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Origin and Fate of Cardiac Mesenchyme

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The development of the embryonic heart is dependent upon the generation and incorporation of different mesenchymal subpopulations that derive from intra- and extra-cardiac sources, including the endocardium, epicardium, neural crest, and second heart field. Each of these populations plays a crucial role in cardiovascular development, in particular in the formation of the valvuloseptal apparatus. Notwithstanding shared mechanisms by which these cells are generated, their fate and function differ profoundly by their originating source. While most of our early insights into the origin and fate of the cardiac mesenchyme has come from experimental studies in avian model systems, recent advances in transgenic mouse technology has enhanced our ability to study these cell populations in the mammalian heart. In this article, we will review the current understanding of the role of cardiac mesenchyme in cardiac morphogenesis and discuss several new paradigms based on recent studies in the mouse. *Developmental Dynamics 237:2804–2819, 2008.* © 2008 Wiley-Liss, Inc.

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INTRODUCTION

The development of the heart from precardiac mesodermal cells into a four-chambered pump that supports a pulmonary and systemic blood circulation is a fascinating process. It involves the differentiation of cardiac precursors into a variety of cell types, including the myocardium, the endocardium, and the cardiac mesenchyme. Each of these cell types contributes in its own specific way to the formation of the cardiac chambers, conduction system, and a highly specialized valvuloseptal apparatus (Lamers and Moorman, 2002; Abu-Issa and Kirby, 2007).

In recent years, our understanding of the origin, development, and fate of the respective cell populations has broadened significantly. This is due, in large measure, to the emergence of sophisticated transgenic mouse technologies. Specifically, the development of Cre-lox recombination strategies (Soriano, 1999) has brought about a breakthrough in our ability to study cell-fate in the murine heart (de Lange et al., 2004) and has provided the means to generate tissue-specific gene knockout mice (Park et al., 2006; Goddeeris et al., 2007). The use of these mouse models, in addition to histological and experimental embryological approaches (Thompson and Fitzharris, 1979; Kirby et al., 1985; Manner, 1993; Perez-Pomares et al., 2002a) including cell-labeling studies (Poelmann and Gittenberger-de Groot, 1999), has helped identify the major cell populations that contribute to the different stages of development of the four-chambered heart. These tools were critical in establishing the currently accepted paradigm that the heart is derived from precardiac mesodermal cell populations found in the primary and secondary heart fields (PHF and SHF, respectively; Srivastava, 2006; Abu-Issa and Kirby, 2007).

Proper formation of the heart is critically dependent upon mesenchymal cell contributions from both intra- and extra-cardiac sources. This includes mesenchyme that originates from the endocardium, cardiac neural crest, epicardium, and the second heart field. In this review, we will discuss the origin and fate of these different subpopulations, and provide some updated insights into their role in cardiac development.

HEART DEVELOPMENT: A BIRDSEYE VIEW

Formation of the precardiac mesoderm is generally considered to be the

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first step in cardiac development. In this process, a specific subset of mesodermal cells, generated during embryonic gastrulation, migrate anterolaterally to form two bilateral heart-forming regions (Fig. 1A1). Differentiation of these precardiac mesodermal cells results in the formation of myocardial and endocardial progenitors (Lough and Sugi, 2000) which, combined, form the so-called primary heart field (PHF). As the embryo folds, these two regions coalesce to form a cardiac crescent (Fig. 1A2). Fusion of the cardiac crescent at the embryonic midline results in the formation of the primary heart tube (Fig. 1A3; Abu-Issa and Kirby, 2007; Moorman et al., 2007). This, more or less, linear heart tube is initially suspended, over its entire length, from the rest of the embryo by the dorsal mesocardium (Fig. 1B). The PHF-derived heart tube consists of a myocardial outer mantle that is separated from an inner endocardial tube by an acellular matrix, generally referred to as the cardiac jelly (CJ; Eisenberg and Markwald, 1995). As development proceeds, the primary heart tube elongates at both the arterial and venous pole. During this event, the tubular heart loops toward the right (Manner, 2004). To accommodate this growth and remodeling, the dorsal mesocardium gradually disintegrates in the mid-section (Drake et al., 2006), leaving the heart tube eventually only connected to the rest of the body by means of the remaining dorsal mesocardium at the caudal and cranial aspects of the heart (Fig 1A4,C). Inhibition of the disintegration of the dorsal mesocardium by, for instance, administration of VEGF at early embryonic stages, results in perturbation of looping (Drake et al., 2006).

A series of recent studies has demonstrated that the four-chambered heart only partly derives from the PHF and that recruitment of cells that find their origin in another pool of cardiac mesodermal precursor cells, dubbed the secondary heart field (SHF), also plays a significant part in heart development. Whether the PHF and SHF are truly two discrete entities is still a matter of debate. More comprehensive descriptions of the developmental aspects of the PHF and SHF is beyond the scope of this study, but can be found in several recent review articles (Abu-Issa and Kirby, 2007; Moorman et al., 2007). In the context of this study, however, it is important to note that the cells that originate from the SHF have a distinct characteristic pattern of gene expression, for example, they express the LIM homeodomain expression factor Islet 1 (Isl1), and contribute to heart development at a relatively late stage (Kelly et al., 2001; Cai et al., 2003; Verzi et al., 2005). The SHF-specific gene expression profile, as well as the fact that cells of the SHF contain transcriptional elements that up-regulate expression of genetic markers, such as the AHF-Mef2C construct, allows determination of contribution and fate of the SHF cells (Kelly et al., 2001; Cai et al., 2003; Verzi et al., 2005). From studies in recent years, the concept has emerged that the PHF primarily contributes to the atria, atrioventricular junction, and left ventricle. The outflow tract, right ventricle, and components of the venous pole are now generally considered to be derivatives of the SHF (Srivastava, 2006).

As the heart is going through its looping stages, and continues to grow by the addition of cells from the SHF, the respective chambers concomitantly expand as a result of relatively high levels of proliferation of the chamber myocardium (Moorman and Christoffels, 2003). As a result of these remodeling events, the future segments of the heart become recognizable and acquire their more definitive positions. It is noteworthy that at this stage (approximately embryonic day [E] 5–9 in mouse, Hamburger and Hamilton [HH] stage HH16–HH17 in chick, 4 weeks in human) all the developing components of the heart are still basically arranged in series and the appropriate segmental alignment still needs to be established (Fig. 1D).

As looping is occurring, the cardiac jelly that initially is found distributed more or less uniformly throughout the tube, starts to accumulate in the atrioventricular junction (AVJ) and outflow tract (OFT; Fig. 1D). At the same time, the jelly starts to disappear from other parts of the heart, and the endocardium becomes virtually plastered against the myocardium of the developing chambers. The local swellings of cardiac jelly in AVJ and OFT are commonly referred to as "cushions." They are rich in extracellular matrix (ECM) components, and will subsequently be populated to varying degrees by different subpopulations of cardiac mesenchyme (Schroeder et al., 2003). As will be detailed below, the origin and extent of colonization of the different mesenchymal subpopulations have a profound influence upon the fate of these tissues and their specific contributions to valvuloseptal morphogenesis. We will first describe the respective mesenchymal structures from an anatomical/morphological perspective and discuss their general function during cardiac morphogenesis. In following sections we will then, in more detail, discuss the embryonic origin of the mesenchymal cells found in these structures, some of their characteristics, and their fate.

CARDIAC MESENCHYMAL STRUCTURES

AV Cushions

The mesenchymal structures most frequently studied in the developing heart are undoubtedly the AV cushions. It is well established that they play an essential role in the formation of the AV valves and the AV septal components (Schroeder et al., 2003; Wessels and Sedmera, 2003; Kruithof et al., 2007). The superior and inferior cushions (Fig. 2A,A′,D), also known as the "major" cushions, are the first to form. Their development is followed by the appearance of two "lateral" cushions, which are found in the left and right side of the AV junction respectively (Wessels et al., 1996; de Lange et al., 2004; Fig. 2B,B′,D). Historically, the major AV cushions have been the primary focus of attention in most descriptive and experimental studies. It is important to realize that they are the tissues generally isolated for the study of epithelial-to-mesenchymal transformation (EMT) using the collagen gel assay (Runyan and Markwald, 1983). Therefore it needs to be noted that our current insights into the molecular regulation of EMT, as derived from these in vitro studies, mainly pertain to the major cushions and that we know virtually nothing about the mechanisms that govern the, delayed, formation of the lateral cushions. To the best of our knowledge, no information has been pub-

lished on spatiotemporal differences in expression of factors known to be involved in regulation of EMT (growth factors, transcription factors, receptors) in either the AV myocardium or the AV endocardium that could underlie this phenomenon.

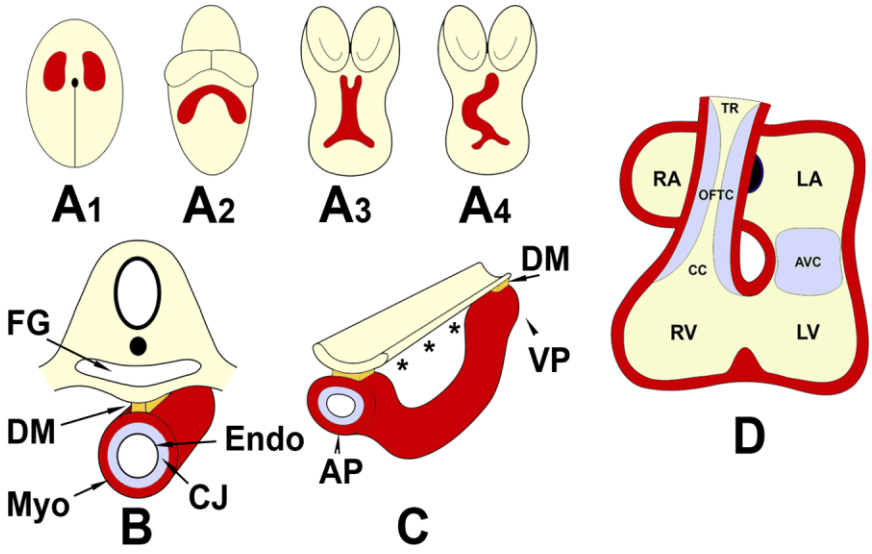

Fig. 1. Early stages of heart development. A schematic cartoon depicting the characteristic early stages in heart development. Ventral views of the embryo are depicted in A1–A4. **A1:** Bilateral heart fields form after progenitor cells have migrated from the primitive streak. **A2:** Fusion of the bilateral heart fields at the midline creates a cardiac crescent. **A3:** Fusion of the cardiac crescent leads to the formation of the linear heart tube. **A4:** Disintegration of the dorsal mesocardium enables the heart tube to undergo its characteristic D-loop. **B:** The tubular heart at the stage depicted in A3 is suspended from the rest of the embryo by the dorsal mesocardium **C:** After disintegration of the dorsal mesocardium in the mid-section, the heart tube remains attached to the embryo by means of its dorsal mesocardial connections at the arterial and venous poles. **D:** By embryonic day (E) 8.5–9 (mouse), the respective components of the embryonic heart are still aligned in series. AVC, atrioventricular Cushions; AP, arterial pole; DM, dorsal mesocardium; Endo, endocardium; FG, foregut; LA, left atrium; LV, left ventricle; Myo, myocardium; OFTC, outflow tract cushions; RA, right atrium; RV, right ventricle.

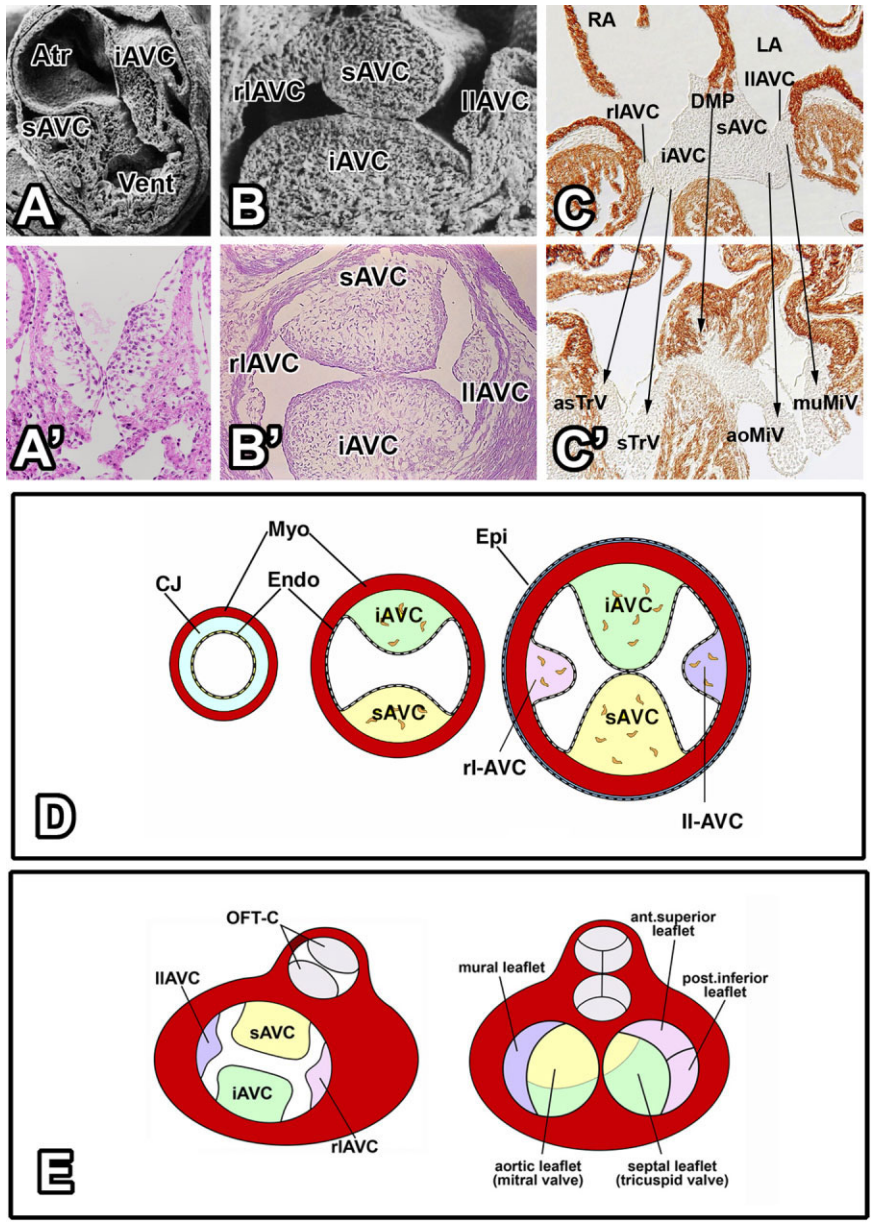

Fig. 2. The atrioventricular (AV) cushions. **A,A′:** Scanning electron micrograph and histological section of an embryonic day (E) 10.5–11 mouse heart, show the major AV cushions. **B,B′:** By E13, the major cushions have begun to fuse and the right and left lateral (rlAVC, llAVC) cushions are now discernible. **C:** This transverse section shows the AV mesenchymal complex formed by the superior and inferior AV cushions (sAVC, iAVC) with the DMP, as well as the lateral cushions which do not fuse with the complex. **C′:** An E14.5 mouse heart showing the contributions of the different AV mesenchymal tissues from (C) to the respective valvar leaflets. **D:** This cartoon depicts the formation of the AV endocardial cushions. Cardiac jelly accumulates first in the dorsal and ventral aspect of the heart tube to form the major AV cushions. The lateral cushions form after the major cushions. **E:** Fate of the major and lateral AV cushions in the mature heart. The sAVC (yellow) contributes primarily to the aortic leaflet of the mitral valve, and the iAVC (green) contributes largely to the septal leaflet of the tricuspid valve. The rlAVC (pink) and llAVC (purple) contribute to the mural leaflets of the tricuspid and mitral valves, respectively. aoMiV, aortic leaflet of the mitral valve; asTrV, anterosuperior leaflet of the tricuspid valve; DMP, dorsal mesenchymal protrusion; Epi, epicardium; iAVC, inferior AV cushion; llAVC, left lateral AV cushion; muMiV, mural leaflet of the mitral valve; rlAVC, right lateral AV cushion; sAVC, superior AV cushion, other abbreviations as in Figure 1.

Each individual AV cushion plays a specific and crucial role in valvuloseptal development. Nonetheless, the lateral AV cushions are often ignored in textbooks. The right lateral cushion contributes, in combination with the atrioventricular canal myocardium, to the formation of the anterosuperior leaflet and posterior leaflet of the tricuspid valve, while in the left AV junction, the left lateral cushion is involved in the formation of the mural leaflet of the mitral valve (Fig. 2C,C′,E; Lamers and Moorman, 2002; Wessels and Sedmera, 2003; de Lange et al., 2004). It is of note that the derivatives of these lateral cushions can be involved in many congenital and acquired valve abnormalities, including, but not restricted to, Ebstein's anomaly, AV valve insufficiency, valve prolapse, and mitral/tricuspid stenosis. Despite their prominent size in early development, the major cushions play a relatively minor role in formation of the AV valve leaflets. The superior AV cushion, also known as the anterosuperior or ventral cushion, contributes to the aortic leaflet of the mitral valve, while the inferior AV cushion, also know as the posteroinferior or dorsal cushion, takes part in the formation of the septal leaflet of the tricuspid valve, a relatively late event in valvuloseptal morphogenesis. In fact, the chief contribution of the fusing major AV cushions (Fig. 2B,B′,C) appears to be in the formation of the AV mesenchymal complex (Snarr et al., 2007b). This complex forms in the midline of the common AV canal and leads to the separation of the left and right AV communications (Fig. 2C,D). Recent work has clearly demonstrated that, in addition to the major AV cushions, proper AV septation also requires the contribution of two additional mesenchymal structures; the mesenchymal cap on the leading edge of the primary atrial septum and the dorsal mesenchymal protrusion (DMP; Snarr et al., 2007b).

Mesenchymal Cap of the Primary Atrial Septum

Compared with the major AV cushions, the "cap" is a relatively small mesenchymal structure. It initially forms as a small cushion-like tissue on the anlagen of the primary atrial septum (Fig. 3A,B). As the septum is growing, it becomes a prominent mesenchymal ridge on the leading edge of the myocardial part of the septum (Fig. 3C,D) (Arrechedera et al., 1987; Wessels et al., 2000). As the septum elongates and descends into the atrial cavity, the cap helps to close the primary atrial foramen, thereby separating left from right atrium. Anteriorly, the cap is contiguous with the superior AV cushion, while posteriorly it is in mesenchymal continuity with the dorsal mesenchymal protrusion (DMP; Wessels et al., 2000; Snarr et al., 2007b) (Fig. 3J). The expansion of the major AV cushions, in combination with their fusion with the cap and the DMP, results in the formation of the AV mesenchymal complex (Fig. 3K). The formed AV mesenchymal complex not only separates the left from the right AV junction, it also serves as the anchor for the muscular components of the atrial (Fig. 3K) and ventricular septa.

Dorsal Mesenchymal Protrusion

The DMP is a body of mesenchyme at the venous pole of the heart (Wessels et al., 1996, 2000) that is contiguous with the mesoderm found between the foregut and the dorsal mesocardium (Fig. 3G–I). This mesenchymal tissue, or at least part of it, was originally described by His as the spine of the vestibule or "spina vestibuli" (His, 1880). In his work, this German embryologist describes it as a triangular mesenchymal wedge that extends into the ventral aspect of the heart from the dorsal wall of the atrial cavity (His, 1880). In a series of immunohistochemical studies performed on the human heart, we introduced the term dorsal mesenchymal protrusion (DMP), as it more accurately reflected its histological and anatomical characteristics (Wessels et al., 2000). We demonstrated that this mesenchymal structure (Fig. 3G–I), which is closely associated with the dorsal mesocardium, is contiguous with the cap of the primary atrial septum (PAS) and the AV cushion mesenchyme. Furthermore, we demonstrated that the developing pulmonary vein, which initially is a mid-line structure (Fig. 3E,F) becomes eventually located at the left-most margin of the DMP as this tissue starts to wedge into the atrial cavity (Fig. 3G–I; Webb et al., 1998; Wessels et al., 2000). In more recent studies in the mouse, we have shown that the DMP is an integral part of the AV mesenchymal complex (Fig. 3J,K; Snarr et al., 2007a,b). The clinical relevance of increasing our knowledge of the development of the DMP is indicated by several studies that strongly indicate that perturbation of DMP development may be one of, if not the most, important factors in the etiology of atrial and atrioventricular defects (Sharratt et al., 2003; Snarr et al., 2007a; Wirrig et al., 2007; Goddeeris et al., 2008).

OFT Cushions

After the rearrangement of the cardiac jelly, two prominent elongated cushions can be distinguished within the developing outflow tract of the mammalian heart (Fig. 4). They are most commonly referred to as the septal and parietal ridges (Ya et al., 1998; Perez-Pomares et al., 2003). Within these ridges we can distinguish proximal and distal components. The proximal mesenchymal components (i.e., at the junction with the right ventricle), are also referred to as the conal cushions (Fig. 4A,B,E). They fuse during development, forming the conal septum that separates the aortic and pulmonary outlet components (Fig. 4C). Muscularization of this, initially mesenchymal septum, results in the formation of a muscular outlet-septum (Fig. 4C,D) (van den Hoff et al., 1999; Kruithof et al., 2003; Moralez et al., 2006). This muscularization eliminates the mesenchymal continuity between the proximal part of the septal ridge and the superior AV cushion (Christoffels et al., 2000). The more distally located OFT mesenchymal tissues (i.e., near the aortic sac) are commonly referred to as the truncal ridges (Fig. 4A,B,E). The remodeling of the distal-most components result in the formation of the aortic and pulmonary semilunar valves (Fig. 4D). In addition, the truncal ridges are also involved, in combination with the cardiac neural crest (see below), in the formation of the aorticopulmonary septum. Reminiscent of the situation in the AV junction, two less prominent mesenchymal OFT ridges can be observed

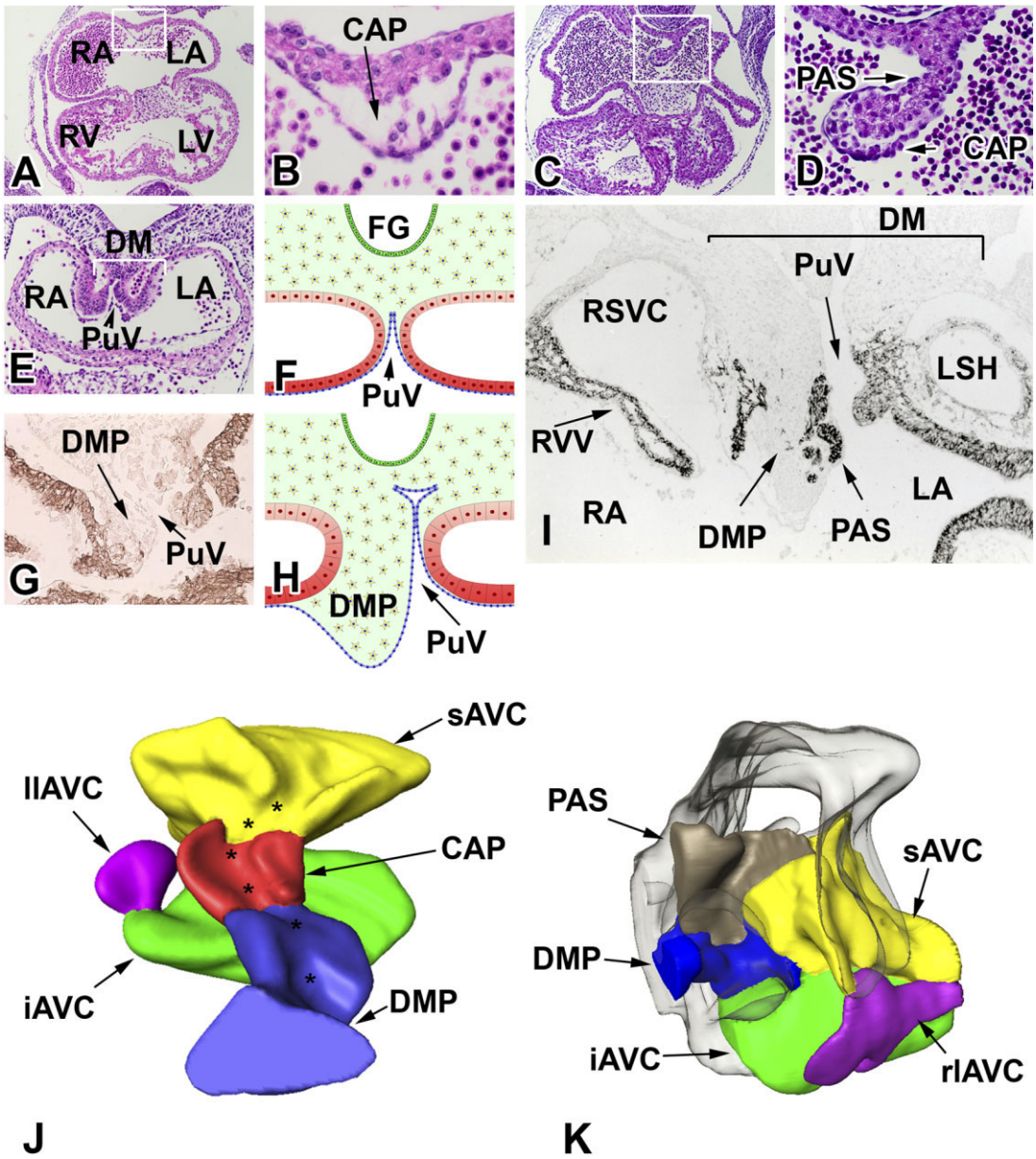
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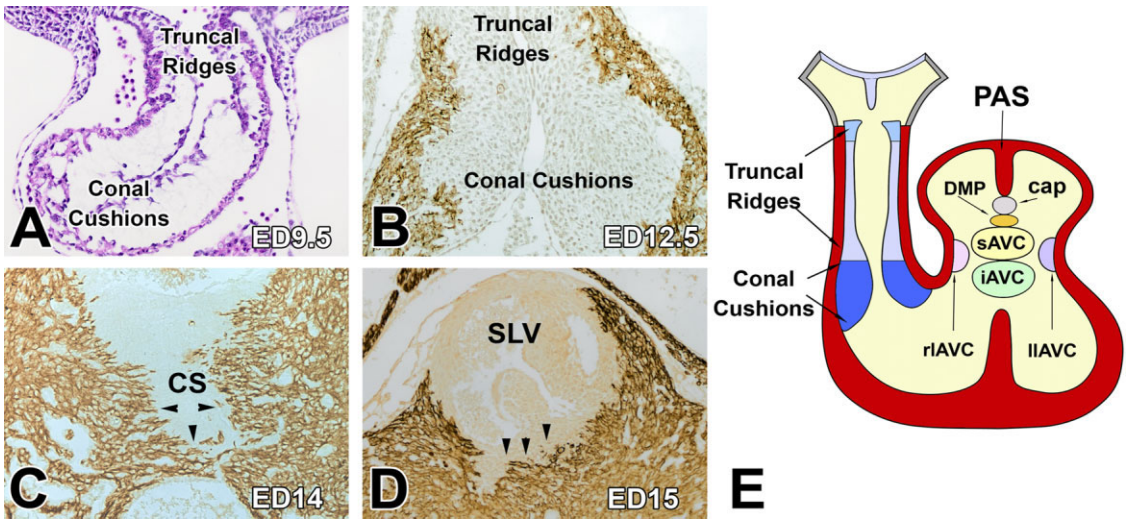
Fig. 3.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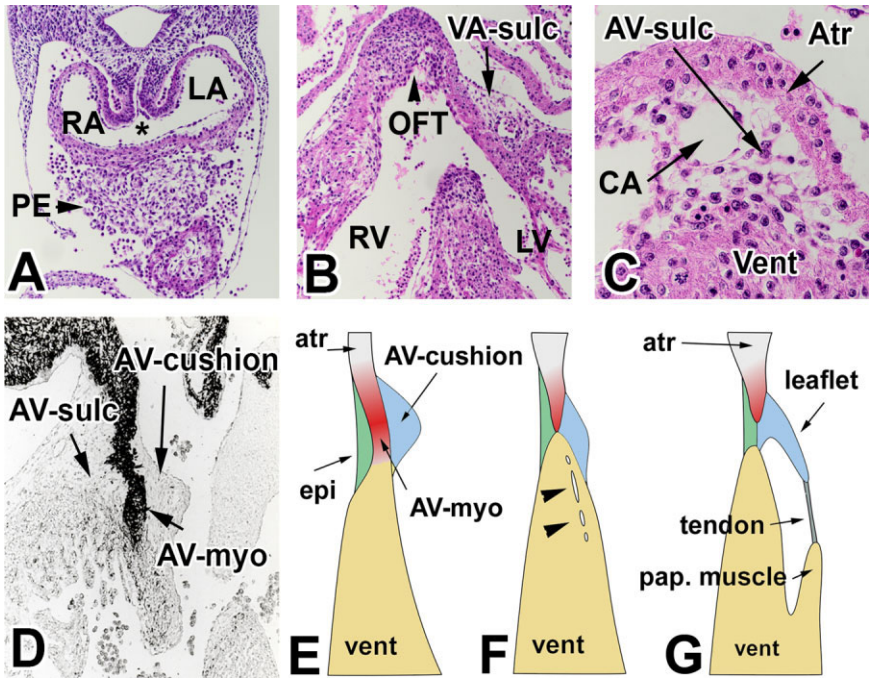

Fig. 5. The epicaridum and subepicardial mesenchyme. **A:** A transverse section of an embryonic day (E) 9.5 mouse embryo showing the proepicardium located ventrally of the venous component of the heart. **B:** Subepicardial mesenchyme accumulated at the ventriculoarterial junction of an E12 mouse heart. **C:** Histological section through the atrioventricular (AV) junction of an E12 mouse heart showing the development of a major coronary artery. **D:** This section of a human heart in the seventh week of development, stained for atrial myosin heavy chain, illustrates the relationship of the mesenchyme of the AV sulcus to the AV myocardium and AV cushion mesenchyme. **E–G:** This cartoon demonstrates how AV sulcus mesenchyme and AV cushion mesenchyme fuse at the lower boundary of the embryonic AV myocardium resulting in "electrical insulation" of atrial and ventricular myocardium.

Fig. 3. The mesenchymal cap on the primary atrial septum (cap) and the dorsal mesenchymal protrusion (DMP). **A,B:** Transverse sections of an embryonic day (E) 9.5 demonstrating the cap as a developing mesenchymal structure on the anlagen of the primary atrial septum. **C,D:** The mesenchymal cap located on the leading edge of the primary atrial septum at E10.5 B,D: Higher magnifications of the boxed areas in A and C, respectively. E–I: The DMP forms within the dorsal mesocardium at the venous pole of the heart. **E–H:** The location of the developing pulmonary vein (PuV) shifts from an initial midline position at E9.5 (E,F) to become a left-sided structure by E11 (G,H). The section in G was immunostained for MLC2a. **I:** Histological, immunostained for atrial myosin heavy chain (Wessels et al., 2000) of a human embryonic heart during the seventh week of development demonstrating the wedged position of the DMP. **J:** AMIRA three-dimensional (3D) reconstruction showing a dorsal view of the DMP (blue) and the other atrioventricular (AV) mesenchymal tissues E11.5 in the mouse. Note the groove within these tissues (asterisks) that marks the location of the myocardial part of the PAS (removed for reconstruction). **K:** The 3D reconstruction of AV mesenchymal tissues at E13. The DMP forms a wedge between the major AV cushions and forms the base of the PAS. At this stage, the atrial cap cannot be distinguished any longer from the other endocardially derived mesenchymal tissues. CAP, mesenchymal cap on the primary atrial septum; LSH, left sinus horn; PAS, primary atrial septum; PuV, pulmonary vein; RSVC, right superior vena cava, RVV, right venous valve.

Fig. 4. The outflow tract (OFT) cushions. **A:** Two elongated cushions of cardiac jelly can be seen in the OFT at embryonic day (E) 9.5. Within these cushions we can distinguish proximal (conal cushions) and distal components (truncal ridges). **B:** By E12.5, the OFT cushions have been mesenchymalized and fuse to form a mesenchymal outlet septum. **C:** Muscularization of the conal septum (CS) produces a muscular outlet septum. **D:** At E15, the fused conal cushions are largely replaced by cardiomyocytes (arrowheads). The distal-most parts of the truncal ridges contribute to the formation of the semilunar valves (SLV). **E:** Cartoon depicting the respective mesenchymal tissues in the developing heart. Abbreviations as in previous figures.

in addition to the major (septal and parietal) OFT ridges. These structures are known as the intercalated ridges (Kramer, 1942). As is the case with the lateral AV cushions, their development has received little attention.

Subepicardial Mesenchyme

During the late stages of looping, the coelomic splanchnopleura gives rise to a recognizable cluster of cells generally referred to as the proepicardium (Fig. 5A). In mammals, these cells comprise a distinct subset of the mesothelium of the septum transversum, evident for instance from the expression of WT1 (Moore et al., 1999). Subsequently, cells from the proepicardium reach the myocardial surface of the heart and form an epithelial cell layer, known as the epicardium. During the early stages of epicardial development, an extracellular matrix-rich, subepicardial space, separating the epicardial epithelium from the underlying myocardium, can be seen in many places. This subepicardial space shows resemblances with the cardiac jelly found between the endocardium and myocardium at early stages, but, in contrast to the situation in the cardiac jelly in early stages, the subepicardial space always contains mesenchymal cells. These cells are derived from an epicardial EMT, which again, is remarkably similar to the EMT found in the cushion tissues. Insight into the regulation of epicardial EMT is slowly emerging. At later stages of development, most of the epicardium becomes virtually plastered against the myocardium (cf. endocardium of cardiac jelly). There are, however, a few important areas where subepicardial mesenchyme accumulates, namely in the ventriculoarterial junction (Fig. 5B), at the interventricular groove, and at the AV junction (Fig. 5C). The accumulated and condensed epicardial mesenchyme in these sulci is involved in the formation of major components of the coronary network. The role of epicardially derived cells (EPDCs) in this process is well established (Perez-Pomares et al., 2002a; Lie-Venema et al., 2007). Another important function of the epicardial AV sulcus is in the establishment of the fibrous annulus that separates the myocardium of the atrial and ventricu-

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lar chambers (Fig. 5D–G). In a series of remodeling events at the AV junction, the mesenchymal tissues of the epicardial sulcus and components of the AV cushion tissues fuse at the lower boundary of the embryonic AV myocardium thereby electrically insulating, with the exception of the central axis of the AV conduction system, the atrial and ventricular myocardial structures (Wessels et al., 1992, 1996). This is a critical process in heart development, as incomplete separation of atrial and ventricular working myocardium may result in accessory AV pathways as observed in Wolff-Parkinson-White syndrome (Pritchett et al., 1980).

ORIGIN AND FATE OF CARDIAC MESENCHYME

The delineation of different mesenchymal cell populations during development in the respective structures described above has been crucial to understanding the origin, fate and function of cardiac mesenchyme. Most of our early insight has come from model systems that are particularly amenable to experimental manipulation such as chick and quail embryos. In recent years our knowledge has been enhanced by the use of mouse models in which Cre-recombinase expression is driven by a promoter in a tissue-specific manner. Crossing these mice with a ROSA26 reporter (R26R) mouse causes irreversible lacZ expression in all cells, as well as in all daughter cells, that are expressing Cre (Soriano, 1999). This has enabled genetic lineage tracing in mice at any time point after recombination. These mice have also proven invaluable in producing conditional knock-out animals. When used in combination with a transgenic mouse, in which a target gene of interest is flanked by loxP sites, deletion of the target gene can be achieved in cells that express Cre. In the following sections, we will discuss the major different sources and fate of cardiac mesenchyme that have been identified through the use of these tools.

Endocardially Derived Mesenchyme

AV cushions.

Thirty years ago, hallmark studies by Markwald and colleagues, revealed that the, initially acellular, AV cushions become mesenchymalized as a result of an EMT of the endocardial cells that are lining the cushions (Markwald et al., 1977; Bolender and Markwald, 1979). Most of our early insights into the process of endocardial EMT come from in vitro collagen assays, developed to assess EMT after cardiac explants containing cushion endocardium are placed on three-dimensional collagen type I gels (Bernanke and Markwald, 1982). This widely applied technique allows for the quantification of EMT by determining the subsequent invasion of the collagen gels by endocardially derived cells (ENDCs). Using these assays, it was demonstrated that signals from the adjacent AV myocardium are required for normal EMT to occur (Eisenberg and Markwald, 1995). Several AV-expressed growth factors have been identified that play an important role in AV cushion formation (Schroeder et al., 2003). These include bone morphogenetic proteins 2 and 4 (BMP2, BMP4; Lyons et al., 1990; Yamagishi et al., 1999; Yamada et al., 2000; Sugi et al., 2004), transforming growth factor-beta 2 (TGFβ2; Dickson et al., 1993; Brown et al., 1996; Romano and Runyan, 2000; Camenisch et al., 2002), TGFβ3 (Potts et al., 1991; Nakajima et al., 1998; Nakajima et al., 1999), vascular endothelial growth factor (VEGF; Dor et al., 2001), and fibroblast growth factor (FGF; Sugi et al., 1995). Histological and in vitro studies clearly showed that AV cushion mesenchyme is principally generated through endocardial EMT, a paradigm that was confirmed in studies using endocardial-specific Cre mice in combination with Rosa26R reporter mice. Specifically, the studies using mice expressing Cre-recombinase driven by the promoter of the endothelial specific receptor tyrosine kinase, Tie2, have shown that (virtually) all mesenchymal cells in the AV cushions (major and lateral), as well as the cells of the mesenchymal cap on the primary atrial septum, are endocardially derived (Fig. 6A–D). These studies also demonstrated that the postnatal fate of ENDCs is limited strictly to the fibrous tissues that constitute the leaflets of the AV valves (de Lange et al., 2004). Of the four different mesenchymal subpopulations that make up the AV mesenchymal complex, the DMP is the only one that does not contain Tie2 expressing mesenchymal cells (Fig. 6C,D), indicating that the DMP mesenchyme has a different developmental origin (Mommersteeg et al., 2006; Snarr et al., 2007b).

Additional understanding of endocardial cell biology and AV cushion development has been achieved by conditional knock-out strategies with endothelial-Cre mice as described above. These studies have elucidated important endocardial-specific roles for a series of genes in pathways that govern cushion formation, including Gata4 (Rivera-Feliciano et al., 2006), Sox9 (Lincoln et al., 2007), Nf1 (Gitler et al., 2003), HB-EGF (Nanba et al., 2006), TGFβ (Jiao et al., 2006), and BMP (Song et al., 2007).

OFT cushions.

Given the overall histological resemblance between the AV cushions and the elongated mesenchymal cushions of the OFT, it was, and sometimes still is, assumed that the mesenchyme populating the OFT cushions has a similar origin as that of the AV cushions. However, Tie2-cre studies, in combination with numerous elegant studies demonstrating the contribution of the cardiac neural crest to OFT development (see below), have established that the contribution of endocardially derived cells to the mesenchymal population of the OFT is, in fact, very restricted and that ENDCs are basically only found at the most proximal portion of the conal cushions and in the most distal part of the truncal ridges (Fig. 6E,F), where they together with cardiac neural crest-derived cells (CNDCs) and second heart field-derived cells contribute to the formation of the leaflets of the semilunar valves. One of the unresolved questions in relation to OFT development is what are the mechanisms that regulate the compartmentalization of ENDCs and CNDCs in the developing cushion tissues of the OFT (Fig. 6E–H).

Cardiac Neural Crest and OFT Development

The cardiac neural crest is located between the otic placode and fourth somite. Cells derived form this part of the neural crest are known to play an important role in cardiovascular de-

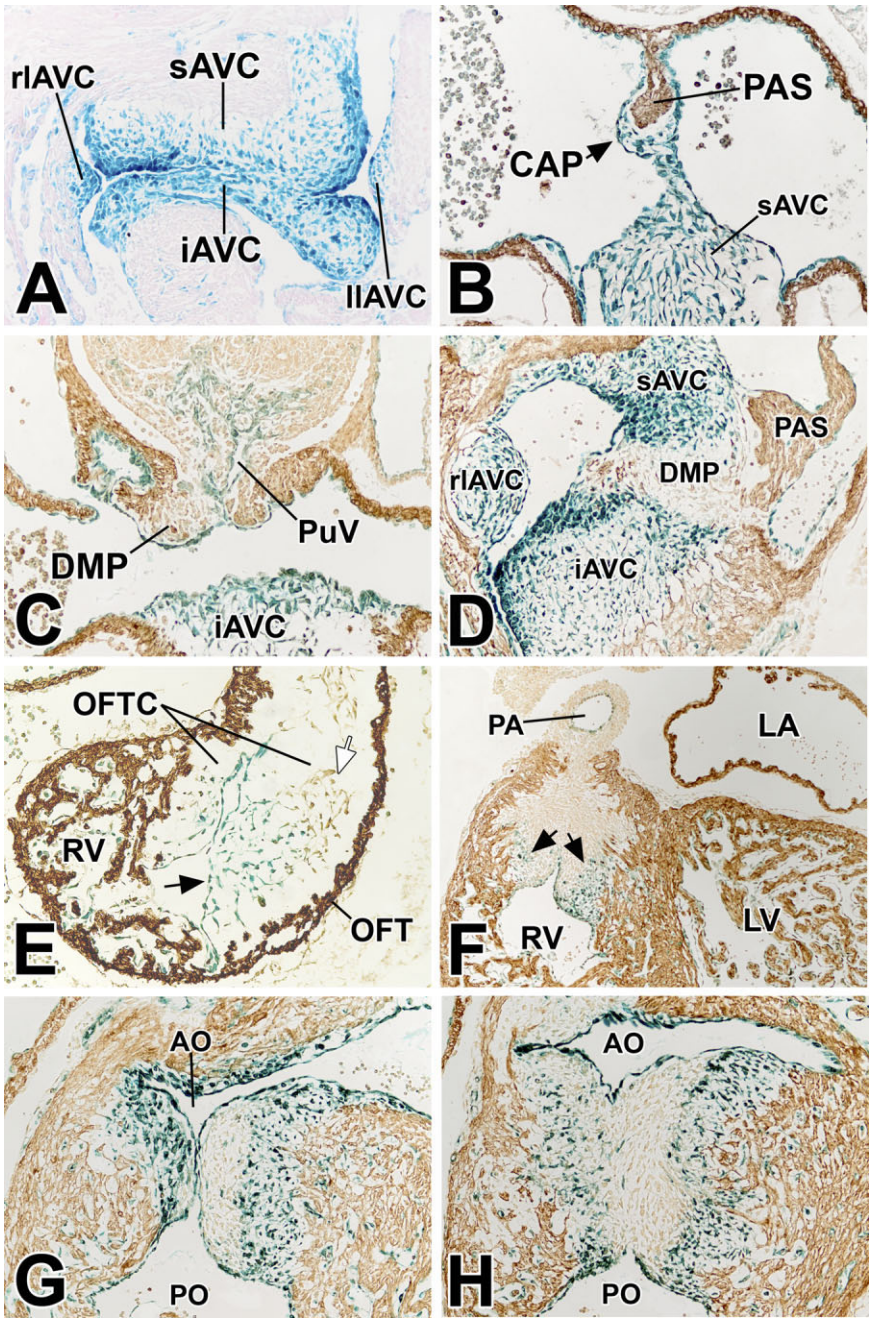

Fig. 6. Endocardially derived mesenchyme. Histological sections from Tie2-Cre\R26R mice. All sections are either eosin counterstained or immunostained for sarcomeric actin. **A:** A transverse section through an embryonic day (E) 13 heart showing that (virtually) all cells of the major and lateral atrioventricular (AV) cushions are Tie2-Cre positive. **B:** A transverse section through an E10.5 heart shows that the CAP on the primary atrial septum (PAS) is also endocardially derived. **C:** The panel shows a section caudal to that in B and illustrates that the dorsal mesenchymal protrusion (DMP) is not an endocardially derived structure. **D:** The AV mesenchymal complex at E13. Note the DMP is the only Tie2-Cre negative mesenchyme. **E:** Endocardially derived mesenchyme in the outflow tract (OFT) at ED10.5 shows lacZ staining in the proximal region of the OFT cushions (i.e. conal cushions). **F:** Transverse section of an E13 heart shows the restriction of the lacZ-positive cells to the most proximal region of the outlet septum. **G,H:** Oblique-transverse sections through the conal cushions at ED13 shows the compartmentalization of lacZ-positive (ENDCs) and lacZ-negative mesenchyme. AO, aortic outlet; PA, pulmonary artery; PO, pulmonary outlet; CAP, mesenchymal cap on the primary atrial septum.

velopment for more than 20 years (Hutson and Kirby, 2003). After undergoing a neuroectodermal EMT transformation, these "ecto-mesenchymal" cardiac neural crest derived cells (CNDCs) migrate toward the heart, passing through the third, fourth, and sixth pharyngeal arches and contribute to the septation of the OFT as well as the development of the semilunar valves (Nakamura et al., 2006; Hutson and Kirby, 2007). CNDCs also contribute to the development of the great arteries, glandular tissues of the neck, and the parasympathetic and sympathetic innervation of the heart (Verberne et al., 1998; Hildreth et al., 2007; Hutson and Kirby, 2007). The first major insight into the importance of these cells to cardiac development came from avian studies performed in the early 1980s. Creating one of the first experimental models of congenital heart disease, Kirby and colleagues showed, that perturbation of the CNC region resulted in defective aorticopulmonary septation, deficient cardiomyocyte function, and mis-patterning of the great arteries, as well as various noncardiovascular related anomalies (Kirby et al., 1983, 1985). The avian system also proved to be valuable in facilitating the first studies of mapping the fate of CNDCs. Using techniques such as quail-to-chick chimeras (Phillips et al., 1987; Miyagawa-Tomita et al., 1991) and retroviral labeling of CNDCs with lacZ (Poelmann et al., 1998), the specific tissue contributions from these cells could now be determined. Not surprisingly, the destinations of the CNDCs coincided with location of the cardiovascular defects observed in the neural crest ablation experiments (Hutson and Kirby, 2003). These studies provided an important foundation for our current knowledge of the neural crest contribution to cardiovascular development. Until the late 1990s, quail and chick were the model systems of choice to study the fate of CNDCs in the context of congenital heart disease. Important insights into the fate and function of CNDCs in mammalian systems were obtained after, for instance the discovery of the expression of the gap junction protein Connexin 43 (Cx43), in the presumed cardiac neural crest-derived mesenchyme (Lo et al., 1997). Generation of a transgenic mouse in which lacZ expres-

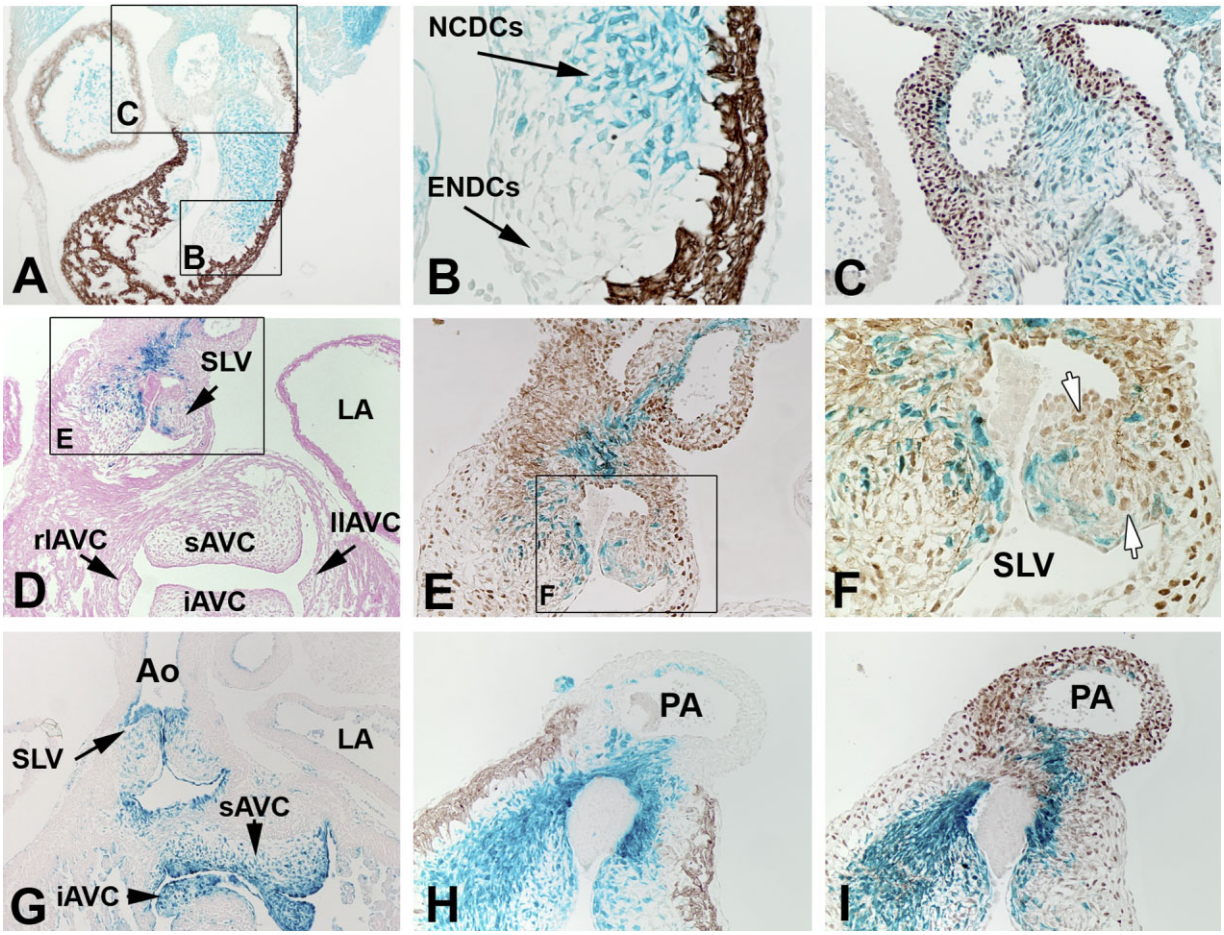

Fig. 7. Neural crest-derived mesenchyme. Histological sections from Wnt1-Cre\R26R mice. Sections are eosin counterstained or immunohistostained for MF20, unless otherwise indicated. **A:** At embryonic day (E) 11, extensive colonization of the outflow tract (OFT) cushions by cardiac neural crest derived cells (CNDCs) can be seen. **B:** Higher magnification of the boxed region (lower) in (A), demonstrating the boundary between CNDCs and endocardially derived cells (ENDCs) in the conal cushions. **C:** Higher magnification of boxed region (upper) shown in A. This section was immunostained for Isl1 and illustrates the Isl1+ and lacZ− cells present in the developing walls of the outlet. **D:** Transverse section through an E13 heart showing the CNDCs contribution to the semilunar valves, and the absence of CNDCs in the atrioventricular (AV) cushions. **E,F:** Higher magnifications of the boxed region in D. Isl1 staining is found in the root of the great vessels and also in the developing leaflets of the semilunar valves (white arrow). **G:** Tie2-Cre\R26R section comparable to that shown in D. The ENDC and CNDC cell populations in the AV cushions and semilunar valves are mutually exclusive. **H,I:** These panels demonstrate the Isl1-positive myocardium of the OFT at embryonic day (E) 13, as well as the Isl1+ and Wnt1-Cre–negative cells of the wall of the pulmonary artery. Abbreviations are the same as in previous figures.

sion was driven by the Cx43 promoter in neural crest cells, but not in myocardial cells, provided the first mammalian model of reporter-gene labeled CNDCs (Waldo et al., 1999). The advent of Cre-lox technology provided an important opportunity to improve and expand upon CNDC fate studies in the mouse by facilitating early, irreversible labeling of neural crest cells, and allowing for determination of CNDC location throughout any time point of development after recombination (Jiang et al., 2000). Multiple neural crest fate-mapping mouse models have since been generated, with the most commonly used models using the promoters of Wnt1, Pax3, and P0 to drive Cre-recombinase

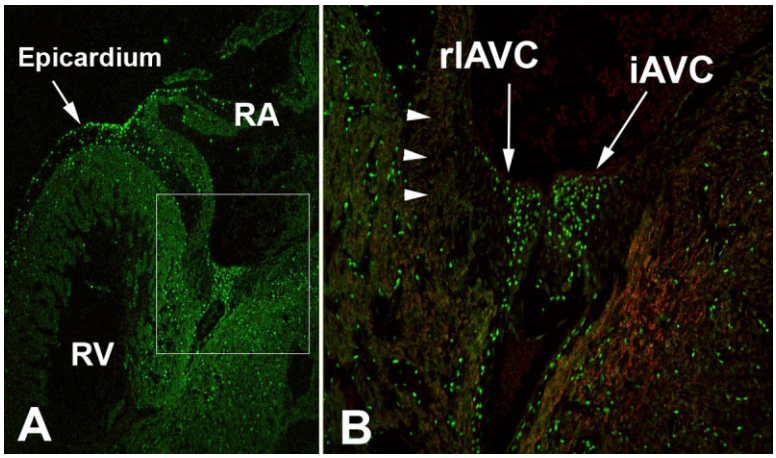

Fig. 8. Epicardially derived mesenchyme. **A,B:** These panels show QCPN staining of a quail-to-chick proepicardial explant chimera at stage Hamburger and Hamilton stage 36. The panels show the significant contribution of the (quail) epicardially derived cells to the atrioventricular (AV) cushion mesenchyme. B is a higher magnification of the boxed region in A. Abbreviations are the same as in previous figures.

(Lee et al., 1997; Jiang et al., 2000; Engleka et al., 2005). While discrepancies between the different models have been reported, all of the models corroborate an overall similar cell fate pattern in the mouse as that described in the chick (Jiang et al., 2000; Nakamura et al., 2006; Hutson and Kirby, 2007). In the developing OFT, CNDCs make significant contributions to the aorticopulmonary septum (APS) and the truncal ridges (Jiang et al., 2000; Engleka et al., 2005; Nakamura et al., 2006).

NEURAL CREST CELLS AND THE TRUNCAL RIDGES

The truncal ridges contribute to the formation of the semilunar valves and the aorticopulmonary septum. These tissues are heavily populated by CNDCs (Fig. 7A–C; Hutson and Kirby, 2007). The specific role for CNDCs in the development of these ridges, however, is not clear. This is complicated by the fact that these tissues also receive mesenchymal contributions from endocardial EMT (see above and Fig. 7G) and from the SHF (Fig. 7E,F,I; Sun et al., 2007) It is important to note that CNDCs as well as SHF cells contribute to the semilunar valves and to the vessel walls of the great arteries at their root (Fig. 7E,F,I; Waldo et al., 2001). The intimate spatiotemporal development of CNDCs in the truncal ridges with SHF-derived cells, underscores the importance of understanding the regulation of, and signaling between, these two different cell populations.

NEURAL CREST CELLS AND THE CONAL CUSHIONS

Fusion of the conal cushions (Fig. 6E–H) results in the formation of a mesenchymal septum that separates the outlets of the left and right ventricles. The most proximal part of the conal septum is composed of cells derived from the endocardium (Fig. 6E,F), whereas the more distally located mesenchyme is predominantly from the neural crest (Fig. 7A,B). Intermingled with these cell populations, mesenchymal cells are found that derive from the second heart field. There are no quail-to-chick chimera studies that have provided evidence that cells derived from the epicardium contribute to this mesenchymal structure, nor has this issue been addressed in recent papers on the fate of epicardial cells in the mouse (Cai et al., 2008; Zhou et al., 2008). It is worth mentioning that it has been illustrated that the epicardium at the surface of the avian OFT actually derives from cephalic pericardium located in the vicinity of the aortic sac, rather than from the proepicardium itself (Gittenberger-de Groot et al., 2000; Perez-Pomares et al., 2003). The proximal part of the mesenchymal septum is subsequently replaced by cardiomyocytes that form the muscular outlet septum. Very little is understood, however, about the mechanism by which this process occurs. Two major paradigms have been proposed: myocardialization, which describes an active in-growth of existing myocardium flanking the outlet septum, and mesenchymal-to-myocardial transdifferentiation of the OFT cushion mesenchyme. Abnormal development of CNDCs has been associated with perturbation of myocardialization (Waller et al., 2000). While it is clear from fate-mapping studies that neither neural crest cells nor endocardially derived cells differentiate into cardiomyocytes (Jiang et al., 2000; de Lange et al., 2004; Nakamura et al., 2006), the role of CNDCs in the development of the muscular outlet septum still remains elusive, especially because the majority of neural crest cells undergo apoptosis during mid-to-late gestation. It has been proposed that CNDCs may play an important role in signaling or cell–cell interactions between the different cell types in the developing OFT. Such interactions have been suggested to occur between CNDCs and cells from the SHF (Goddeeris et al., 2007; Cooley et al., 2008), and between CNDCs and ENDCs (Komatsu et al., 2007). Obtaining further insight into these interactions, and into the interplay between CNDCs and myocardial cells will undoubtedly advance our understanding of the role of CNDCs in OFT development.

The (pro)Epicardium

After the formation of the epicardial epithelium from the proepicardium, an EMT results in the formation of subepicardial mesenchyme (Perez-Pomares et al., 1997; Dettman et al., 1998). The EPDC is very invasive of nature. Cell-fate studies, in particular in the avian heart, have shown that a subpopulation of EPDCs migrate into the ventricular myocardial walls where they either become interstitial fibroblasts or further differentiate into coronary endothelial and coronary smooth muscle cells (Perez-Pomares et al., 2002b). Proepicardial explant studies, in which the proepicardium of quail donor embryos are explanted into stage-matched chick recipients, have demonstrated that EPDCs also migrate into, and mingle with, the endocardially derived mesenchyme of the AV cushions (Fig. 8; Dettman et al., 1998; Gittenberger-de Groot et al., 1998; Manner, 1999; Wessels and Perez-Pomares, 2004). Very little is known about the specific function of this subset of EPDCs. It appears that most, if not all, EPDCs in the cushions eventually die as virtually no epicardially derived cells are found in the more mature valves (de Lange et al., 2004).

The importance of EPDCs to cardiac development was illustrated by a series of studies in which the development of the proepicardium was experimentally inhibited. These in ovo manipulations, in which the proepicardium was either removed, or in which cells from the proepicardium were prevented to reach the surface of the heart in a timely manner, resulted in a spectrum of cardiac malformations including thinning of the ventricular wall, anomalies of the coronary system, and dysplasia of the AV cushions (Perez-Pomares et al., 2002b). The migration and further development of the epicardial cell layer relies on the communication between the epicardium and the underlying myocardium. It is therefore not surprising that perturbation of genes involved in this interaction results in defects reminiscent of those found in the experimental avian models. For instance, mice in which the expression of the myocardial genes VCAM1 (Kwee et al., 1995) and FOG-2 (Tevosian et al., 2000), and mice in which the expression of the epicardial genes WT1 (Moore et al., 1999) and alpha-4 integrin (Yang et al., 1995) is knocked out are all characterized by abnormal coronary development and, in most cases, hypoplastic ventricular myocardium. This has led to the general con-

cept that in the early stages of epicardial development selected genes expressed in the myocardium and the epicardium are important for the establishment of the epicardial lining of the heart and that this epicardium and the EPDCs, are, in turn, of importance for further development, maturation, and proliferation of the underlying myocardium.

Until recently, virtually everything known about the fate of EPDCs was resulting from studies in the avian heart (Manner, 1999; Wessels and Perez-Pomares, 2004; Lie-Venema et al., 2007). Quail-to-chick proepicardial explant studies demonstrated that proepicardially derived cells from the quail donor contribute to a variety of cell types, including coronary endothelium, coronary smooth muscle cells, and a subpopulation of AV cushion mesenchymal cells (Dettman et al., 1998; Wessels and Perez-Pomares, 2004). When explanted on collagen gel, proepicardial cells can undergo myocardial differentiation (Kruithof et al., 2006). However, the in ovo proepicardial explant studies never revealed a contribution of proepicardially derived cells to myocardial structures (Manner, 1999; Wessels and Perez-Pomares, 2004). The proepicardial cell fate studies in the avian heart sharply contrast the findings of recent studies in the murine system. Using two independently generated "epicardial-cre" mice (Cai et al., 2008; Zhou et al., 2008) both groups demonstrate that epicardially derived cells contribute to a subpopulation of cardiomyocytes, concentrated in the interventricular septum. In addition, both studies suggest that the contribution of epicardially derived cells to the coronary endothelium is minimal at most. This apparent discrepancy between the developmental origin of the coronary endothelium may partly be explained by the presence of Flk1-expressing, Tbx18-negative, endothelial precursors in the proepicardium at ED9.0 (Cai et al., 2008).

Similar to the situation in the developing avian heart (Manner, 1999; Wessels and Perez-Pomares, 2004; Lie-Venema et al., 2007), the murine studies also suggest a contribution of the EPDCs to the mesenchyme of the AV cushions (Cai et al., 2008; Zhou et al., 2008). These results are somewhat difficult to reconcile with the Tie2-Cre/ROSA26R endocardial cell fate studies in which all cushion mesenchymal cells appear to be of endocardial origin (de Lange et al., 2004; Snarr et al., 2007b).

Second Heart Field

Right ventricle (RV) and outflow tract (OFT).

As described above, a series of seminal papers, published over the last 10 years, has led to the, now generally accepted, concept that the RV and OFT myocardium develops from cardiogenic mesodermal precursor cells from the so-called second heart field (SHF). SHF cells have been shown to be located in a region that is anterior and medial to the primary heart field (PHF) found in the cardiac crescent (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001; Verzi et al., 2005). In recent years, studies of the SHF have been facilitated by the discovery of several genes which have SHF-specific expression patterns. Fibroblast growth factor 10 (FGF10), was identified by the analysis of a transgenic mouse with a insertional mutation in the FGF10 enhancer region, (Kelly et al., 2001). Characterization of the expression patterns of an nlacZ insert, driven by FGF10 regulatory elements indicated expression of FGF10 in the pharyngeal mesoderm, OFT and RV, observations that were confirmed by in situ hybridization (Kelly et al., 2001). Other genes, including the LIM homeodomain transcription factor Islet 1 (*Isl1*), were also shown to have a similar expression pattern within SHF cells (Cai et al., 2003; Sun et al., 2007). The characteristic expression of Isl1 in cells of the SHF is a helpful tool in establishing the contribution of the SHF to the developing heart. Immunohistochemical Isl1 labeling allows for the detection of SHF cells that are recently added to the heart. SHF cells that have been added at earlier stages cannot be detected this way as they have already lost their Isl1 expression as a result of further differentiation. It is important to note that when using Isl1-cre mice in lineage tracing experiments, both these populations are labeled and indistinguishable (Sun et al., 2007). In Figure 9 it is demonstrated that SHF cells not only contribute to the myocardial wall of the elongating OFT, but also to a subpopulation of mesenchyme in the developing OFT cushions (Fig. 9A–C,G). It is not completely clear whether these mesenchymal cells from SHF origin migrate into the cushions (cf CNDCs) or appear in the cushions as a result of an EMT from SHF-derived endocardial cells. It is important to note that the cardiac phenotype of the *Isl1*-deficient mouse includes severe abnormalities of the OFT (Fig. 9A–C), and RV (Cai et al., 2003) but that the mechanisms that lead to these malformations have not yet been elucidated. Further advances in the field were made by the identification of an Isl1-dependent SHF-restricted enhancer found for the myocyte enhancement factor gene 2c (Mef2c; Dodou et al., 2004; Verzi et al., 2005). Generation of transgenic mice with the expression of Cre driven by a SHF-expressing element have allowed for more detailed study of gene deletion specifically within the SHF. These studies have generated important insight into the molecular pathways that regulate SHF contribution to the heart, including that of canonical Wnt/β-Catenin (Ai et al., 2007; Lin et al., 2007), fibroblast growth factor 8 (Fgf8; Park et al., 2006), SHH (Lin et al., 2006; Goddeeris et al., 2007), and BMPs (Yang et al., 2006).

Dorsal mesenchymal protrusion (DMP).

To date, the majority of the published studies on the SHF have focused on its role in the development of the arterial pole of the heart. Although several gene-expression and fate-mapping studies indicated a contribution of the SHF contributed to the cardiac inflow (Cai et al., 2003; Kelly, 2005; Christoffels et al., 2006; Sun et al., 2007), until recently, very little was known about the specific role of SHF derivatives to the development of this part of the heart. Moreover, no mechanisms had been proposed to account for the inflow tract anomalies that are manifest in the absence of specific SHF-related genes (Abu-Issa et al., 2002; Cai et al., 2003; Lin et al., 2006, 2007; Yang et al., 2006; Ai et al., 2007).

As described above, our studies using the Tie2-Cre/R26R endothelial fate mapping tool, established, by

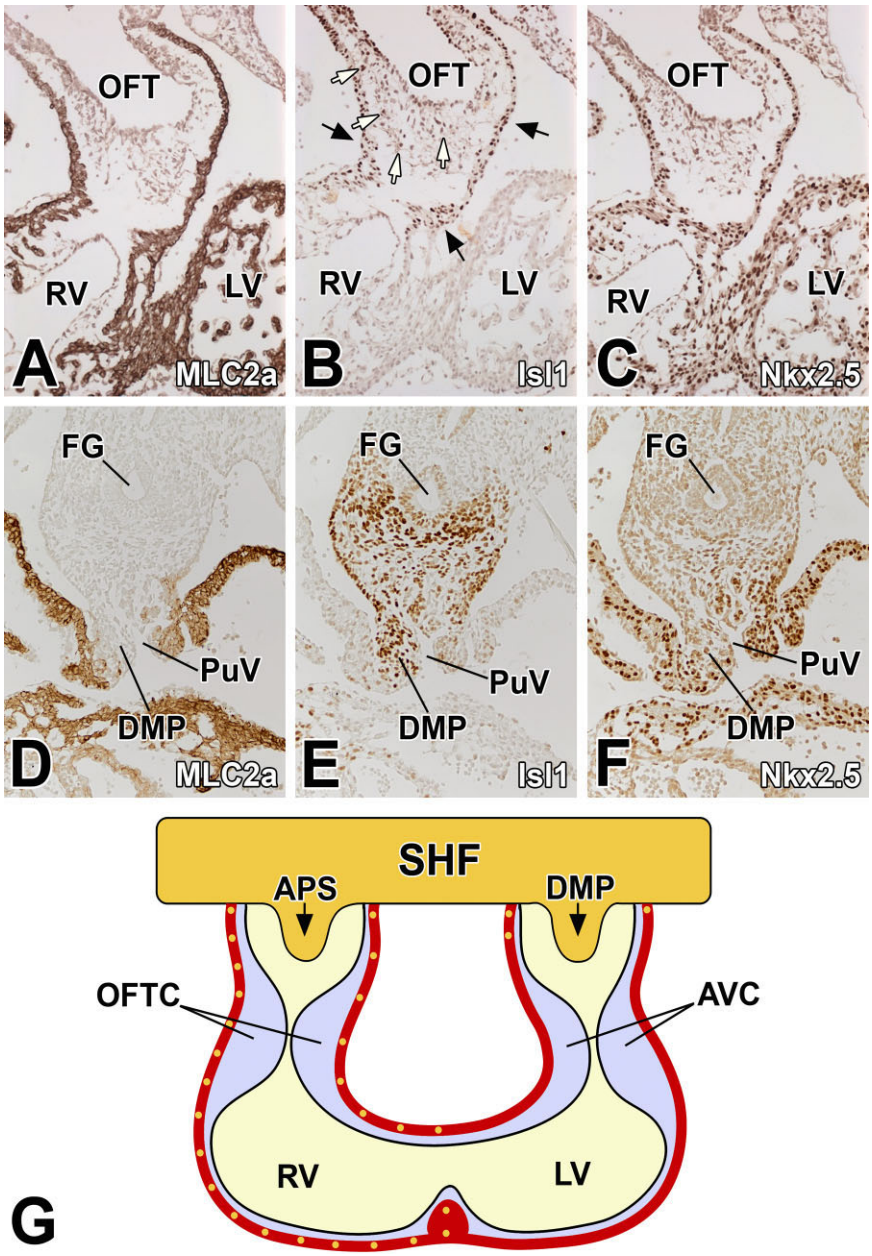

Fig. 9. Second heart field-derived mesenchyme. A–C: Transverse serial sections through an embryonic day (E) 11 mouse heart. **A,C:** The myocardium of the heart is delineated by immunostaining for MLC2a (A) and Nkx2.5 (C). **B:** Isl1 expression is found in the outflow tract (OFT) myocardium (black arrows) as well as in the OFT cushion mesenchyme (white arrows). Panels D–F: Show the SHF contribution to the venous pole of the heart. **D,E:** Immunostaining for MLC2a and Nkx2.5. Note that these markers are not expressed in the DMP. **F:** expression of the SHF marker Isl1 is observed in the foregut mesoderm as well as in the DMP which is protruding into the atrial cavity. **G:** This cartoon depicts the Isl1 positive tissues (orange) that contribute to both the arterial and venous poles of the heart. Abbreviations are the same as in previous figures.

elimination, that the DMP was not an EMT derived population of mesenchyme (Mommersteeg et al., 2006; Snarr et al., 2007b). In subsequent studies, we and others then determined that the DMP was in fact a mesenchymal derivative of the SHF (Fig. 9D–F), thus demonstrating an important role for the SHF in the formation of the atrioventricular complex (Snarr et al., 2007a; Goddeeris et al., 2008). Furthermore, we established that, in contrast to the fate of the superior and inferior AV cushions, which form the aortic leaflet of the mitral valve and septal leaflet of the tricuspid valve respectively (de Lange et al., 2004), the DMP mesenchyme undergoes a mesenchymal to myocardial differentiation (Snarr et al., 2007a), thereby forming the myocardial base of the primary atrial septum (Kim et al., 2001; Snarr et al., 2007b).

While there are many differences between the development of the cardiac outflow and inflow, it is worth mentioning that the SHF is involved in septation at both ends of the heart (Fig. 9G). In a previous study, we showed the abundant expression of Isl1 in the aorticopulmonary septum (Snarr et al., 2007a), the expression in the DMP is also well documented. It is therefore not surprising that maldevelopment of the SHF leads to abnormalities at either end of the heart.

PERSPECTIVES: TAKING CARDIAC MESENCHYME FROM BENCH TO BEDSIDE

First insights into the importance of the DMP and SHF to congenital malformations came, in retrospect, from earlier histological studies on atrioventricular septal defects (AVSDs) also known as AV canal defects and endocardial cushion defects. AVSDs are a commonly diagnosed congenital heart malformation, found in 3–7.5% of all persons with congenital heart defects (Pierpont et al., 2000; Calabro and Limongelli, 2006), and in 25% of people with Down syndrome (DS; Maslen, 2004). A Complete AVSD is characterized by an ostium primum atrial septal defect (ASD), a ventricular septal defect (VSD), and a common AV valve, leaving a persistent AV canal. For many years the etiology of this defect was largely attributed to maldevelopment of the endocardially derived AV cushion mesenchyme, which prompted the use of the term "endocardial cushion" defect, which is still used today (Rajagopal et al., 2007). Analysis of AVSDs in humans with DS and in the Trisomy 16 (Ts16) mouse model for DS provided compelling evidence that these defects may be associated with perturbed development of the mesenchyme from the cardiac inflow region corresponding to the DMP (Webb et al., 1999; Blom et al., 2003). Further characterization of DMP development in the Ts16 mouse, using SHF markers, showed clearly

that abnormal development of the SHF-derived DMP was associated with the AVSD (Snarr et al., 2007a). These findings were subsequently confirmed in other mouse models of AVSD (Goddeeris et al., 2008), and shown to be consistent with the inflow phenotypes of many SHF-deleted genes (Abu-Issa et al., 2002; Cai et al., 2003; Lin et al., 2006, 2007; Yang et al., 2006; Ai et al., 2007). This novel role for the SHF in cardiac development, contributing to AV septation by means of the DMP, represents an important paradigm shift in the etiological mechanisms of the so-called endocardial cushion defect. While the possibility that disrupted endocardial cushion development plays a role in the pathogenesis of AVSDs cannot be excluded, it is clear that cardiac mesenchyme outside of the endocardial cushions, specifically the DMP, is important for normal AV septation.

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