal mesocardium (Chang, 1931), sinus venosus tissue (Van Praagh and Corsini, 1969), and mesenchymatous tissue (van Gils, 1981). Given the histological characteristics of the mesenchyme, i.e., it is a mesenchymal tissue that protrudes into the dorsal aspect of the atrial cavity, we have dubbed it the “dorsal mesenchymal protrusion (DMP),” as this more accurately describes its specific features relevant to the development and architecture of the heart (Wessels et al., 2000).

In the course of development, the DMP is continuous with the mesenchymal cap on the leading edge of the primary atrial septum (Webb et al., 1998b; Anderson et al., 1999; Wessels et al., 2000). While it is generally accepted that normal AV septation includes the complete fusion of the DMP and mesenchymal cap with the superior and inferior AV cushions, resulting in the formation of the AV mesenchymal complex and the closure of the primary atrial foramen (Webb et al., 1998a; Anderson et al., 1999), the origin of the DMP and the extent of its contribution to the AV mesenchymal complex have been controversial (Tasaka et al., 1996; Webb et al., 1998b; Wessels et al., 2000; Kim et al., 2001). Studies of the DMP in heart development have previously relied upon anatomical relationships and histological analyses of the DMP and surrounding tissues (Webb et al., 1998a,b; Anderson et al., 1999; Wessels et al., 2000; Kim et al., 2001; His, 1880). The importance of understanding the origin and fate of the DMP is underscored by indications of a potential role for the DMP in atrioventricular septal defect (AVSD) pathology (Tasaka et al., 1996; Webb et al., 1999; Blom et al., 2003). The lack of specific markers and/or genetically modified mouse models that would allow the discrimination of the DMP from other mesenchymal tissues has long limited the analysis of development and fate of this mesenchymal population. Recently, it was shown that by using the endocardial fate mapping Tie2-Cre/ ROSA26RlacZ mouse system, the murine DMP could be identified as a non-endocardial-derived population of mesenchyme during early development of the primary atrial septum (Mommersteeg et al., 2006). However, the fate of the DMP, after fusion of the AV cushions, still remains poorly understood. As defects in the fusion of the AV cushions and formation of the AV mesenchymal complex have been associated with AVSDs, we sought to understand the relationship of the DMP to the fused mesenchymal tissues in the AV mesenchymal complex. Utilizing the same Tie2-Cre/ROSA26RlacZ mouse system to discriminate the DMP from endocardial derived mesenchyme, in combination with 3-dimensional reconstruction techniques, we performed a comprehensive spatiotemporal

analysis, determining the contribution of the DMP to the AV mesenchymal tissues before and after fusion of the major AV cushions (embryonic day (ED)10.5–16). Our reconstructions show that, during development, the DMP assumes a wedged position in the posterior aspect of the fusing superior and inferior AV cushions. Hence, we conclude that the DMP plays a hitherto unrecognized, but significant, role in the formation of the mature AV mesenchymal complex.

RESULTS

Mating of Tie2-Cre and ROSA26 lacZ reporter mice generated embryos in which cells derived from the endothelial lineage constitutively expressed lacZ. Staining for lacZ in ED10.5 embryos identified the endocardial-derived cells (ENDCs) in the AV cushion mesenchyme (Fig. 1A). At this stage, a noticeable cluster of lacZ-positive mesenchyme is also seen on the leading edge of the primary atrial septum (Fig. 1A,B). This tissue represents the mesenchymal cap, previously described in the mouse and human (Webb et al., 1998b; Anderson et al., 1999; Wessels et al., 2000). This mesenchymal cap extends caudally to the point of continuity between the developing primary atrial septum (PAS) and the right and left pulmonary ridges (Fig. 1C–E). Continuing further caudally, a lacZ-negative protrusion of mesenchyme is observed on the right pulmonary ridge and is continuous with the dorsal mesocardium and the pulmonary mesenchyme surrounding the developing lung buds (Fig. 1D–F). This intracardiac, Tie2-Cre-negative, mesenchyme is the dorsal mesenchymal protrusion (DMP). The pulmonary vein can be seen developing within this mesenchyme as previously described (Webb et al., 1998b) (Fig. 1E,F).

At ED11.5, the major AV cushions are approaching each other, but have not yet fused (Fig. 2A). The lacZ-negative DMP is found at the base of the PAS (Fig. 2B). On other sections, the DMP can be distinguished as the mesenchymal population juxtaposed to, and continuous with, the inferior AV cushion, while at the same time being continuous with the mesenchymal cap on the PAS (Fig. 2C,D). Stained sections of the heart closer to the AV junction show that the mesenchymal cap is also contiguous with the superior AV cushion (Fig. 2E,F). Thus, the inferior AV cushion, DMP, mesenchymal cap of the PAS, and the superior AV cushion are in complete mesenchymal continuity even though the major cushions have not yet fused. In addition, it is interesting to note that while, just like the other above-mentioned tissues, the DMP is endocardially-lined, virtually no blue cells (i.e., ENDCs) are found within its mesenchymal cell population (Fig. 2F).

Based on the serial sections shown in Figure 2, an AMIRA 3D reconstruction analysis was performed to gain further insight into the spatial relationships between the different mesenchymal tissues (Fig. 3). A superior view of the heart at ED11.5 (Fig. 3A,B) shows the DMP as an extension of the mediastinal mesenchyme protruding ventrally into the dorsal wall of the atrial chamber and the dorsal aspect of the primary atrial septum (Fig. 3B). This particular orientation also clearly demonstrates the continuity of the DMP with the mesenchymal cap on the PAS, the cap itself extending further ventrally along the inferior aspect of the primary atrial septum and merging with the superior AV cushion. From the inferior view, our 3D model illustrates the non-fused superior and inferior AV cushions (Fig. 3C,D). The DMP is connected to both the inferior AV cushion and the mesenchymal cap,

bridging the space between the endocardial-derived tissues (Fig. 3C,D). The spatial relationships between the respective AV mesenchymal tissues are further illustrated in Figure 3E–F. In Figure 3E, the mesenchymal tissues (and part of the PAS) are viewed from the left lateral side showing how the DMP and mesenchymal cap together form an arch over the primary atrial foramen. Figure 3F is a postero-superior view of this mesenchymal bridge (with the PAS removed).

By ED13, the mesenchymal tissues of the AV complex (i.e., the major AV cushions, the mesenchymal cap, and the DMP) have fused, resulting in closure of the primary atrial foramen and the formation of a separate left and right AV communication (Fig. 4A). Within the fused mesenchymal tissues, the DMP constitutes a sub-population of lacZ-negative, non-endocardial-derived, mesenchymal cells wedged between the dorsal aspect of the superior and inferior AV cushions (Fig. 4B–D). Within this region of the DMP, but not in the populations of ENDCs, muscularization is observed (Fig. 4E). As previous studies had reported increased numbers of apoptotic cells within our region of interest (Abdelwahid et al., 2001, 2002; Cheng et al., 2002), we set out to determine where programmed cell death occurred with respect to the DMP and ENDCs. TUNNEL labeling identified a discrete region of apoptosis at the interface between the DMP and the inferior AV cushion. In addition, a trail of apoptotic cells was seen within the body of the DMP (Fig. 4F).

Based on serially sectioned embryos at ED13, AMIRA 3D models were generated to determine the relative contribution of endocardial- and non-endocardial-derived tissues to the AV mesenchymal complex (Fig. 5). In the histological sections, the (fused) superior and inferior AV cushions could be distinguished by contour (Fig. 5A,B). However, as the mesenchymal cap on the PAS has, at this stage, now completely fused with AV cushion mesenchyme, the cap cannot be identified as a separate entity anymore, and, hence, in the reconstructions is incorporated into the cushion tissues. A posterosuperior view of the mesenchymal AV tissues (Fig. 5C; orientation comparable to Fig. 3F) illustrates the relationship of the DMP to the endocardial-derived major (superior and inferior) and left and right lateral AV cushions. Combined, Figure 5C and 5D–F (a right lateral view) clearly demonstrate the central wedged position of the non-endocardial-derived DMP within the AV mesenchymal complex.

From ED15 onward, the individual mesenchymal tissues cannot be distinguished using standard histological methods anymore. However, the Tie2-Cre/ROSA26RlacZ mouse system still allows the recognition of the DMP as a population of lacZ-negative mesenchyme, continuous with the endocardial-derived, lacZ-positive mesenchyme of the major AV cushions. At ED15, the DMP is situated near the attachment of the right and left venous valves (Fig. 6A,C). As in the previous stage, muscularization is observed within the DMP (Fig. 6B,D). At later stages, virtually the entire region of the DMP is muscularized (data not shown).

DISCUSSION

Significant strides in our understanding of the DMP, and its potential importance to heart development, were made in studies utilizing techniques such as standard histology,

immunohistochemistry, and electron microscopy (Tasaka et al., 1996; Webb et al., 1998b; Anderson et al., 1999; Wessels et al., 2000; Kim et al., 2001). These studies revealed important associations between the development of the DMP and the atrial and AV septal structures (Tasaka et al., 1996; Webb et al., 1998b; Anderson et al., 1999; Wessels et al., 2000; Kim et al., 2001). The importance of the DMP was further emphasized by work linking abnormal DMP development to AV septal defects in human cases and animal models of Down Syndrome (Tasaka et al., 1996; Webb et al., 1999; Blom et al., 2003). Due to the intimate relationship of the DMP with the endocardial-derived cushion tissues, determining the extent of the DMP in relation to these tissues has been a subject of much disagreement (Tasaka et al., 1996; Webb et al., 1998b; Wessels et al., 2000; Kim et al., 2001). Thus, studies to delineate the DMP have long been hampered by the absence of molecular tools to mark the DMP specifically and by the fact that experimental cell-fate tracing techniques, such as those used to study the contribution of the neural crest (Poelmann et al., 1998; Waldo et al., 1999) and the epicardium (Gittenberger-de Groot et al., 1998; Perez-Pomares et al., 2002) to heart development, are not (yet) feasible for this particular structure.

The advent of Crelox technology has, however, made available genetic tools to study this topic in more detail. Recently, our colleagues in Amsterdam used the Tie2-Cre and Rosa26 reporter mice to identify the DMP as a non-endocardial-derived population of mesenchyme (Mommersteeg et al., 2006), thereby supporting the earlier observations made in the human heart (Wessels et al., 2000). This clarified an important contention as to the DMP's origin, and to its previous morphological description as an atrial spine (Mommersteeg et al., 2006). We utilized the same genetic tools to ascertain the cellular contribution of the DMP to the AV mesenchymal tissues throughout AV septal development.

DMP Contribution to Non-Fused AV Cushion Mesenchyme

At earlier stages of development (ED10.5–ED11.5), our results confirmed previous findings that the DMP is a non-endocardial-derived population of mesenchyme closely associated with the developing AV endocardial cushions. As shown in our reconstructions (Fig. 3), the DMP bridges a connection between the endocardial-derived inferior AV cushion and mesenchymal cap. Several studies have suggested an important role for the DMP in atrial septation and closure of the primary atrial foramen (Tasaka et al., 1996; Webb et al., 1998a, 1999; Anderson et al., 1999; Blom et al., 2003). This is supported by our reconstructions, which illustrate the DMP as a principal mesenchymal component making up the base of the primary atrial septum and being contiguous with the endocardial-derived mesenchymal cap. The roles that these two different populations of mesenchyme play in atrial septation are not well understood and are under investigation.

It was noted previously, and confirmed in our studies, that the DMP, just like all other AV mesenchymal tissues, is enclosed by the endocardial lining of the heart (Mommersteeg et al., 2006). The DMP, however, is the only mesenchymal tissue in this region devoid of endocardial-derived cells. In fact, without the presence of a physical boundary, endocardial- and non-endocardial-derived cell populations are found side by side underneath the Tie-2-cre marked, endocardial lining. This generates intriguing questions. Specifically, if the DMP

mesenchyme is not generated by endocardial EMT, where do these mesenchymal cells come from and how is the process of EMT restricted to specific endocardial populations in this segment of the heart? Additional experimentation, including the development of other cell-fate tracing approaches, comparative analysis of known EMT-related gene expression patterns within this region, and the identification of DMP-specific gene products (e.g., through microarray analysis), may provide further insight into the development of the mesenchymal tissues in this area.

DMP Contribution to AV Mesenchymal Complex

Fusion of the mesenchymal components of the AV junction to form the AV mesenchymal complex is an important step in closure of the primary atrial foramen and proper AV septation (Webb et al., 1998a; Anderson et al., 1999). The significance of the extracardiac DMP to this process has been suggested by studies on human Down Syndrome fetuses and Trisomy 16 mice. These studies reported associations between abnormal accumulation of DMP mesenchyme and AV septal defects (Tasaka et al., 1996; Webb et al., 1999; Blom et al., 2003). To clarify the role of the DMP in animal models of congenital heart defects (CHD), the contribution of the DMP to the fused mesenchymal complex needs to be well defined. Using the tools of Tie2-cre lineage tracing and 3D reconstruction, we defined the spatial boundaries of the DMP before and after major AV cushion fusion and determined the relative contributions of the individual mesenchymal components of the AV complex. Our results show that the DMP makes an important contribution to the fusing major AV cushions, forming a mesenchymal wedge between the dorsal aspect of the superior and inferior AV cushions.

To specifically determine the involvement of the DMP in the etiology of cardiac defects in models for CHD, it is feasible to introduce the Tie2-Cre and ROSA26RlacZ constructs into the mouse model of choice (e.g., Ts16, TGF beta2 k.o. mouse). However, given the complexity of this approach, it would be desirable to obtain DMP-specific markers that would allow the delineation of the DMP in these experimental settings.

Muscularization in the DMP

Interestingly, at ED 13 we found multiple foci of muscularization in the DMP, but not in the endocardial-derived mesenchyme, confirming earlier reports that the mesenchyme in the venous pole of the heart is an active site of muscularization (Kim et al., 2001; Kruithof et al., 2003; Christoffels et al., 2006; Mommersteeg et al., 2006). Importantly, we did not find myocardial gene expression in ENDCs, which confirms previous findings that indicate that ENDCs do not transdifferentiate into cardiomyocytes in vivo (de Lange et al., 2004). Thus, a growing body of evidence suggests that mesenchymal-to-myocardial transdifferentiation in the venous pole of the heart is restricted to the mesenchyme derived from the DMP. Interestingly, previously we have demonstrated that, when cultured in vitro, AV cushion mesenchyme can differentiate into myocardium (van den Hoff et al., 2001; Kruithof et al., 2003). At the time, this led us to postulate that this was an in vitro phenomenon. In hindsight, however, it is possible that co-isolated DMP mesenchyme was responsible for these observations. Notwithstanding, there is convincing evidence that ENDCs play a role in the regulation of muscularization. For example, knock-out studies of ENDC-expressed

genes (e.g., NF1, TG-Fbeta2) are characterized by aberrant muscularization phenotypes (Sanford et al., 1997; Lakkis and Epstein, 1998; Bartram et al., 2001; Molin et al., 2003), including defects in muscularization at the base of the atrial septum.

Apoptosis at the DMP/Cushion Interface

As areas of apoptosis within the general area of the dorsal mesocardium and the AV cushions had been reported previously (Poelmann and Gittenberger-de Groot, 1999; Abdelwahid et al., 2001, 2002; Cheng et al., 2002), we set out to determine the exact location of the apoptotic cells within the defined region of the AV mesenchymal complex as delineated by our histological and genetic markers. While a few apoptotic cells were found in the DMP, the majority of apoptotic cells were found at the interface between the DMP and the inferior AV cushion, specifically within the lacZ-positive ENDC population. We cannot exclude the possibility that (some of) the TUNNEL-stained cells are non-endocardial-derived cells that are intermingled with ENDCs. The significance of this specific region of apoptosis is unclear. Earlier studies have suggested the possibility that apoptosis might occur at tissue junctions in response to mechanical stress generated by the physical interactions of adjoining cells (Cheng et al., 2002). Alternatively, the process of apoptosis might play a role in setting boundaries to limit migration/intermingling of the respective cell populations. While these explanations seem plausible, we do not see the same distinct region of apoptosis at the interface between the DMP and the superior AV cushion (data not shown).

In addition to the apoptosis at the DMP/inferior AV cushion interface, there was also a trail of apoptotic cells from the dorsal mesocardium to the DMP mesenchyme (Fig. 4F). Studies in the avian system have demonstrated that some neural crest-derived cells that migrate into the dorsal mesocardium eventually undergo apoptosis (Poelmann and Gittenberger-de Groot, 1999). Furthermore, cell-fate tracing studies using the Wnt1-Cre mouse (de Lange et al., 2004; Nakamura et al., 2006) showed some neural crest-derived cells migrating through the dorsal mesocardium in the mouse. Importantly, these studies have ruled out a significant material contribution of the neural crest to the DMP. We cannot exclude, however, the possibility that they may indeed represent a subpopulation of neural crest cells in the dorsal mesocardium.

In conclusion, we have demonstrated that the DMP constitutes a prominent mesenchymal component in the AV mesenchymal complex and we have shown that, during development, the DMP, in combination with the mesenchymal cap of the primary atrial septum, fuse with the major AV cushions to close the primary atrial foramen and to form the AV mesenchymal complex. We believe that this new insight into the mechanism of AV septation could provide novel ideas related to the etiology of congenital cardiac malformations in the venous pole of the heart that can be investigated using the genetic tools now available and new techniques to be developed in the future.

EXPERIMENTAL PROCEDURES

Mice

The Tie2-Cre (C57Bl/6) and TgR(ROSA26)26SOR mice have been described previously (Soriano, 1999; Kisanuki et al., 2001). Isolation of embryonic tissues and assessment of developmental stages expressed in embryonic days were performed following established protocols (Waller and Wessels, 2000).

LacZ Staining and Immunohistochemistry

Whole embryos were fixed on ice in 4% paraformaldehyde for 40 min (ED10–12) or removed hearts for 1 hr (ED13–adult). Specimens were washed in a 0.02% sodium deoxycholate, 0.01% NP-40/ PBS solution overnight at 4°C. Washed specimens were stained with Xgal solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, and 1 mg/ml X-gal (5-bromo-4-chloro-3-indoyl β -D-galactopyranoside) at 37°C for 2–3 days. Stained specimens were embedded in paraffin and sectioned at 5 μ m. Immunohistochemistry was performed on 5- μ m thick sections as previously described (Waller and Wessels, 2000). A monoclonal antibody against Sarcomeric Actin (Sigma, A2172) was used to delineate myocardial tissues. TUNEL staining using ApopTag peroxidase in situ detection kit (Chemicon International) was performed on 5- μ m sections according to protocol.

Three-Dimensional Reconstruction

Two 3-dimensional models of the AV junction were generated using AMIRA software (v3.1.1; TGS Template Graphics Software, Inc.). Serial sections (5 μ m) were photographed using a Polaroid DMC1 camera at 10 $\times$ magnification (1,600 $\times$ 1,200 pixels at 128 pixels/100 μ m) producing 56 (ED 11.5) and 64 (ED13) slices. Slices were aligned in AMIRA using the sum of least squares alignment algorithm. Additional alignment corrections were made visually using the contours of the lacZ-positive mesenchyme as landmarks. Segmented volumes were smoothed using a 3D Gaussian filter mask of 3–5 voxels for 5–10 iterations. Surface generation within AMIRA was accomplished using the Generalized Marching Cube algorithm.

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Fig. 1.

The atrioventricular mesenchymal tissues at ED 10.5. Shown are transverse sections of a lacZ-stained Tie2-Cre/R26R embryo at ED 10.5 that are immunostained for sarcomeric actin. **A:** Low magnification of a section through the AV junction. **B:** The boxed area in A is shown at a higher magnification. These panels demonstrate the lacZ staining of endocardial and endocardial-derived cells in the endocardial derived mesenchymal cap on the primary atrial septum and the endocardial derived superior AV cushion. **C:** The mesenchymal cap continues caudally to the left and right pulmonary ridges. **D–F:** The dorsal mesenchymal protrusion is seen as a continuation of the mesenchyme of the dorsal mesocardium, its mesenchymal cells being characterized by the absence of lacZ staining. **E,F:** F is higher magnification of boxed area in E. E and F demonstrate how the pulmonary vein develops at the left lateral margin of the DMP, adjacent to the myocardium of the left pulmonary ridge. Note that the endocardium lining the DMP, as well as the endothelium lining of the primitive pulmonary vein, are lacZ positive. CAP, mesenchymal cap; DM, dorsal mesocardium; DMP, dorsal mesenchymal protrusion; iAVC, inferior AV cushion; LA, left atrium; LPR, left pulmonary ridge; LV, left ventricle; PAS, primary atrial septum; PIT, pulmonary pit; PM, pulmonary mesenchyme; PuV, pulmonary vein; RA, right atrium; RPR, right pulmonary ridge; RV, right ventricle; sAVC, superior AV cushion.

Fig. 2.

The dorsal mesenchymal protrusion at ED 11.5. The frontal sections of a Tie2-Cre/R26R embryo at ED 11.5 are shown. **A:** An eosin-counterstained section. **B–F:** The serial sections are immunolabeled for sarcomeric actin to allow delineation of the myocardial tissues of the heart. The sections shown in A and B serve to demonstrate the arrangement of the atrial structures. A specifically demonstrates the lacZ-positive staining of endocardial-derived cells populating the superior and inferior AV cushions. **B:** The primary atrial septum in relation to the DMP, and the relationship of the dorsal mesenchymal protrusion to the dorsal mesocardium (proper) and the non-stained mediastinal mesenchyme (MM) of the body wall. **C,D:** D is a higher magnification of C. These panels illustrate how the Tie2-Cre-positive mesenchymal cap is continuous with the Tie2-Cre

negative mesenchyme of the dorsal mesenchymal protrusion. **E:** All the individual components of the AV mesenchymal complex are clearly shown. Note the lacZ-negative DMP adjoining the inferior AV cushion. **F:** A higher magnification of E.

Both the DMP (black arrow) and iAVC (white arrow) are lined by contiguous Tie2-Cre positive endocardial cells (small arrows). CAP, mesenchymal cap; DM, dorsal mesocardium; DMP, dorsal mesenchymal protrusion; iAVC, inferior AV cushion; LA, left atrium; llAVC, left lateral AV cushion; LSH, left sinus horn; PAS, primary atrial septum; RA, right atrium; RVV, right venous valve; sAVC, superior AV cushion.

Fig. 3.

3D Reconstructions of the atrioventricular mesenchymal tissues at ED 11.5. Panels shown in this figure are 3D-AMIRA reconstructions of the atrial and atrioventricular component of the ED 11.5 heart depicted in Figure 2. **A,B:** A superior view with the upper portion of the atria removed. To reveal the mesenchymal structures of the AV mesenchymal complex, the atrial myocardium was rendered semi-transparent in B. **C,D:** The reconstructions that are shown in A and B, respectively, have been rotated to allow an inferior view of the heart. C and D illustrate the relationship of the AV cushions with the DMP and mesenchymal cap. **E:** Left lateral view. **F:** Posterosuperior view. E and F show the respective components of the AV mesenchymal complex and the primary atrial septum (E only) in more detail. Note the bridging arch made by the DMP

connecting the endocardial-derived CAP with the inferior AV cushion over the primary atrial foramen (white arrow). **F:** The AV mesenchymal complex with the myocardial component of the primary atrial septum removed. Note the groove (white dotted line) that indicates the location of this part of the septum in relation to the AV mesenchymal tissues. CAP, mesenchymal cap; DMP, dorsal mesenchymal protrusion; iAVC, inferior AV cushion; LA, left atrium; llAVC, left lateral AV cushion; LSH, left sinus horn; MM, mediastinal mesenchyme; PAS, primary atrial septum; RA, right atrium; sAVC, superior AV cushion.

Fig. 4.

The contribution of the dorsal mesenchymal protrusion to the atrioventricular mesenchymal complex at ED 13. **A:** A transverse section of an ED13 embryo (see also E and F), immunostained for sarcomeric actin. The AV mesenchymal tissues have fused at this stage and separate left and right AV connections have been established. This section nicely demonstrates the significant contribution of the DMP to the AV mesenchymal complex. A larger magnification of the boxed area in A is depicted in E. **B–D:** D is a higher magnification of C. These panels show sections from other ED13 specimens, sectioned in different orientations.

These panels show more clearly that, within the AV mesenchymal complex, the DMP is a population of lacZ-negative mesenchymal cells that is wedged between the superior and inferior AV cushions. **E:** Compare with A. The active muscularization that is taking place within the DMP (arrowheads). **F:** A sister-section of E processed for TUNNEL labeling to

identify apoptotic cells. This panel shows a discrete region of apoptosis at the boundary of the DMP and inferior AV cushion (white arrowheads), most apoptotic cells being located within the region of the Tie2-cre labeled cells. In addition, a trail of isolated apoptotic cells is located within the body of the DMP (black arrows). DM, dorsal mesocardium; DMP, dorsal mesenchymal protrusion; iAVC, inferior AV cushion; LAVJ, left AV junction; LSH, left sinus horn; LV, left ventricle; PAS, primary atrial septum; RA, right atrium; RAVJ, right AV junction; rlAVC, right lateral AV cushion; RV, right ventricle; RVV, right venous valve; sAVC, superior AV cushion.

Fig. 5.

3D Reconstructions of the atrioventricular mesenchymal tissues at ED 13. **A:** A section of a lacZ-stained ED 13 Tie2Cre/R26R heart, immunolabeled for sarcomeric actin. Although the major AV cushions are in the process of fusing, the superior and inferior AV cushions can be distinguished by their contours. **B:** A 3D reconstruction based on the histological information on the AV mesenchymal tissues as provided by serial sections of the heart in A. **C:** A posterosuperior view of the AV mesenchymal complex shows the central location of the DMP with respect to the endocardial-derived major (sAVC, iAVC) and lateral (lIAVC, rIAVC) AV cushions. It is important to note that the mesenchymal cap, at this stage, is indistinguishable from the sAVC. **D–F:** The model is positioned to provide a left lateral view of the AV mesenchymal complex. **E:** The DMP can be seen to extend ventrally, forming a small wedge in between the sAVC and iAVC. **F:** The relationship of the DMP and fused mesenchymal components with the PAS (atrial myocardium semi-transparent). This view clearly illustrates the DMP as a major non-endocardial-derived mesenchymal component of the base of the PAS. **D:** A perspective on the location of the DMP and AV mesenchymal complex with respect to the atrial myocardium. The RVV attaches to the complex near the DMP. DMP, dorsal mesenchymal protrusion; iAVC, inferior AV cushion; lIAVC, left lateral AV cushion; PAS, primary atrial septum; rIAVC, right lateral AV cushion; RVV, right venous valve; SAF, secondary atrial foramen; sAVC, superior AV cushion.

Fig. 6.

The atrioventricular mesenchymal complex at ED 15. **A,C:** Transverse sections of an ED15 embryo illustrate the DMP as the lacZ-negative mesenchyme continuous with the endocardial derived mesenchyme near the attachment of the left and right venous valves. **B,D:** Higher magnifications of the boxed areas in A and C, respectively. These panels show the considerable muscularization that is occurring within the DMP. AVC, AV cushion; DMP, dorsal mesenchymal protrusion; LSCV, left superior caval vein; PAS, primary atrial septum; PuV, pulmonary vein; RA, right atrium; RVV, right venous valve.