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The Pathogenesis of Atrial and Atrioventricular Septal Defects with Special Emphasis on the Role of the Dorsal Mesenchymal Protrusion

Laura E. Briggs[1], **Jayant Kakarla**[2], and **Andy Wessels**[1,*]

[1]Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, 173 Ashley Avenue, Charleston, South Carolina 29425, USA

[2]Institute of Genetic Medicine, Newcastle University, Newcastle Upon Tyne, United Kingdom

Abstract

Partitioning of the four-chambered heart requires the proper formation, interaction and fusion of several mesenchymal tissues derived from different precursor populations that together form the atrioventricular mesenchymal complex. This includes the major endocardial cushions and the mesenchymal cap of the septum primum, which are of endocardial origin, and the dorsal mesenchymal protrusion (DMP), which is derived from the Second Heart Field. Failure of these structures to develop and/or fully mature results in atrial septal defects (ASDs) and atrioventricular septal defects (AVSD). AVSDs are congenital malformations in which the atria are permitted to communicate due to defective septation between the inferior margin of the septum primum and the atrial surface of the common atrioventricular valve. The clinical presentation of AVSDs is variable and depends on both the size and/or type of defect; less severe defects may be asymptomatic while the most severe defect, if untreated, results in infantile heart failure. For many years, maldevelopment of the endocardial cushions was thought to be the sole etiology of AVSDs. More recent work, however, has demonstrated that perturbation of DMP development also results in AVSD. Here, we discuss in detail the formation of the DMP, its contribution to cardiac septation and describe the morphological features as well as potential etiologies of ASDs and AVSDs.

Keywords

atrioventricular septal defect; AVSD; atrial septal defect; ASD; dorsal mesenchymal protrusion; DMP; ostium primum defect; ostium secundum defect; second heart field; SHF

Introduction

Cardiac septation encompasses a complicated series of events in which structures derived from various precursor populations interact and fuse with one another to functionally and physically separate the different compartments of the four-chambered heart. Given the complexity of the process it may not be surprising that it is incomplete in a significant portion of the human population. In this review, we describe the anatomy of the

*To whom correspondence should be addressed: wesselsa@musc.edu, (843) 792-8183.

atrioventricular (AV) junction, the origin and fate of tissues involved in septation, and will also discuss cardiac malformations that occur when the septation process goes awry.

Anatomy of the Four-Chambered Heart

The fully formed heart is a midline, four-chambered organ positioned within the middle mediastinum. Its two atria, each of which contains a venous component, a body, an appendage, and an AV valve vestibule, are physically and functionally separated by an atrial septum. The atrial septum is comprised of several components including the septum primum (SP) anchored by a muscular base and the septum secundum (SS) (Fig. 1A). The muscular base of the septum primum forms the floor, or antero-inferior rim, of the oval fossa while the septum secundum contributes to its superior margin.

Defects in the Atrioventricular Septal Complex

Patent Foramen Ovale (PFO)

In the adult heart, the superior and inferior caval veins return de-oxygenated venous blood from systemic circulation to the right atrium whereas the pulmonary vein delivers oxygenated, arterial blood from the pulmonic circulation to the left atrium. During fetal development, however, gas exchange occurs in the placenta and oxygenated blood is carried back to the fetus through the umbilical vein. After bypassing hepatic circulation via the ductus venosus, oxygenated blood enters the inferior caval vein where it joins, but streams separately from, deoxygenated blood returning from lower portions of the developing fetus (Barclay et al., 1939; Edelstone and Rudolph, 1979; Lind and Wegelius, 1949). At the junction of the right atrium and the inferior caval vein, a flap of tissue termed the Eustachian valve directs the more dorsally located, oxygenated blood across an interatrial communication termed the foramen ovale. The superior boundary of this communication is formed by the septum secundum while the septum primum acts as a flap valve of the foramen, allowing unidirectional shunting of oxygenated blood from the right to left atrium. The left ventricle and ascending aorta then deliver this blood to the two tissues with the highest oxygen demand, the developing brain and heart.

Shortly after birth and the establishment of pulmonic gas exchange, increased relative left to right atrial pressure pushes the septum primum against the septum secundum. Once in contact, these two structures fuse, resulting in permanent closure of the foramen ovale and two fully partitioned atria. Thus, the foramen ovale, whether patent or closed, is important for the delivery of oxygenated blood to tissues; the fetal patency of the foramen ovale ensures that vital structures receive blood with a higher oxygen content, while its closure postnatally helps maintain arterial oxygen saturation by preventing the mixture of venous and arterial blood.

In approximately 20–34% of the population, the septum primum and septum secundum fail to fuse, resulting in patency of the foramen ovale (Fig. 1B). The presence of a patent foramen ovale (PFO) does not necessarily imply active communication between the two atrial chambers. In fact, as long as left atrial pressure exceeds that of the right atrium and if the flap valve is of sufficient size to cover the boundaries of the oval fossa, the PFO remains functionally closed. Under certain hemodynamic conditions, however, the PFO may open and thus enable thrombi, air or any other substance to pass from venous to arterial circulation. It is by this mechanism that a PFO may become pathological and result in conditions such as cryptogenic stroke, migraine with aura, and decompression illness (Anzola et al., 1999; Choong et al., 2008; Dowson et al., 2008; Kim and Girardi, 2008; Sztajzel et al., 2002; Wilmshurst et al., 1989).

Ostium Secundum Defect

If the septum primum is of insufficient size to cover the entire oval fossa, and/or if the septum secundum fails to fully form, an ostium secundum defect will result (Fig. 1C). The clinical course of patients diagnosed with an isolated ostium secundum defect is often benign and may be associated with few, if any, functional limitations. Larger, more severe ostium secundum defects may lead to right atrial volume overload due to persistent left-to-right shunting and, as a result, increased risk of complications such as atrial fibrillation and pulmonary hypertension (Clark and Kugler, 1982; Engelfriet et al., 2007; Ruschhaupt et al., 1984). Eventually, increased pulmonic resistance can cause right-sided pressure to increase; subsequently, blood is shunted from right-to-left (Eisenmenger syndrome) and systemic oxygenation decreases (Engelfriet et al., 2007).

Ostium Primum Defect

The ostium primum defect is a less common, but more severe, possible consequence of aberrant septation. Although interatrial shunting is also observed in ostium primum defects, these malformations are now considered to be part of the family of abnormalities known as atrioventricular septal defects (AVSD) and therefore, will be discussed in the next section.

Atrioventricular Septal Defect (AVSD)

AVSDs represent a spectrum of cardiac malformations and include three subtypes: incomplete AVSD, transitional AVSD and complete AVSD (Jacobs et al., 2000). While all AVSDs are characterized by a common AV valve, the variants differ with respect to the level at which shunting between chambers is permitted as well as the morphology of the common AV valve (Anderson et al., 1998a).

Incomplete AVSDs (Fig. 1D) are characterized by the presence of distinct mitral and tricuspid annuli, or left and right valvar orifices (Jacobs et al., 2000). In transitional AVSDs, fusion of the anterior and posterior bridging leaflets results in a single valvar annulus; however, attachment of these leaflets to the ventricular septum creates, in effect, two distinct valvar orifices. In contrast, complete AVSDs (Fig. 1E) are characterized by the presence of a single, common AV valve orifice (Jacobs et al., 2000). All three variants generally demonstrate interatrial shunting due to defective septation between the inferior margin of the septum primum and the atrial surface of the common atrioventricular valve; however, shunting at the ventricular level will also be permitted if the bridging leaflets are attached to the leading edge of the atrial septum (Anderson et al., 1998a) (Fig. 1E). In transitional AVSDs, attachment of the common valve to the ventricular septum assists in separation of the right and left ventricle while in complete AVSDs, shunting at the ventricular level is freely permitted through a defect between the ventricular surface of the common valve and the interventricular septum (Jacobs et al., 2000).

Within these defects, the size of the ventricles is variable but important in determining whether the patient is a candidate for biventricular surgical repair (Bharati and Lev, 1973; Jegatheeswaran et al., 2010). In balanced AVSDs, both ventricles are appropriately sized while in unbalanced AVSDs, one ventricle is so small that the patient must be managed as having a functionally univentricular heart (Bharati and Lev, 1973; Jegatheeswaran et al., 2010). Complete AVSDs may also be classified according to the Rastelli classification system, which is based on the morphology, degree of bridging and chordal attachments of the superior leaflet (Rastelli et al., 1966).

The clinical presentation of AVSD is not uniform and depends on the size of the defect, the degree of shunting as well as the presence of other malformations. In general, untreated complete AVSD results in congestive heart failure within the first few months of life while

the signs and symptoms of incomplete AVSD are subtler and may not manifest until later in life (Minich et al., 2010). Management of these defects requires surgical correction, which may be complicated by the presence of other cardiac abnormalities including dysplastic AV valve leaflets, isomeric atrial appendages, and tetralogy of Fallot or double outlet right ventricle (Shuhaiber et al., 2009).

Etiology of Abnormal Septation

Cardiac malformations, including septal defects, often occur in the context of a syndrome and in association with other abnormalities (Table 1). Holt-Oram syndrome, which results from mutations in *TBX5*, is characterized by atrial and ventricular septal defects as well as upper limb abnormalities (Basson et al., 1997; Li et al., 1997). Heterotaxy syndromes, or left-right laterality defects, often include septal defects as well as isomerism of the atrial appendages and may result from dysregulation of pathways involved in left-right patterning, such as *PITX2*, *SHH* and *NODAL* (Cunningham et al., 1998; Isaac et al., 1997; Levin et al., 1995; Piedra et al., 1998). 3p deletion syndrome, while rare, results in severe defects including AVSD as well as other congenital heart defects, microcephaly, low birth weight, and hypotonia; prior work has implicated *CRELD1* and/or *CRELD2* as the cause of atrioventricular septal defects in this deletion syndrome (Green et al., 2000; Robinson et al., 2003; Rupp et al., 2002).

The most common genetic syndrome, Down syndrome (trisomy 21), is strongly associated with atrioventricular septal defect; approximately one-quarter of children with Down syndrome possess this malformation and roughly two-thirds of complete AVSD occur in association with trisomy 21 (Barlow et al., 2001). However, not all children with Down syndrome possess the defect, suggesting that its pathogenesis is multifactorial and may involve multiple genetic modifiers and/or an environmental component.

Genetic mutations may also result in non-syndromic septal defects, defects that occur in isolation or in absence of phenotypic features typically associated with a syndrome (Table 2). Non-syndromic AVSDs, which are estimated to occur in approximately one per 10,000 live births, are thought to occur as a result of multifactorial inheritance or sporadic mutations (Carmi et al., 1992; Sheffield et al., 1997). *GATA4, CRELD2, BMP4, ALK2 and CFC1* are all genes suggested to play a role in non-syndromic AVSD (Garg et al., 2003; Maslen, 2004; Roessler et al., 2008; Smith et al., 2009). Ostium secundum defects have been linked to mutations in *GATA4,* its binding partners *NKX2-5* and *TBX5*, and the downstream transcription target of *GATA4* and *TBX5*, *MYH6* (Biben et al., 2000; Ching et al., 2005; Garg et al., 2003; Okubo et al., 2004; Sarkozy et al., 2005; Schott et al., 1998).

Between 1981 and 1989, a large case-control study, the Baltimore-Washington Infant Study, was conducted to assess risk factors for congenital cardiovascular defects. This study, which included 363 infants with AVSD (210 of whom were also diagnosed with Down syndrome), provided invaluable information regarding the contribution of non-inherited risk factors to the pathogenesis of septal defects. The results of this study, as well as others, are summarized here and in table 3. Although the mechanism is not fully understood, pre-gestational diabetes significantly increases the risk of both syndromic (Loffredo et al., 2001b) and non-syndromic complete AVSD (Loffredo et al., 2001a). Other potential risk factors for AVSD include urinary tract infection during the first trimester of pregnancy (Cleves et al., 2008), maternal use of ibuprofen (Ferencz et al., 1997), moderate to heavy maternal cigarette use (Ferencz et al., 1997; Malik et al., 2008), maternal use of cocaine (Ferencz et al., 1997) and maternal exposure to paint, varnishes, and ionizing radiation (Cleves et al., 2008; Ferencz et al., 1997; Malik et al., 2008). Ostium secundum defects have been linked to pre-pregnancy obesity (Gilboa et al., 2010), maternal use of β blockers

(Caton et al., 2009), alcohol (Tikkanen and Heinonen, 1991), cigarette use (Kallen, 1999), and maternal age greater than 34 (Ferencz et al., 1997).

Much like their morphological features, classification, and clinical presentation, the etiology of septal defects is complex. These defects may arise from perturbation of any one component of the AV septal complex and development of each component is governed by multiple molecular pathways. In order to better understand the pathogenesis of these complicated defects, it's important to review formation of the heart, the development of the AV canal and the components that comprise the AV septal complex.

Early Heart Development

In the vertebrate embryo, the heart is the first organ to become functional; its development commences at gastrulation with the formation of precardiac mesoderm. Cells composing this middle embryonic germ layer then migrate anterolaterally to establish bilateral primary heart fields. The two heart fields eventually combine to form the cardiac crescent and, as the mesodermal cells differentiate into myocardial and endocardial progenitors, the primary heart tube (Abu-Issa and Kirby, 2007). The linear tube is suspended from the foregut endoderm by the dorsal mesocardium and is oriented such that the arterial pole (outflow) is cranial to the venous pole (inflow), with bilateral venous tributaries draining into the latter (Drake et al., 2006; Manner, 2004). The two poles of the linear tube soon receive contributions from a second cardiogenic population; this population of cells is termed the Second Heart Field (SHF) and its addition leads to pronounced elongation of the linear tube (Kelly and Buckingham, 2002; Mjaatvedt et al., 2001; Moorman et al., 2007; Snarr et al., 2007a; Waldo et al., 2001; Zaffran and Kelly, 2012).

Soon thereafter, the central portion of dorsal mesocardium suspending the linear tube disintegrates, enabling the primary heart tube to loop to the right (Manner, 2004). Looping of the primary heart tube results in a more cranially and dorsally positioned inflow region. This inflow region, or venous pole of the primary heart tube, gives rise to the symmetrical common atrium which receives blood from the developing embryo through bilateral venous tributaries, the right and left superior caval veins.

At this point, the common atrium is separated from the left ventricle by the AV canal but the various components are still arranged in series. In order to progress from this "looped-tube" structure to a more mature, four-chambered configuration, changes must occur along the inner and outer curves of the looped heart. Along the outer curve, serial ballooning gives rise to the apical portions of the right and left ventricles (Davis, 1927; Lamers and Moorman, 2002; Moorman et al., 2003). It is through remodeling events along the inner curvature and at its junction with the AV canal and the outlet component that the atrial cavities come to be shared between ventricles. While the left atrium is able to communicate with the left ventricle from the outset, communication between the right atrium and the right ventricle requires expansion of the AV canal, the right side of which becomes incorporated into the right atrium as the vestibule of the tricuspid valve (Wessels et al., 1992).

Within just one embryonic day in murine development, the atrial entry point of the caval veins shifts from a caudal to dorsal position (Soufan et al., 2004). The junction between the systemic venous tributaries and the developing right atrium can be visualized by the appearance of the venous valves, bilaminar structures that merge cranially to form the septum spurium in the roof of the right atrium (Anderson et al., 2006). Simultaneously, the systemic venous tributaries are enveloped by primary myocardium as they are incorporated into the pericardial cavity (Soufan et al., 2004).

Eventually, the caval veins are further enclosed by primary myocardium and more caudally, canalization of the pulmonary pit leads to communication between the pulmonary vein and atrial cavity (Soufan et al., 2004). It should be noted that mouse and man differ with respect to the venous pole of the heart. In humans, the left caval vein regresses with the proximal portion giving rise to the coronary sinus while the rest of it forms the ligament of Marshall and the oblique vein (Wessels and Sedmera, 2003); if it fails to do so, persistent left superior caval vein results. In the mouse, however, persistence of the left superior caval vein is normal; the vessel is joined by the azygous vein as well as several cardiac veins before it drains into the right atrium (Wessels and Sedmera, 2003).

Components of Atrial and Atrioventricular Septation

Atrioventricular cushions

The primary heart tube consists of an outer layer of myocardium separated from an inner layer of endocardium by an acellular, extracellular matrix or, cardiac jelly. Around 8.5ED, when the linear tube begins to loop, the myocardium in the outflow tract and at the junction of the atria and left ventricle begins to secrete extracellular matrix components, including chondroitin sulfate and hyaluronan (DeVlaming et al., 2012; Kruithof et al., 2012; Manasek, 1970; Manasek et al., 1973). The production of lipophilic glycosaminoglycans results in localized formation of AV, or endocardial, cushions (DeVlaming et al., 2012; Kruithof et al., 2012; Manasek, 1975; Markwald et al., 1977; Markwald and Smith, 1972). The endocardial cells lining these cushions are unique in that they respond to growth factors released by underlying myocardium; after receiving these inductive cues, they undergo an epithelial-to-mesenchymal transition (EMT) and then migrate away from the surface and into the cushions (DeVlaming et al., 2012; Kruithof et al., 2012; Mjaatvedt et al., 1987; Runyan and Markwald, 1983).

This process of epithelial-to-mesenchymal transition results in the mesenchymalization of the two major endocardial cushions of the AV canal: the superior (ventral) endocardial cushion that is associated with the inner curvature of the looped heart and the inferior (dorsal) endocardial cushion that forms along its outer curvature. The two major cushions then expand and eventually fuse, separating the heart into left and right AV orifices. Following development of the major endocardial cushions, the lateral cushions appear later as lateral swellings of mesenchyme at the junction between the atria and ventricles (de Lange et al., 2004; Wessels et al., 1996). Each of the four cushions plays an important role in AV valvuloseptal development; the superior and inferior AV cushions contribute to the aortic leaflet of the mitral valve and the septal leaflet of the tricuspid valve (Snarr et al., 2008). The lateral cushions also make significant contributions to the AV valves; the right lateral cushion contributes to the anterosuperior and inferior leaflets of the tricuspid while the left lateral cushion is involved in formation of the mural leaflet of the mitral valve (Snarr et al., 2008).

Despite their significant contribution to valvuloseptal development, the lateral AV cushions have been largely ignored in most experimental studies and thus, relatively little is known regarding the molecular mechanisms that govern their delayed formation and maturation. Further studies comparing development of the major and lateral cushions are certainly needed as recent work indicates that the two are fundamentally different (Wessels et al., 2012).

Proper formation of the major cushions, which are centrally located between the interventricular septum and interatrial septum, is critical to cardiac septation as all components of the AV septal complex eventually fuse to these structures. Cushion abnormalities are observed in a number of genetically modified mouse models, including

neurofibromin-1, retinoid X receptor alpha, and hyaluronan synthase-2 knockout mice (Camenisch et al., 2000; Gruber et al., 1996; Lakkis and Epstein, 1998). While defects of the endocardial cushions are variable with respect to etiology and morphological features, they are usually severe and result in embryonic lethality.

The Atrial Septa

The septum primum makes its first appearance in the looped heart as a muscular crescent in the roof of the common atrium. This true septal structure is capped on its leading edge by mesenchyme and protrudes into the atrial cavity toward the fusing endocardial cushions (Figs. 2A– D′). As it proceeds toward the superior and inferior endocardial cushions, the left and right atrial chambers communicate via the ostium primum. Eventually, the mesenchymal cap of the septum primum fuses with the endocardial cushions (Fig. 2C′), but before it does so, fenestrations form in the dorsal portion of the septum, at the site of its attachment to the atrial roof (Figs. 2C′, D′). This results in formation of the ostium secundum and the preservation of interatrial communication (Figs. 2E, E′). Fusion of the septum primum to the AV cushions and concomitant detachment from the roof of the atrial cavity enables the muscular septum primum to act as the flap valve of the oval foramen (Figs. 2E, E′).

Following formation of the ostium secundum, an additional structure begins to develop at its cranial margin, the so-called septum secundum. In the developing human, the septum secundum is not a true septum; rather, it is formed by an infolding of dorsal atrial myocardium. This septum secundum does not become pronounced until the second trimester, by which time the right pulmonary veins have migrated to reach their definitive position in the atrial roof. By forming the superior boundary of the oval fossa, the septum secundum provides a structure against which the flap valve can close in postnatal life. In the mouse, the septum secundum, although small, is a true septum (Figs. 2E, E′). Thus, closure of the ostium secundum is largely accomplished by fusion of the cranial portion of the septum primum with the atrial wall.

Prior work indicates that the septum primum is a left-sided structure as the left-sided markers creatine kinase-B (CK-B) and Pitx2c are expressed within its myocardium (Meno et al., 1998; Wessels et al., 2000). Consistent with the importance of Pitx2c to left-sided development, the interatrial septum fails to properly develop in mice deficient for this transcription factor (Figs. 5B, D, D′). Absence of PITX2C results in isomerism of the right atrial appendages, AVSD, and abnormalities of the ventricular outflow tracts (Liu et al., 2002) (Figs. 5B, D, D′). The molecular mechanisms that drive the formation of the septum primum from a left-sided precursor population, however, are not fully understood.

In humans, mutations in several genes, including *NKX2-5, GATA4 and TBX20*, have been linked to atrial septal defects and patency of the foramen ovale (Garg et al., 2003; Hirayama-Yamada et al., 2005; Kirk et al., 2007). Mice carrying heterozygous *Nkx2-5* mutations demonstrate defective morphogenesis of the atrial septum, resulting in ostium secundum atrial septal defects and, secondary to increased mechanical stress, postnatal aneurysm of the septum primum (Biben et al., 2000). Adult mice heterozygous for *Tbx20*, which genetically and/or physically interacts with both *Nkx2-5* and *Gata4*, also demonstrate abnormalities of the atrial septum, persistent foramen ovale, aneurysm of the septum primum, and dilated cardiomyopathy (Brown et al., 2005; Stennard et al., 2003; Stennard et al., 2005). Heterozygous *Gata4* mice on an inbred C57 genetic background display severe cardiac anomalies including ostium secundum defect, AVSD and ventricular septal defect. Interestingly, these defects are not observed in mice on a mixed genetic background (Bisping et al., 2006; Pu et al., 2004; Rajagopal et al., 2007). Other mouse models with

defective development of the septum primum include *Pdgfrα*$^{-/-}$ and *podoplanin*$^{-/-}$ mutants (Bax et al., 2010; Mahtab et al., 2009)(Douglas et al., 2009).

Although the septum primum is an important component of the AV septal complex, its development does not, at least upon superficial inspection, seem to be perturbed in all murine models of AVSD. Furthermore, abnormal development of the septum primum is responsible for defects located within the oval fossa (ostium secundum defects), and does not produce the ostium primum defect, indicating that maldevelopment of an additional structure, or structures, may be necessary for the pathogenesis of AVSD.

Dorsal Mesenchymal Protrusion

Nomenclature—The looping primary heart tube is attached to the developing embryo by mesocardial tissue located at its venous and arterial poles (Drake et al., 2006). Soon after looping is complete, the dorsal mesocardium persisting at the venous pole provides the portal of entry for an extracardiac population of mesenchyme to protrude into the common atrium (Figs. 3A, B). Like the endocardial cushions and cap of the septum primum, this structure, the dorsal mesenchymal protrusion (DMP), contributes to the AV mesenchymal complex (Snarr et al., 2007b; Wessels et al., 2000). The DMP resembles the structure first identified by Wilhelm His the elder as the “spina vestibuli” (His, 1880), or vestibular spine. Based on a series of immunohistochemical studies, we introduced the term DMP as it more accurately reflects the anatomical and histological characteristics of the structure (Wessels et al., 2000).

The DMP is Derived From the Second Heart Field—Two bilateral primary heart fields coalesce to form the linear heart tube (Abu-Issa and Kirby, 2007). Over the last decade, an additional or “second” cardiogenic mesodermal precursor population, located anteromedial to the primary heart field in the cardiac crescent, has been identified as an important component of heart development (Abu-Issa and Kirby, 2007; Kelly and Buckingham, 2002; Mjaatvedt et al., 2001; Moorman et al., 2007; Snarr et al., 2007a; Waldo et al., 2001; Zaffran and Kelly, 2012). This population, termed the second heart field (SHF), significantly contributes to the arterial pole of the developing heart (Abu-Issa and Kirby, 2007; Cai et al., 2003; Kelly and Buckingham, 2002; Mjaatvedt et al., 2001; Verzi et al., 2005; Waldo et al., 2001; Zaffran and Kelly, 2012).

While the significance of the SHF to arterial pole development has been established for many years, more recent studies have revealed that this population of cells is also involved in development of the DMP (Snarr et al., 2007a). Earlier cell fate studies demonstrated that the DMP, unlike the mesenchyme of the AV cushions and cap of the septum primum, is not derived from the endocardium (Snarr et al., 2007b). Subsequent work revealed expression of the SHF-marker Isl1 within the developing DMP (Figs 3C, D).

Relationship to other AV Septal Components—After cardiac looping is complete, an endothelial invagination into the dorsal mesocardium demarcates the future orifice of the pulmonary vein (Lamers and Moorman, 2002). This site is flanked by the mesocardial reflections, two symmetrical structures that are also referred to as the pulmonary ridges (Soufan et al., 2004; Webb et al., 1998; Wessels et al., 2000) (Fig. 3A). The SHF population that gives rise to the dorsal mesenchymal protrusion resides just dorsal to these mesocardial reflections, in between the foregut and atrium, and can be visualized as a highly proliferative, ISL1 expressing population of mesenchyme (Snarr et al., 2007a) (Fig. 3A).

Soon thereafter, around 10.5ED, this prong of mesenchyme, which is continuous with the mesenchymal cap of the septum primum cranially, protrudes into the common atrium, to the right of the developing pulmonary vein (Snarr et al., 2007b; Webb et al., 1998) (Figs. 3B,

D). By 11.5ED, all mesenchymal components of the AV septal complex have formed and the stage is set for their interaction (Webb et al., 1998). At this point, the major AV cushions are in close proximity but have not yet fused. Therefore, it is the DMP alone that bridges the gap between two endocardially-derived tissues; it is continuous with the inferior AV cushion ventrally as well as the mesenchymal cap of the septum primum cranially (Snarr et al., 2007b; Wessels et al., 2000) (Fig. 4A). The cap, in turn, is contiguous with the superior AV cushion (Snarr et al., 2007b) (Fig. 4A). While all three mesenchymal tissues that contribute to the AV septal complex are in continuity and are beginning to fuse, the ostium primum has yet to fully close (Fig. 2D, D′).

By 13ED, however, fusion of the major cushions, the DMP and the cap of the septum primum results in closure of the primary atrial and the formation of separate right and left valvar orifices (Snarr et al., 2007b). Using the Tie2-Cre/R26R reporter system, the DMP can be visualized within this mesenchymal mass of mostly endocardially-derived tissues as a lacZ-negative, non-endocardial-derived population of mesenchymal cells wedged between the superior and inferior AV cushions dorsally (Snarr et al., 2007b) (Fig. 4B). Around this developmental stage, the cells comprising the DMP begin to undergo a mesenchymal-to-myocardial differentiation which is associated with a decrease in the level of ISL1 expression and an increase in expression of NKX2-5 (Snarr et al., 2007a) (Figs. 3E–G). This mesenchymal-to-myocardial differentiation of SHF-derived cells which, within the developing heart, only occurs in cells derived from the DMP, is important for maturation of the AV septal complex as it is the mechanism by which the muscular base of the septum primum, the rim of the oval fossa, is formed (Snarr et al., 2007a).

Potential Mechanisms Leading to Abnormal Development of the DMP—Prior studies have clearly indicated that the DMP is an essential component of the AV septal complex and thus, AV septation, but the molecular mechanisms underpinning its proper development and maturation are not fully understood. What is known, however, is that within less than half a day of murine embryonic development, a relatively large population of SHF cells protrudes from the area surrounding the foregut, past the mesocardial reflections and into the common atrium (Snarr et al., 2007b; Webb et al., 1998). This rapid expansion of cells is associated with intense proliferation of the SHF cells that ultimately give rise to the DMP (Fig. 4C). If this precursor population fails to proliferate, then the DMP may be hypoplastic (Fig. 4D). If the DMP fails to form, or if its proportions are abnormally small, it will be unable to partition the dorsal-inferior region of the AV junction.

Aberrant apoptosis is another mechanism that could contribute to maldevelopment of the DMP. At 13ED, after the DMP has fused with the inferior AV cushion, TUNEL staining demonstrates that programmed cell death occurs at the interface of these two mesenchymal tissues and within the DMP itself (Snarr et al., 2007b). If apoptosis were to occur prematurely, either within the DMP or within its SHF precursor population, the contribution of the DMP to the AV septal complex may be absent or abnormally small (Fig. 4E). Such apoptosis may also contribute to the formation of the so-called vestibular atrial septal defect (Sharratt et al., 2003).

The exact mechanism(s) by which the DMP protrudes into the common atrium have yet to be elucidated but may involve cell migration. Collective cell migration, the process by which a group of cells is transported to a new location, is important for organogenesis as well as numerous other developmental processes. While both the distance these cells travel as well as the number of cells involved is highly variable, an extracellular cue is generally required to initiate cell migration (Affolter and Weijer, 2005). Growth factors, cytokines and/or proteins from the extracellular matrix can act as this stimulus and may originate from a neighboring cell, and/or the extracellular matrix. These cues then initiate intracellular

signaling pathways, including Rho, Rac and other members of the small GTPase family. Small GTPases facilitate migration by stimulating extension of lamellipodia, turnover of cell-substrate adhesion, and cell body contraction (Allen et al., 1997; Nobes and Hall, 1995; Zhao et al., 2000). If the cue that stimulates migration is not released and/or received, or if any other step involved in this complicated process is altered, collective cell migration will not occur and therefore, incomplete cardiac septation will result.

NKX2-5 is widely expressed in the developing heart but notably, is absent from the DMP as this group of ISL1-positive cells protrudes into the atrial cavity. After the DMP has fused with the other components of the AV septal complex, the DMP begins to muscularize (Snarr et al., 2007a). At this point, although many cells of this SHF-derived population are still ISL1 positive, a significant number also express NKX2-5 (Snarr et al., 2007a). Eventually, ISL1 expression is completely abolished as the DMP terminally differentiates into a myocardial phenotype, thereby forming the muscular base of the septum primum (Snarr et al., 2007a). In order for the DMP to migrate from the area surrounding the foregut to its final position within the heart, it must maintain a mesenchymal phenotype. If the cells that form the DMP prematurely differentiate into a myocardial phenotype, they may be unable to migrate into the atrial cavity and contribute to the AV septal complex (Goddeeris et al., 2008) (Fig. 4F).

Mouse Models of DMP Maldevelopment—For many years, maldevelopment of the endocardial cushions was thought to be the primary etiology of atrioventricular septal defects. More recently, however, analysis of AVSDs in mouse models, as well as observations in human fetuses with Down Syndrome (Blom et al., 2003), have revealed that defective development of the DMP also contributes to the pathogenesis of these defects.

AVSD is part of the spectrum of cardiac malformations observed in the Trisomy 16 mouse. (Epstein et al., 1985; Miyabara et al., 1982; Waller et al., 2000; Webb et al., 1999). Hypoplasia of the DMP has been implicated in the pathogenesis of this malformation in this mouse model (Snarr et al., 2007a; Webb et al., 1999). Although the mesenchymal cap of the septum primum is both substantial and continuous with the superior endocardial cushion in these mutants (Webb et al., 1999), septation is deficient caudally, where the DMP normally bridges the space between the cap and the inferior endocardial cushion. Consequently, these mice demonstrate AVSD as well underdevelopment of the septum primum. While the process of septation is obviously abnormal in trisomic specimens, the bridging leaflets of the common atrioventricular valve can be fused to one another, suggesting that fusion of the superior and inferior endocardial cushions is not necessarily aberrant in these mice (Webb et al., 1999). The cushions are, however, abnormal with respect to shape and volume and thus, may still play a role in the pathogenesis of AVSDs in the Ts16 mouse (Hiltgen et al., 1996; Webb et al., 1996; Webb et al., 1999).

Although polysomy in both the mouse (Ts16) and human (Ts21) is associated with AVSD, abnormalities involving the ventricular outflow tracts are frequently observed in only the murine version and include common arterial trunk, double outlet right ventricle, interrupted aortic arch and overriding aorta (Waller et al., 2000). Furthermore, the lesions seen in man and mouse differ with respect to valvar morphology as well as the connection between the atrioventricular junction and the ventricles (Anderson et al., 1998b).

The additional cardiac lesions observed in the murine model of Down syndrome may result from fundamental differences in which genes are triplicated. For example, the most widely used murine model of Down syndrome, Ts65Dn, is trisomic for only part of the Hsa21 syntenic region on Mmu16 (Li et al., 2007). Furthermore, the Ts65Dn mouse is trisomic for a region on Mmu17 that does not correspond to any region on Hsa21(Li et al., 2007).

Regardless of whether the Ts16 mouse is a perfect model for studying cardiac malformations observed in the context of Down's syndrome, morphological analysis of this transgenic mouse has provided invaluable insight into the role of the DMP in the pathogenesis of AVSD.

Recently, the Morrisey lab described defective development of the DMP and resultant AVSD in *Wnt2*$^{-/-}$ mutants (Tian et al., 2010). Prior work had demonstrated that Wnt signals through β-catenin to maintain ISL1 positive SHF progenitors at the anterior, or arterial, pole of the heart (Ai et al., 2007; Cohen et al., 2007; Kwon et al., 2007; Lin et al., 2007). Through subsequent experiments, it was determined that the ligand WNT2 regulates expansion of SHF progenitors at the venous pole of the heart, through a β-catenin dependent signaling mechanism (Tian et al., 2010). Importantly, this ligand is expressed in a manner that is spatiotemporally consistent with development of the DMP but is not expressed within the epicardium, endocardium, or myocardium of the AV canal or ventricles (Tian et al., 2010). Global deletion of *Wnt2* leads to diminished numbers of ISL1+ SHF progenitors and reduced proliferation within the DMP precursor population; consequently *Wnt2*$^{-/-}$ mutants demonstrate hypoplasia of the DMP and AVSD (Tian et al., 2010). Interestingly, treatment of pregnant females with LiCl, an activator of Wnt/β-catenin signaling (Klein and Melton, 1996), at a stage consistent with DMP development rescues DMP proliferation in *Wnt2*$^{-/-}$ mutants, as well as the AVSD phenotype, and increases survival by approximately 5-fold (Tian et al., 2010). These results indicate that *Wnt2* signaling is required for proliferation and expansion of the SHF population that gives rise to the DMP and confirms what had been described in the Ts16 mouse, that maldevelopment of the DMP is associated with AVSD.

In addition to defective development of the atrial septum, *Pdgfr*α$^{-/-}$ embryos also display abnormal development of the DMP. PDGFR-α is expressed within the SHF at the venous pole of the heart and is proposed to interact with NKX2-5 (Bax et al., 2010; Prall et al., 2007). Consistently, *Pdgfr*α$^{-/-}$ embryos demonstrate a hypoplastic DMP and increased expression of NKX2-5, suggesting that loss of PDGFR-α results in premature myocardial differentiation of the DMP (Stennard et al., 2003).

Using global knockout mice to study molecular mechanisms governing DMP development is often hampered by early embryonic lethality, which may be extracardiac in origin. Furthermore, due to lack of specificity, global knockouts are less informative for understanding molecular mechanisms that drive formation of a particular structure; if the DMP fails to develop in a traditional knockout, is it due to altered signaling within the DMP itself or does it stem from altered signaling in a nearby structure (i.e. mesocardial reflections) that contributes to DMP development through paracrine signaling? Many of these issues have been circumvented by the advent of site-specific recombinase technology, which has enabled the creation of transgenic mice whose genomes may be altered in a tissue and/or temporal-specific manner.

Gene expression within the SHF-derived DMP can be manipulated specifically through use of the *Mef2c*-AHF-Cre mouse, a transgenic mouse in which cre-recombinase is expressed within the SHF (Figures 4G and G′). This approach was used by Goddeeris et al. to determine the role of sonic hedgehog (Shh) signaling in the developing DMP (Goddeeris et al., 2008). Prior studies have implicated this signaling pathway in cardiac septation as *Shh*$^{-/-}$ mutants demonstrate AVSD (Goddeeris et al., 2008). Furthermore, loss of *Shh* from the pharyngeal endoderm, which is juxtaposed to the dorsal mesocardium during development of the DMP, also results in AVSD (Goddeeris et al., 2007).

First, to determine whether AV septation was dependent upon Shh signaling within the myocardium and/or endocardium, the Shh receptor smoothened (Rajagopal et al.) was

conditionally deleted respectively, through generation of *TnT-cre;Smo$^{flox/-}$* and/or *Tie2-cre;Smo$^{flox/-}$* mutants. These mutants did not demonstrate defective septation, indicating that AV septation is not dependent on myocardial and/or endocardial *Shh* signaling (Goddeeris et al., 2008). However, AVSDs are observed upon deletion of *Smo* from the developing DMP (Figs. 5E– H′), further illustrating the important role this structure plays in AV septation (Goddeeris et al., 2008).

While the contribution of the DMP to the AV septal complex at 11.5ED was less extensive in *Mef2c-AHF-cre; Smo$^{flox/-}$* mutants when compared to controls, neither proliferation nor apoptosis within the dorsal mesocardium differed significantly between groups (Goddeeris et al., 2008). Eventually the DMP muscularizes, but only after it has migrated into the atrial cavity and fused with the other components of the AV septal complex (Snarr et al., 2007a). Interestingly, Goodeeris et al. found ectopic myocardialization within the dorsal mesocardium of *Mef2c-AHF-cre; Smo$^{flox/-}$* mutants. Furthermore, they describe decreased migration of explanted dorsal mesocardium upon treatment with the small molecular Smo inhibitor cyclopamine (Goddeeris et al., 2008). These results indicate that *Shh* signaling may be required to prevent premature mesenchymal-to-myocardial transition of the DMP and/or its proper migration.

Summary

Over the course of fetal development, the heart transforms from little more than a linear tube to a complex, four-chambered organ. Partitioning of these chambers requires the proper spatiotemporal formation, interaction, and fusion of several structures including the septum primum with its mesenchymal cap, the septum secundum, the atrioventricular cushions, and the dorsal mesenchymal protrusion.

The most severe consequence of abnormal septation is the AVSD. For decades, perturbation of endocardial cushion development was thought to be the primary etiology of these malformations, so much so that the term “endocardial cushion defect” has become synonymous with AVSD. A growing body of evidence, however, has demonstrated that perturbation of the DMP can also lead to AVSD. It is easy to understand how this structure may have been overlooked; the anatomical features of the venous pole of the developing heart are often difficult to identify and/or interpret, even in *normal* embryos. As our ability to manipulate the murine genome and analyze the 3-dimensional architecture of the developing heart improves, the role this structure plays in both future, and established, mouse models of AVSD requires further study as do the molecular mechanisms underpinning its proper formation and maturation.

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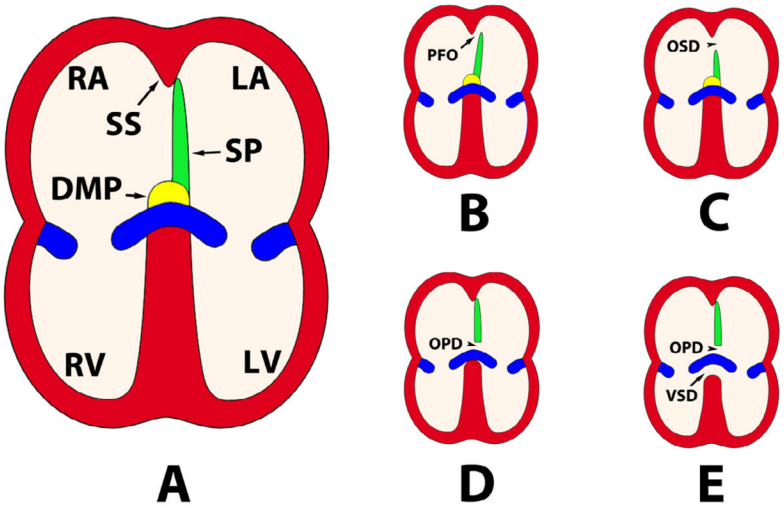

Figure 1. Illustration of Atrial and Atrioventricular Septal Defects

In Figure 1A, a mature, properly septated heart is depicted. The septum primum (green) has fused to the septum secundum (red), resulting in closure of the ostium secundum while interaction of the DMP (yellow), septum primum and major endocardial cushions (blue) results in closure of the ostium primum (OP). In Figure 1B, a patent foramen ovale is illustrated. Here, although the septum primum has not fused to the septum secundum, under normal hemodynamic conditions the two remain in contact to functionally partition the atria. If right atrial pressure were to exceed that of the left atrium, however, right-to-left shunting would result. In Figure 1C, an ostium secundum defect (OSD) is depicted; the flap valve (green) of the ostium secundum is not of sufficient length to prevent atrial communication. Figure 1D depicts an incomplete AVSD, a malformation in which atrial shunting is freely permitted due to persistence of the ostium primum. Complete AVSDs (1E) are characterized by both atrial and ventricular shunting is permitted. RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; DMP, dorsal mesenchymal protrusion; SP, septum primum; SS, septum secundum; OSD, ostium secundum defect; OPD, ostium primum defect; PFO, patent foramen ovale; VSD, ventricular septal defect

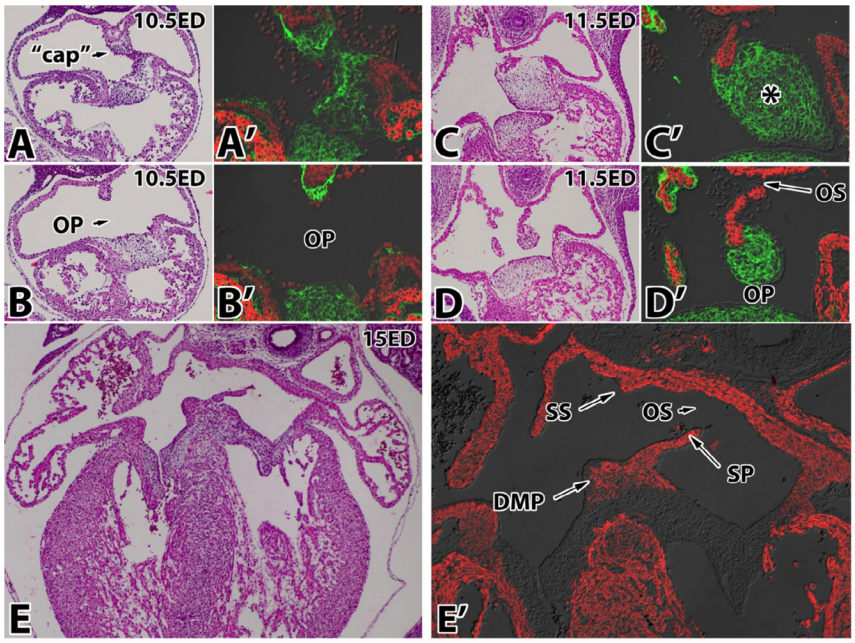

Figure 2. Interaction of the mesenchymal cap of the septum primum and the endocardial cushions

At 10.5ED, the AV canal is positioned to the left of midline (2A). The septum primum is capped by mesenchyme and protrudes toward the major endocardial cushions (2B). At this stage, the ostium primum is patent while the ostium secundum has yet to develop (2B). By 11.5ED, the mesenchymal cap of the septum primum has fused with the sAVC (*2C′). The ostium primum has yet to fully close while the ostium secundum is already forming, as demonstrated by the fenestrations in the septum primum (2D′). Figures 2E and 2E′ depict a specimen at 15ED; all components of the AV septal complex have fused. The septum secundum has formed, the ostium primum is fully closed and right to left shunting is permitted through the ostium secundum only. The septum primum acts as the flap valve of the foramen ovale and shortly after birth, will fuse with the septum secundum to fully partition the atria. MC, mesenchymal cap; SP, septum primum; SS, septum secundum; OP, ostium primum; OS, ostium secundum; DMP, dorsal mesenchymal protrusion

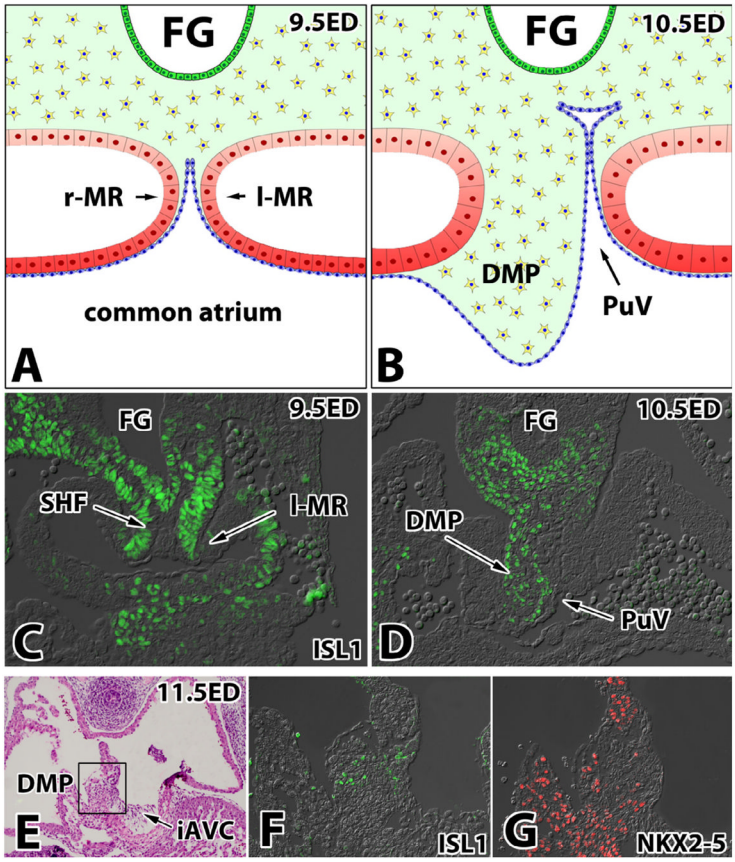

Figure 3. The DMP is derived from the SHF and eventually muscularizes to form the floor of the oval fossa

At 9.5ED (3A, 3C), the SHF that gives rise to the DMP is located just dorsally to two symmetrical mesocardial reflections that flank the orifice of the primitive pulmonary vein. By 10.5ED (3B, 3D), the DMP protrudes past these reflections, to the right of the developing pulmonary vein and into the common atrium. The precursor population that gives rise to the DMP (3C) as well as the DMP itself (3D) can be visualized by staining with the known SHF marker Isl1. The DMP, which is contiguous with the mesenchymal cap of the septum primum has fused with the iAVC (3E) at 11.5ED. In order to form the muscular base of the oval fossa, the DMP must myocardialize; at 11.5ED, the DMP is just beginning to undergo mesenchymal-to-myocardial transition and as such, Isl1 expression (3F) is beginning to decrease while expression of NKX2-5 (3G) is upregulated. SHF, second heart field; DMP, dorsal mesenchymal protrusion; FG, foregut; iAVC, inferior atrioventricular cushion; PuV, pulmonary vein; r-MR, right mesocardial reflection; l-MR, left mesocardial reflection

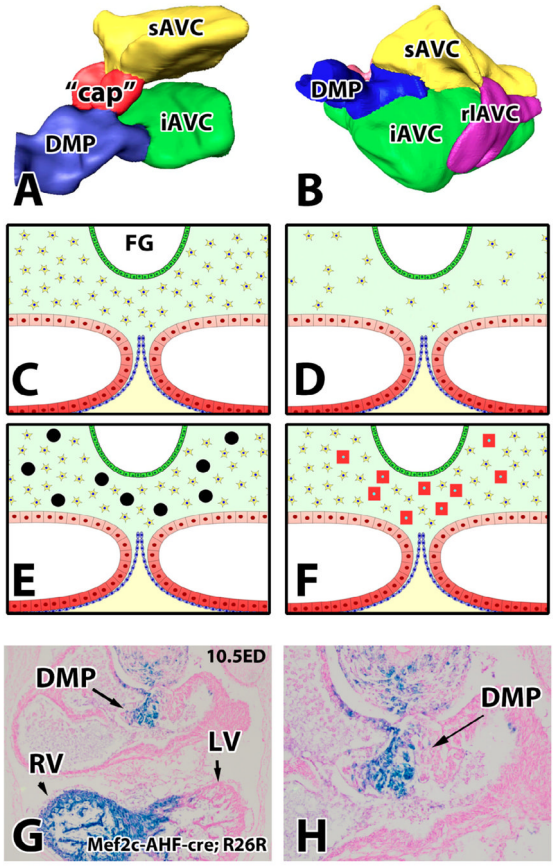

Figure 4. Relationship of the DMP to other Septal Components and Potential Mechanisms Leading to its Maldevelopment

Figure 4A depicts the relationship of the DMP to the other mesenchymal components of the AV septal complex at 11.5ED. The DMP (blue) is continuous with the mesenchymal cap (MC, red) of the septum primum cranially as well as the inferior atrioventricular cushion (iAVC, green) ventrally. The cap of the septum primum is also in continuity with the superior atrioventricular cushion (sAVC, yellow). Thus, the sAVC and iAVC, which have yet to fuse, are continuous through the mesenchymal cap of the septum primum and the DMP. At 13ED (4B), all components of the septal complex have fused and the DMP is wedged between the sAVC (yellow) and iAVC (green) dorsally. At this point, the mesenchyme derived from the cap of the septum primum cannot be distinguished from that of the sAVC. Figure 4C depicts the proliferation of SHF cells that ultimately give rise to the DMP at 9.5ED. The DMP may fail to properly form due to decreased proliferation of these cells (4D), increased apoptosis (4E), or premature mesenchymal-to-myocardialization (4F). Figures 4G– H demonstrate that the *Mef2c*-AHF-cre transgene is expressed within the developing DMP. DMP, dorsal mesenchymal protrusion; iAVC, inferior atrioventricular cushion; sAVC, superior atrioventricular cushion; rlAVC, right lateral atrioventricular cushion; RV, right ventricle; LV, left ventricle; FG, foregut

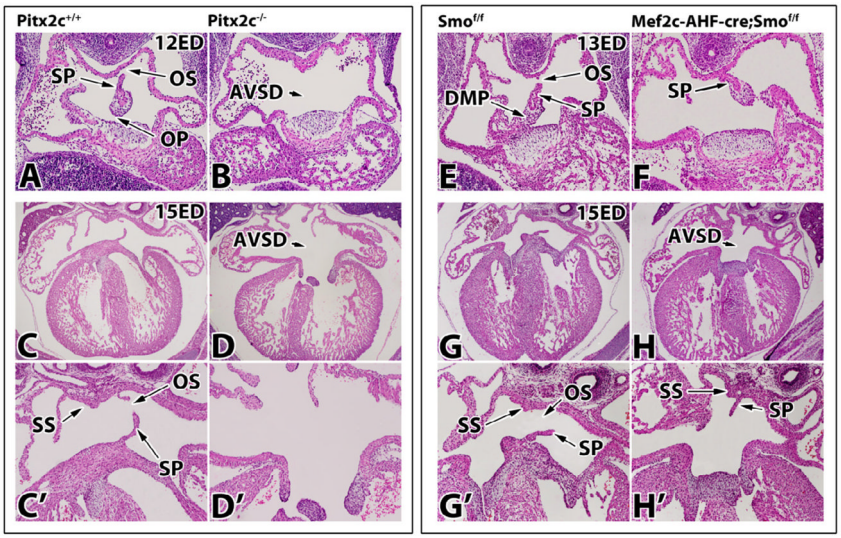

Figure 5. Murine Models of Atrioventricular Septal Defect

Figures 5A– D′ depict Pitx2c$^{-/-}$ specimens and wild-type controls. At 12ED in a normal mouse heart (5A), the septum primum with its mesenchymal cap can be seen protruding toward the endocardial cushions. At this stage, both the ostium primum and secundum are patent but the ostium primum is in the process of closing. In Pitx2c$^{-/-}$ specimens at 12ED (5B), however, no septum primum is observed and as such, the atria communicate freely. By 15ED in the normal mouse (5C, C′), the ostium primum has fully closed while the ostium secundum is patent. The right and left venous valves, which demarcate the orifice of systemic venous return, can be observed within the right atrium. In the Pitx2c$^{-/-}$ specimen at 15ED (5D, D′), the septum primum is absent and, consistent with right atrial isomerism, two sets of venous valves (one in each atrium) can be observed. In this model of AVSD, an ostium primum enables atrial shunting while a ventricular septal defect permits shunting at the ventricular level as well (5D, D′).

Figures 5E– H′ depict deletion of Smoothened from the anterior heart field, including the DMP, (Mef2c-AHF-cre; Smo$^{f/f}$) (5F, 5H, 5H′) and wild-type controls (Smo$^{f/f}$) (5E, 5G, 5G′). Here, at 13ED, the septum primum may be observed within the conditional knockout (5F); however, the ostium primum is still patent and the ostium secundum has not formed. Even two days later in developent, the primary septum is still attached to the roof of the atria and a large ostium primum defect can be observed in these mutants (5H, H′). MC, mesenchymal cap; SP, septum primum; SS, septum secundum; OP, ostium primum; OS, ostium secundum; iAVC, inferior atrioventricular cushion; sAVC, superior atrioventricular cushion; AVSD, atrioventricular septal defect

Table 1

	Syndrome	Gene	OMIM #	References
OSD	Alagille 2	NOTCH2	OMIM: 610205	(McDaniell et al., 2006)
	Axenfield-Rieger	PITX2	OMIM: 602482	(Cunningham et al., 1998; Zetterqvist et al., 1971)
	Cardio-facio-cutaneous	KRAS, BRAF, MEK1, MEK2	OMIM: 115150	(Armour and Allanson, 2008; Niihori et al., 2006; Reynolds et al., 1986)
	Fryns		OMIM: 229850	(Bamforth et al., 1989; Lin et al., 2005)
	Holt-Oram	TBX5	OMIM: 142900	(Glauser et al., 1989; Holt and Oram, 1960; Newbury-Ecob et al., 1996; Sletten and Pierpont, 1996; Smith et al., 1979; Terrett et al., 1994)
	Ivemark		OMIM: 208530	(Calabro et al., 1988; Moller et al., 1967)
	Noonan	PTPN11	OMIM: 163950	(Burch et al., 1993; Croonen et al., 2008; Sznajer et al., 2007)
	Ritscher-Schinzel		OMIM: 220210	(Orstavik et al., 1998)
	Rubinstein-Taybi	CREBBP, EP300	OMIM: 180849	(Stevens and Bhakta, 1995)
	Patau	Ts13		(Musewe et al., 1990)
AVSD	CHARGE	CHD7	OMIM: 214800	(Issekutz et al., 2005)
	Down	Ts21	OMIM: 190685	(Freeman et al., 1998; Park et al., 1977; Stoll et al., 1998)
	Ellis-van Creveld	EVC, EVC2	OMIM: 225500	(Kamesui et al., 1997; McKusick et al., 1964; Ruiz-Perez and Goodship, 2009; Tompson et al., 2007)
	Ivemark		OMIM: 208530	(Moller et al., 1967)
	Kaufman-McKusick	MKKS	OMIM: 236700	(Owens et al., 2009)
	Ritscher-Schinzel		OMIM: 220210	(Hoo et al., 1994; Ritscher et al., 1987)
	Smith-Lemli-Opitz	DHCR7	OMIM: 270400	(Ryan et al., 1998)
	3p	CRELD, CALL	OMIM: 606217	(Drumheller et al., 1996; Green et al., 2000; Kozma et al., 2004; Robinson et al., 2003)

Table 2

	Gene	OMIM #	References
OSD	ACTC1	OMIM: 102540	(Matsson et al., 2008; Monserrat et al., 2007)
	CFC1	OMIM: 605194	(Ozcelik et al., 2006; Wang et al., 2011)
	GATA4	OMIM: 600576	(Garg et al., 2003; Hirayama-Yamada et al., 2005; Reamon-Buettner and Borlak, 2005; Tomita-Mitchell et al., 2007)
	MYH6	OMIM: 160710	(Ching et al., 2005)
	NKX2-5	OMIM: 600584	(Benson et al., 1999; Gutierrez-Roelens et al., 2006; Reamon-Buettner and Borlak, 2004a; Schott et al., 1998)
	TBX5	OMIM: 601620	(Garg et al., 2003; Reamon-Buettner and Borlak, 2004b)
	TBX20	OMIM: 606061	(Kirk et al., 2007; Posch et al., 2010)
	ZIC3	OMIM: 300265	(Ware et al., 2004)
AVSD	ALK2	OMIM: 102576	(Smith et al., 2009)
	BMP4	OMIM: 112262	(Posch et al., 2008)
	CRELD1	OMIM: 607171	(Robinson et al., 2003; Zatyka et al., 2005)
	GATA4	OMIM: 600576	(Reamon-Buettner and Borlak, 2005)
	NKX2-5	OMIM: 600584	(Inga et al., 2005; Reamon-Buettner and Borlak, 2004a)
	TBX5	OMIM: 601620	(Reamon-Buettner and Borlak, 2004b)

Table 3

	Non-Inherited Modifiers	Type	Odds Ratio (95% CI)	References
OSD	Advanced age		1.6 (1.0 – 2.5)	(Ferencz et al., 1997)
	Alcohol		1.9 (1.0 – 3.4)	(Tikkanen and Heinonen, 1991)
	β-blockers		2.6 (1.3 – 4.4)	(Caton et al., 2009)
	Cigarette		1.63 (1.04 – 2.57)	(Kallen, 1999)
	Obesity		1.29 (1.07 – 1.55)	(Gilboa et al., 2010)
	Thalidomide			(Smithells and Newman, 1992)
AVSD	Cigarette	Non-syndromic	2.5 (1.21 – 5.19)	(Ferencz et al., 1997; Malik et al., 2008)
	Cocaine	Non-syndromic	3.45 (1.05 – 11.40)	(Ferencz et al., 1997)
	Diabetes	Non-syndromic	20.6 (5.6 – 76.4)	(Loffredo et al., 2001a)
		Syndromic	22.8 (7.4 – 70.5)	(Loffredo et al., 2001b)
	Ibuprofen	Syndromic	2.49 (1.42 – 4.34)	(Ferencz et al., 1997)
	Ionizing radiation	Non-syndromic	4.54 (1.36 – 15.18)	(Ferencz et al., 1997)
	Maternal illness	Non-syndromic	2.29 (1.11 – 4.73)	(Cleves et al., 2008)
	Paint	Syndromic	1.77 (1.19 – 2.63)	(Ferencz et al., 1997)
	Varnish	Non-syndromic	4.54 (1.36 – 15.18)	(Ferencz et al., 1997)