A novel CRELD1/VEGF genetic interaction in heart disease and development.

By

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CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation of

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ABSTRACT

Atrioventricular septal defects (AVSD) are highly heritable congenital heart malformations, yet little is known about their genetic etiology. The first single AVSD genetic risk factor to be discovered is a unique gene called *CRELD1*, which was identified by the Maslen laboratory in 2003. It is now well established that missense mutations in *CRELD1* are associated with AVSD; however, how CRELD1 contributes to disease is not known. To establish the extent to which CRELD1 influences cardiovascular development, we created and I characterized a constitutive *Creld1*-knockout mouse (*Creld1*$^{(-/-)}$). During this first characterization of CRELD1 function in vivo, I found that elimination of CRELD1 expression resulted in death by embryonic day 12.5 and that before death there were numerous abnormalities, including cardiovascular and endocardial cushion defects. This is significant because defects in atrioventricular endocardial cushions are a potential cause of AVSD. Furthermore, I found that the underlying mechanisms appear to include increased apoptosis, particularly in the heart and branchial arches; poor embryonic and yolk sac vascularization; as well as abnormal placental development. This suggests that CRELD1 can affect heart development by both direct and indirect causes; direct by increasing apoptosis in the developing heart and indirect by causing placental abnormalities and global vascular insufficiencies.

In contrast to the *Creld1*$^{(-/-)}$ mouse, the *Creld1*-heterozygous mouse (*Creld1*$^{(+/-)}$) was viable and normal, which is consistent with *CRELD1*-missense mutations being incompletely penetrant for AVSD in humans and evidence that AVSD etiology is multifactorial. Thus, we began to consider the existence of other AVSD-risk modifiers that might affect CRELD1-induced risk. Specifically we investigated a role for VEGF, a known regulator of endocardial cushion development, for its involvement in AVSD etiology. When we did this we indeed found that the *VEGF-634C* polymorphism, a common polymorphism known to cause VEGF over-expression, was strongly associated with AVSD. Furthermore, we found that *CRELD1*-missense mutations were always fully penetrant for AVSD when the *VEGF-634C* polymorphism was also present. Based on this, I hypothesized that a CRELD1-functional deficiency in combination with VEGF over-expression could cause AVSD. I then tested if a CRELD1-deficiency can interact with VEGF over-expression to cause abnormal endocardial cushion development using an endocardial cushion explants assay. I did this by culturing *Creld1*$^{(-/-)}$, *Creld1*$^{(+/-)}$ and *Creld1*$^{(+/+)}$ mouse endocardial cushions with increasing levels of purified VEGF protein and found that, while a CRELD1 deficiency alone was not enough to alter endocardial cushion development, the addition of VEGF did cause abnormal endocardial cushion development. This supports the existence of CRELD1/VEGF causative AVSD-risk profile, which is the first AVSD-risk profile to be proposed.

PREFACE

The vast majority of diseases are complex and caused by the interaction of numerous risk factors (Craig 2008). However, few studies investigate the interplay of risk factors, so despite the frequency of complex diseases, causative risk profiles are rarely known (Craig 2008). Here I investigate the existence of a causative risk profile for atrioventricular septal defects (AVSD), a clinically significant congenital heart malformation of unknown etiology. Identifying risk profiles for AVSD is important because AVSD are associated with increased morbidity and mortality (Boening et al. 2002, Stulak et al. 2010), which may be due to the existence of persistent underlying genetic defects. Therefore, the identification of genetic risk profiles could help both the prediction and ongoing management of AVSD cases.

AVSD occur in 2.4-3.1/10,000 live births (Craig 2006) and are thought to arise from defects in endocardial cushion formation during embryogenesis, which subsequently causes abnormal heart valve and septa formation. Past studies using animal models have successfully identified numerous genetic pathways critical for endocardial cushion development; however, the relevance of these pathways or the successful identification of other pathways relevant to isolated human AVSD cases is scarce (Maslen 2004). This may be due to the complex nature of AVSD. Nevertheless, in 2002 we described the first

single genetic risk factor found to be associated with AVSD in humans, a gene now named *CRELD1* (Robinson et al. 2003, Rupp et al. 2002).

Our description of CRELD1 was also the first description of the CRELD family of proteins, thus upon identification nothing was known about the role of CRELD1 in heart development or even if CRELD1 could affect endocardial cushion formation. Accordingly, in this thesis I present the first characterization of CRELD1 during mammalian development by characterizing a *Creld1*-knockout mouse model. Additionally, I include early exploration into the action of CRELD1, including the ability of CRELD1 to affect apoptosis, proliferation, epithelial-to-mesenchymal transformation and global gene expression.

This thesis also explores a potential role for VEGF as a modifier of AVSD risk because while *CRELD1*-missense mutations are strongly associated with AVSD (Robinson et al. 2003) they are incompletely penetrant, suggesting that additional modifiers are needed to breach the disease threshold. Accordingly, we tested for and found an exclusive association between the *VEGF-634C* polymorphism (a polymorphism that causes VEGF over-expression) (Awata et al. 2002, Awata et al. 2005, Lambrechts et al. 2003, Petrovic et al. 2007), *CRELD1*-missense mutations and AVSD. This discovery of a genetic profile specific for AVSD prompted me to hypothesize that CRELD1 and VEGF are coalescing to

disrupt endocardial cushion development and cause AVSD. I then confirmed a CRELD1/VEGF interaction by using an endocardial cushion explants assay, making the CRELD1/VEGF interaction the first AVSD risk profile described.

Chapter 1: Here I will lay the foundation for this dissertation. First I will cover the basics of heart development: what causes an AVSD (defects in endocardial cushion formation) and how the culturing of endocardial cushions can aid in the study of AVSD origins.

Chapter 2: In chapter 2, I will discuss the clinical impact and current treatment of AVSD; and discuss the long-term risks associated with repaired AVSD.

Chapter 3: Next, I will briefly cover what is known about congenital heart defect risk factors. Then I will discuss the complex nature of AVSD genetics and what is known about CRELD1 as an AVSD risk factor and what is not known about the role of CRELD1 in the pathogenesis of AVSD. Lastly, I will introduce VEGF as a modifier of endocardial cushion development and why we believe that the *VEGF-634C* over-expression polymorphism could be a modifier of AVSD risk.

CHAPTER 4: Chapter 4 provides a detailed explanation of the approach and methods used to test my hypothesis that a combination of VEGF-over-expression and CRELD1-deficiency can cause AVSD.

CHAPTER 5: This chapter is focused on the role of CRELD1 in development and demonstrates that CRELD1 could cause AVSD through the characterization of the *Creld1*-knockout mouse. The mouse characterization revealed that elimination of CRELD1 expression caused death by embryonic day 12.5. Examination before death found numerous abnormalities, including abnormal endocardial cushions. This is significant because defects in atrioventricular endocardial cushions are a potential cause of AVSD. The underlying mechanisms included increased apoptosis, particularly in the heart and branchial arches; poor embryonic and yolk sac vascularization; as well as abnormal placental development. Additionally, microarray assays found that *Creld1*-knockout caused massive gene dysregulation, but especially notable was that a CRELD1-functional deficiency was associated with VEGF up-regulation.

CHAPTER 6: This chapter concerns the role of VEGF in AVSD etiology. Presented is an association study that shows, for the first time, that the *VEGF-634C* polymorphism (a polymorphism that causes VEGF up-regulation) is associated with AVSD. Additionally, we demonstrate that the *VEGF-634C* allele exclusively co-segregates with *CRELD1*-missense mutations in AVSD cases. From these results, I then hypothesize that a CRELD-

functional deficiency plus VEGF over-expression can disrupt endocardial cushion formation and thus is an AVSD risk profile. I then test and support this hypothesis using an endocardial cushion explant assay, a classic system for monitoring endocardial cushion growth ex vivo.

CHAPTER 7 & 8: These chapters include an in-depth discussion of how these results fit into the literature at large. Especially emphasized is how these results expand our knowledge of CRELD1 basic biology. Also discussed are the potential implications of our findings, as the CRELD1/VEGF profile is the first genetic risk profile ever described for AVSD. Additionally, I will suggest a few natural next steps.

CHAPTER 1:

INTRODUCTION TO HEART DEVELOPMENT

Significance of congenital heart defects

Congenital heart defects occur in 9/1,000 live births (American Heart Association Retrieved 30 July 2010), which makes them the most common form of live-birth defect. At one time congenital heart defects were mostly lethal in childhood but now routine surgical repair has greatly increased survival to adulthood. However, the increased survival has created a new medical challenge: An emerging and novel adult population living with repaired cardiovascular defects, which may be as large as 1,300,000 individuals in the United States (American Heart Association Retrieved 30 July 2010).

This novel population of adults living with repaired heart defects has created both direct and indirect strains on our medical infrastructure. Additionally, these patients are increasingly acknowledged not to be cured, as was once thought. Instead, patients with repaired congenital heart defects often require additional operations for progressive valvular dysfunction, arrhythmias, or for other causes; and thus

require lifelong comprehensive care (Dearani et al. 2007), the costs of which exceed 2.2 billion dollars for inpatient congenital heart defect surgical repair alone (American Heart Association Retrieved 30 July 2010).

In addition to increasing the prevalence of congenital heart defect patients, the increased survival due to surgical repair may also increase the incidence of new cases in the future. Congenital heart defects are well known to exhibit heritability (Insley 1987); therefore, we expect that the incidence of congenital heart defects will only increase as adults living with repaired heart defects have children of their own. This proposed increase in heart defect incidence is an alarming hypothesis, considering how common congenital heart defects are already. Just *how* common is put in perspective when we consider that, even with surgical repairs, nearly twice as many children die from congenital heart disease as die from all forms of childhood cancers combined (American Heart Association Retrieved 30 July 2010). Obviously, to ignore this growing patient population would be a mistake.

We cannot hope to improve the care of congenital heart defect patients beyond reactive or palliative care, such as surgery, unless we understand the cause of congenital heart defects. Therefore, this thesis is dedicated to identifying the cause of heart defects, so that someday more personalized and efficient care can be

offered to this growing patient population. More specifically, this thesis will focus on identifying the genetic risk factor associated with a subset of congenital heart defects called atrioventricular septal defects (AVSD).

Heart development

However, before we can address AVSD genetics, we must first understand how the heart develops and what errors in development can cause an AVSD.

The first cardiac progenitors are found in the primary heart field

The earliest population of cardiac progenitors compose the primary heart field. This field arises during gastrulation from the anterior splanchnic mesoderm and will give rise to the cardiac crescent, which will ultimately become the left ventricle and atria (Anderson et al. 2002). Specification of this tissue to a cardiovascular fate is dependent on signaling from neighboring tissues, including positive signaling from the bone morphogenetic protein family (Schultheiss et al. 1997); and both positive and negative signaling from the Wnt/wingless proteins (Schneider and Mercola 2001, Pandur et al. 2002, Marvin et al. 2001). This is accompanied by the expression of cardiac specific transcription factors,

especially tinman/Nkx2-5 or Csx (Bodmer 1993, Azpiazu and Frasch 1993). Once specified, the primary heart field migrates medially to form two linear heart tubes, which will eventually fuse to form a single-chambered heart tube composed of an inner layer of endothelial cells surrounded by an outer layer of myocardial cells, between which is a layer of extracellular matrix rich in proteoglycans and glycosaminoglycans called the cardiac jelly (Harvey 2002). Initially this continuous "straight heart tube" is suspended along its length by a dorsal mesocardium but it quickly liberates itself from the body wall and undergoes rightward looping. By the fourth week in human development, it will begin to beat and pump blood. From there, the tube undergoes ballooning and begins septation to form the mature four-chambered heart.

A second source of cardiac progenitors: the secondary heart field

The primary heart field forms the initial heart tube and heavily contributes to the left ventricle of the mature heart. However, the portions of the heart tube that will contribute to the right ventricle, outflow tract and atria arise primarily from a second source of cardiac precursors known as the secondary heart field. This secondary heart field forms from the pharyngeal mesoderm located medial to the cardiac crescent. From here, this field contributes to the heart tube by migrating into the outflow and inflow poles of the developing heart, where it

then helps the expansion and development of the heart tube. The primary and secondary heart fields share heart-specific expression profiles such as the homeobox gene *Nkx2-5* but can also be distinguished by the unique expression of other transcription factors, as the primary heart field expresses the T-box transcription factor Tbx5 and the bHLH transcription factor Hand1; in contrast, the secondary heart field is marked by the expression of Hand2, the LIM-homeodomain transcription factor Isl1 and Fgf10 (Garry and Olson 2006). These distinct expression profiles allow for the selective genetic manipulation of the fields, which enables experimenters to test the origins of heart components.

A third source of cardiac progenitors: cardiac neural crest

A series of seminal transplantation and cell labeling experiments in avian embryos in the early 1980s revealed a third source of cardiac cells (Kirby et al. 1983, Kirby 1987, Bockman and Kirby 1984, Creazzo et al. 1998). These studies found that specific ectodermal cell populations located between the mid-otic placode and the third somite of the dorsal neural tube contributed to the outflow tract of the heart. Loss of these neural crest cells caused cardiovascular abnormalities of the outflow tract and aortic arch, including double outlet right ventricle, persistent truncus arteriosus and interrupted aortic arch (Kirby et al. 1983, Kirby 1987, Bockman and Kirby 1984, Creazzo et al. 1998). Accordingly, this

neural crest cell population was termed the cardiac neural crest. Subsequent mammalian fate-mapping studies using markers of cardiac neural crest fate, such as Connexin 43, Wnt1 or Pax3 have confirmed the role of the cardiac neural crest cells in the heart. Specifically, it has been found that the cardiac neural crest contributes significantly to the endocardial cushions of the outflow tract, which is a structure vital for the septation of the mature aorta and pulmonary arteries (Waldo et al. 1999, Epstein et al. 2000). However, cardiac neural crest contributions appear limited, and there is no evidence that they contribute to the atrioventricular endocardial cushions that form the atrioventricular valves and parts of the atrioventricular septa.

Division of heart tube into a four-chambered heart

In the normal heart, the atrial septum is connected to the ventricular septum by atrioventricular fibrous annulus tissue or the atrioventricular septum (defined as the region between the apical mitral and atrial tricuspid valve). Loss of this tissue is the defining feature of AVSD. To understand what causes this loss of septum contiguity we must first understand how the atrioventricular septa and valves normally form, a process that begins at approximately week 5 in human development [embryonic day 9 (E9) in mice] and is heavily contributed to by the endocardial cushions.

Atrial septum

The atrial septum is composed of contributions from the septum primum, the septum secundum and the endocardial cushions. Atrial septation begins when the septum primum, a muscular crescent, extends from the atrial roof towards the center of the heart and towards the developing endocardial cushions. However, before the septum primum merges its mesenchymal cap with the endocardial cushions, there is a temporary space between the two structures called the ostium primum (also called the primary atrial foramen), which allows blood from the right atrium to flow directly into the left atrium (Abdulla et al. 2004). This right-to-left shunt is vital in fetal circulation, during which time the most highly oxygenated blood arrives not from the lungs and into the left side of the heart (as in postnatal life); but from the placenta and through the right side of the heart. Therefore, before the ostium primum is closed and filled in with fibrous septal tissue derived from the mesenchymal cap and endocardial cushions, a secondary foramen or foramen ovale opens near the primary septum's origin (Dudek 2006).

Simultaneous to the fusion of the septum primum with the endocardial cushions and to the opening of the secondary foramen, a second septum (the septum secundum) begins to extend from the right side of the septum primum. This second septum will eventually cover the foramen ovale but importantly it does

not seal the hole. Thus, blood continues to flow between the right and left atrium, until birth, when the right-to-left shunt is no longer needed (Figure 1). This foramen ovale is sealed postnatally when the first breaths cause the pulmonary pressure to decrease and the pressure in the left side of the heart to increase. This forces the septum primum against the septum secundum, functionally sealing the foramen ovale, which then fuses closed, leaving only a remnant known as the fossa ovalis.

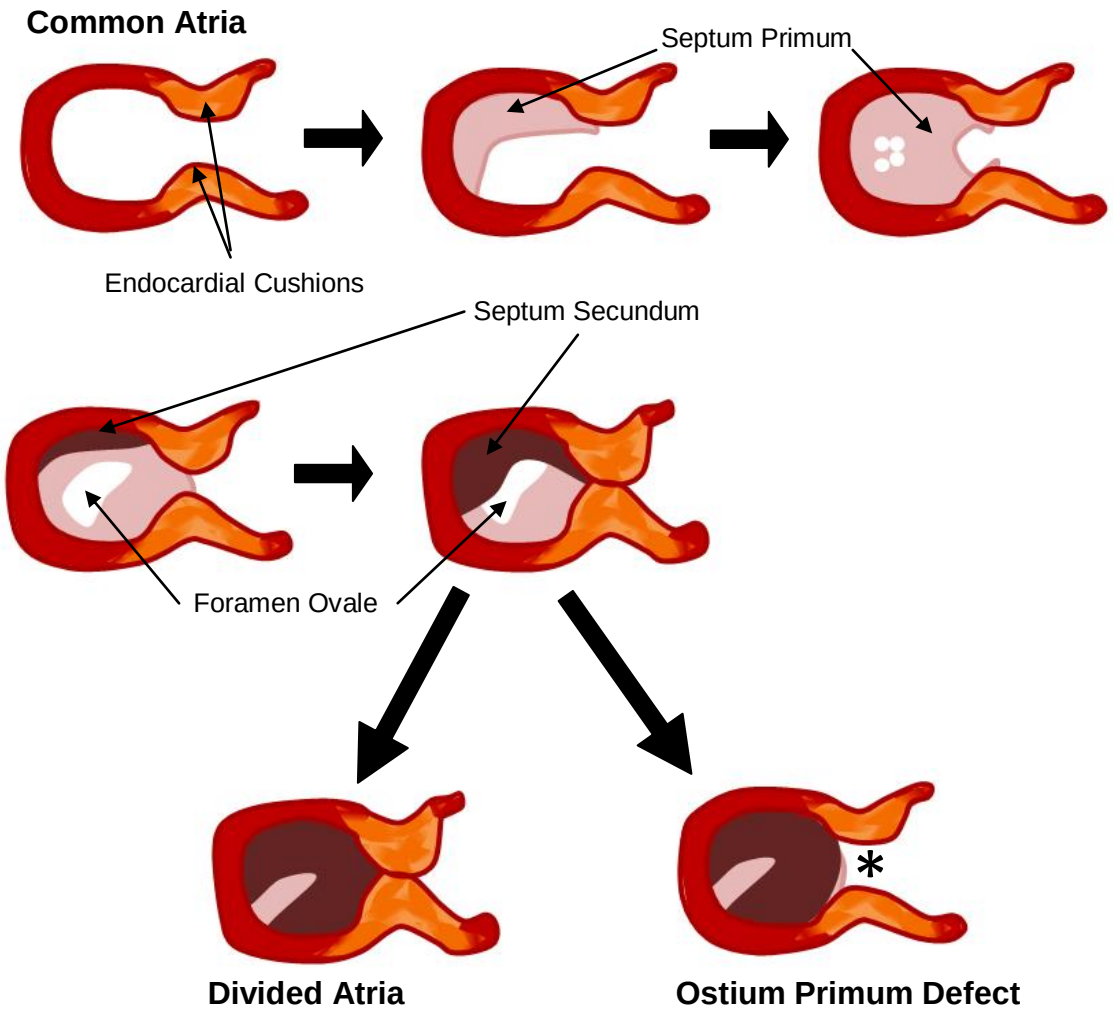

Common Atria
Septum Primum
Endocardial Cushions
Septum Secundum
Foramen Ovale
*
Divided Atria
Ostium Primum Defect

Figure 1: Illustration of how the atrial septum forms.

Above is an illustration demonstrating how the common atrial chamber is divided, as viewed as a sagittal cross section of the right atrium looking towards the left atrium. The common atria is divided into a left and right atrium by contributions from two septa (the septum primum and the septum secundum) and contributions from the endocardial cushions. If the atrioventricular endocardial cushions do not fuse with the septum primum during development an ostium primum defect occurs (denoted by asterisk). This is the type of atrial septal defect found in AVSD. Details about atrial septal formation can be found in the main text. Figure adapted from Abdulla et al. (Abdulla et al. 2004).

Ventricular septum

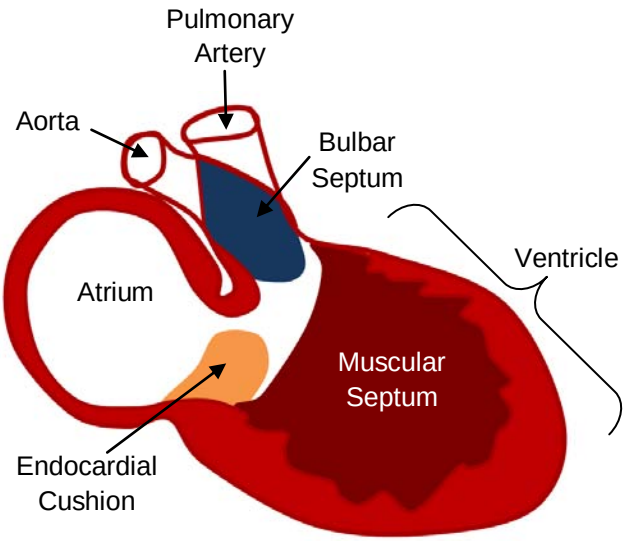
Also during the 4th week of human development the two primitive ventricles become septated. The ventricular division begins as a muscular fold, extending from the anterior wall to the floor of the ventricles, grows towards the atrioventricular valves as a concave ridge. Second to this, trabeculations coalesce in the inlet region and grow into the ventricular cavity. These septal primordia (the muscular ridge and trabeculation) then fuse to form the bulk of the muscular interventricular septum, which is now in contact with the outflow septum. However, completion of the muscular septum still leaves a large opening called the interventricular foramen between the two ventricles. Closure of the interventricular foramen depends on contributions from membranous tissues (Figure 2). This includes contributions from the endocardial cushions, and the right and left bulbar ridges (Abdulla et al. 2004, Satpathy 2008).

Errors in the atrioventricular septum

Errors in any part of ventricular or atrial septum formation can result in a ventricular septal defect (VSD) or an atrial septal defect (ASD), respectively; and are classified based on their anatomical location within the septa. Accordingly, membranous ventricular septum (Figure 2) and ostium primum (Figure 1) defects are the ventricular and atrial septum defects found in AVSD. Membranous

ventricular septum defects can be caused by errors in the endocardial cushions' ability to create the upper membranous ventricular septum; similarly, ostium primum defects are traditionally thought to be caused by a failure of the endocardial cushions to fuse with the septum primum. Thus, particular attention is paid to the atrioventricular endocardial cushions when studying the development of AVSD.

Interventricular Septum Formation



Successful Interventricular Septum Formation

Membranous Septal Defect

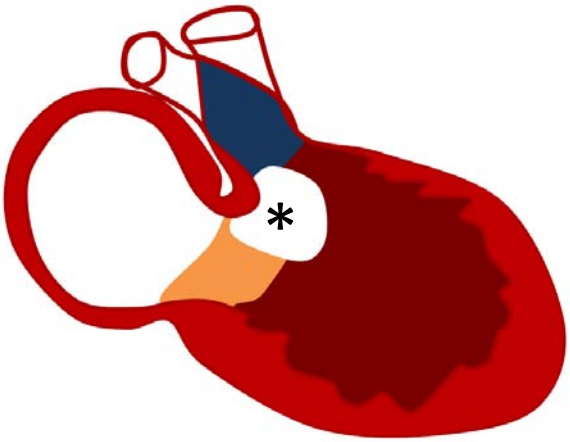


Figure 2: Membranous Ventricular Septal Defect.

The muscular septum forms the bulk of ventricular septum. However, the top portion of the ventricular septum is filled in with membranous tissue derived from the bulbar septum and the endocardial cushions. Defects in the fusion of the muscular septum with the endocardial cushions or an absence of the endocardial cushions can cause membranous septal defects found in AVSD (denoted by asterisks). For details see main text. Figure adapted from Abdulla et al. (Abdulla et al. 2004).

Atrioventricular valve formation

The atrioventricular valves are also almost entirely derived from the atrioventricular endocardial cushions (de Lange et al. 2004, Lincoln et al. 2004), with some contribution from the atrioventricular myocardium (de Lange et al. 2004, Gaussin et al. 2005), as undermining of the myocardium helps form the papillary muscles and chordae tendinea of the valves.

The earliest valves are derived from the endocardial cushion. Thus the early valves consist of mesenchymal leaflets and extracellular matrix, primarily hyaluronic acid (Camenisch et al. 2000) and versican (Henderson and Copp 1998). The signaling involved in the formation of the early leaflets is incompletely understood but includes signaling by calcium, VEGF and nuclear factor of activated T cells (NFAT) (Wagner and Siddiqui 2007). The valves then begin to mature between E15.5 and E18.5 in mouse. Maturation includes increased cell density and proliferation, accompanied by areas of condensation, which are reminiscent of bone formation. A process associated with increased fibronectin expression, which is thought to bridge the leaflet cells with the interstitial collagen network and increased N-cadherin expression, which is thought to promote cellular adhesion. Then from E18.5 onwards, the leaflet (at the level of the papillary muscle) elongates parallel with heart growth (Kruithof et al. 2007).

Next the primitive valve remodels to form structurally resilient adult valves. At first (E15.5 and E18.5) hyaluronic acid, other acidic mucopolysaccharides or glycosaminoglycans, and versican are homogenously expressed in the mitral mural leaflet (except in the areas of condensation). But starting at postnatal day 6.5 (N6.5) in mouse these extracellular matrix proteins (as seen with Alcian Blue staining) become restricted to the atrial side of the leaflet. This atrial side will become the atrialis layer and provides the adult valve with elasticity. This is then accompanied by the accumulation of stress-resistant extracellular matrix proteins such as collagen 1 and Collagen V. This accumulation first occurs at the anchoring points to the annulus fibrosus and papillary muscles, before spanning the entire length of adult leaflets at the ventricular side. This ventricular side will be known as the fibrosa layer and will provide the mature leaflet's strength (Kruithof et al. 2007). Between the atrialis and fibrosa layers is the spongiosia layer, which provides cushioning.

While the timeline of valve formation is established, the signaling involved in heart valve remodeling and maturation is not well understood, likely because there is a scarcity of mice with valve defects that are viable past the first half of gestation (Stainier et al. 2002). Therefore, more is known about early valve development, namely the early formation of the atrioventricular endocardial cushions.

AV-endocardial cushion defects can cause AVSD

The atrioventricular endocardial cushions contribute to the membranous ventricular septum, are critical to closing the ostium primum in the atrial septum and form large portions of the atrioventricular valves. Therefore, defects in endocardial cushion formation can be responsible for the constellation of defects seen in an AVSD. This is why AVSD are sometimes called endocardial cushion defects.

Endocardial cushion development

Endocardial cushions first start to form during the fourth week of human development in two discrete segments of the heart tube: at the junction of the future atria and ventricles (the atrioventricular canal); and in the outflow tract. Endocardial cushion development begins as a swelling of the heart tube's cardiac jelly. Exactly how this extracellular matrix expansion is controlled is unknown but a few pathways have been implicated. In mouse, versican, a chondroitin sulfate proteoglycan, is necessary for cardiac jelly production, as an absence of versican inhibits cell invasion into the endocardial cushions (Yamamura et al. 1997). Additionally, hyaluronic acid synthase 2 (has2), a high-molecular weight glucosaminoglycan polymer that is thought to have an architectural and cell adhesion role in the extracellullular matrix (Stainier et al. 2002), is necessary for cardiac jelly production, ventricular trabeculation and endocardial cushion formation (Camenisch et al. 2000).

Successful swelling of the cardiac jelly is then followed by a subset of the endothelial cells in the outflow tract or atrioventricular junction undergoing a transformation into mesenchymal cells, via a mechanism called epithelial-to-mesenchymal transformation (EMT). Successful EMT is partially dependent on the competency of the cushion endothelial cells to respond to EMT inductive signals, as neighboring endothelial cells in the ventricle do not respond similarly (Runyan and Markwald 1983). The signals important for inducing these competent endothelial cells to undergo EMT arise from the neighboring myocardium and swollen cardiac jelly and include signaling from BMPs, Wnts, Notch, TGF-β, VEGF, hyaluronic acid, neurofibromin, NFAT, Connexin 45, fibronectin, transferin, slug and EGF (Wagner and Siddiqui 2007, Gaussin et al. 2002, de la Pompa et al. 1998, Ranger et al. 1998, Kumai et al. 2000, Nakajima et al. 2000, Romano and Runyan 1999). More specifically, the activation and delamination of the endothelial cells in endocardial cushion formation is associated with increased Bmp, VEGF, Notch, Slug and TGF-β signaling. And the subsequent mesenchymal cell migration into the cardiac jelly is associated with increased VEGF and hyaluronic acid signaling, although abnormally high VEGF signaling can also inhibit EMT (Dor et al. 2003). Lastly, proliferation of this cell population is balanced by positive regulators such as Wnt and BMP; and negative regulators such as EGF and Neurofibromin (Wagner and Siddiqui 2007). The net result is a subset of endothelial cells undergoing hypertrophy, breaking

homotypic cell-adhesive contacts, undergoing cytoskeletal changes, extending filopodia and invading the matrix-rich cardiac jelly (Figure 3), where they expand and remodel to eventually form the valves and septa. However, how all these necessary factors interact is not as well studied and the exact sequence of events involved in endocardial cushion EMT is unknown. However, one model proposes that TGF-β2 signaling and inhibitory regulative G-protein (Gi) signaling is required in combination with Slug to initiate EMT, while TGF-β3 and BMP are important in the later EMT stages (Stainier et al. 2002).

Successfully activated mesenchymal cells can be marked by their expression of α smooth muscle actin (Nakajima et al. 1997), cell surface β 104 galactosyltransferase, Msx-1, Mox-1 or type 1 pro-collagen (Stainier et al. 2002). But these are only markers of transformation. The only mesenchymal marker that has also been shown to be critical in cushion EMT is vinculin (Stainier et al. 2002). The fact that so few markers have also been shown to be necessary for cushion development, likely speaks to the robustness and genetic redundancy inherent to heart development, which hints at the complexity of endocardial cushion development and AVSD genetics.

In the outflow tract the endothelial cells that contribute to the endocardial cushions are derived from both the cardiac neural crest and secondary heart

field (specifically anterior heart field) (Cai et al. 2003, Buckingham et al. 2005, Verzi et al. 2005, Abu-Issa and Kirby 2007). Similarly, there is some suggestion that the endothelial cells that contribute to the atrioventricular endocardial cushions might also be derived from numerous heart fields. As a recent study found that when BMP4, a gene essential for cardiac myocyte specification and endocardial cushion development (Delot 2003, Schneider et al. 2003), is knocked out of the anterior heart field, membranous ventricular septal defects result (McCulley et al. 2008). However, this could also be due to paracrine effects due to an absence of BMP4 in the anterior heart field. So it remains to be conclusively shown whether any other fields, besides the primary heart field, contribute to the atrioventricular endocardial cushions.

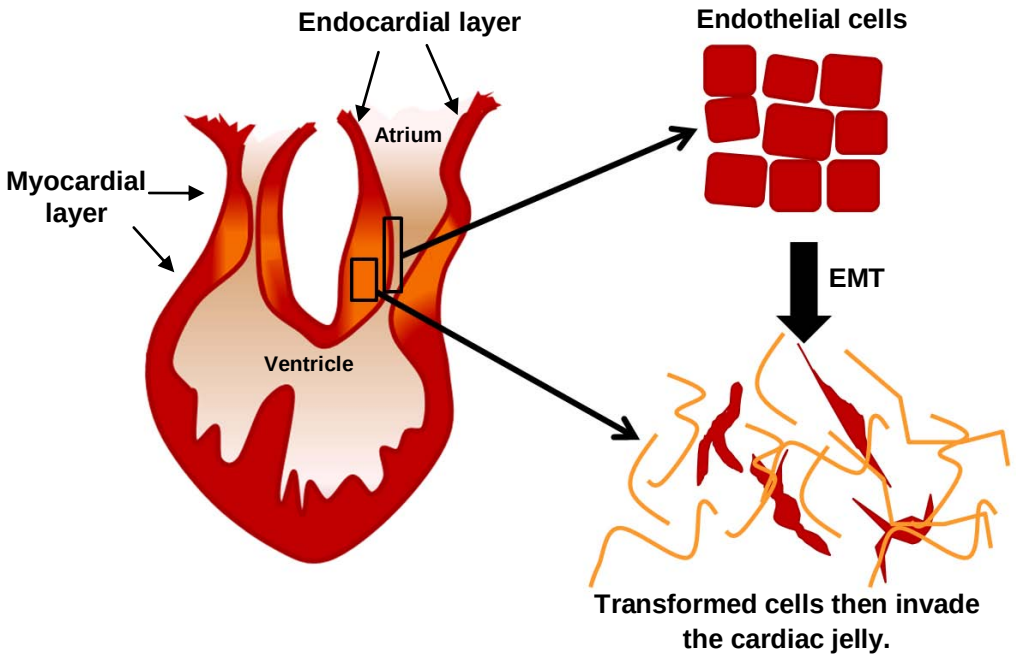


Figure 3: Diagram illustrating EMT during cushion formation.

Diagramed above is a cross section of an E10.5 developing mouse heart. The endocardial cushions are shown in orange. The endocardial cushions are composed of a population of endothelial (endocardial) cells that have undergone an epithelial-to-mesenchymal transformation (EMT) and invaded the extracellular matrix (or cardiac jelly) found between the endocardium and myocardium layers of the heart tube.

Endocardial cushion can be cultured

Due the pivotal role that endocardial cushions play in the development of AVSD, there is much interest in identifying and characterizing molecules important in endocardial cushion development, as it is proposed that the genes that affect endocardial cushion development may be risk factors for AVSD. This has prompted intense investigation of endocardial cushion development, including the creation of an ex vivo assay, which makes cushions more accessible to manipulation and allows for continued monitoring of early cushion growth.

Roger Markwald's laboratory was the first to successfully explant the atrioventricular endocardial cushions of avian embryos onto a 3-D collagen-matrix; specifically cushions from Hamburger and Hamilton stage 12 to 18, which covered the time from initial enlargement of the cardiac jelly to EMT. When they did this they found that the myocardium continued to beat and that the cushion endothelial cells were able to undergo EMT, migrate in three dimensions and "seed" the matrix (Figure 4). Furthermore, they found that the neighboring ventricular endothelial cells could not seed the matrix (Runyan and Markwald 1983), suggesting that the cushion endothelial cells are uniquely competent to undergo EMT. Most importantly they found that the atrioventricular cushion

formation in 3-D culture mimicked the histological sequence seen in vivo (Bernanke and Markwald 1982).

The explant culture system was then employed with mouse atrioventricular endocardial cushions by Todd Camenisch. The chick and mouse explants were found to be comparable, as 26 ± 1 somites in either species reliably produced atrioventricular and outflow tract cushions that transformed and invaded the collagen matrix. However, there is some difference between the dynamics of EMT in the two species, if the cushions are explanted before transformation (<28 somites). Explanted <28 somite avian cushion endothelial cells initially leave the explants as a monolayer and then only a subset of cells transform and seed the matrix; while the <28 somite mouse cushion endothelial cells appear to transform and seed the matrix directly from the explants. Some of the difference in EMT between the species may be due to subtle differences in reactivity, as the avian cushions express both TGFβ2 and TGFβ3 during EMT initiation, while in mouse cushions TGFβ3 is not detected during EMT initiation. In avian cushions, TGFβ2 mediates initial cell–cell separation of activated endocardium, while TGFβ3 is necessary for the subsequent invasion (Camenisch et al. 2002).

Despite this subtle difference, both the avian and mouse explant culture systems mimic in vivo EMT events, including transformation and invasion of mesenchymal cells; and therefore this system can be used to monitor cushion development. Accordingly, in this thesis, when I test whether potential AVSD risk factors can indeed affect early valve and septa formation I will culture endocardial cushions on a 3-D collagen matrix to monitor EMT.

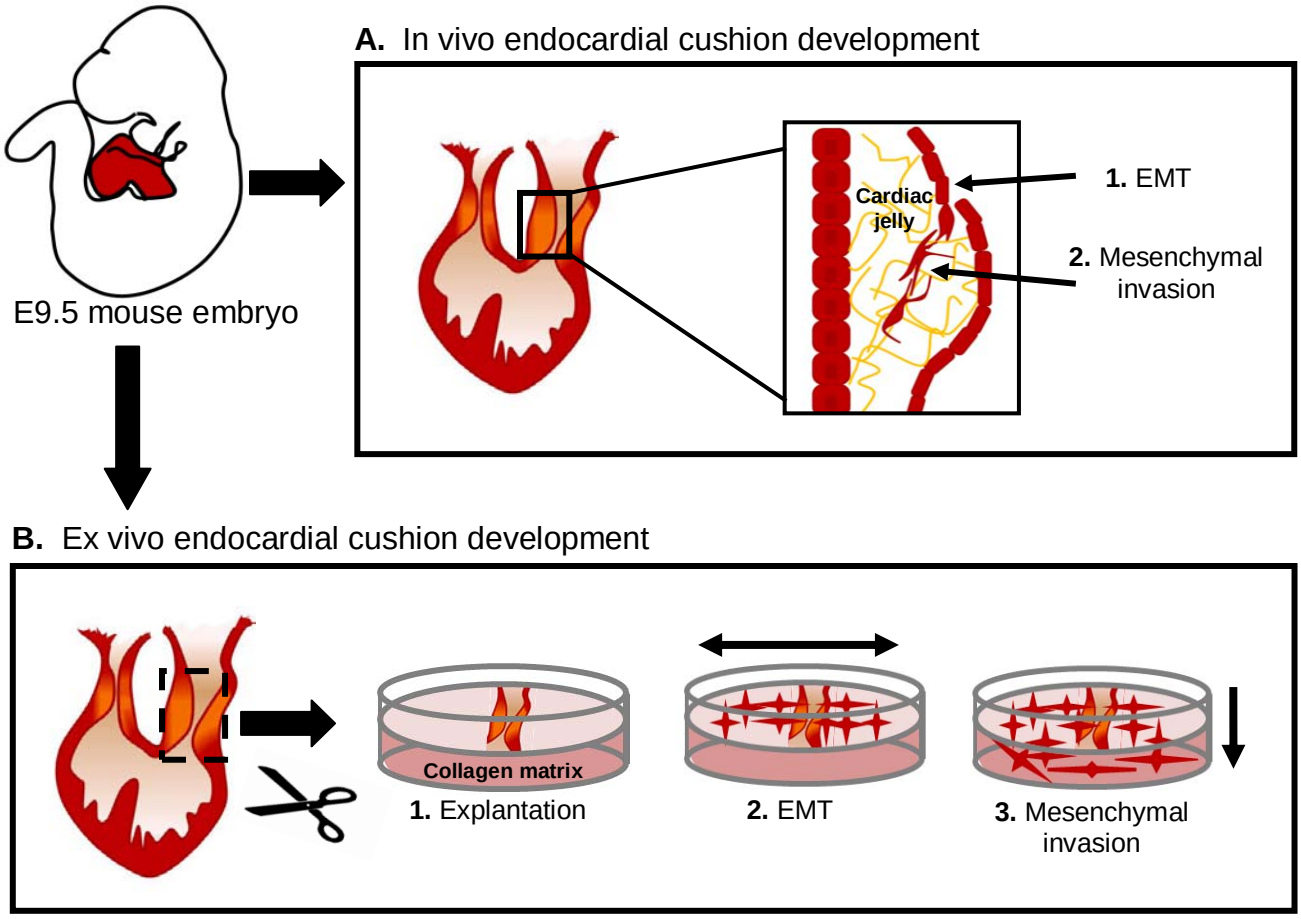


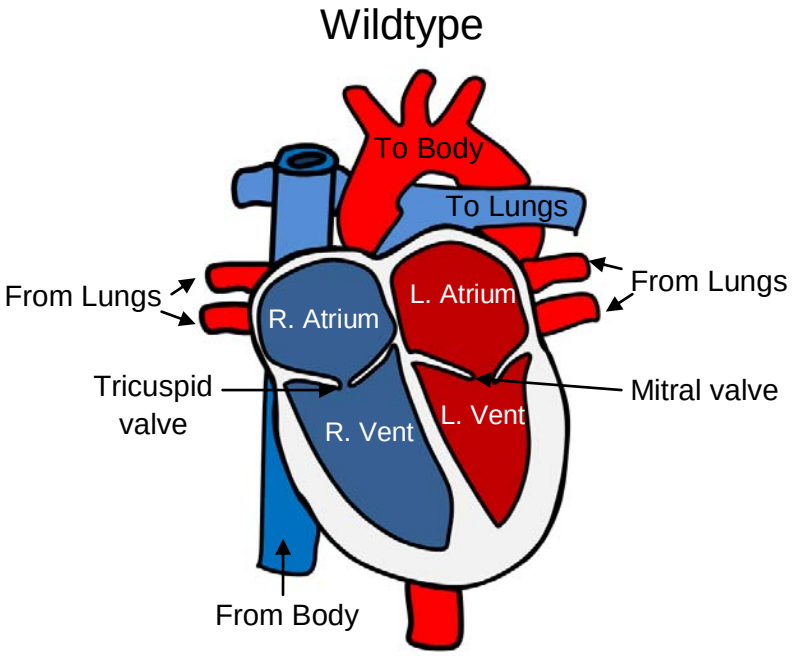
Figure 4: The 3-D collagen-matrix assay is a proxy for development.

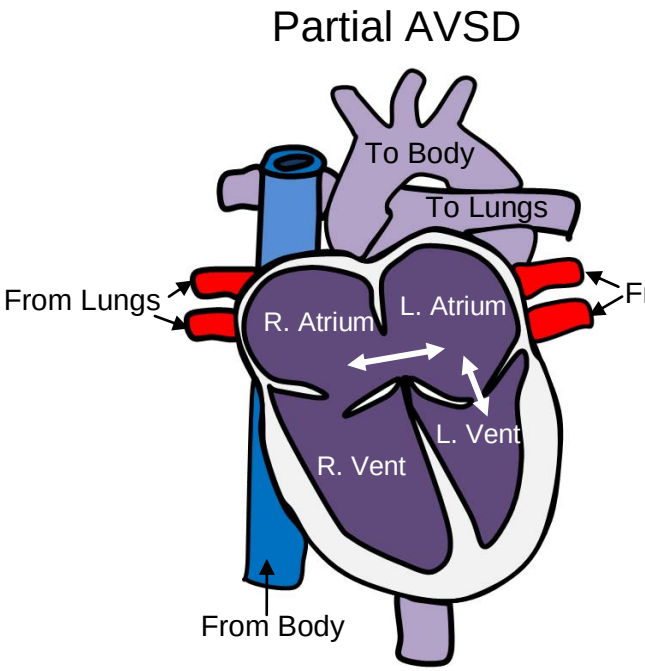
At E9.5 in mouse development the endocardial cushions are still only swellings of the cardiac jelly, as EMT has not occurred. However, EMT will occur over the next day and transformed endothelial cells will be seen invading the cardiac jelly as mesenchymal cells **(Panel A)**. If however, the atrioventricular canals are explanted at E9.5, then the transformed cells can be seen migrating across and into the provided 3-D collagen matrix **(Panel B)** instead of the cardiac jelly.

CHAPTER 2:

CLINICAL IMPACT AND CURRENT TREATMENT OF AVSD

There are two types of AVSD: partial and complete AVSD, both of which involve errors in endocardial cushion formation and as a class occur in 2.4-3.1/10,000 live births (Craig 2006). Both partial and complete AVSD cases are characterized by the absence of a continual septum in the heart, which causes the left and right valves (mitral and tricuspid, respectively) to merge into a single common hybrid valve with five leaflets, two of which ride over the truncated ventricular septum and are thus shared by both the right and left ventricles (bridged leaflets). In complete AVSD cases both the membranous atrial and ventricular septum are affected. In contrast, partial AVSD cases are primarily a failure of the membranous atrial septum and not of the ventricular septum. Accordingly, in complete AVSD cases the common valve has a single annulus, thus resulting in a single heart orifice. Partial AVSD cases have separate mitral and tricuspid annuli within the common valve and abnormal communication primarily between the atrium and through a cleft mitral valve (if present) (Figure 5).


Wildtype
To Body
To Lungs
From Lungs
From Lungs
L. Atrium
R. Atrium
Tricuspid valve
Mitral valve
L. Vent
R. Vent
From Body


Partial AVSD
To Body
To Lungs
From Lungs
From Lungs
R. Atrium
L. Atrium
L. Vent
R. Vent
From Body

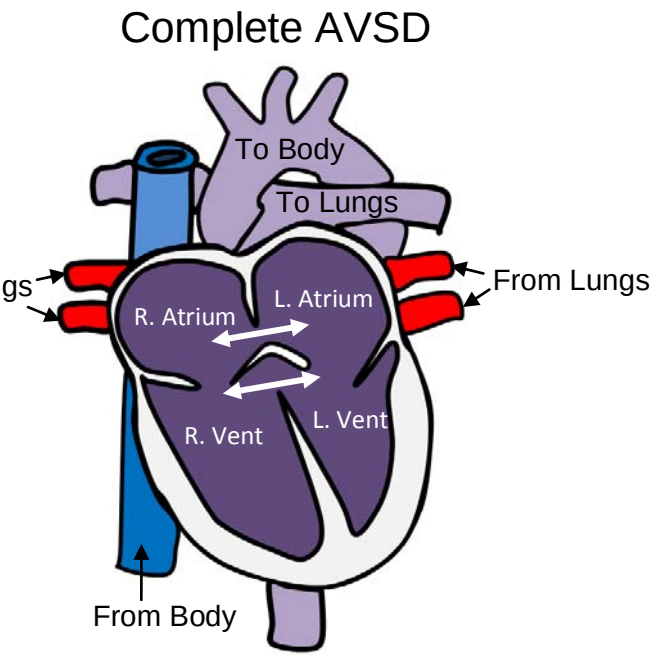

Complete AVSD
To Body
To Lungs
From Lungs
From Lungs
R. Atrium
L. Atrium
L. Vent
R. Vent
From Body

Figure 5: Diagram of AVSD.

Complete and partial AVSD both involve the incomplete septation of the heart and common atrioventricular valve. Complete AVSD involve holes in both the atrial and ventricular septa; and a common atrioventricular valve with a common annulus. Partial AVSD involve a patent ostium primum in the atrial septum and a common valve with separate annuli. In both cases these errors are thought to arise from endocardial cushion defects, hence they are grouped together as AVSD. Additionally, in both cases these abnormalities permit the mixing of oxygenated blood (pictured in red) and de-oxygenated blood (pictured in blue), as shown with white arrows. This mixing of oxygenated and de-oxygenated blood (pictured in purple) greatly reduces the efficiency of the heart. Right atrium, R. Atrium; Left Atrium, L. Atrium; Right Ventricle, R. Vent; Left Ventricle, L. Vent.

AVSD can cause pulmonary complications

Loss of contiguity between the atrial and ventricular septum, such as seen in AVSD, allows mixing or "shunting" of the blood from the left and right sides of the heart. In a complete AVSD or partial AVSD with mitral regurgitation, this allows an excess volume of blood to flow from the systemic (left side) circulation to the pulmonary circulation (right side) in a process called a left-to-right shunt. This causes increased pulmonary blood flow (Vincent et al. 1984) and increased pressure across the lung capillaries, which can cause pulmonary edema and abnormal pulmonary function. If left untreated this may lead to a failure of the pulmonary vasculature to remodel during early infancy, which causes abnormal pulmonary vascular bed reactivity, fixed pulmonary hypertension and eventually irreversible pulmonary vascular disease (Haworth 1998).

AVSD can cause metabolic abnormalities

Sixty percent of hospitalized children with left-to-right shunts have acute and chronic malnutrition (Cameron et al. 1995). Why this would be is uncertain but may be due to an increased metabolic expenditure caused by the increased respiratory effort and myocardial work common to left-to-right shunts. This is supported by a study showing that infants with large left-to-right shunts have

increased oxygen consumption, which is normalized after surgical correction (Puhakka et al. 1993).

AVSD can cause congestive heart failure

Untreated AVSD often causes heart failure, a condition in which the heart can no longer pump enough blood to meet the demands of body. Congestive heart failure usually develops by the second month of life in infants with AVSD. However, if there is significant regurgitation of blood through the common atrioventricular valve, coarctation of the aorta or ventricular imbalance, cardiac failure may begin within the first week of life (Craig 2006). Therefore, if untreated many with AVSD die in infancy and those who do survive often develop pulmonary vascular disease and eventually die with Eisenmenger syndrome (Craig 2006), a syndrome defined by cyanosis, pulmonary hypertension and erythrocytosis. It is Eisenmenger syndrome, with its associated cyanosis that is behind the term “blue baby” ascribed to infants with cyanotic heart conditions like AVSD.

Diagnosing AVSD

Signs of AVSD may include mitral regurgitation; congestive heart failure; or the

asynchronous closure of the aortic valve and the pulmonary valve, which can be heard as a wide fixed splitting of S2 during auscultation. Additionally, a chest X ray may find bi-ventricular enlargement. An echocardiogram can then be used to show the pathophysiology and Doppler echocardiograms can determine the presence of mitral, tricuspid or common valve regurgitation.

AVSD treatment

The natural history of AVSD is similar to VSD but unlike muscular VSD, AVSD do not undergo spontaneous narrowing or closure. Therefore, in order to avoid the complications inherent to septal holes, AVSD must be treated with pharmaceuticals and surgery.

The drugs used to care for AVSD are often the same used to care for congestive heart failure, such as diuretics like lasix (furosemide) to address the edema associated with poor circulation; and angiotensin converting enzyme (ACE) inhibitors, such as captopril and digoxin, to reduce the activity of the renin-angiotensin-aldosterone pathway, which is abnormally high in left-to-right shunt patients due to the inability of the heart to meet the body's needs (Buchhorn et al. 2001) . These medical treatments can mitigate AVSD symptoms and may

allow infants to wait for surgery until they are older. However, there is no medical cure for congestive heart failure.

Initial surgical treatment may include pulmonary artery banding to restrict blood flow to the pulmonary system and prevent permanent lung damage before corrective heart surgery is performed. Most complete surgical heart repairs then occur at 3-6 months for infants with a complete atrioventricular septal defect and at 6-18 months for infants with a partial atrioventricular septal defect (Dudek 2006). These complete surgical repairs include patching the holes in the atrial or ventricular septa, usually with manmade materials such as Dacron or Gore-Tex; and dividing and reconstructing the common atrioventricular valve.

Recovery after surgical repair of a partial AVSD is usually brief and most are home within 4-5 days; additionally, survival after surgery is greater than 97% (Dudek 2006). However, repair of complete atrioventricular septal defects is more frequently associated with complications, including increased pre-surgical pulmonary vascular resistance, which can necessitate mechanical ventilation. Still, most report only a 5-7 day hospital stay and a more than 90 percent surgery survival rate (Dudek 2006) with a complete AVSD. After surgery,

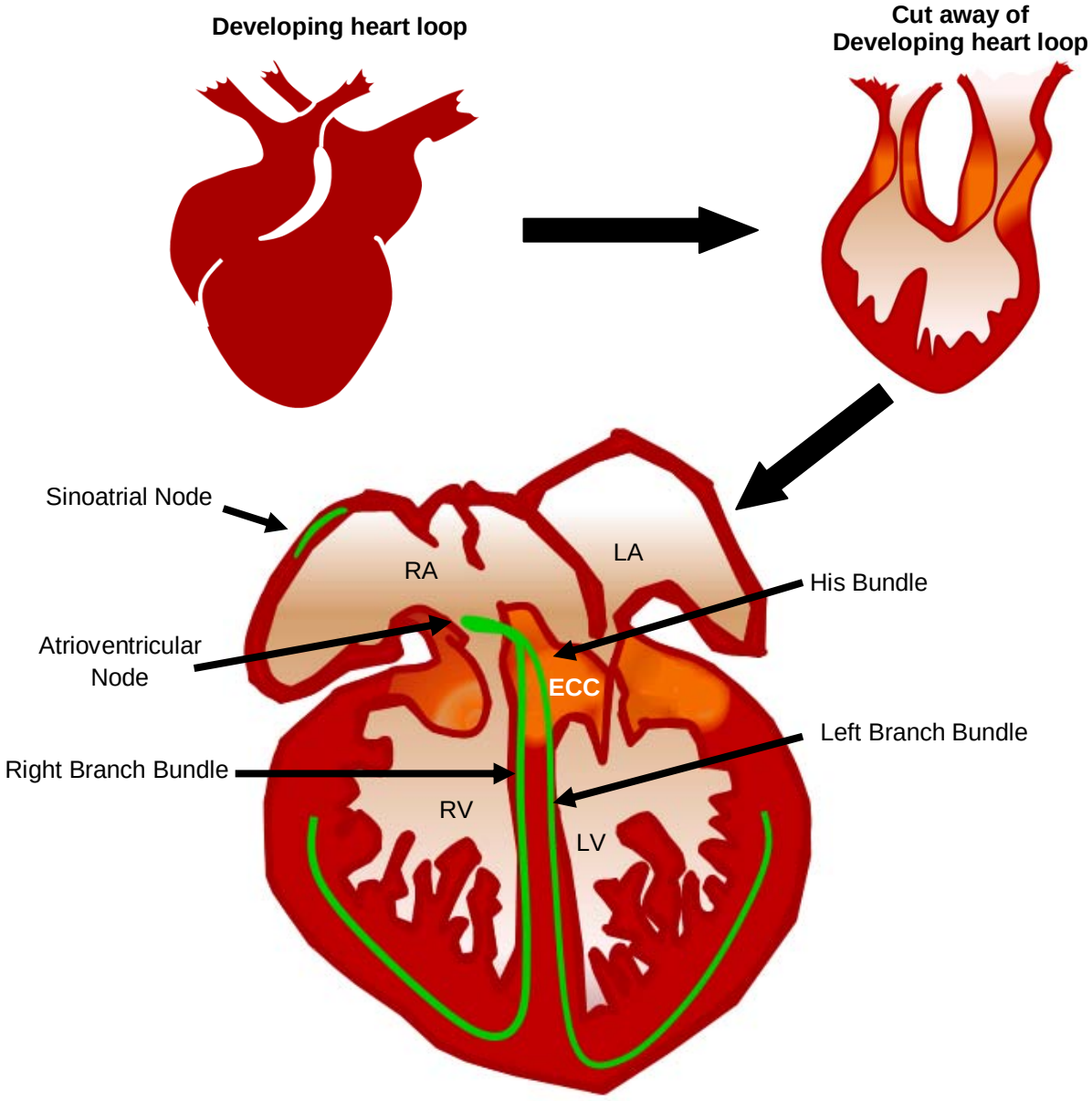
most repaired-AVSD patients are medication free, although continued lifetime follow up with a cardiologist is recommended.

Repaired-AVSD long-term risks

We are only now able to appreciate the long-term outcome of patients living with AVSD, as the first reported surgical correction of a complete AVSD occurred as recently as 1954 (Lillehei et al. 1955). Now instead of being thought of as "cured", AVSD patients are increasingly acknowledged to still be at high risk (Boening et al. 2002, Stulak et al. 2010). These risks include complications following surgical repair, including leaky mitral valves, stenosis of the aorta, or problems with electrical conduction. While stenosis of the aorta and mitral valve regurgitation can be repaired with additional surgeries, the electrical problems are a more serious complication. This is reflected in the fact that patients surviving operations for common congenital heart defects are at a greater risk than their age-matched controls for late-sudden death (Silka et al. 1998).

The association between congenital heart defects, like AVSD, and sudden death may be because the conduction tissues critical to proper heart contractions are located very near to the atrioventricular septum and the atrioventricular valves

(Figure 6). Thus surgical repairs to this area may cause scarring in these delicate tissues. Alternatively, the same genes involved in patterning valves and septa may be important in patterning innervations and thus AVSD could be associated with inborn electrical problems. This is supported by the fact that the actual position of an AVSD dictates the position of the atrioventricular conduction tissues, as the presence of an AVSD forces displacement of the atrioventricular node posteriorly and the His bundle inferiorly (Moss and Allen 2008). Then again, the same genes important in valve and septa formation may simply also be important for heart health on a cellular level; as many of the genes needed to maintain adult health, including adult cardiovascular health, are the same genes involved in development (Combs and Yutzey 2009, Nemir and Pedrazzini 2008, Oka et al. 2007). Whatever the reason, though, the long term survival rate after a successful AVSD surgery is between 80-90% at 10 years and only 65% after 20 years (Drose 1998).



Mature septated heart

Figure 6: Diagram of the endocardial cushions' contributions to the mature heart.

The heart begins as a continuous tube that is divided into a mature four-chambered heart through contributions from the valve and septa anlagen or primordium called endocardial cushions (pictured in orange and labeled "ECC"). It is these endocardial cushions that will form much of the mature heart valves and septa. Accordingly, errors in the formation or maturation of endocardial cushions are thought to cause AVSD, which is the malformation or complete absence of valves and septa. It is notable that the region contributed to by the endocardial cushions is very close to the cardiac conduction system. Pictured is the sinoatrial node**,** which is the heart's pacemaker. Normally, electrical stimulus is generated by the sinoatrial node located in the right atrium that then travels to the atrioventricular node, where it pauses before continuing down the bundle of His to the ventricles, which divides and provides stimuli to both ventricles. This pause is important to insure that the atria finish emptying before the ventricles contract. Inhibition of the conduction system may cause heart block. Thus the close proximity of the conduction system to the endocardial cushion tissue may be why congenital heart defects are strongly associated with sudden death. ECC, endocardial cushions; LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium.

Understanding AVSD genetics may advance future patient care

By identifying the cause of human AVSD, we hope that patient care can progress beyond purely reactive and palliative care. If we understood the cause of AVSD, one of the first things we could offer patients is genetic counseling about the odds of AVSD transmission within their family based on their unique risk profiles, which we are currently unable to do. Additionally, we could develop more relevant animal and cell culture models, which could be used to investigate why AVSD patients are at a higher risk for sudden death. If we understood the cause of these sudden deaths we might be able to prevent them. This prevention might include altering surgical procedures to better avoid conduction tissue or proactively implanting defibrillators or pacemakers into patients deemed inherently high risk. Moreover, creating relevant disease models should allow for the screening of drugs engineered for use in AVSD patients, instead of just borrowing medications from the congestive heart failure population. And lastly, the Holy Grail would be to prevent and cure AVSD. To this end, gene therapy is the most optimistic approach; however, performing in utero gene therapy is fraught with peril, from technical challenges to ethical considerations. More practically, understanding what factors (genetic or environmental) can cause AVSD may allow us to identify high-risk mothers that could decrease their chance of having a child with AVSD by adopting lifestyle changes. Still, the best AVSD treatments or preventions may remain unforeseen until we know what causes AVSD and are able to directly address the long-term underlying risks that accompany membranous septal defects and a common atrioventricular valve.

CHAPTER 3:

CONGENITAL HEART DEFECTS RISK FACTORS

Considering the burden that congenital heart defects present to both patients and society-at-large, it is important to understand the underlying molecular causes of AVSD. Only by understanding the molecular cause of AVSD can we ever hope to predict the transmission and progression of AVSD, which is the first step to providing more personalized and efficient care for this population. But before we can explore the genetics of AVSD we need to familiarize ourselves with what risk factors are associated with other congenital heart defects affecting the atrioventricular valves and septa.

External risk factors

External factors (chemical, physical, biological) are thought to cause of 10% of congenital defects (De Santis et al. 2004). Thus, it is plausible that external factors may play a role in AVSD etiology. Supporting this notion is the fact that some drugs are known heart teratogens. For example, isotretinoin, a drug used to treat acne and an analogue of vitamin A, can cause heart defects. Studies in humans have found isotretinoin use during the first trimester of pregnancy causes miscarriage in 22% of

mothers and malformations in 18% of fetuses studied, including conotruncal heart defects and aortic-arch abnormalities (Lammer et al. 1985). Additionally, there is some suggestion that lithium, which is used to treat bipolar disorder, is associated with cardiac defects. Retrospective and case studies have found, out of a total of 284 cases of infants exposed to Lithium during gestation, 32 had abnormalities, 22 of which included cardiac abnormalities, including Ebstein's anomaly, patent ductus arteriosus (PDA) and tricuspid regurgitation (Yacobi and Ornoy 2008).

External factors such as maternal health can also affect heart development, as maternal diabetes mellitus is associated with a five-fold increase in the risk of congenital heart defects in human infants (Wren et al. 2003), including endocardial cushion defects, persistent truncus arteriosus and ventricular septal defects. Additionally and in specific regard to this thesis, the Baltimore-Washington Infant Study found that infants of diabetic mothers are more susceptible to developing an AVSD (Loffredo 2000). Consistent with these human studies, the embryos of diabetic mice are disposed to develop persistent truncus arteriosus and ventricular septal defects (Kumar et al. 2007). Why this association exists is not well understood but it has been shown that expression of Bmp4, Msx1 and Pax3 (all genes involved in the cardiac neural crest) is reduced in the defective mouse hearts born to diabetic dams; and that maternal diabetes is associated with reduced proliferation and increased apoptosis in the endocardial cushions and myocardium of murine embryos born to

diabetic dams (Kumar et al. 2007). However, how diabetes mellitus is affecting these developmental changes is still only speculation.

High altitude also appears to be a risk factor for congenital heart defects. A study of 32,578 Tibetan children, between the ages of 4-18 living at 2,535; 3,600 and 4,200 meters found that congenital heart defect morbidity rose with increasing altitude, with 5.45 cases per thousand at 2,535 meters, 6.80 cases per thousand at 3,600 meters and 9.79 cases per thousand at 4,200 meters. Additionally, the constitution of congenital heart defects differs between the altitudes (Jin et al. 2008). This huge epidemiology study is convincing evidence that altitude can affect heart development and has prompted proposals that altitude and oxygen saturation can affect heart development enough to affect human disease rates. This has spurred investigation into how high altitude could affect heart patterning, including investigation of hypoxia-responsive genes such as VEGF in heart development (Dor et al. 2001).

Genetic risk factors

Congenital heart defects are suspected to have a genetic component, as the prevalence of congenital heart defects in the family members of patients with isolated congenital heart disease is 2%–16% versus the 0.4%–0.8% found in the

general population, suggesting heritability (Campbell 1973, Loffredo et al. 2004, Samanek 2000, van der Velde et al. 2005). Accordingly, below are some genes that have been found associated with congenital heart defects.

Familial autosomal-dominant cases

Although numerous genes are known to be necessary for animal model heart development, only a few mutations in these genes have been related to human congenital heart disease, many of which are only found in a few rare families who exhibit familial autosomal-dominant congenital heart defects, e.g. *NKX2-5*, *GATA4*, *MYH6* and *NOTCH* (Joziasse et al. 2008). Mutations in the heart-specific transcription factor *NKX2-5* have been identified in families with inherited autosomal dominant atrial septal defects, atrioventricular block, ventricular septal defect or AVSD (Reamon-Buettner and Borlak 2004, Benson et al. 1999). Mutations in another transcription factor, *GATA4* have been found associated with autosomal dominant congenital heart defects in two families who had atrial septal defects, ventricular septal defects, atrioventricular septal defects, pulmonary valve thickening, or insufficiency of the cardiac valves (Hirayama-Yamada et al. 2005, Garg et al. 2003). Mutations in a structural protein, alpha-myosin heavy chain (*MYH6*), are proposed to be causative in a large family with dominantly inherited atrial septal defect (Ching et al. 2005). Mutations in the signaling protein *NOTCH1* are associated with a spectrum of developmental

aortic valve anomalies and severe valve calcification in non-syndromic autosomal-dominant human pedigrees (Garg et al. 2003) and missense mutations in *JAGGED1* (a NOTCH ligand) have been found in a large kindred with autosomal dominant tetralogy of Fallot (Eldadah et al. 2001).

The successful discovery of genetic variants associated with familial heart defects is a proof-of-principle that genes are associated with heart defects. The reason that so many genetic risk factors have been identified in familial congenital heart defect cases (which are rare) is because linkage analysis can be used to discover familial genes. Thus, rare familial congenital heart defects are a valuable resource for identifying new genetic risk factors. Furthermore, risk factors found in familial cases may also be relevant to the larger sporadic or syndromic heart defect population. This is supported by the fact that *GATA4* mutations have also been found associated with isolated congenital heart defects cases, including an atrial septal defect, a ventricular septal defect and tetralogy of Fallot (Tomita-Mitchell et al. 2007).

Syndromic cases

It appears that about one third of congenital heart defect patients also have dysmorphic features, suggesting that their defects are part of a syndrome, such as trisomy 21, Noonan's syndrome or Holt-Oram syndrome (Formigari et al.

2009). Accordingly, it is postulated that the genetic risk factors involved in syndromes might also be involved in congenital heart defect risk. In support of this, *TBX5* a gene that when mutated can cause Holt-Oram syndrome [a syndrome that includes a variety of heart defects including atrial septal defects, ventricular septal defects and conduction-system defects (Basson et al. 1997)] is also the first single-genetic risk factor found to be associated with isolated congenital heart defects, including ventricular septal defects, atrial septal defects and AVSD (Reamon-Buettner and Borlak 2004). Furthermore, another syndrome, heterotaxy (a disorder of left-right symmetry that is often associated with cardiac defects) has been postulated to share risk genes with congenital heart defects, as AVSD may result from misalignment of atria over the ventricles either during heart looping or septation. Supporting this hypothesis, a mutation in the first gene associated with heterotaxy (MIM 306955), the zinc finger transcription factor (*ZIC3*), has been found in a patient that did not have heterotaxy but did have an atrial septal defect and pulmonic stenosis (Ware et al. 2004). Thus *TBX5* and *Zic3* are proof-of-principle that risk factors originally identified in syndromes may also prove relevant to the often more common isolated congenital heart defect population.

Mouse models

CITED2, which encodes a CREBBP/EP300 interacting transcriptional modulator, is essential for heart development in mice. Thus it was speculated that mutations in CITED2 might cause heart defects in humans. Therefore, CITED2 was sequenced in a population composed of numerous heart defect where CITED2 alterations were found, including in one patient with a sinus venosus atrial septal defect and abnormal pulmonary venous return; one patient with an atrial septal defect of the secundum type; one patient with a perimembranous ventricular septal defect; two patients with tetralogy of Fallot; one with situs inversus totalis, transposition of the great arteries, and perimembranous ventricular septal defect; one patient with perimembranous VSD and secundum atrial septal defect; and one patient with perimembranous ventricular septal defect (Sperling et al. 2005). Thus, genes identified in mouse models as essential to heart development have been shown to be relevant to human disease.

The variety of defects associated with CITED2 is notable and consistent with the range of congenital heart defect phenotypes associated with other single genetic risk factors for isolated heart defects, such as CITED2, GATA4, NKX2-5 and TBX5. Such heterogeneity suggests that modifiers must exist that can affect the penetrance and expressivity of these risk factor genes. However, risk factor

combinations are rarely explored and no reliable causative risk profiles have ever been described for any congenital heart defect population. This is a shortcoming I seek to address in my thesis.

AVSD inheritance

Most AVSD are associated with Down syndrome

Sixty percent of AVSD cases are associated with trisomy 21 (Down syndrome). Accordingly, compared to the euploid population, the Down syndrome population is at a 2,000-fold increased risk of developing AVSD (Maslen et al. 2006), an association that suggests that AVSD susceptibility genes exist on chromosome 21. This has spurred the investigation of chromosome 21 for AVSD risk factors. Particular focus has been placed on the Down syndrome "critical region" located on the distal half of the long arm of human chromosome 21, which contains most of the gene transcribing sites on chromosome 21 and thus, is thought to contain the genes essential to producing Down syndrome symptoms (Shapiro 1999). Accordingly, some have proposed that DSCAM (Down syndrome cell adhesion molecule), a gene that is located in the Down syndrome critical region and is expressed in the heart during cardiac development, might be an AVSD risk gene (Barlow et al. 2001); however, this has not been confirmed. In fact, to date, no single AVSD risk gene on chromosome 21 has been identified

as an AVSD risk gene. This is likely due to the inherent limitations associated with this method of gene discovery, as mapping risk genes to chromosome 21 relies on the existence of a robust study population of partial chromosome 21 cases that are free of other genomic re-arrangements, which may confound the phenotype. However, such simple partial chromosome-21 cases are exceedingly rare.

To get around the limitations of gene mapping in the human Down syndrome populations, some have turned to mouse models. In mice, approximately two thirds of the orthologs of the 243 known human chromosome 21 (Hsa 21) genes are on mouse chromosome 16 (Mmu 16) (Benson and Sund 2010). Thus, Mmu 16 trisomic mice have been made in the hopes of modeling Down syndrome; however, these mice are not viable, as they are also partially trisomic for three other human chromosomes (Hsa 3, 21, 22). Therefore, the most useful Down syndrome mouse models are only trisomic for the portions of Mmu 16 that are orthologous to Hsa21. These models are therefore said to have partial trisomy or segmental trisomy (Benson and Sund 2010). Popular partial segmental strains include the Ts65Dn (Reeves et al. 1995) or Tc1Cje models. Alternatively some have created transchromosomic mice (Tc1) that are aneuploid for the human chromosomes 21, as this method would most closely recapitulate the gene dosage in humans. However, this last method is fraught with technical

challenges, including difficulties in achieving male germline transmission, somatic retention and chromosome integrity (O'Doherty et al. 2005).

Although none of the mouse models perfectly mimic human genetics, Down syndrome mouse models do exhibit a number of Down syndrome characteristics, including synaptic plasticity and learning deficits; abnormal craniofacial morphology; hampered T lymphocyte activation; and atrioventricular canal defects (O'Doherty et al. 2005). Thus, these mouse models may prove useful for mapping the chromosome 21 genes associated with the variety of abnormalities found in Down syndrome, including AVSD risk genes. Additionally, these mice models may also prove useful for identifying other (non-chromosome 21) risk factors, as the penetrance of the cardiac defects can depend on the Down syndrome mouse strain background (Benson and Sund 2010). Thus these Down syndrome mouse models may serve as AVSD-susceptibility models that can be used to genetically investigate whether other candidate risk factors can modify AVSD penetrance or expressivity.

Similarly, while it is notable that 40% of Down syndrome children have congenital heart defects and about 45% of those cases are AVSD (Seale and Shinebourne 2004), it is equally notable that most Down syndrome children do

not have any heart malformations. This means that in humans three copies of chromosome 21 is not enough to cause an AVSD but instead other risk factors are also needed. Accordingly, AVSD genetics are thought to be complex, possibly contributed to by a combination of risk factors, including contributions from chromosome 21, external factors or non-chromosome 21 genetic variations.

AVSD also regularly occur in the euploid population

AVSD are common in the euploid population and while a few euploid cases seem to be transmitted in an autosomal dominant fashion with incomplete penetrance (McKusick 1998), this is the exception. Instead, the vast majority of euploid AVSD cases do not have any appreciable inheritance pattern and occur as sporadic events. Sporadic inheritance patterns are the hallmark of a complex disease; accordingly, we believe that AVSD are the result of a combination of risk factors, possibly both genetic and environmental. However, such heterogeneity makes finding risk factors a challenge because it lowers the statistical power of identifying any one risk factor within a study population. This may explain why many genes have been identified as critical for heart development but few genes have been successfully associated with human AVSD. To minimize the statistical weakness inherent to studying heterogeneous populations, our laboratory used

strict inclusion/exclusion criteria when creating our patient population to minimize as much genetic heterogeneity as possible.

CRELD1 is the first AVSD risk factor

CRELD1 is a candidate gene from the *AVSD2* risk locus

Previous linkage mapping studies identified two AVSD risk loci in humans, *AVSD1* and *AVSD2*. *AVSD1* was mapped in a family with autosomal dominantly inherited AVSD to 1p31-p26 (Sheffield et al. 1997), however the risk gene in this region has still not been located. The *AVSD2* locus was mapped in children with a very rare syndrome called 3p minus syndrome. This syndrome is caused by the absence of the distal part of the short arm of chromosome 3 and can cause numerous abnormalities, including low birth weight; psychomotor and mental retardation; hypotonia; microcephaly; ptosis; micrognathia; telecanthus; and congenital heart defects. About one third of children with 3p minus syndrome have AVSD, suggesting an AVSD risk factor must exist in this region (MIM, 606217). This prompted the mapping of an AVSD risk locus (*AVSD2*) to 3p24.2-25, which contains 153 genes (Green et al. 2000). The Maslen laboratory then screened this region for an AVSD risk gene.

When the Maslen lab screened the *AVSD2* locus, they found an unknown gene in this region that is expressed in the heart (Robinson et al. 2003, Rupp et al. 2002), implying that the gene had the potential both temporally and spatially to cause an AVSD when mis-regulated. Initially this gene was named 'cirrin' but it was quickly renamed by the Human Genome Nomenclature committee to Cysteine-rich with EGF-like domain (CRELD) 1.

CRELD1 is the founding member of the CRELD-family of proteins (Rupp et al. 2002), a unique family of proteins, whose function is unknown and of which bioinformatics analyses did not reveal much additional information. The CRELD family is defined by a unique CRELD domain (previously called the 'WE' domain). This CRELD domain is a glutamate and tryptophan rich domain that is highly conserved and located near the amino (N)-terminus, which is predicted to be extracellular, while the carboxy (C)-terminus consists of a two-pass transmembrane domain that is thought to anchor the protein to the cell membrane (Figure 7). With no knowledge about the function of CRELD1 we turned directly to human association analysis to test if CRELD1 could be the *AVSD2* risk factor.

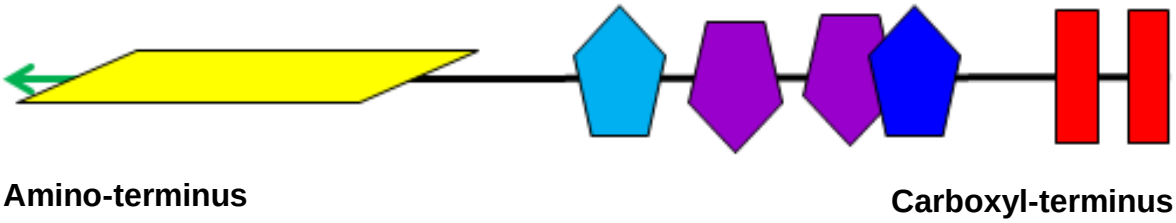


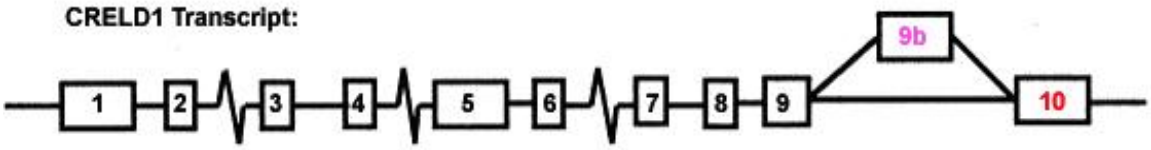
Figure 7: CRELD1 structure.

CRELD1 is predicted to be cell-surface protein, which is bound to the cell membrane by a two-pass transmembrane domain (pictured in red). The CRELD family is characterized by a number of domains. CRELD1 has repeated EGF-like domains, including an EGF-like 1 domain that is pictured in aqua and an EGF-like 2 calcium binding domain that is pictured in blue. A unique motif called the CRELD domain (pictured in yellow) is rich in tryphtophan (W) and glutamate (E) residues and was thus formally called the "WE" domain. There are also two tandemly repeated FU 1 domains (pictured in purple), which are furin-like cysteine rich regions typically involved in signal transduction by receptor tyrosine kinases that use receptor aggregation. There is also a poorly conserved proline-rich N-terminus that is predicted to be a signal peptide (pictured as a green arrow). These CRELD1 featured were determine by UniProtKB.

CRELD1-isoforms

CRELD1 can also be alternatively spliced, most notably into the CRELD1-9b isoform. CRELD1 is normally affixed to the cell surface by a two-pass transmembrane domain (Rupp et al. 2002) but when *CRELD1* utilizes a cryptic exon embedded in intron 9 (exon 9b) the transmembrane domain is eliminated and replaced by a unique carboxyl-terminus (Rupp et al. 2002) (Figure 8). This resulting product is no longer tethered to the cell membrane but is instead secreted into the extracellular space. However, the extracellular domain of CRELD1 remains unaltered. Thus, we speculate that this soluble CRELD1-9b form might work as a CRELD1-ligand decoy; however, this has not been confirmed as a CRELD1 ligand has not been identified.

A.


CRELD1 Transcript:
1
2
3
4
5
6
7
8
9
9b
10

B.

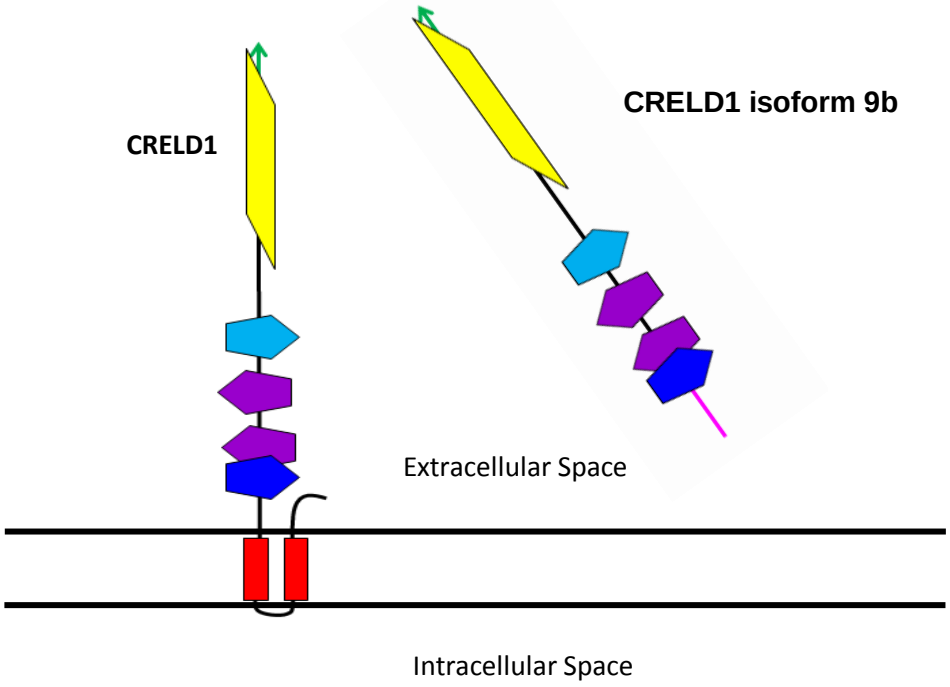

CRELD1
CRELD1 isoform 9b
Extracellular Space
Intracellular Space

Figure 8: Illustration of the major soluble CRELD1 isoform.

A: Pictorial representation of the intron/exon boundaries of CRELD1. The solid lines represent introns, the open boxes are exons. Exons are labeled 1 through 10. The major CRELD1 transcript uses exon 10 (in red); the alternatively spliced product, CRELD1-9b, uses the cryptic exon 9b (in pink) located in intron 9. **B**: A pictorial representation of CRELD1 and CRELD1-9B, illustrating the relative location of the soluble CRELD1-9B to the membrane-bound CRELD1. CRELD1 is usually anchored to the cell's membrane by a pair of transmembrane domains. The alternatively spliced CRELD1-9b lacks these transmembrane domains, allowing CRELD1-9B to be secreted. Note that the extracellular domains remain identical; therefore, the 9B isoform would be a capable competitor of CRELD1.

CRELD1-missense mutations are associated with AVSD

To test if *CRELD1* is associated with AVSD the Maslen lab performed a case control study. When we did this we found that almost 8% of sporadic AVSD cases (including some with heterotaxy) and 5% of Down syndrome-associated AVSD cases are heterozygous for a *CRELD1*-missense mutation (Robinson et al. 2003, Rupp et al. 2002, Maslen et al. 2006). Importantly, we never found any *CRELD1*-missense mutations in over 400 chromosomes from unrelated but race-matched controls. This highly specific genetic association between *CRELD1*-missense mutations and AVSD suggests that mutations in *CRELD1* increase the risk of developing an AVSD, a finding that was subsequently confirmed by other investigators (Zatyka et al. 2005, Sarkozy et al. 2005, Guo et al. 2010). This makes *CRELD1*-missense mutations the first single genetic risk factor ever associated with sporadic AVSD.

The CRELD1 mutations found included an alanine to proline substitution at amino acid 286, a glutamate to lysine substitution at amino acid 325, an arginine to cysteine substitution at 329 and a glutamate to lysine substitution at residue 414. Three of these mutations p.A286P, p.E325K and p.R329C are in conserved domains, including the FU1 and EGF-like 2 domains. The most common mutation, R329C, is especially notable because it is a cysteine addition where a

highly conserved arginine residue should be. Addition of a cysteine has the potential to be deleterious in any protein because of its ability to form disulfide bonds and therefore cause aberrant protein folding. Specifically, the R329C substitution is within the highly conserved EGF-like domain (Maslen et al. 2006), a domain that normally contains six cysteines that form disulfide bonds in a C1-C3, C2-C4, C5-C6 pattern. Thus, we hypothesized that the addition of a seventh cysteine in this domain could cause misfolding; or impair binding with itself or ligands. Misfolding was demonstrated by the abnormal migration of the R329C protein in a native gel (Robinson et al. 2003).

What is known about CRELD1 according to existing literature

In the last few years CRELD1 has started to appear regularly in the literature, which may provide insight into its importance and function. CRELD1 has been identified as a marker of lineage commitment and cell fate in mammary epithelial subpopulations (Kendrick et al. 2008); a recent abstract suggests that CRELD1 is an important regulator of polarized cell migration in *Drosophila* ovaries, a process regulated by NOTCH signaling (deCathelineau et al. 2009, Nystul and Spradling 2009); and most recently, a CRELD1 isoform, CRELD1α, was identified as an α1a-adrenoceptor down-regulating protein (Nishimune et al. 2010). Additionally, it has been shown that CRELD1 can directly regulate the pro-

apoptotic factor RTN3 (Xiang and Zhao 2009). However, none of these studies have been confirmed by independent groups and all of these studies were performed in cell culture, so CRELD1 activity in vivo has not been addressed. Accordingly, in this thesis I characterized the role of CRELD1 in vivo, with a specific emphasis on heart development.

AVSD genetics are multifactorial

Monogenic Mendelian inheritance of disease is rare; instead most diseases are thought to be multifactorial or complex (Motulsky 2006), such as heart disease, stroke, diabetes, cancer and most congenital heart defects. Similarly, while AVSD exhibit heritability suggesting the existence of genetic risk factors; AVSD rarely exhibit Mendelian inheritance patterns (Burn et al. 1998, Gill et al. 2003) and even when they do the expressivity varies greatly (Reamon-Buettner and Borlak 2004, Benson et al. 1999, Hirayama-Yamada et al. 2005, Garg et al. 2003), indicating that AVSD genetics are complex. This is consistent with CRELD1 epidemiology, as *CRELD1* mutations are incompletely penetrant for AVSD (Robinson et al. 2003). Thus, I propose that it will take numerous risk profiles to explain the full spectrum of AVSD cases (Figure 9). Additionally, I believe that these risk profiles can be complicated and include both environmental as well as genetic hits; and that these genetic hits may include both rare variants and

common functional polymorphisms. Our challenge now is to identify other risk factors associated with AVSD and to determine what specific combinations of risk factors (in combination with *CRELD1*-missense mutations), or 'risk profiles' are needed to cause AVSD.

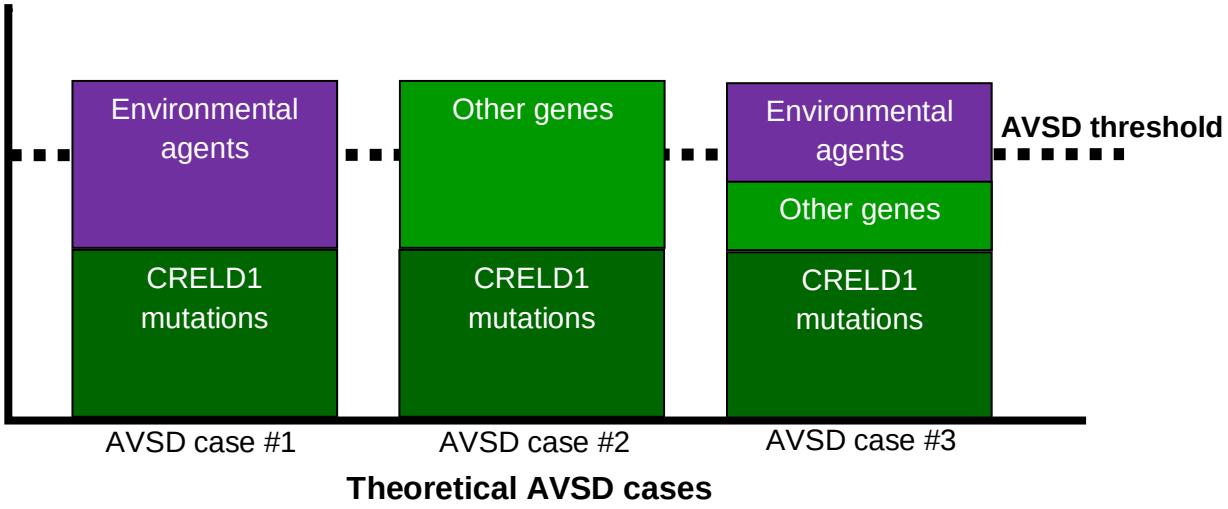


Figure 9: Diagram of the multifactorial nature of AVSD genetics.

The above graph illustrates how multiple unique AVSD risk profiles might lead to the same disease phenotype. The dotted line above represents the "AVSD disease threshold." The bar graphs illustrate theoretically how multiple risk factors might additively contribute to AVSD risk and breach the disease threshold to cause an AVSD. My working hypothesis is that numerous risk factors work in a combinatorial fashion to contribute to AVSD. However, the number of factors needed to breach the disease threshold is dependent on the relative impact of the factors involved and will have to be determined empirically on a factor-by-factor basis.

VEGF may be an AVSD risk factor

Overview of the VEGF-A protein

Vascular endothelial growth factor (VEGF-A) is located on chromosome 6 and was originally purified from bovine pituitary folliculostellate cells and tumor cell line conditioned media (Woolard et al. 2009). It is a member of the superfamily of cysteine knot proteins, which includes VEGF-B, -C, -D, and placental growth factor (PlGF); and can be alternatively spliced to create five isoforms, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$ and $VEGF_{145}$. The main and best studied VEGF molecule is the VEGF-A_{165} isoform and thus, this will be the isoform that I will focus on in this thesis and hereafter just refer to as VEGF.

The mature form of VEGF is secreted as a homodimer (Nicosia 1998) that transduces its signals by binding to two tyrosine kinase receptors: VEGFR1 (alternatively called Flt1) (Shibuya et al. 1990, de Vries et al. 1992) and VEGFR2 (alternatively called Kdr) (Millauer et al. 1993, Mustonen and Alitalo 1995) with high affinity, Kd 1-20 and 75-770 pM respectively (Mustonen and Alitalo 1995). Both VEGFR1 and VEGFR2 receptors are expressed by endothelial cells but do not have redundant functions, as knockout of VEGFR2 blocks mesodermal cell differentiation to angioblasts (Shalaby et al. 1995) and knockout of VEGFR1

induces increased endothelial progenitor production (Fong et al. 1995, Fong et al. 1999). Concordantly, VEGF is known for its ability to induce differentiation, proliferation and migration of endothelial cells; act as an endothelial-cell survival factor; and stimulate neovascularization (Ferrara et al. 1996, Leung et al. 1989, Benjamin and Keshet 1997, Benjamin et al. 1999, Gerber et al. 1999). Accordingly, VEGF also can influence heart development, particularly the ability of endothelial cells to transform and populate endocardial cushions.

VEGF expression is necessary for endocardial cushion development

Vegf is normally up-regulated in the heart tube, specifically in the myocardium that underlies the atrioventricular endocardial cushion during the onset of endocardial cushion formation (E10.5) (Dor et al. 2001), suggesting that *Vegf* has a role in atrioventricular cushion development. Supporting this, knockdown of VEGF in a mouse model causes reduced endocardial cushion mesenchyme at E10.5 (Ferrara et al. 1996).

However, there is one study that made a contrary conclusion (Stankunas et al. 2010). This study inhibited VEGF function with an inducible sFLT (an engineered secreted form of VEGFR1 designed to sequester the VEGFR1 ligands, such as VEGFA, VEGFB, and PlGF) at various stages in development and found that their

inhibition only impacted the outflow tract endocardial cushions but not the atrioventricular canal endocardial cushions (Stankunas et al. 2010). Therefore, Stankunas et al. concluded that VEGF signaling is only essential in the outflow tract endocardial cushions' EMT (Stankunas et al. 2010). However, this study's conclusion is based on incomplete data points. Stankunas et al. found that an E9.0-9.75 inhibition of VEGF could inhibit outflow tract EMT. While the exact timeline of VEGF function in heart development is not known, it is known that outflow tract cushion EMT typically occurs about a day later than atrioventricular cushion EMT (Runyan and Markwald 1983). Therefore, it would seem logical to test atrioventricular EMT by inhibiting VEGF signaling a day before this at E8.0-8.75. However, the earliest time point tested in this study was E8.75. Because, while VEGF has been documented to be up-regulated at E10.5, it may also be necessary earlier to condition endothelial cells for EMT initiation; however, this was not been tested. Therefore, based on others' work (Ferrara et al. 1996, Dor et al. 2001), I conclude that VEGF signaling is necessary for normal atrioventricular endocardial cushion development.

VEGF over-expression inhibits endocardial cushion development

Numerous studies have shown that various levels of VEGF can negatively impact heart development. Premature induction of VEGF expression in the myocardium at E9.5 (using a tetracycline-regulated transgenic system) can prevent

atrioventricular endocardial cushion EMT (Dor et al. 2003). Additionally, if 100 ng/ml of recombinant human VEGF-165 is added to explanted atrioventricular canals at E9.5, EMT is inhibited (Dor et al. 2001). However, the relevance of the Dor et al. explant model to development is debatable, as 100 ng/ml may be as high as a 6,000 fold increase in VEGF expression (if normal at E9.5 is considered the same as an E12.5 heart at 14.92 pg/ml) (Miquerol et al. 2000). Recently, another more biologically relevant study found that only a three- to four-fold over-expression of VEGF in a mouse model can cause embryonic lethality by mid-gestation (E12.5-E14) and heart abnormalities, including thin ventricular walls and outflow tract defects (Miquerol et al. 2000). This suggests that even a minor increase in VEGF can impact development, including cushion development, although, in this case only outflow tract cushion development. In summary, it appears that VEGF over-expression or premature induction can inhibit atrioventricular endocardial cushions; however whether this ability to inhibit atrioventricular endocardial cushion is relevant to normal development or human disease remains to be proven.

VEGF as a mediator of hypoxia

It has long been acknowledged that hypoxia or nutritional insults during gestation contribute to congenital heart defects but the exact molecular mechanism remains unknown (Clemmer and Telford 1966, Ingalls et al. 1952,

Jaffee 1974). However, recently *VEGF* has emerged as a candidate mediator of hypoxic effects on the developing heart, largely because *VEGF* over-expression can impact the heart and *VEGF* can be robustly induced by environmental factors, such as hypoxia and hypoglycemia (Ikeda et al. 1995, Levy et al. 1996). Additionally, both hypoxia and anoxia have been shown to induce precocious VEGF expression specifically in E9.5 endocardial cushion explants to levels comparable to naïve E10.5 explants (43.12 and 45.1 vs. 3.35 pg/ml) (Dor et al. 2003), suggesting that hypoxia may perturb heart development through VEGF up-regulation in the developing endocardial cushions. In support of this hypothesis, the ability of hypoxia to inhibit EMT in heart explants can be reversed by the addition of a soluble inhibitor of VEGF signaling (sFLT) (Dor et al. 2003).

VEGF polymorphisms are implicated in disease

VEGF is involved in both vascular development and in pathological angiogenesis where it is perhaps best known for its pro-angiogenic role in cancer progression. VEGF polymorphisms have also been associated with numerous diseases, including proliferative diabetic retinopathy (Ray et al. 2004), breast cancer (Brower 2003), myocardial infarctions in patients with type 2 diabetes (Petrovic et al. 2007), chronic heart failure prognoses (van der Meer et al. 2005) and

gastric cancer prognoses (Chae et al. 2006). I propose that the involvement of VEGF polymorphisms in multiple complex-disease processes is explained by it being a modifier gene, not as a causative gene. In other words, I propose that VEGF acts in concert with additional risk factors to "unmask" phenotypes induced by other susceptibility genes but variations within VEGF are not sufficient alone to cause disease. Thus VEGF is a promising disease modifier for complex diseases, such as AVSD, which may be caused by a combination of low-penetrance genetic risk factors.

The *VEGF-634C* polymorphism causes over-expression

The *VEGF*-634C polymorphism is located in the 5' untranslated region (UTR) of VEGF. Healthy subjects homozygous for the -634CC polymorphism have significantly higher VEGF serum levels than those individuals with the -634CG genotypes (Awata et al. 2002, Petrovic et al. 2007). This association is recapitulated by in vitro studies that also show higher VEGF levels associated with the -634C allele, compared to the -634G allele (Awata et al. 2005, Lambrechts et al. 2003). These associations may be due to the fact that the *VEGF* -634C allele's relative IRES-B (Internal Ribosomal Entry Site-B) activity is higher than the -634G allele's activity (Lambrechts and Carmeliet 2004), which suggests that *VEGF* post-transcriptional regulation is affected by the -634C polymorphism. Thus, it appears that this polymorphism is functional, which makes it a promising candidate to modify the risk of AVSD. Moreover, others have shown that the

VEGF-634C polymorphism is enriched (0.42 versus 0.21, P < .001) in a heterogeneous congenital heart defect population (Vannay et al. 2006). Therefore we decided to test if the *VEGF*-634C polymorphism could act as a modifier of AVSD risk (Figure 10).

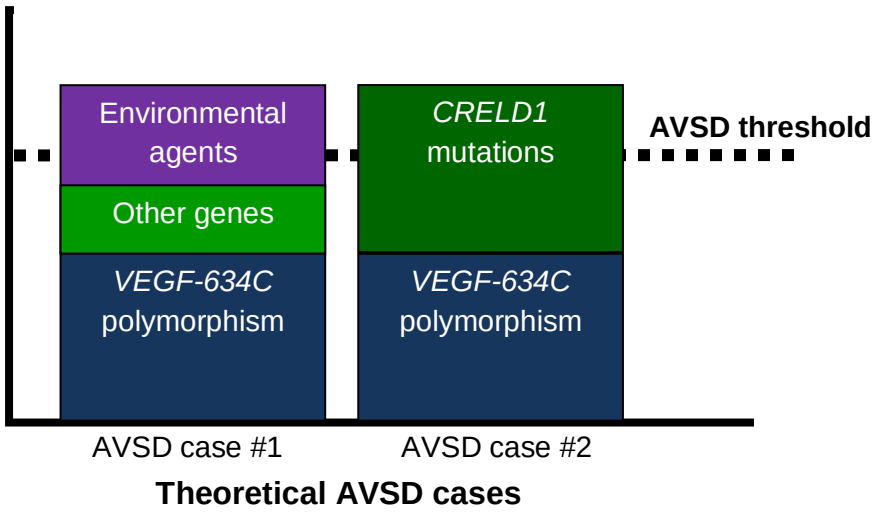


Figure 10: VEGF may contribute to AVSD risk.

The dotted line above represents the "AVSD disease threshold", while the bar graphs illustrate my hypothesis that the *VEGF*-634C polymorphism can contribute to AVSD risk and when the *VEGF*-634C polymorphism is combined with other risk factors can cause AVSD. Accordingly, this thesis will test if the *VEGF*-634C polymorphism is associated with increased AVSD risk in general (case #1) and specifically if the *VEGF*-634C polymorphism in combination with a *CRELD1*-missense mutation can cause AVSD (case #2).

CHAPTER 4:

APPROACH & METHODS

Creation of the *Creld1*-knockout mouse

To knockout *Creld1* we used a modified pACN-1 vector (a gift from Dr. Kirk Thomas, University of Utah, Utah, USA) (Bunting et al. 1999). The pACN-1 vector contains a "self-excising" neomycin cassette that recombines, thereby preventing non-specific promoter effects. This knockout-cassette was positioned within *Creld1* such that 1.9 kilobase (kb) of the 5′ untranslated region (UTR), all of exon 1 and a portion of intron 1 was eliminated upon recombination. The *Creld1*-genomic DNA fragment was obtained by restricting the genomic clone of a 129-strain murine genomic library (OHSU Transgenic Mouse Core Facility, Portland, OR, USA). The replacement construct included an upstream homology arm (5′ relative to the *Creld1*-ATG codon) that spanned approximately 6.3 kb and a downstream homology arm of 3 kb. We also added the selectable marker thymidine kinase downstream of the 3′ arm of homology (a gift from Dr. Scott Stadler of Shriners Hospital for Children, Portland, OR, USA). The completed and sequenced construct was delivered to the Gene Targeted Mouse Service at the University of Cincinnati, Department of Molecular Genetics, Biochemistry and Molecular Biology (Cincinnati, OH, USA). Successfully targeted clones of D15T44 ES cell line (from 129/SvEVTac mice from the Taconic labs) were injected into C57Bl blastocytes to create six chimeric male mice. These six

chimeric mice were bred to NIH Bl/Sw females to generate *Creld1*-heterozygote (*Creld1*$^{(+/-)}$) mice. Full *Creld1*$^{(+/-)}$ were then backcrossed to form and maintain the *Creld1*-knockout (*Creld1*$^{(-/-)}$) colony. See Figure 13.

Mouse colony ethical considerations

The *Creld1*$^{(-/-)}$ mouse strain was generated and is being used under approved IACUC protocols. All personnel, including myself, received instruction in the proper care and handling of laboratory animals. We complied with all institutional and federal policies for the ethical use and treatment of animals; and strictly adhered to the Animal Welfare Act, "Guide for the Care and Use of Laboratory Animals" and all USDA and NIH regulations and standards. Compliance was monitored by members of the Department of Comparative Medicine, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Allele-specific PCR for *Creld1*-knockout mouse genotyping

Primer "KO-F" was designed 5′ of the neo-cassette and paired with a "Reverse" primer (designed in the 3′ homology region of the knockout cassette) to detect a 700 base pair *Creld1*-knockout-allele amplified fragment. A "WT-F" primer was made to bind within exon1 of *Creld1* and also paired with the "Reverse" primer to produce an amplified fragment of 528 base pairs, corresponding to the wild-type genotype. KO-F

primer: CCAGTCAAAAACCACAGAGAGGG, WT-F primer: CATCCTTCTCCCCGAGCTGAG, Reverse primer: GTGTTTCCACCCCCGAAGT. See Figure 14.

Genomic DNA was prepared from tail clips or from embryonic amnions using the QIAGEN DNAeasy kit, per manufacturer's protocol (QIAGEN; Valencia, CA, USA). PCR reaction: 5 μl SuperMixII (Invitrogen; Carlsbad, CA, USA) + 1 μl genomic DNA + 0.5 μl of each 10 μM primer (KO-F, WT-F and Reverse primer) + 2.5 μl water. Cycling Parameters: 94°C x 2 minutes [94°C x 30 seconds, 60°C x 30 seconds, 68°C x 1 minute] x 35 cycles + 68°C x 3 minutes + 4°C hold.

Staging and collection of embryos

Embryonic day 0.5 (E0.5) was determined by vaginal plug. Pregnant mice, with staged embryos, were sacrificed with CO_2 asphyxiation followed by cervical dislocation. Uteri were immediately removed and stored in phosphate buffered saline (PBS) [PBS = 100 mM Tris-HCl, pH 7.4 + 9% sodium chloride] on ice during embryo isolation. Somites were counted to verify embryonic stage. Embryonic yolk sacs were used for DNA isolation. Placentas and embryos were collected and fixed for downstream applications. Pictures of fresh tissue were taken with a Leica MZ12 dissecting microscope, a Q Imaging MicroPublisher 3.3RTV camera and Rincon Version 7.4 imaging software.

Histological sectioning of mouse tissues

After harvesting, embryos were immediately placed in freshly prepared 4% paraformaldehyde (Fisher Scientific F79; Pittsburgh, PA, USA) in PBS and fixed overnight at 4°C; washed in PBS for 3 minutes, five times; and dehydrated using 30 minute washes in an increasing ethanol gradient, 10% -100%. Embryos were then transferred to Cellsafe Biopsy Capsules (Electron Microscopy Sciences; Hatfield, PA, USA) and cleared twice for 15 minutes with xylene (Fisher Scientific X5-4; Pittsburgh, PA, USA). The embryos were incubated at 60°C under vacuum in fresh Paraplast Plus (McCormick Scientific, LLC; Maryland Heights, MO, USA) twice for 1 hour and once overnight. Embryos were then arranged in fresh paraplast and cooled overnight. Embedded embryos were then cut into 8 μm sections and arranged onto Colorfrost/Plus microscope slides (Fisher Scientific; Pittsburgh, PA, USA) and adhered with water and allowed to bake overnight at 37°C, after which slides are ready to use in downstream applications. Alternatively, some of the sectioned embryos (as indicated in the text) and all of the adult mouse tissues were purchased from Zyagen (San Diego, CA, USA).

CRELD1 immunohistochemistry (IHC) of sections

Embryo sections were deparaffinized twice for 15 minutes with xylene and re-hydrated with 5 minute incubations of decreasing ethanol (100% - 10%), followed by a 5 minute PBS wash and epitope retrieval, consisting of a 20 minute boil in 100 mM Tris + 100 mM EDTA. Slides were then cooled and quenched in 3% freshly prepared

H_2O_2 for 30 minutes (Fisher Scientific H325; Pittsburgh, PA, USA) washed for 5 minutes in PBS and blocked in PBS + 0.1% Triton X-100 + 8% bovine serum albumin (BSA) for 1 hour. All subsequent incubations were done in a humidified chamber with 200 μl of reagent per slide and all subsequent washes were done by complete immersion. All steps were performed at room temperature. After blocking, slides were incubated overnight in a 1/150 dilution of goat anti-mCRELD1 antibody (R&D Systems AF4116; Minneapolis, MN, USA) in PBS + .01% Triton X-100 + 4% BSA. Slides were washed 6 times for 5 minutes in PBS + .01% Triton X-100, then incubated for 1 hour in a 1/500 dilution of anti-goat IgG antibody conjugated to horseradish peroxidase (HRP) (Sigma A5420, Sigma-Aldrich Corp.; St. Louis, MO, USA) in PBS + .01% Triton X-100 + 4% BSA. Slides were then washed 6 times for 5 minutes in PBS + .01% Triton X-100. The HRP signal was amplified using the Renaissance Tyramide Signal Amplification Biotin System (PerkinElmer Life and Analytical Sciences Inc.; Waltham, MA, USA), per the manufacturer's protocol. HRP was reacted with 3,3'-diaminobenzidine (DAB) (Pierce 1855910 & 1856090 Thermo Scientific; Rockford, IL, USA) for 20 minutes, followed by three rinses in PBS and a 5 minute fix in 4% paraformaldehyde – the slides were then rinsed with water and allowed to dry. Coverslips were mounted using Permount (Fisher Sp15-500, Fisher Scientific; Pittsburgh, PA, USA). Sections were visualized on a Zeiss Axiophot microscope using an Evolution MP COLOR camera by Media Cybernectics and Image Pro Plus 6.3 imaging software. IHCs, both with and without primary antibody, were performed in

duplicate for each tissue type and stage assessed to assure reproducibility and specificity.

Hematoxylin and Eosin (H&E) staining

Sectioned tissues were deparaffinized twice for 5 minutes with xylene and rehydrated with 2 minute descending ethanol washes (100% - 10%) and a water wash, followed by a 2 minute stain with Gill #1 Hematoxylin (Sigma GHS116, Sigma-Aldrich Corp.; St. Louis, MO, USA) and 15 minute water rinse. The slides were dehydrated with 2 minute ascending ethanol washes (10% - 100%), followed by a 2 minute stain with intensified Eosin Y (Fischer "Protocol" 23-314-630, Fisher Scientific; Pittsburgh, PA, USA). The slides were rinsed with 100% ethanol 5 times, rinsed in xylene and coverslipped with Permount (Fisher Sp15-500, Fisher Scientific; Pittsburgh, PA, USA). H&E staining was performed in triplicate for each genotype, *Creld1*$^{(+/+)}$, *Creld1*$^{(+/-)}$ and *Creld1*$^{(-/-)}$. Tissues were visualized on a Zeiss Axiophot microscope using an Evolution MP COLOR camera by Media Cybernectics and Image Pro Plus 6.3 imaging software.

Alcian Blue and Nuclear Fast Red staining

Sectioned embryos were deparaffinized twice for 5 minutes with xylene and rehydrated with 2 minute descending ethanol washes (100% - 10%) and a water wash; and stained with Alcian Blue solution [1% Alcian Blue 8Gx in a 3% acetic acid solution (pH 2.5)] for 30 minutes, then washed in running water for 2 minutes and

counterstained in Nuclear Fast Red solution [(0.1% Nuclear Fast Red in 5% aluminum sulfate in water), which was filtered and had a few grains of thymol added as a preservative] for 5 minutes and washed in running water for 1 minute. Tissues were dehydrated with 5 minute washes in an increasing ethanol gradient (10% -100%) before being cleared with a xylene wash and coverslipped with Permount (Fisher Sp15-500, Fisher Scientific; Pittsburgh, PA, USA). Staining was performed in triplicate for each genotype, *Creld1*$^{(+/+)}$, *Creld1*$^{(+/-)}$ and *Creld1*$^{(-/-)}$ and visualized on a Zeiss Axiophot microscope using an Evolution MP COLOR camera by Media Cybernectics and Image Pro Plus 6.3 imaging software. To quantitative the number of mesenchymal cells in the cushions, two serial sections of two different embryos were counted.

Whole mount immunohistochemistry of mouse placentas and embryos

Placentas and embryos upon extraction were immediately fixed in 4% paraformaldehyde (Fisher Scientific F79; Pittsburgh, PA, USA) overnight at 4°C. All subsequent washes and incubation were performed with gentle rocking; 1 ml volumes were used for all washes and 500 μl volumes were used for all antibody incubations. Between every step tissues were transferred to a new 1.7 ml microcentrifuge tubes. After fixing, tissues were bleached with freshly prepared 5% H_2O_2 (Fisher Scientific H325; Pittsburgh, PA, USA) at 25°C overnight and then washed with PBS + 1% Triton X-100 (PBS+1%T) for 5 minutes, followed by two incubations in PBS + 3% nonfat milk (PBSM) + 1% Triton X-100 (1%T) for an hour at 25°C. The

tissues were incubated in 10 µg/ml goat anti-human Six3 antibody (Santa Cruz Biotechnology sc-49114; Santa Cruz, CA, USA) or rat anti-PECAM-1 antibody (Santa Cruz Biotechnology sc-101454; Santa Cruz, CA, USA) in PBSM + 0.1%T overnight at 4°C. After incubation, tissues were washed for 5 minutes, 5 times, in PBSM + 0.1%T before incubating in 1:100 anti-goat IgG antibody that is HRP conjugated (Sigma-Aldrich Corp. A5420; St. Louis, MO, USA) or anti-rat IgG HRP antibody (R&D Systems HAF005; Minneapolis, MN, USA) in PBSM + 0.1%T overnight at 4°. After incubation tissues were washed 6 times for 5 minutes in PBSM + 0.1%T. Then tissues were washed with PBS + 0.1%T for 20 minutes. HRP was then reacted with DAB (Pierce 1855910 & 1856090, Thermo Scientific; Rockford, IL, USA) for 20 minutes and washed 3 times with PBS + 0.1%T + 0.2% bovine serum albumin for 5 minutes each, followed by post-fixing with 4% paraformaldehyde for 5 minutes, then transferred to 50% glycerol, then to 70% glycerol and finally stored at 4°C. Tissues were imaged with a Leica MZ12 dissecting microscope, a Q Imaging MicroPublisher 3.3RTV camera and Rincon Version 7.4 imaging software. PECAM-IHCs were performed in triplicate for each *Creld1*-genotype and developmental time point. Six3-IHCs were performed in duplicate for each *Creld1*-genotype.

Measuring placenta areas

Processed placentas were used to minimize spurious placental volume differences caused by variation in blood loss during placenta collection. Placentas that had previously undergone IHC and had been stored in glycerol were measured in glycerol

while being pressed under the weight of a microscope slide. The two-dimensional profile of the placentas were then circumscribed by 'n' points using a Leica MZ12 dissecting microscope, a Q Imaging MicroPublisher 3.3RTV camera and Rincon Version 7.4 imaging software to generate pixel area. Areas were assessed in triplicate for each *Creld1*-genotype and statistical significance was determine with Student's t-test (p-value <.05 was considered significant).

Western blot

CRELD1-9B was probed with a custom antibody from Sigma Genosys (The Woodlands, Texas, USA) generated against the amino acid sequence WKLGSHPHSTYVKMC. Western Blot Protocol: protein extracts (Human Placenta Protein Medley from BD Biosciences; Sparks, MD, USA) were run, transferred, blocked with 5% nonfat milk, probed overnight 1:10,000, and incubated with a goat anti-rabbit secondary (Biorad, 170-6518; Hercules, CA) at 1:20,000. Five five-minute washes were performed between each step. Protein was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific; Rockford, IL, USA), per the manufacturer's protocol.

TUNEL staining

DeadEnd™ Colorimetric TUNEL System (Promega, G7360; Madison, WI, USA) was used to stain apoptotic nuclei dark brown per the manufacturer's protocol. To quantitate the number of TUNEL-positive cells in the AV-canal and glossopharyngeal

arches, mesenchymal cells were counted in three serial sections of representative embryos. Sections were visualized on a Zeiss Axioskop2 motplus microscope with an AxioCam HRM camera and AxioVision 4.6.3 software. TUNEL was performed in triplicate for each *Creld1*-genotype.

Bromodeoxyuridine (BrdU) proliferation assay

Two hours before E10.25 embryos were to be collected, pregnant mice were injected intraperitoneally with 100 mg/kg body weight of BrdU (Sigma-Aldrich Corp.; St. Louis, MO, USA) dissolved in 0.9% sodium chloride and filtered sterile at 0.2 mm. Embryos were then collected, embedded, sectioned, de-paraffinized and re-hydrated as stated above. The slides were washed in PBS + 1% Triton X-100, 3 times for 5 minutes then permeabilized with a 10 minute incubation in 1N HCl on ice, followed by a 10 minute incubation in 2N HCl at room temperature before moving them to 37°C for 20 minutes. The embryos were then washed in room temperature 0.1M borate buffer for 12 minutes, followed by 3 washes for 5 minutes in PBS + 1% Triton X-100 at room temperature. Tissues were quenched with 3% H_2O_2, followed by 3 washes of 5 minutes in PBS and blocked in blocking solution [PBS + 1% Triton X-100 + 1M Glycine + 5% BSA] for 1hr. Then slides were incubated overnight at room temperature with 1/150 anti-rat BrdU antibody (Abcam, ab6326; Cambridge, MA, USA) in the same blocking solution as before and washed 3 times for 5 minutes with PBS + 1% Triton X-100. This was followed by incubation with 1/400 anti-rat IgG HRP antibody (R&D systems, HAF005; Minneapolis, MN, USA) for 1 hour in blocking

solution. Slides were then washed for 5 minutes in PBS + 1% Triton X-100, 3 times, before HRP was reacted with DAB (Pierce, 1855910 & 1856090; Thermo Scientific; Rockford, IL, USA) for 20 minutes and rinsed three times with PBS. Finally, slides were post-fixed in 4% paraformaldehyde for 5 minutes and rinsed with water. Coverslips were mounted using Permount (Fisher Scientific, SP15; Pittsburgh, PA, USA) before being visualized as with the TUNEL staining. BrdU assays were performed in triplicate for each *Creld1*-genotype.

R329C-knockin mouse genotyping

DNA was obtained from Dr. Sheilla Cherry (Roger Reeves Laboratory at Johns Hopkins, Baltimore, Maryland). Allele specific R329 forward primer : GTGAAAACACGGAGGGAGGCTACT (specific for mutant mouse genotype). Allele specific R329C wild-type forward primer: GTGAAAACACGGAGGGAGGCTACC (specific for the wild-type mouse genotype). R329C genotyping universal reverse primer: GCTGCCACAGCTCCAATGAAGAT. 663 basepair fragments will only amplify if corresponding genome is present. Cycling Parameters: 94°C x 2 minutes [94°C x 30 seconds, 72°C x 30 seconds, 72°C x 1.5 minute] x 35 cycles + 72°C x 3 minutes + 4°C hold.

Allele-specific PCR for gender determination

PCR and cycling parameters are the same as in the *Creld1*-knockout mouse genotyping. Sry-F: TGGGACTGGTGACAATTGTC, Sry-R: GAGTACAGGTGTGCAGCTCT. A PCR product of 382 basepairs indicated a male.

Microarray

E9.5 embryonic mouse hearts were collected and RNA was extracted using MELT Total Nucleic Acid Isolation system (Ambion; Austin, TX) per the manufacturer's protocol with optional on-bead DNase digestion. Yolk sacs were genotyped for *Creld1* and gender, as described above. Heart RNA was then pooled by genotype and balanced for gender, then the pooled RNA was equally divided for three separate microarray analyses. Microarray assays were performed in the Affymetrix Microarray Core of the OHSU Gene Microarray Shared Resources (project #267CM). There, labeled target cDNA was prepared, samples were amplified and labeled using the NuGEN Ovation v.2 w/ FLv2 protocol; and hybridized to a Mouse Genome 430 2.0 Gene Chip array in triplicate. Image processing and expression analysis was then performed using Affymetrix GCOS version 1.4.0.036 software. This was followed by our analysis of the _GS.CHP files using GeneSifter (www.genesifter.net) to perform a pairwise analysis (t-test analysis with a Benjamini and Hochberg correction), which was normalized to the median and log transformed.

Study subjects inclusion criteria

Study subjects were recruited through the Oregon Congenital Heart Disease Registry (ORCHD), a population-based registry of all Oregon patients born with one of 14 major heart defects and who have heart surgery before the age of 19. There are about 5,000 patients in the registry, and it includes approximately 75-80% of all cases of congenital heart defects in Oregon, with a 90% follow-up rate on all patients. ORCHD categorizes the registry based on clinical diagnosis of the heart defect from surgical records — this helps to insure homogeneity within phenotypic categories. Inclusion criteria for this study were based on the diagnosis (at surgery) of non-syndromic isolated AVSD, including complete AVSD (also known as complete atrioventricular canal) and partial AVSD (also known as ostium primum atrial septal defect). Both of these defects arise from the improper development of the atrioventricular endocardial cushion and are thought to represent a phenotypic and developmental continuum of AVSD. Therefore, we found it appropriate to group complete and partial AVSD cases together when studying the genetics of AVSD in human populations. There was no evidence of any extra-cardiac anomalies in this cohort. In total we recruited 29 individuals with a complete AVSD and 21 individuals with a partial AVSD. All study subjects were self-reported Caucasian. DNA specimens were obtained from all study subjects by either a peripheral blood draw or saliva collection. Controls are from the Coriell Caucasian Human Variation Control panel (race matched). We did not control for age or gender.

Human ethical considerations

Subjects for this study were recruited through the Oregon Registry of Congenital Heart Defects or by an approved protocol for the investigation of congenital heart disease through The Children's Hospital of Philadelphia. All studies were done with informed consent and conformed to institutional guidelines.

Genotyping for the VEGF -634C allele

Case and control DNA was amplified with Advantage-GC Genomic PCR (Clontech; Mountain View, CA) per the manufacturer's protocol, using 1.1 μl of 25 mM Mg(OAc) and 5.0 μl of GC Melt with the forward amplification primer: CAGACGGCAGTCACTAG and reverse amplification primer: GTG TCTGTCTGTCTGTC. Parameters: 95°C x 2 minutes [95°C x 30 seconds, 59°C x 30 seconds, 68°C x 1 minute] x 30 cycles + 68°C x 5 minutes + 4°C hold. Amplified PCR bands were then purified using Qiagen's QIAquick Gel Extraction Kit (QIAGEN; Valencia, CA, USA). Sanger sequencing was then performed with the forward sequencing primer: GGGGCGCTCGGCCACCA and reverse sequencing primer: CGTCAGCGCGACTGGTCAGC. Significance was calculated using chi square test.

Vegf qPCR

SuperScript III First-Strand Synthesis System (Invitrogen; Carlsbad, CA, USA) was used to make cDNA from the E9.5 mouse hearts' pooled RNA, which was also used

in the microarray assays. VEGF Primers, F:CTGGCCAGGCTCCCGATT R:GATGCCGGTTCCAACCAGAAGTT. GAPDH Primers, F:AAATATGACAACTCACTCAAGATTGTC R:CCCTTCCACAATGCCAAAGT. qPCR reaction: 6.27μl cDNA + 7.5 μl SYBR green (Applied Biosystems; Foster City, CA) + 0.54 F primer (25μM) + 0.54 R primer (25μM). Cycling Parameters: 94°C x 2 minutes [94°C x 30 seconds, 60°C x 30 seconds, 68°C x 30 seconds] x 50 cycles + 68°C x 3 minutes + 4°C hold.

Heart explant assay

With a dissecting scope, atrioventricular canals from mouse embryos, at embryonic day 9.5, were isolated then cut in half longitudinally and placed on a pre-equilibrated 3-D collagen-matrix and cultured (Figure 4) as described before (Dor et al. 2001). Genotypes were then assessed as described above. The endocardial cushions were then allowed to adhere overnight. The next day fresh growth media was added that either was or was not dosed with extra purified mouse-VEGF 120 protein (R&D Systems AF4116; Minneapolis, MN, USA). Successful mesenchymal cell migration across and into the collagen gels was then assessed at 48, 72 and 96 hours after explantation by two blinded independent observers, whose observations were then averaged. Endocardial cells were classified upon their appearance as rounded polygonal cells on the collagen-matrix's surface with intact cell-to-cell junctions. The invasive mesenchymal cells were characterized by the appearance of stellate-shaped cells within or on top of the matrix. Mandatory recounts were enacted if the

independent counts varied by more than 10%. Statistical comparisons between groups are done using Student's t-test.

ELISA assay

VEGF ELISA Kit, Mouse QIA52 (Calbiochem; Darmstadt, Germany) was used on media from explanted heart after 96 hours in culture, per the manufacturer's instructions. ELISA results were quantified to a standard curve made of the provided purified VEGF.

CHAPTER 5:

CRELD1 during development

The CRELD1-protein is found in multiple adult and embryonic tissues

Previously we showed that *Creld1*-RNA is expressed in a wide variety of fetal and adult tissues (Rupp et al. 2002). Subsequently, I performed immunohistochemistry (IHC) experiments and confirmed ubiquitous CRELD1-protein expression in numerous adult mouse tissues (Figure 11). Along these lines, I also confirmed CRELD1-protein expression during mouse embryogenesis by immunohistochemistry. Here the expression is also widespread but strongest in the developing brain, somites and heart (Figure 12). This ubiquitous expression of CRELD1 suggests that CRELD1 is important in the development or homeostasis of numerous tissue types.

Expression in the heart included expression in the developing endocardial cushions (Figure 16), which are primordial heart structures that give rise to components of the adult heart valves and septa and as mentioned previously, endocardial cushion dysregulation is thought to cause AVSD. Therefore, the observation of CRELD1-protein expression within the endocardial cushions supports our hypothesis that CRELD1 is vital for proper valve and septa formation and is thus involved in AVSD etiology. However, this still needed to be directly tested.

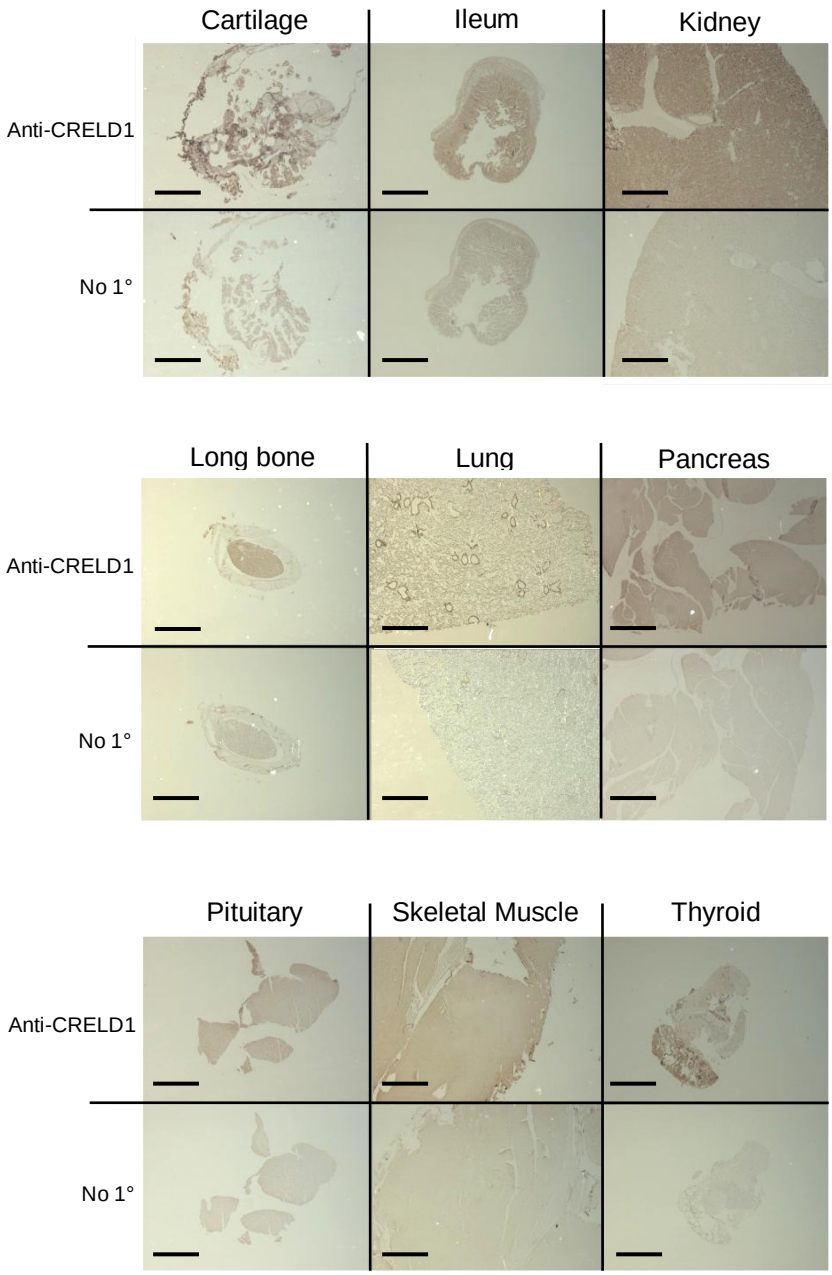


Figure 11: CRELD1-IHC of adult mouse tissues.

CRELD1-immunohistochemistry (IHC) of adult mouse tissues, which were purchased from Zyagen. The top row of each of panels was probed with anti-CRELD1 and the bottom row of each panel (No 1°) is the negative control, which was performed without a primary antibody. Scale bar represents 1mm.

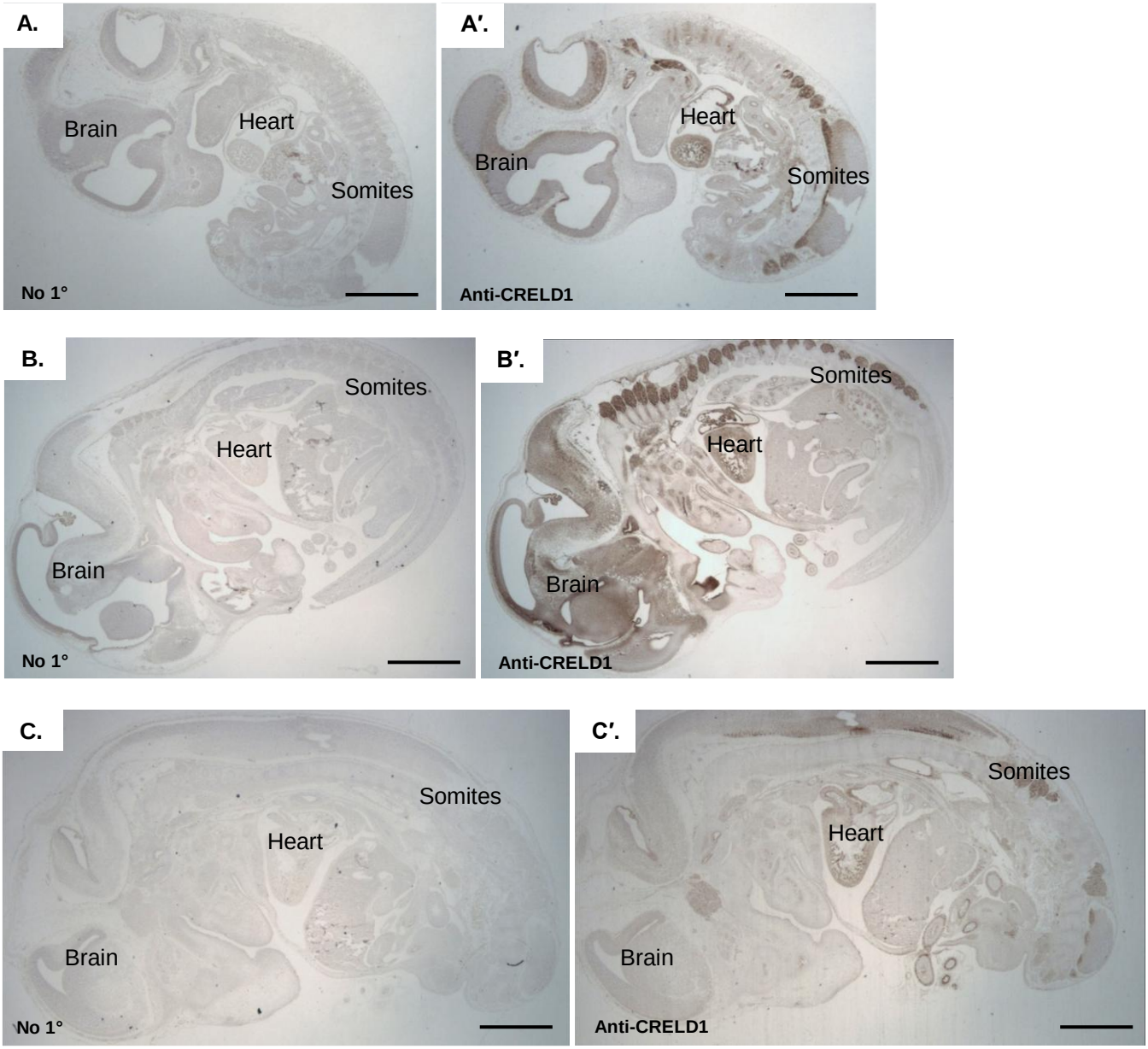


Figure 12: CRELD1-IHC of sagitally sectioned wild-type mouse embryos.

A & A′: CRELD1-IHC of a embryonic day 10.5 (E10.5) mouse embryo (anti-CRELD1) and negative-IHC (no 1°). **B & B′**: CRELD1-IHC of an E12.5 mouse embryo (anti-CRELD1) and negative-IHC (no 1°).**C & C′**: CRELD1-IHC of an E13.5 mouse embryo (anti-CRELD1) and negative-IHC (no 1°). Scale bars represent 1mm.

Creld1-knockout mouse

To test the necessity of CRELD1 in development, we created a *Creld1*-knockout mouse by positioning a self-excising cassette within a *Creld1*-genomic fragment such that 1.9 kb of the 5′ untranslated region (5′ UTR), all of exon 1 and a portion of intron 1 was eliminated upon recombination (Figure 13); thus, causing loss of *Creld1* transcripts. Allele-specific PCR was then used to genotype offspring (Figure 14). Successful elimination of *Creld1* was then confirmed by northern blot analysis (Figure 15) and by immunohistochemistry (Figure 16).

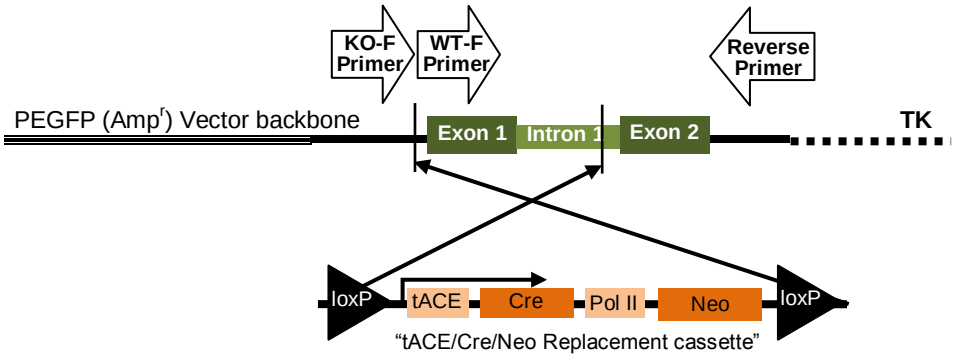


Figure 13: A schematic diagram of the *Creld1*-knockout targeting vector.

A schematic of the *Creld1*-targeting vector, which included an upstream homology arm (5' relative to the *Creld1*-ATG codon) that spanned approximately 6.3 kb and a downstream homology arm of 3 kb. Recombination was driven by the self-excising tACE/Cre/Neo cassette, which disrupted 1.9 kb of the 5' untranslated region (UTR), exon 1 and part of intron 1 of the mouse *Creld1* gene. Also illustrated are the locations of the allele-specific primer binding sites that were used for genotyping, see Methods section. Thymidine kinase, TK; testis-specific angiotensin-converting enzyme (tACE) promoter, tACE; cre-recombinase, cre; RNA polymerase II, PolII; neomycin, Neo.

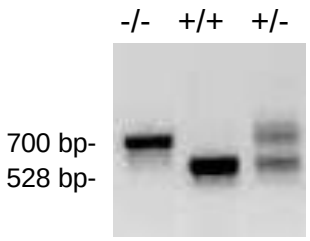


Figure 14: Genotyping *Creld1*-knockout mouse by allele-specific PCR.

PCR with the KO-F primer and Reverse primer generates a 700 base pair (bp) fragment specific to the *Creld1*-knockout (-/-). PCR with WT-F primer and Reverse primer generates a 538 base pair PCR product corresponding to the wild-type allele (+/+). Heterozygous mice display both copies (+/-).

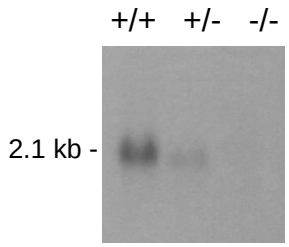


Figure 15: Northern blot showing reduction or absence of *Creld1*-RNA expression.

Northern blot showing an absence of the 2.1 kb *Creld1*-RNA in the *Creld1*-knockout mouse (-/-) and reduced expression in the *Creld1*-heterozygote (+/-). Wildtype (+/+).

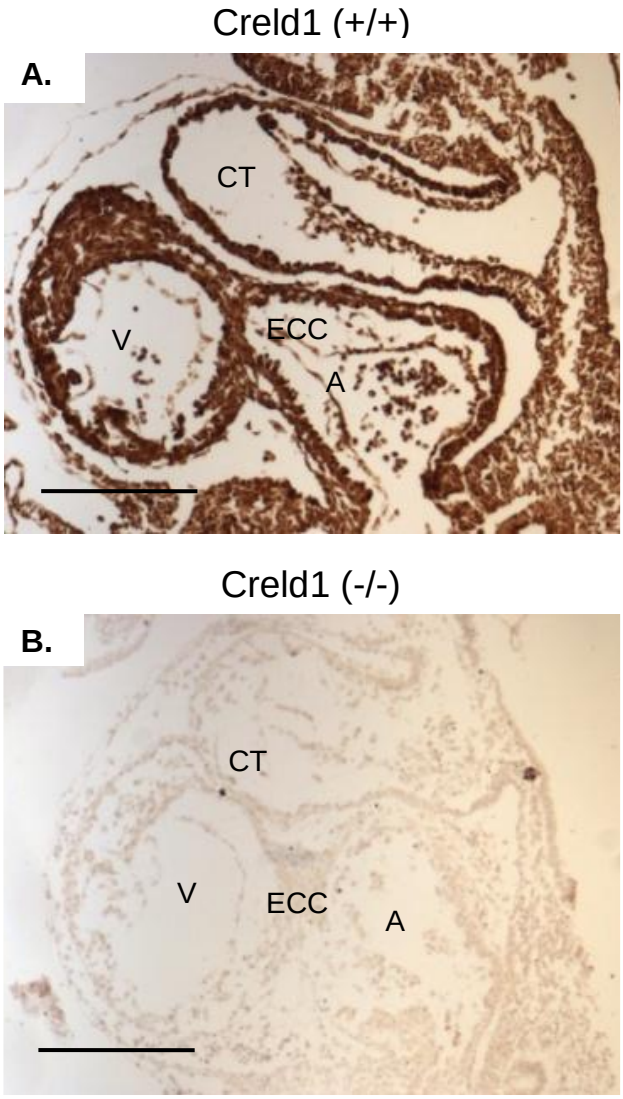


Figure 16: CRELD1-IHC of a sagittally sectioned E10.5 wild-type mouse heart.

A: CRELD1-IHC of a sectioned E10.5 wild-type mouse heart showing CRELD1-protein expression in the developing heart, including the endocardial cushions. **B**: CRELD1-IHC of an E10.5 Creld1$^{(-/-)}$ mouse heart, which demonstrates the specificity of the CRELD1-IHC, which was shown in Figures 11 & 12. Conotruncal endocardial cushions, CT; common ventricle, V; common atrium, A; atrioventricular endocardial cushions, ECC. Scale bars represent 200 μm.

Knockout of *Creld1* resulted in embryonic lethality

We assessed 345 pups from *Creld1*$^{(+/-)}$ intercrosses and found that knockout of *Creld1* was incompatible with life, as no *Creld1*$^{(-/-)}$ pups were born. In contrast, *Creld1*$^{(+/-)}$ pups were born in the expected Mendelian ratio of 2 heterozygotes to every one wildtype (Table 1). Next we examined litters during development to determine when the *Creld1*$^{(-/-)}$ embryos were dying (Table 2). At E12.5 we found little *Creld1*$^{(-/-)}$ embryonic tissue, suggesting earlier death. A day earlier at E11.5, while we found *Creld1*$^{(-/-)}$ tissue in the expected Mendelian ratio (1:3), the embryos appeared dead. Death at ~ E11.5 was supported when I examined younger embryos and found E10.5 and E9.5 *Creld1*$^{(-/-)}$ embryos alive and in the expected Mendelian ratio (1:3). Ratio conformance was tested by chi-square analyses (E9.5, $\chi^2 = 4.232$, p-value = 0.1205; E10.5 embryos, $\chi^2 = 1.800$, p-value = 0.4066; E11.5 embryos, $\chi^2 = 0.880$, p-value = 0.6440).

This suggests that there was no significant *Creld1*$^{(-/-)}$ attrition before E12.5. However, the study lacked the power to detect a discrepancy of 3-5% and considering the large number of embryos we assessed (In total, 675 embryos E10.5 or younger) and that we consistently observed lower than expected numbers of *Creld1*$^{(-/-)}$ embryos, it is still possible that some *Creld1*$^{(-/-)}$ embryos are absent before E10.5. However the more relevant question is: Is CRELD1 essential for heart development and can a CRELD1-deficiency cause an AVSD?

Live pups					
+/+ Male	+/+ Female	+/- Male	+/- Female	-/- Male	-/- Female
61 (18%)	57 (17%)	106 (31%)	121 (35%)	0 (0%)	0 (0%)
34%		66%		0%	

Table 1: Pup survival.

Pup survival was tallied 3 weeks post birth. 67 litters with an average of 4.64 pups per litter were assessed. Complete knockout of *Creld1* was lethal, while *Creld1*$^{(+/+)}$ and *Creld1*$^{(+/-)}$ mice were found in Mendelian ratios. There was no significant difference in gender ratios.

	Genotypes		
Embryonic Day	-/-	+/-	+/+
9.5	51 (20.4%)	125 (50%)	74 (29.6%)
10.5	92 (22.2%)	214 (51.6%)	109 (26.3%)
11.5	12 (24%)	28 (56%)	10 (20%)
12.5	3[a] (15%)	11 (55%)	6 (30%)

Table 2: Incidence of *Creld1*-genotypes during embryogenesis.

This table records the incidence of *Creld1*-genotypes during embryogenesis. E10.5 and E9.5 *Creld1*$^{(-/-)}$ embryos were observed alive at nearly Mendelian ratios (1 wildtype: 2 heterozygotes: 1 knockout). However, by E12.5 very little embryonic tissue could be identified and by E13.5 no intact *Creld1*$^{(-/-)}$ embryos were ever found. [a.] The E12.5 Creld1$^{(-/-)}$ embryos were all dead and being reabsorbed at this stage.

E10.5 *Creld1*$^{(-/-)}$ embryos were malformed

To determine why knockout of *Creld1* results in embryonic lethality I began a comprehensive characterization study. When I did this I found that by E10.5 the *Creld1*$^{(-/-)}$ embryos had numerous structural and vascular abnormalities (Figure 17).

Especially notable was the poor vascularization in the yolk sacs of the *Creld1*$^{(-/-)}$ embryos that contrasted with both the *Creld1*$^{(+/+)}$ and *Creld1*$^{(+/-)}$ littermates, which had well-vascularized yolk sacs by E10.5 (Figure 17 B & D versus F). The primitive vascularization of the *Creld1*$^{(-/-)}$ yolk sac gave it an appearance reminiscent of an "orange peel," a phenotype previously described in the delta-like 4 ligand (a Notch partner) knockout mice (Gale et al. 2004).

To follow up on the vascular abnormalities, I performed PECAM immunohistochemistry, which revealed further vascular abnormalities in the cephalic venous plexus of the *Creld1*$^{(-/-)}$ embryos (Figure 18 A, B & C). By E10.5 the *Creld1*$^{(+/+)}$ and *Creld1*$^{(+/-)}$ embryos had well developed and well-organized cephalic vascular trees, while the *Creld1*$^{(-/-)}$ embryos' vasculature remained more primitive and failed to arborize into higher order branching structures.

PECAM immunohistochemistry of the E10.5 *Creld1*$^{(-/-)}$ embryos also revealed gross defects in the head, an underdeveloped heart and disorganization of the neural tube (Figure 18). Most notable was the underdeveloped olfactory placodes and forebrain, which appears to have nearly fused telecephalons (Figure 18 G, H & I).

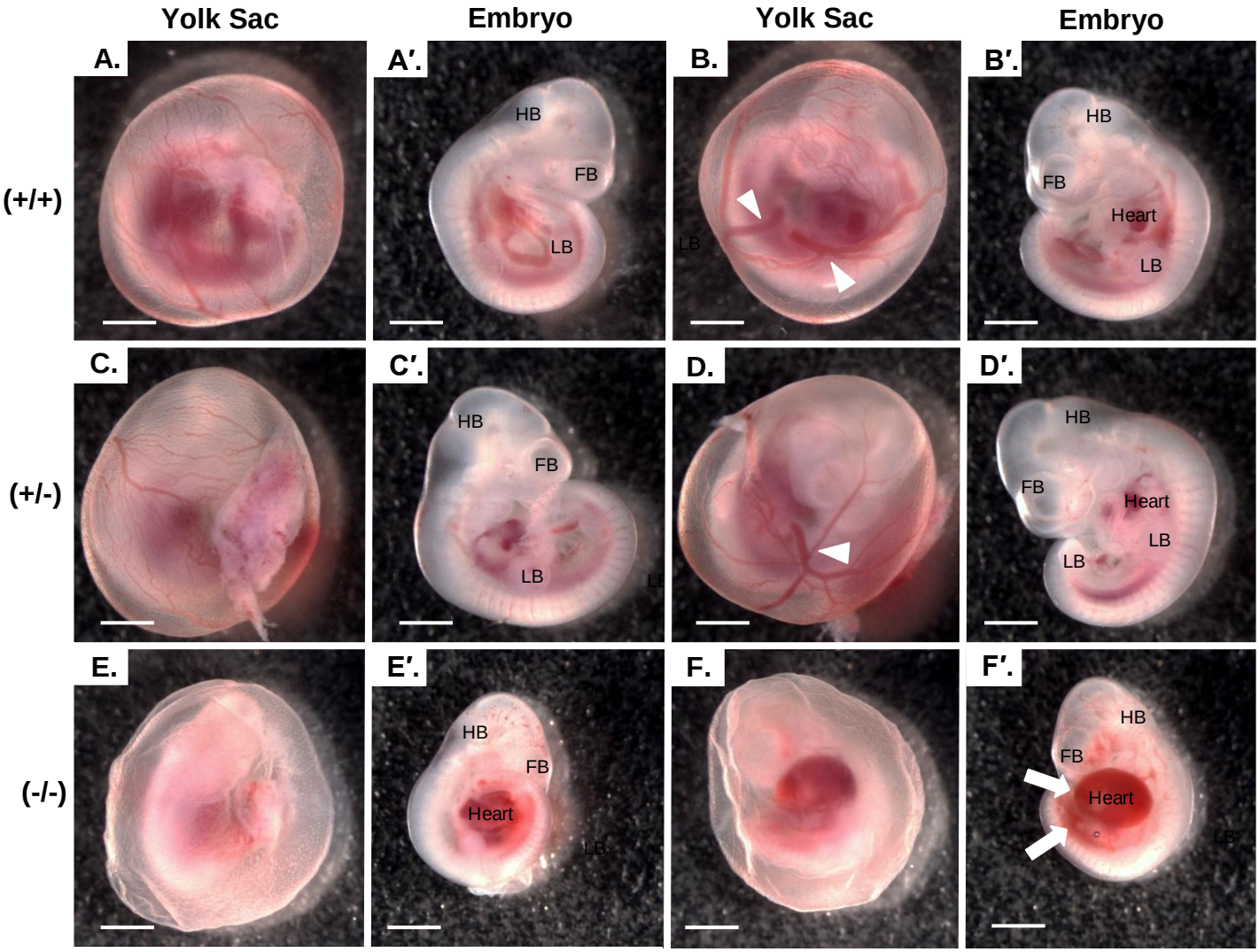

Yolk Sac
Embryo
Yolk Sac
Embryo
A.
A′.
B.
B′.
(+/+)
HB
FB
LB
Heart
C.
C′.
D.
D′.
(+/-)
E.
E′.
F.
F′.
(-/-)

Figure 17: Representative images of fresh, whole E10.5 embryos.

A & B: *Creld1*$^{(+/+)}$ embryos; right-side and left side, respectively. **C & D**: *Creld1*$^{(+/-)}$ embryo; right-side and left side, respectively. **E & F**: *Creld1*$^{(-/-)}$ embryos; right-side and left side, respectively. **A, B, C, D, E & F**: Images of embryos still within their intact yolk sacs. **A′ ,B′ ,C′ ,D′ ,E′ & F′**: Images of embryos with yolk sacs removed. White arrowheads indicate the well developed conducting arteries in the *Creld1*$^{(+/+)}$ and *Creld1*$^{(+/-)}$ yolk sacs (**B & D)**. This is in contrast to the yolk sacs of the *Creld1*$^{(-/-)}$ littermates, which lack large vessels **(E & F)**. Also notable in the *Creld1*$^{(-/-)}$ embryo is hemorrhaging and gross enlargement of pericardial sac, as marked by the white arrows in panel **F′**. Forebrain, FB; hindbrain, HB; limb buds, LB. Scale bars represents 1mm.

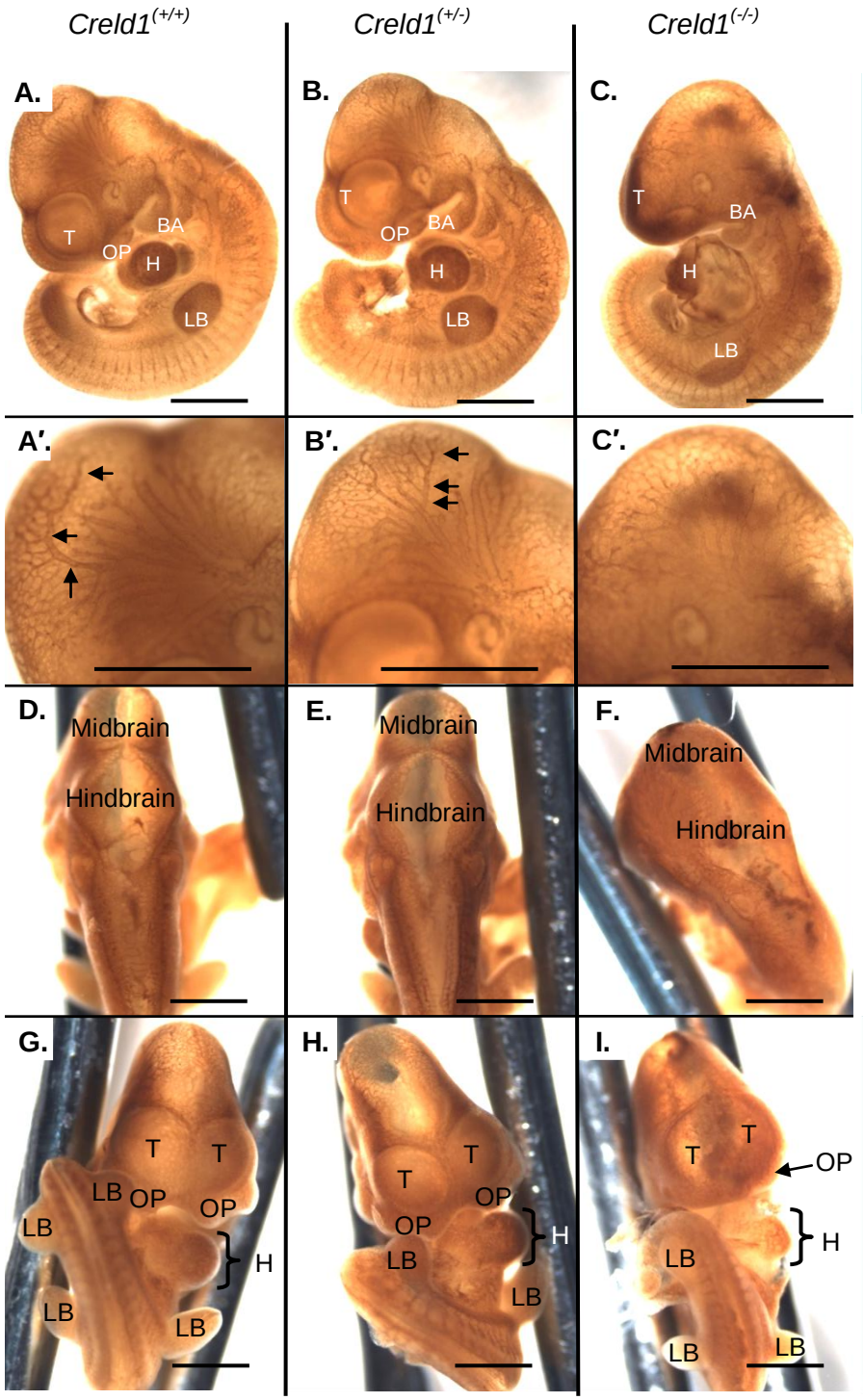

Creld1(+/+)
Creld1(+/-)
Creld1(-/-)
A.
B.
C.
T
BA
OP
H
LB
A′.
B′.
C′.
D.
E.
F.
Midbrain
Hindbrain
G.
H.
I.
T
OP
H
LB

Figure 18: PECAM-IHC of whole E10.5 embryos.

A, D & G: The first column is a *Creld1*$^{(+/+)}$ embryo. **B, E & H**: The second column is a *Creld1*$^{(+/-)}$ embryo. **C, F & I**: The third column is a *Creld1*$^{(-/-)}$ embryo. Cranial abnormalities were observed in the *Creld1*$^{(-/-)}$ embryos, which had underdeveloped telencephalons and olfactory placodes (**A & B** versus **C**; and **G & H** versus **I**). *Creld1*$^{(-/-)}$ embryos' neural tubes were also disorganized (**D & E** versus **F)**; as was the vasculature. Black arrows indicate the well-developed cephalic venous plexus of the *Creld1*$^{(+/+)}$ and *Creld1*$^{(+/-)}$ embryos (**A' & B'**), this is in contrast to the *Creld1*$^{(-/-)}$ plexus that remained more primitive (**C'**). Limb buds, LB; olfactory placode, OP; branchial arch, BA; telencephalon, T; heart, H. Scale bars represent 1mm.

Creld1$^{(-/-)}$ embryonic hearts had endocardial cushion defects

At E9.5 the *Creld1*$^{(-/-)}$ embryos were grossly indistinguishable from wild-type littermates, as their hearts were beating and had normal endocardial cushion development (Figure 19). However, by E10.5 the *Creld1*$^{(-/-)}$ hearts started to display abnormalities. Histological examination of E10.5 hearts revealed that both the conotruncal and atrioventricular (AV) endocardial cushion of *Creld1*$^{(-/-)}$ embryos had fewer cells and less extracellular matrix (Figure 20 & Figure 21). Additionally, *Creld1*$^{(-/-)}$ hearts had thinner myocardium, poor trabeculation and sparse cellularization; suggesting poor overall heart development.

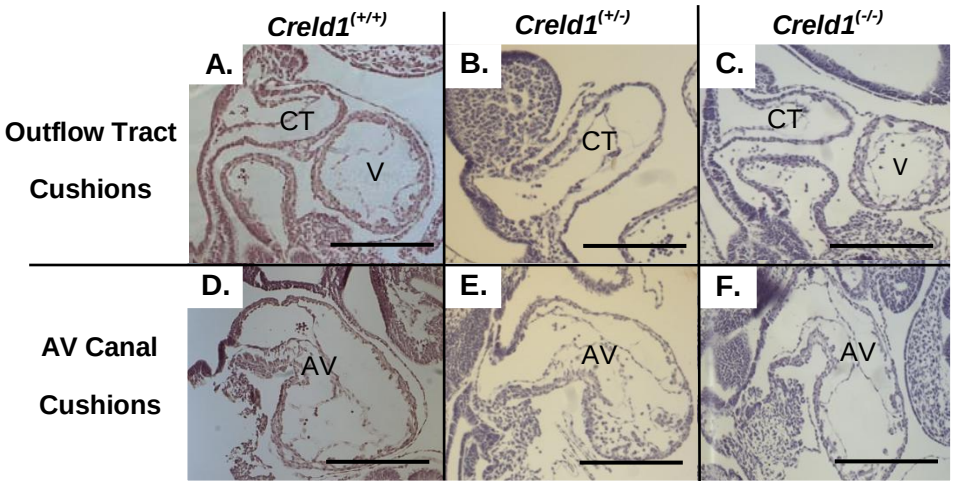
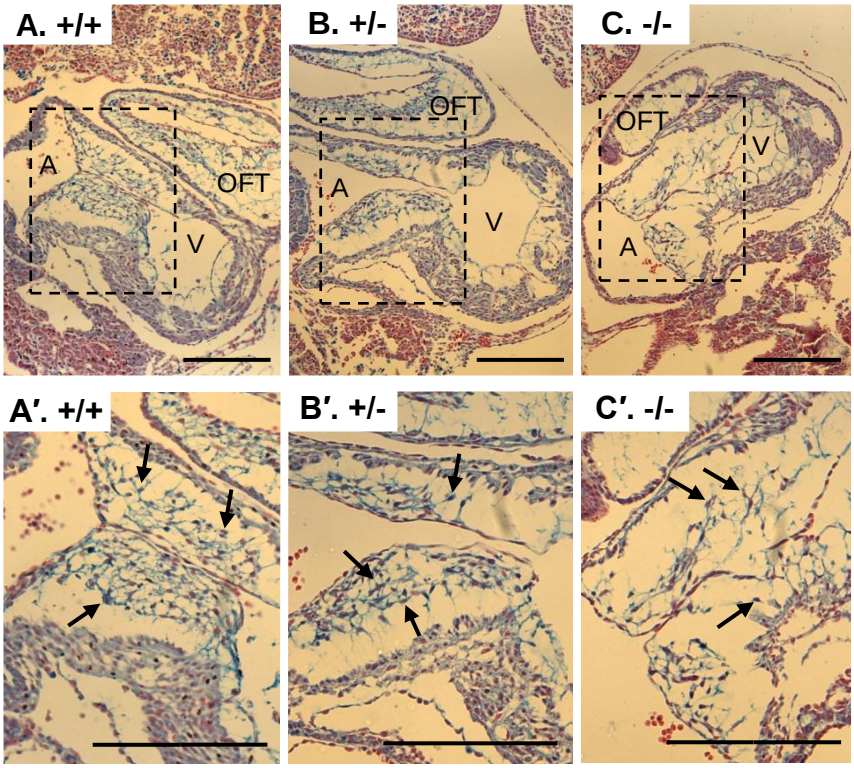


Figure 19: H&E stained sections of E9.5 mouse hearts.

H&E stained sections of E9.5 mouse hearts showed no defects in the atrioventricular or outflow tract cushions of *Creld1*-deficient hearts. **A, B & C**: Top row, sectioned outflow tracts or conotruncal cushions (CT) of E9.5 *Creld1*$^{(+/+)}$ **(A)**, *Creld1*$^{(+/-)}$ **(B)** and *Creld1*$^{(-/-)}$ **(C)** embryos. **D, E & F**: Bottom row, sectioned atrioventricular cushions (AV) of the common atrioventricular canal of E9.5 *Creld1*$^{(+/+)}$ **(D)**, *Creld1*$^{(+/-)}$ **(E)** and *Creld1*$^{(-/-)}$ **(F)** embryos. Conotruncal endocardial cushions, CT; common ventricle, V; atrioventricular endocardial cushions, AV. Scale bar represents 200μm


A. +/+
B. +/-
C. -/-
A
OFT
V
A′. +/+
B′. +/-
C′. -/-

D.

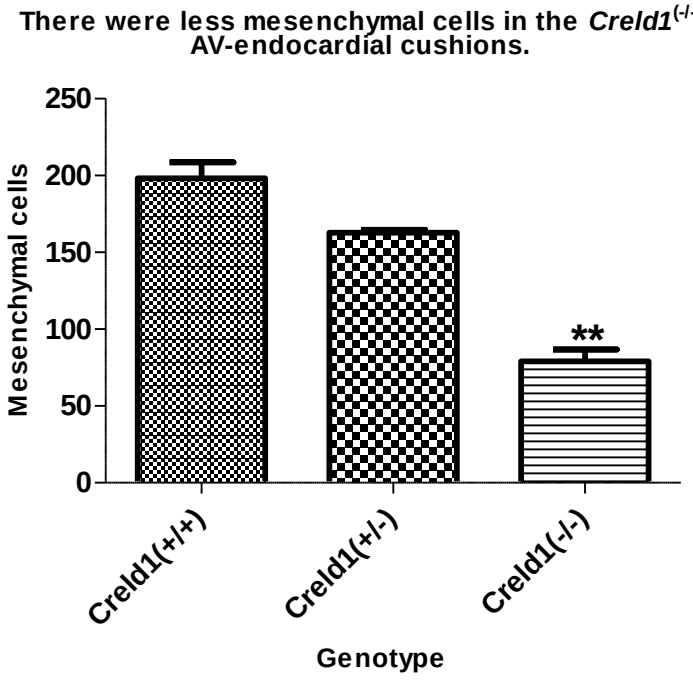
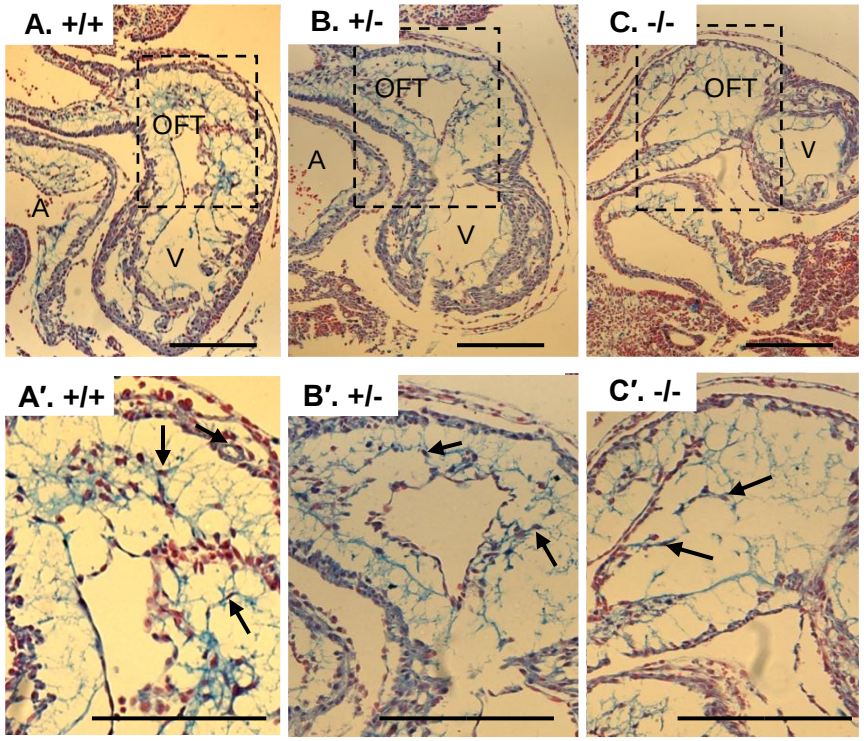

There were less mesenchymal cells in the Creld1(-/-) AV-endocardial cushions.
Mesenchymal cells
250
200
150
100
50
0
**
Creld1(+/+)
Creld1(+/-)
Creld1(-/-)
Genotype

Figure 20: Alcian Blue and Nuclear Fast Red staining of E10.5 AV-canals.

Alcian Blue was used to stain the extracellular matrix (hyaluronic acid, versican and other acidic mucopolysaccharides or glycosaminoglycans) and Nuclear Fast Red was used to stain the cells in E10.5 atrioventricular canals. **A**: *Creld1*$^{(+/+)}$ atrioventricular canal. **B**: *Creld1*$^{(+/-)}$ atrioventricular canal. **C**: *Creld1*$^{(-/-)}$ atrioventricular canal. **A', B' & C':** Enlarged images of the outlined portions of panels **A, B & C**. Notable are fewer mesenchymal cells and less extracellular matrix in the *Creld1*$^{(-/-)}$ panels (**C & C'**). Representative mesenchymal cells in the endocardial cushions are denoted by arrows. **D:** Mean and SEM of the mesenchymal cells observed in the AV-endocardial cushions. ** = p value ≤ 0.001. Outflow tract, OFT; atrioventricular, AV; common ventricle, V; common atrium, A. Scale bar represents 200 μm.


A. +/+
OFT
A
V
B. +/-
OFT
A
V
C. -/-
OFT
V
A′. +/+
B′. +/-
C′. -/-

D.

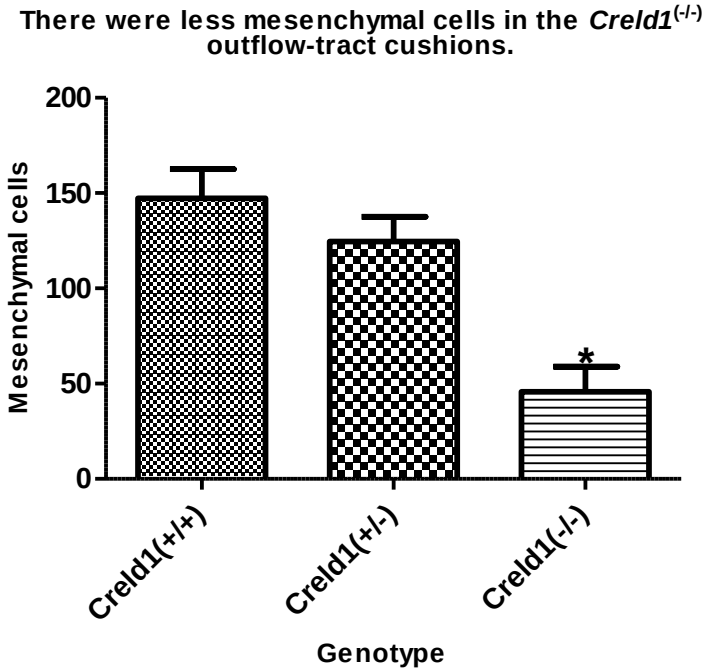

There were less mesenchymal cells in the Creld1(-/-) outflow-tract cushions.
Mesenchymal cells
200
150
100
50
0
*
Creld1(+/+)
Creld1(+/-)
Creld1(-/-)
Genotype

Figure 21: Alcian Blue and Nuclear Fast Red staining of E10.5 outflow tracts.

Alcian Blue was used to stain the extracellular matrix and Nuclear Fast Red was used to stain the cells in E10.5 outflow tracts. **A**: *Creld1*$^{(+/+)}$ outflow tract. **B**: *Creld1*$^{(+/-)}$ outflow tract. **C**: *Creld1*$^{(-/-)}$ outflow tract. **A', B' & C':** Enlarged images of the outlined portions of panels **A, B & C**, respectively. Notable are fewer mesenchymal cells and less extracellular matrix in the *Creld1*$^{(-/-)}$ panels(**C & C'**). Representative mesenchymal cells in the endocardial cushions are denoted by arrows. **D:** Mean and SEM of the mesenchymal cells observed in the outflow-tract endocardial cushions. * = p value ≤ 0.01. Outflow tract, OFT; common ventricle, V; common atrium, A. Scale bar represents 200 μm.

Creld1$^{(-/-)}$ placentas had abnormal gene expression and morphology

There is an emerging appreciation for the role of the placenta in cardiovascular health. It is well known that the placenta is indispensible for the maternal/fetal nutrient exchange. Additionally, epidemiological studies have shown that environmental factors such as maternal nutrition (Shaw et al. 1999, Botto et al. 2000, Loffredo et al. 2001) are important for proper cardiovascular development and that poor placental communication can cause heart defects in chicks (Hogers et al. 1999, Hogers et al. 1997, Broekhuizen et al. 1999). This knowledge has logically led to the undertaking of numerous epidemiological and animal model studies that now strongly suggest that altered placental vigor can increase the risk of both congenital heart defects and adult cardiovascular problems, two topics that are well reviewed elsewhere (McMillen et al. 2008, Thornburg et al. 2008). But despite circumstantial evidence for its existence, to date, no one has identified a genetic risk factor for human AVSD that impinges upon placenta development.

Considering the widespread expression of CRELD1, including the placenta (Figure 22) and the role of the placenta in heart development, I considered it essential to make at least a cursory analysis of placental morphology during our characterization of CRELD1 function in development. My decision to do this is notable because investigation of placental morphology is usually ignored during knockout mouse characterizations, even though “the vast majority of embryonic lethal phenotypes

that are due to loss-of-function mutations are associated with placental defects" (Natale et al. 2006).

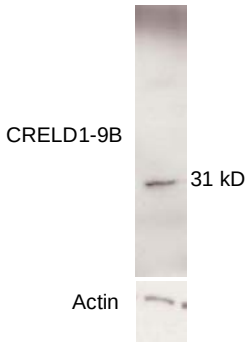


Figure 22: CRELD1 is expressed in the placenta.

A soluble isoform of CRELD1, CRELD1-9B is found in the placenta.

Immunohistochemistry of whole placentas for PECAM, a marker specific for endothelial cells, revealed more PECAM-protein expression in the E9.5 *Creld1*$^{(-/-)}$ placentas examined (Figure 23a), whereas *Creld1*$^{(+/-)}$ placentas were indistinguishable from wildtype. We also observed that the E9.5 *Creld1*$^{(-/-)}$ placentas had poor structural integrity. Upon extraction, the *Creld1*$^{(+/+)}$ and *Creld1*$^{(+/-)}$ placentas maintained a spherical form but the E9.5 *Creld1*$^{(-/-)}$ placentas always laid flat after embryo extraction and were more prone to breakage upon handling (Figure 23a).

By E10.5 the placentas had regained normal PECAM-protein expression levels (Figure 23b). However, at this stage the *Creld1*$^{(-/-)}$ placentas were generally smaller than their *Creld1*$^{(+/-)}$ or *Creld1*$^{(+/+)}$ littermates, as the two-dimensional pixel areas of *Creld1*$^{(+/+)}$ placentas were 650 ± 42.9 pixels^2; *Creld1*$^{(+/-)}$ placentas were 632 ± 27.24 pixels^2; and *Creld1*$^{(-/-)}$ placentas were 593.3 ± 38.47 pixels^2 (Figure 24). While the differences in the placental areas were not statistically significant, it is possible that the earlier aberrant PECAM-protein over-expression is reflective of erroneous cell specification and that this stunts placental growth a day later.

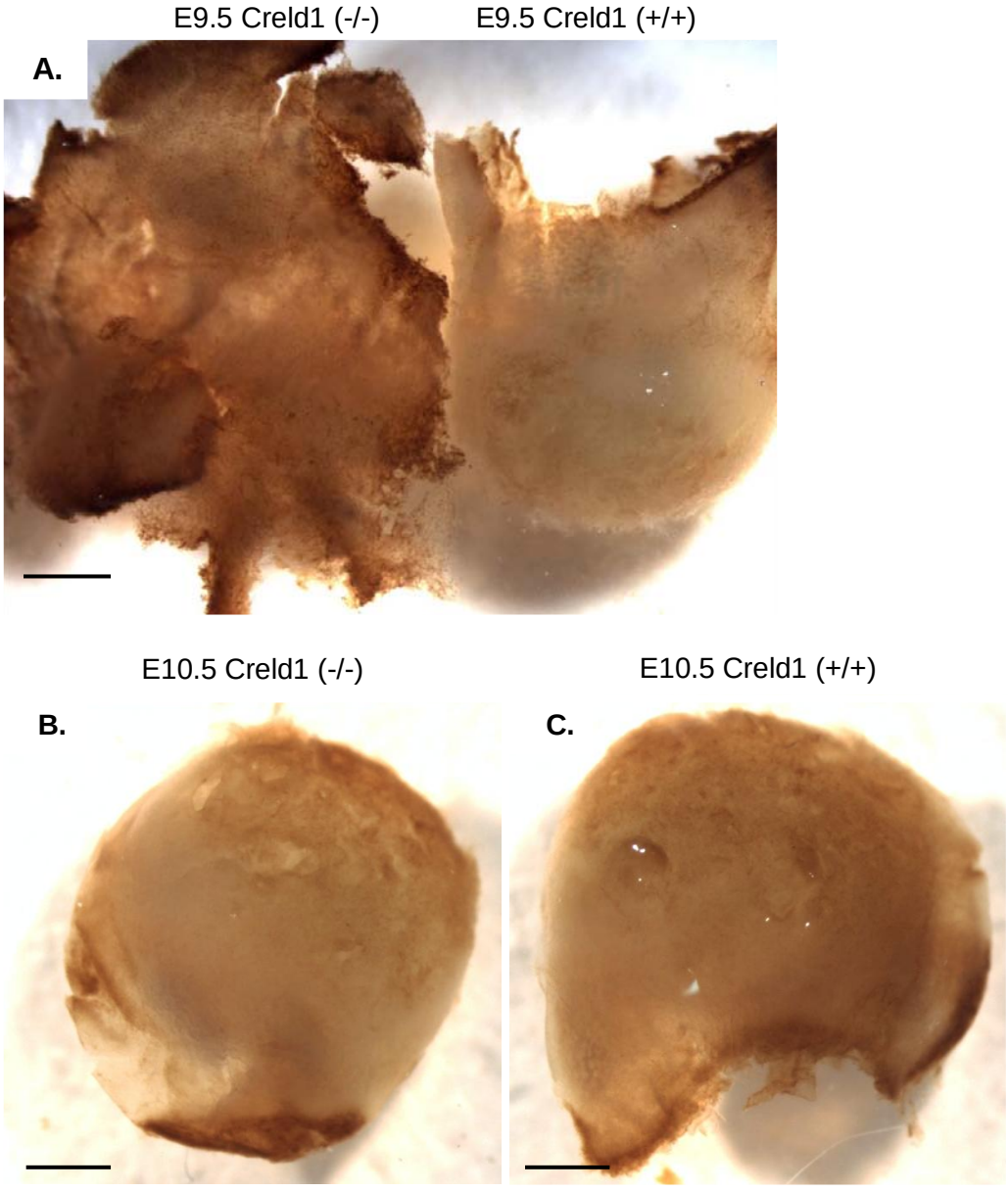

E9.5 Creld1 (-/-)
E9.5 Creld1 (+/+)
A.
E10.5 Creld1 (-/-)
E10.5 Creld1 (+/+)
B.
C.

Figure 23: Placenta morphology and PECAM expression.

PECAM-protein was consistently more highly expressed in the E9.5 *Creld1*$^{(-/-)}$ placentas but by E10.5 PECAM-protein expression returned to normal levels. **A**: E9.5 *Creld1*$^{(-/-)}$ placenta (**pictured left**) in comparison to E9.5 *Creld1*$^{(+/+)}$ placentas (**pictured right**). **B**: PECAM-IHC of E10.5 *Creld1*$^{(-/-)}$ placentas were indistinguishable from the E10.5 *Creld1*$^{(+/+)}$ placentas (**C**). *Creld1*$^{(+/-)}$ placentas were indistinguishable from *Creld1*$^{(+/+)}$ placentas. Scale bar represents 1 mm. Each genotype and stage was assessed in triplicate.

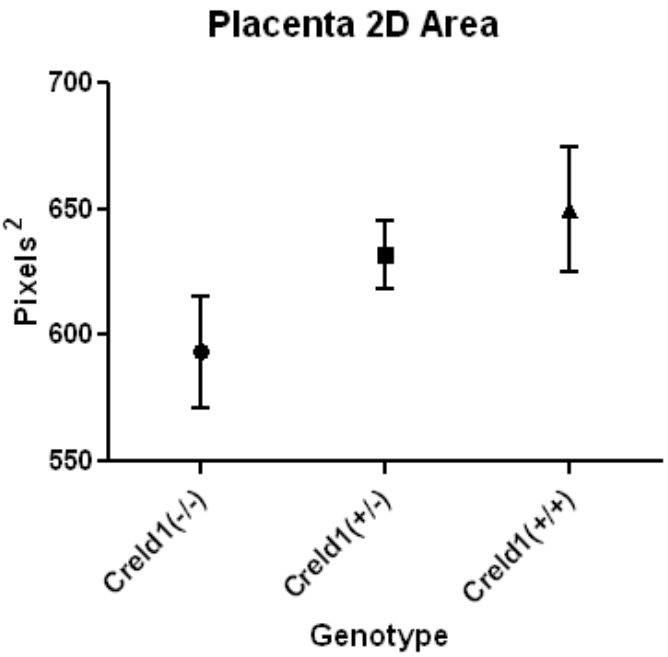
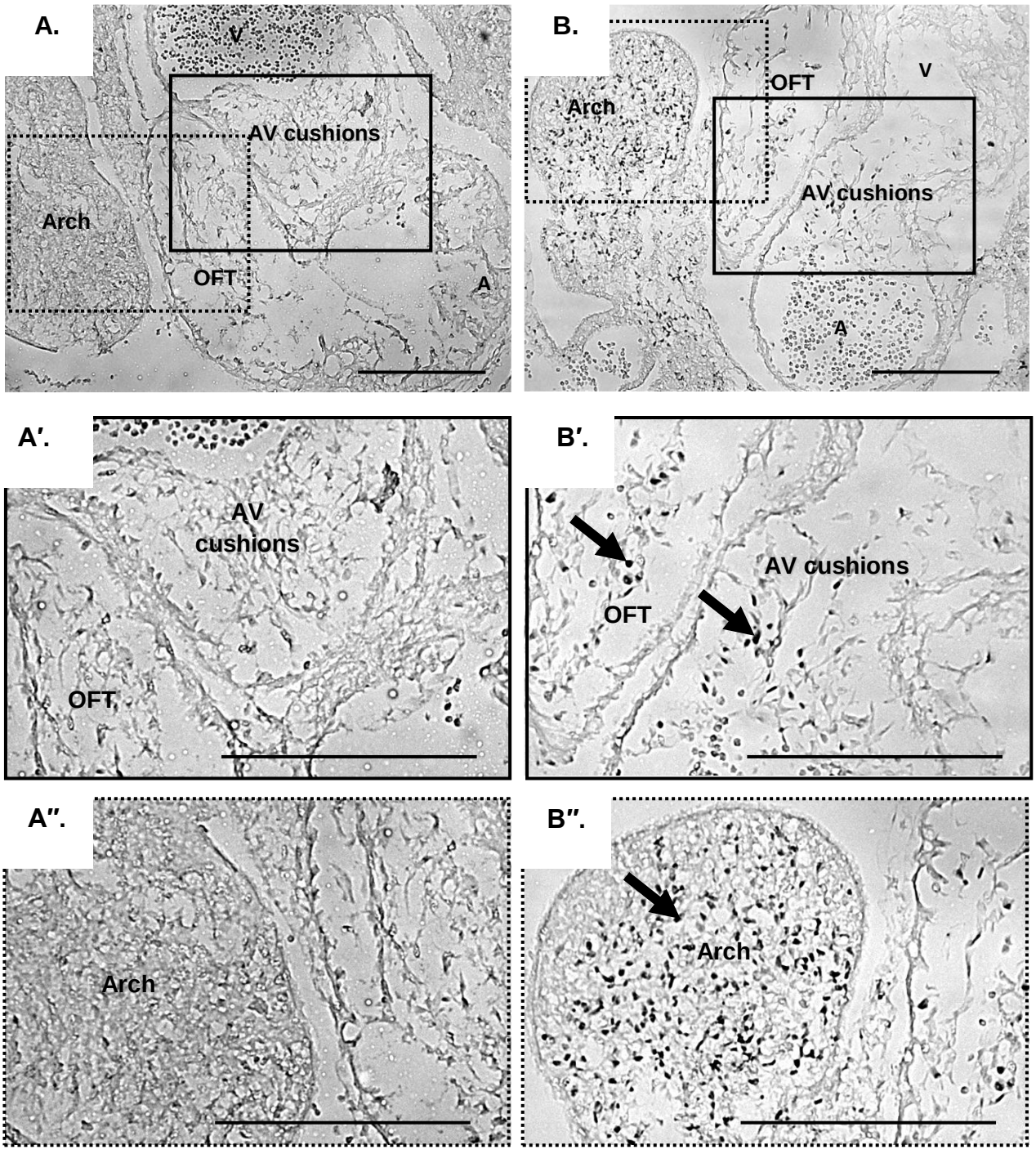


Figure 24: Knockout of *Creld1* stunted placental growth.

At E10.5 the *Creld1*-deficient placentas tended to be smaller. N=3 for each genotype.

Creld1$^{(-/-)}$ embryos exhibited increased apoptosis

To address the mechanism by which an absence of CRELD1 causes embryonic abnormalities and lethality I investigated the balance of cell proliferation and apoptosis in the *Creld1*$^{(-/-)}$ embryos. When I did this, I found no change in the levels of cell proliferation in E10.25 embryos, as determined by bromodeoxyuridine (BrdU) incorporation (data not shown). I did, however, observe a large increase in the number of apoptotic cells in E10.5 *Creld1*$^{(-/-)}$ embryos. This increased apoptosis was observed in the *Creld1*$^{(-/-)}$ hearts including in the myocardium and endocardial cushions but also elsewhere, with especially high levels in the pharyngeal arches (Figure 25). Therefore, it appears that an abnormally high level of apoptosis may be responsible for the paucity of cells in the *Creld1*$^{(-/-)}$ endocardial cushions and the overall poor growth of *Creld1*$^{(-/-)}$ embryos.


A.
V
AV cushions
Arch
OFT
A
B.
OFT
V
Arch
AV cushions
A
A′.
AV
cushions
OFT
B′.
AV cushions
OFT
A″.
Arch
B″.
Arch

C.

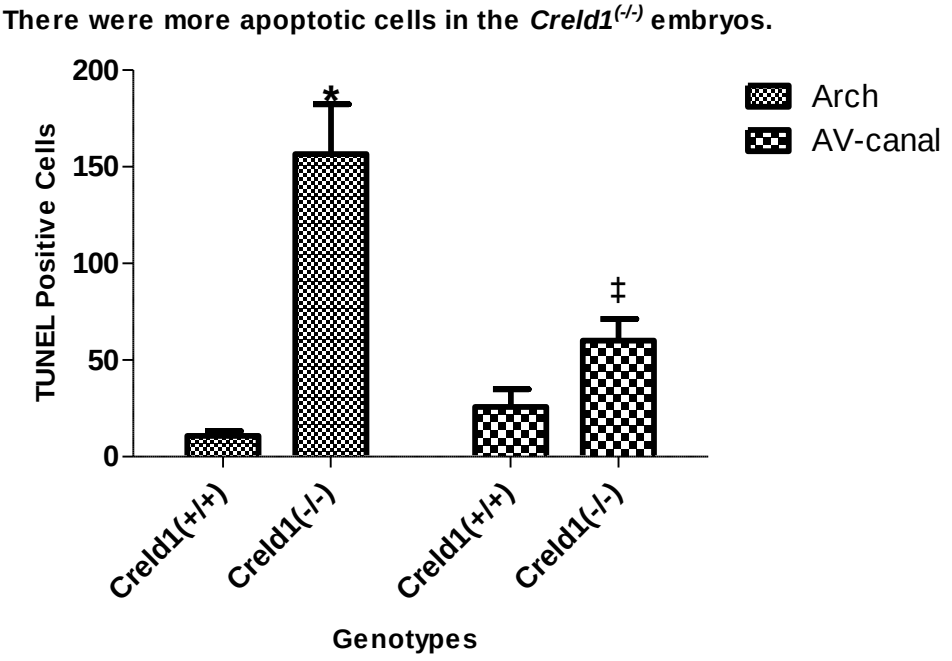


Figure 25: TUNEL-stain is increased in E10.5 *Creld1*$^{(-/-)}$ embryos.

Increased numbers of TUNEL-positive cells were seen in E10.5 *Creld1*$^{(-/-)}$ embryos, **B panels**. This is in contrast to the *Creld1*$^{(+/+)}$ littermates, **A panels**. Some representative TUNEL positive cells, seen as black, are indicated by arrows. **A′:** Magnification of *Creld1*$^{(+/+)}$ atrioventricular and outflow tract cushions shown in panel **A**. **A″**: Magnification of the *Creld1*$^{(+/+)}$ glossopharyngeal arch shown in panel **A**. **B′:** Magnification of the *Creld1*$^{(-/-)}$ atrioventricular and outflow tract cushions shown in panel **B**. **B″:** Magnification of the *Creld1*$^{(-/-)}$ glossopharyngeal arch shown in panel **B**. **C**. Mean and SEM of the mesenchymal cells observed in the glossopharyngeal arches and AV-canals. * = p value ≤ 0.01, ‡ = p value ≤ 0.05. Glossopharyngeal arch, Arch; outflow tract, OFT; atrioventricular endocardial cushions, AV cushions; common atrium, A; common ventricle, V. Scale bar represents 200μm.

CRELD1 is not necessary for EMT

Considering the scarcity of mesenchymal cells in *Creld1*$^{(-/-)}$ endocardial cushions, I decided to assess how CRELD1 could affect endocardial cushion development using a heart explant assay. To do this I explanted and cultured E9.0 mouse endocardial cushion on a 3-D collagen matrix, which is a classic culturing method that allows for EMT quantification, as successfully transformed cells can be seen migrating across and into the supportive 3-D collagen matrix and therefore can be counted. When I cultured under endogenous levels of VEGF (<15 ng/ml) I found no difference in the numbers of mesenchymal cell migrating from the canals of various *Creld1* genotypes [*Creld1*$^{(+/+)}$, *Creld1*$^{(+/-)}$ and *Creld1*$^{(-/-)}$] (Figure 26). Thus, I concluded that CRELD1 is not necessary for EMT to proceed, nor did it apparently affect mesenchymal cell migration or proliferation.

However, the fact that there was no difference in the levels of mesenchymal cells that migrated out of explants of various *Creld1* backgrounds is in striking contrast to what occurs in vivo, where the endocardial cushions of *Creld1*$^{(-/-)}$ are malformed (Figure 27). This highlights the obvious – that while culturing tissue can help us explore what is possible, even the best of systems never perfectly mimics in vivo conditions. Thus we must consider other modifiers of cushion growth, specifically modifiers that might affect how CRELD1 can affect the explanted hearts.

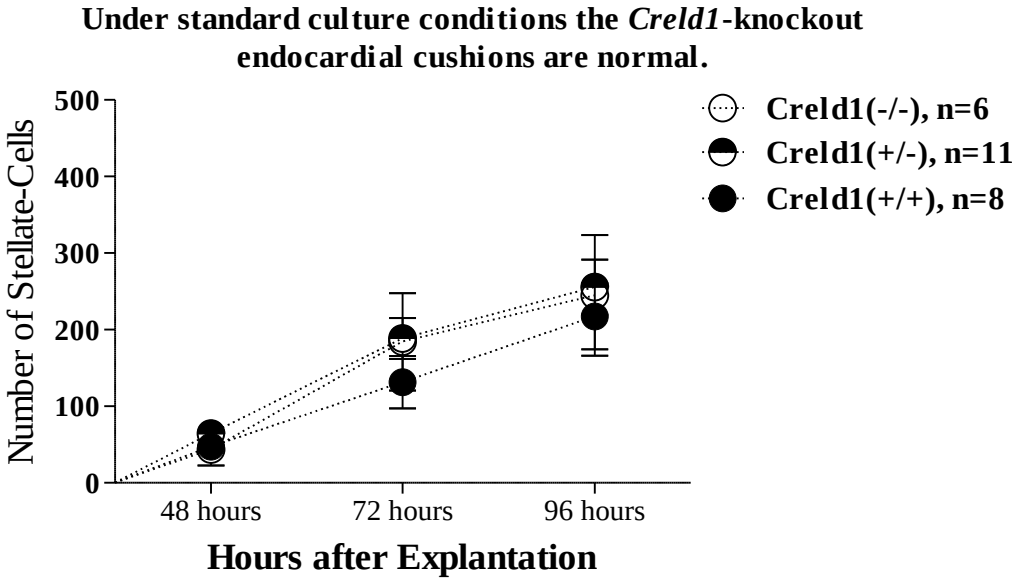


Figure 26: Explants grow the same under endogenous VEGF levels.

Under endogenous levels of VEGF, the stellate cell counts (number of mesenchymal cells) from the explanted *Creld1*$^{(-/-)}$, *Creld1*$^{(+/-)}$ and *Creld1*$^{(+/+)}$ atrioventricular (AV) canals were indistinguishable at all time points.

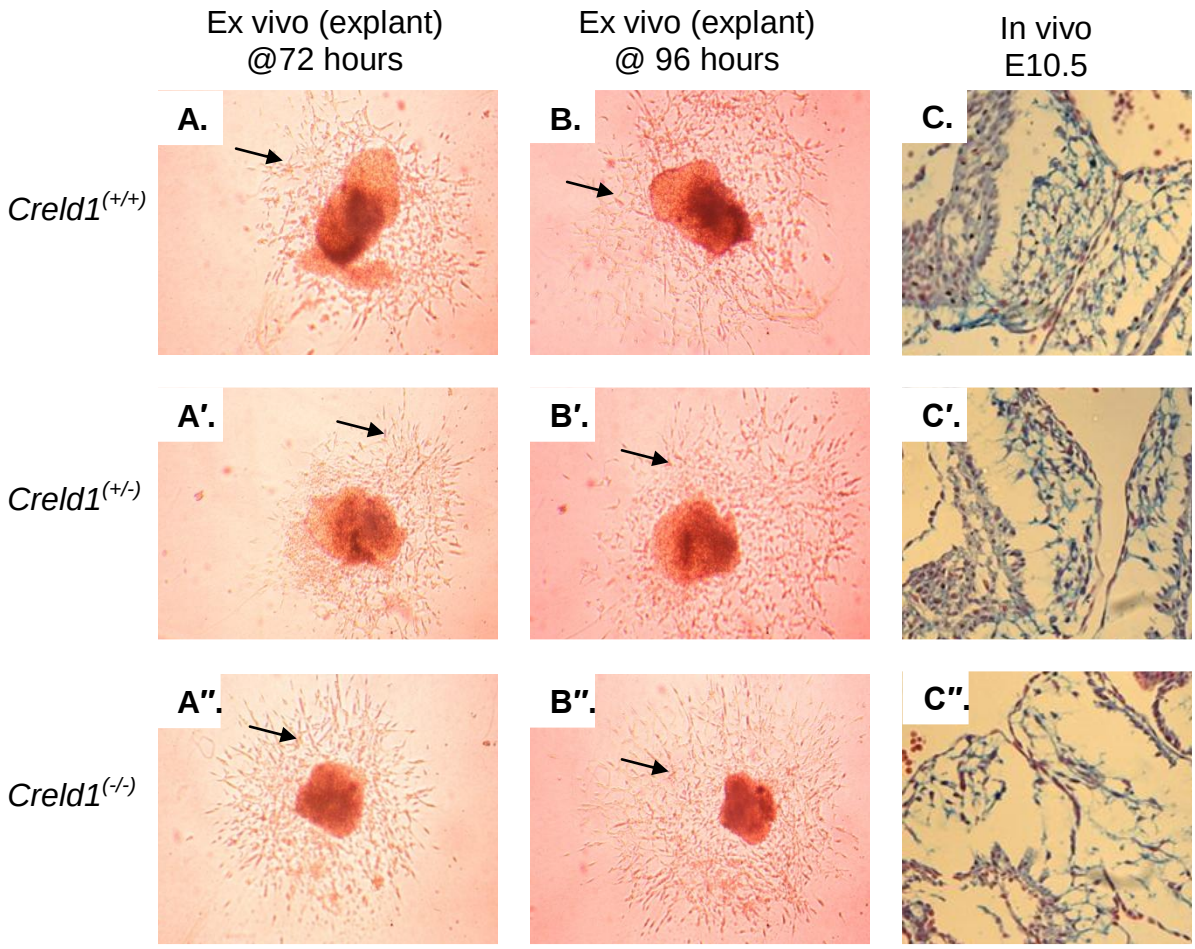


Figure 27: In vivo and in vitro heart phenotypes differ.

A & B: There was no difference in the number of mesenchymal cells seen migrating out of endocardial cushions explanted at E9.0, regardless of the *Creld1* genotype. Representative mesenchymal cells are denoted by arrows. **C:** However, in vivo the *Creld1*$^{(-/-)}$ endocardial cushions have noticeable fewer mesenchymal cells, as seen in the Alcian Blue/Nuclear Fast Red sections (also shown in Figure 18).

Knockout of one *Creld1* allele does not produce a significant phenotype

Creld1$^{(+/-)}$ mice were born and survived in the expected Mendelian ratio (Table 1). Additionally, gross and histological examinations did not reveal any major septal or valve defects in *Creld1*$^{(+/-)}$ mice; and the mass of adult *Creld1*$^{(+/-)}$ and *Creld1*$^{(+/+)}$ hearts were indistinguishable (Figure 28). Similarly our collaborator's laboratory never found a septal defect in the 45 postnatal day 0 (P0) pups they examined (Dr. Roger Reeves, personal communication). However, we did find that 3/12 (25%) of *Creld1*$^{(+/-)}$ adult-mouse hearts (>6 months of age) had a patent foramen ovale (PFO). This is in contrast to 1/9 (11%) of *Creld1*$^{(+/+)}$ adult hearts with a PFO (Figure 29). However, this increased PFO incidence is not statistically significant.

Still debatable, though, is if this slight increase in PFO incidence is biologically significant. PFOs result from the incomplete closure of the foramen ovale, a small communication in the embryonic heart that, during development, allows for the most highly oxygenated blood (returning from the placenta) to proceed directly to the metabolically hungry embryonic head. Upon birth, when the most highly oxygenated blood is received via the lungs, the foramen ovale normally closes to ensure that the most highly oxygenated blood, again, is preferentially shunted to the brain. However, in 10 - 35% of people the foramen ovale remains open (patent) (Fisher et al. 1995). The biological significance of this continued communication

remains debated but it has been suggested that PFOs contribute to migraine headaches and strokes (Sommer 2009). More research is still needed to determine if these associations are real. Meanwhile, I will consider a PFO at face value, as a small hole in the atrial septum, which may reflect a problem with the septum primum, which may or may not affect its ability to properly fuse with the endocardial cushions and cause an AVSD.

The fact that the *Creld1*$^{(+/-)}$ mice are normal is consistent with AVSD being multifactorial. After all, in humans *CRELD1*-missense mutations are incompletely penetrant; therefore, we expected that the absence of a single *Creld1* allele would not be enough to cause an AVSD in our mouse model. Accordingly, we continued to look for other AVSD-risk modifiers.

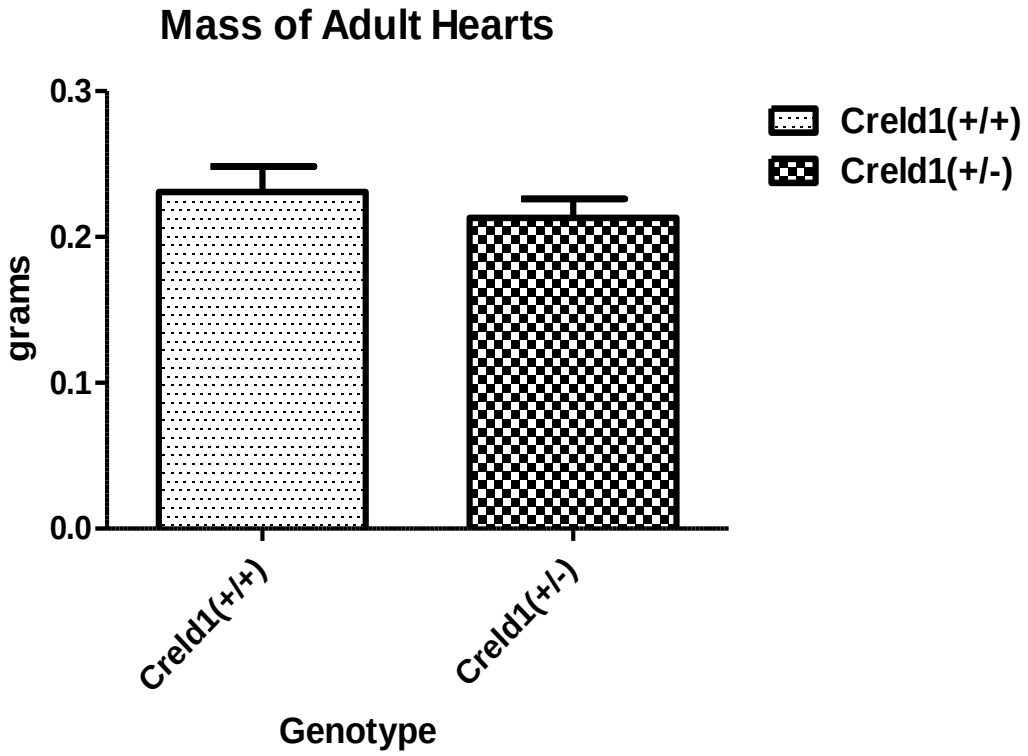


Figure 28: Mass of adult hearts.

The mass of adult mice (>6 month old) hearts did not vary according to *Creld1* genotype, n=10 for each genotype.

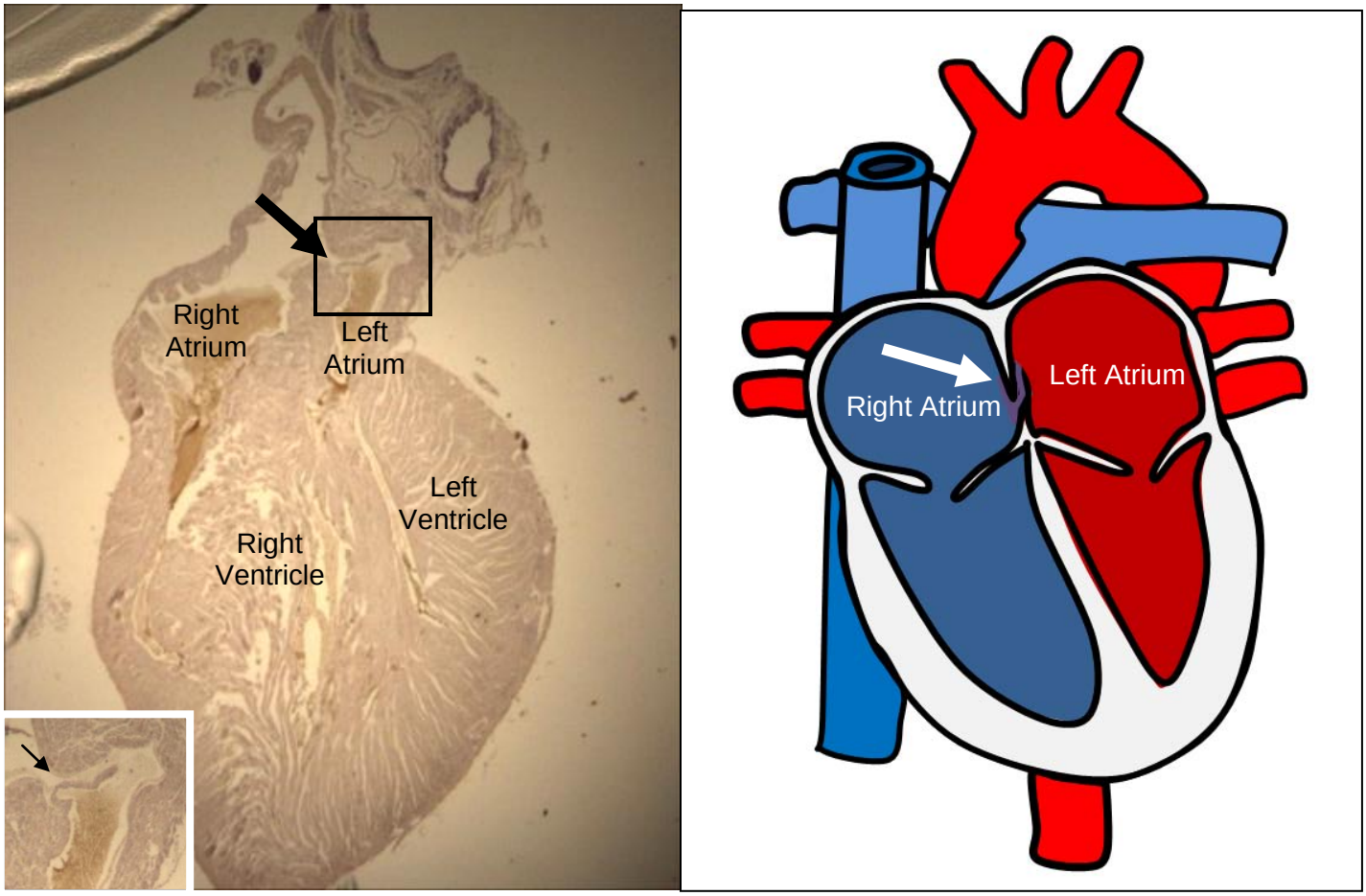


Figure 29: Example of a *Creld1*$^{(+/-)}$ heart with a PFO.

On the left is a representative H&E stained section of a whole adult *Creld1*$^{(+/-)}$ mouse heart sectioned coronally. Arrow indicates a small communication between the atrium, which is likely a PFO. On the right is a diagram of a PFO, which can be seen as a small communication between the right and left atrium and causes the mixing of oxygenated and deoxygenated blood. Arrow indicates PFO.

Creld1-deficiencies affect gene expression

To understand how CRELD1 is exerting its effects on the developing embryo, I compared the total RNA expression of E9.5 *Creld1*-knockout (*Creld1*$^{(-/-)}$) and *Creld1*-knockin (*Creld1*$^{(R329C/R329C)}$) mouse hearts to *Creld1*$^{(+/+)}$ hearts using gene expression microarray analysis. [The *Creld1*$^{(R329C/R329C)}$ mouse is a knock-in mouse created by our collaborators at Johns Hopkins that contains a knock-in of the most common human *CRELD1*-missense mutation associated with AVSD and is phenotypically normal (personal communication, Roger Reeves)]. Microarray analysis revealed a long list of genes (464 genes) that underwent at least a 4-fold change in response to a *Creld1*-deficiency. However, despite, or perhaps due to the large number of genes affected by *Creld1*-disruption I was not able to identify any single biochemical signaling pathway (using GeneSifter's Gene Ontologies) for which CRELD1 was necessary. However, I did find a 4–fold up-regulation of *Vegf* expression that I was able to confirm as a 3-fold over-expression with qPCR (Figure 30).

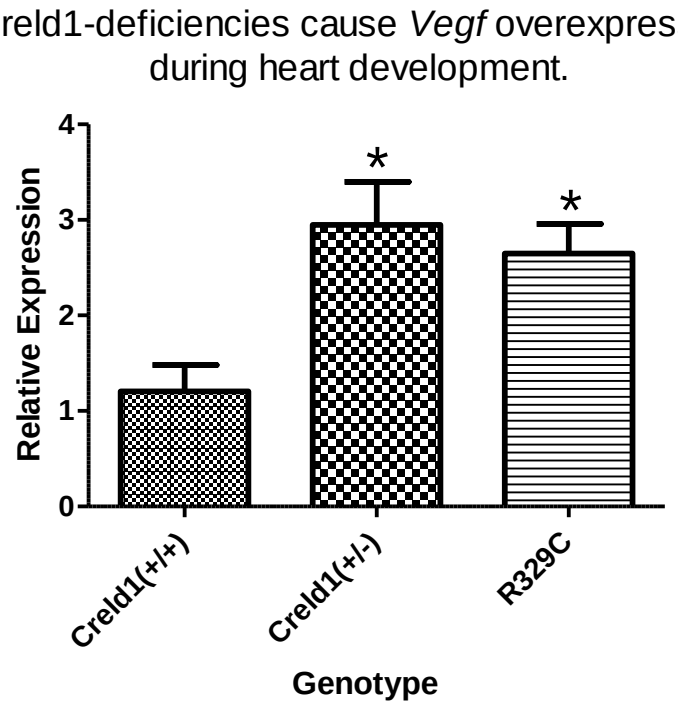


Figure 30: A CRELD1-deficiency is associated with *Vegf* over-expression.

Vegf-RNA was almost 3-fold higher in E9.5 *Creld1*-deficient [$Creld1^{(-/-)}$ and $Creld1^{(R329C/R329C)}$] mouse hearts. * = *p value* ≤ 0.01.

CHAPTER 6:

CRELD1/VEGF risk profile

VEGF-634C polymorphism is associated with AVSD

We genotyped AVSD cases (see Methods for patient population information), Coriell Caucasian Human Variation controls and the unaffected family members of AVSD cases for the *VEGF*-634C polymorphism and found a significant association between the *VEGF*-634C polymorphism and idiopathic cases of AVSD (Table 3). Additionally, we were able to corroborate that the *VEGF*-634C polymorphism can cause increased VEGF-protein expression (Figure 31).

	Controls	AVSD cases
VEGF 634G	83 (83%)	57 (62%)
VEGF 634C	17 (17%)	35 (38%) *
Total alleles	100	92

Table 3: The *VEGF*-634C polymorphism is associated with AVSD

The *VEGF*-634C polymorphism was enriched in the idiopathic AVSD population compared to the Coriell Caucasian Human Variation Control population *(*= *p value* ≤ 0.001).

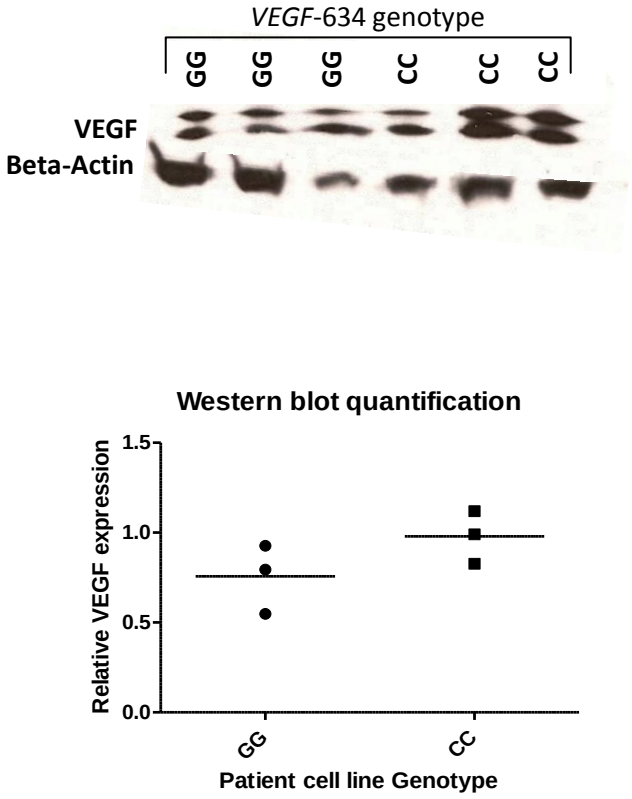


Figure 31: The -634C polymorphism is associated with increased VEGF over-expression.

Western blot analysis of three lymphoblast cell lines derived from individuals either homozygous for *VEGF*-634CC or *VEGF*-634GG polymorphism (six lines total) found that a *VEGF*-634C polymorphism is associated with VEGF over-expression (one-tailed t-test = p value ≤ 0.1).

CRELD1 mutations and the *VEGF*-634C variant co-segregate in AVSD

To test if the *VEGF* -634C polymorphism could be modifying the AVSD risk associated with *CRELD1*-missense mutations, we stratified our *VEGF* genotyping data by the incidence of *CRELD1*-missense mutations. When we did this, we found that *all* AVSD cases that have a *CRELD1*-missense mutation also carried the *VEGF*-634C polymorphism (5/50 AVSD cases), while in contrast the unaffected parents and siblings of cases *never* carried both a *CRELD1* mutation and the *VEGF*-634C polymorphism. Instead unaffected carriers of *CRELD1*-missense mutation always carried the wild-type *VEGF*-634GG polymorphism (Table 4).

It is notable that we have family information for two of the cases who carried a *CRELD1*-missense mutation and the *VEGF*-634C polymorphism. In these two cases the *CRELD1* mutation was inherited from one parent and the *VEGF*-634C polymorphism from the other parent, so that only the affected individuals carried both alterations. In one of those families there were also two unaffected siblings, neither of which carried both the *CRELD1*-missense mutation and the *VEGF*-634C polymorphism (Figure 32).

This data suggests that while neither a *CRELD1*-missense mutation nor the *VEGF*-634C polymorphism is sufficient to cause an AVSD alone, the two genetic alterations together greatly increase the risk of developing an AVSD. Next, I will show how I used our mouse model to test this interaction.

	CRELD1-missense mutation carriers	
	Unaffected	Affected
VEGF-634GG	12	0
VEGF-634GC	0	5

Table 4: *CRELD1* mutations/*VEGF*-634C polymorphism co-segregate in AVSD.

All the AVSD cases with a *CRELD*-missense mutation also carried the *VEGF*-634C polymorphism (5/50 or 10% of AVSD cases examined), while the unaffected parents and siblings of cases who carried a *CRELD1*-missense mutation *never* carried the *VEGF*-634C polymorphism.

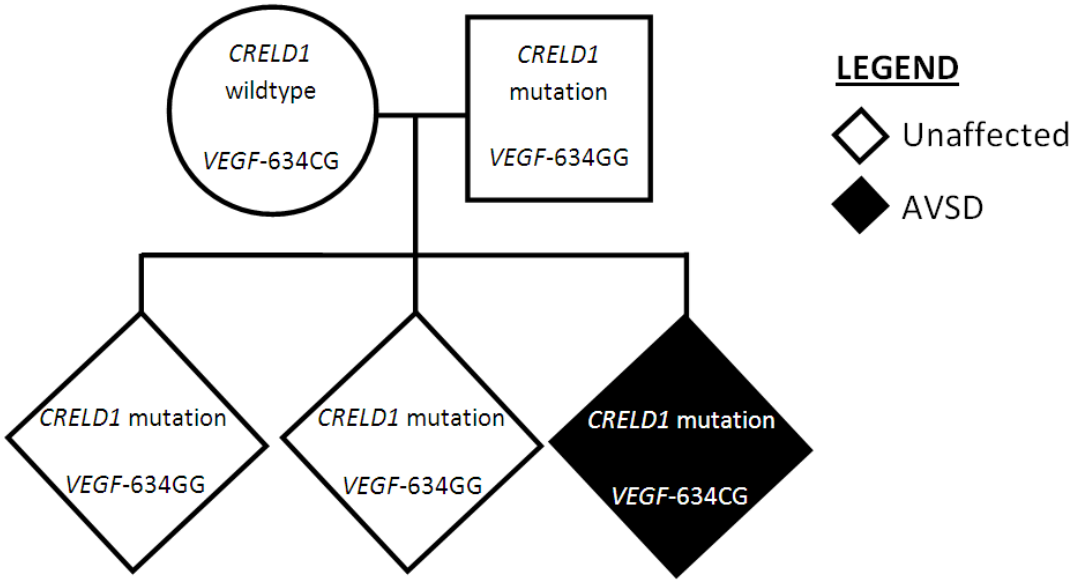


Figure 32: Unaffected family members never had two hits.

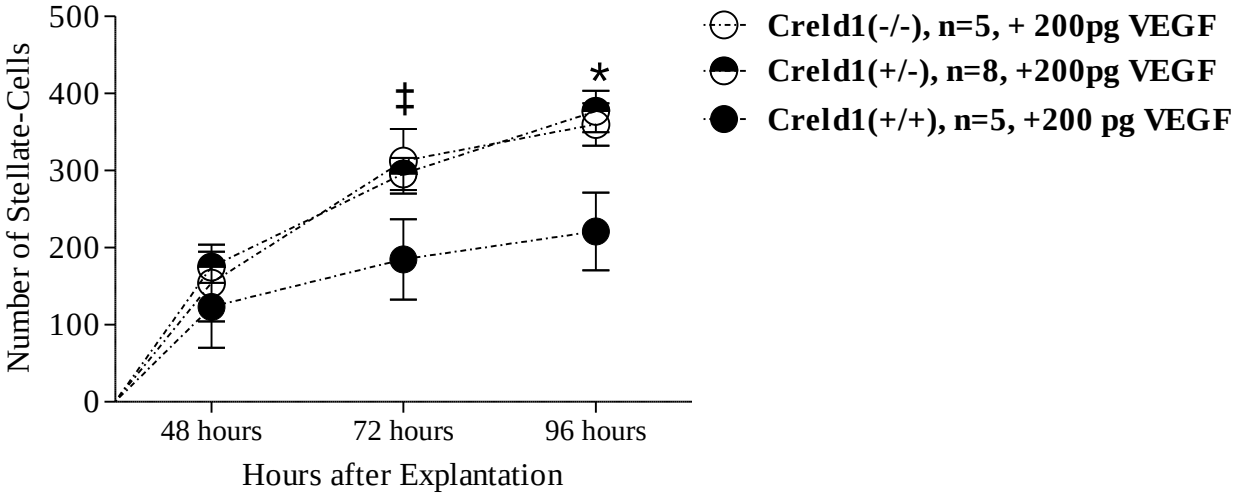
We genotyped an AVSD case's immediate family members and found that the unaffected family members carried *either* a *CRELD1*-missense mutation or a *VEGF*-634C polymorphism but never both; while, the combination of a *CRELD1*-missense mutation and the *VEGF*-634C polymorphism was only associated with the AVSD case (filled). Normalcy was confirmed in the unaffected family members (unfilled) by echocardiographs.

The exclusive co-segregation of *CRELD1*-missense mutations with the *VEGF*-634C polymorphism in idiopathic AVSD cases suggested to me that a combination of *CRELD1*-missense mutations and the *VEGF*-634C polymorphism can cause AVSD. I extrapolated this to mean that a CRELD1-functional deficiency in combination with VEGF over-expression can cause AVSD. This extrapolation was based on the fact that the *CRELD1*-missense mutations are predicted to be detrimental (Robinson et al. 2003) and thus cause a CRELD1-deficiency; and that the *VEGF*-634C polymorphism causes VEGF over-expression (Awata et al. 2005, Lambrechts et al. 2003, Petrovic et al. 2007).

VEGF and CRELD1 interact in cushion explant assay

Individually both CRELD1 and VEGF can affect endocardial cushion development; therefore, I decided to test if a *Creld1*-deficiency can interact with VEGF over-expression in the endocardial cushions. When I subjected explanted endocardial cushion to higher VEGF-protein levels (200 pg/ml) in an attempt to mimic the over-expression associated with the human *VEGF*-634C polymorphism, I found that the *Creld1*-deficient explants [both *Creld1*$^{(+/-)}$ and *Creld1*$^{(-/-)}$] had increased mesenchymal cell migration compared to the wild-type explants, which in contrast were unaffected by the addition of 200 pg/ml of VEGF (Figure 33). This confirms, in a heart development model, that a CRELD1 deficiency can interact with VEGF over-expression, as was implied in the epidemiology studies.

A. The response of explanted endocardial cushions to 200 pg/ml of VEGF.



B. The response of explanted endocardial cushions to 200 pg/ml of VEGF.

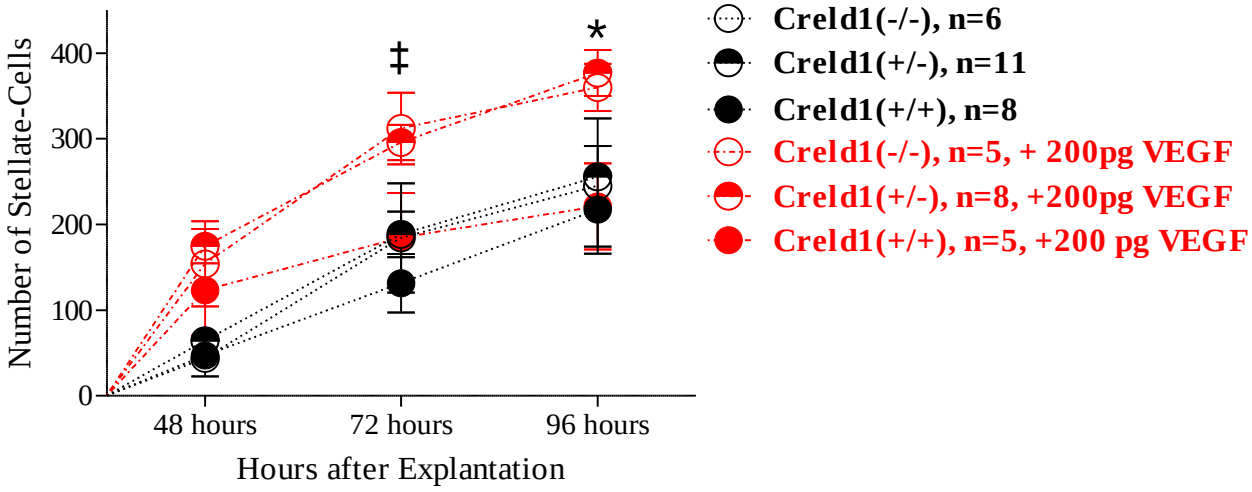


Figure 33: *Creld1*-deficient explants grow differently with VEGF addition.

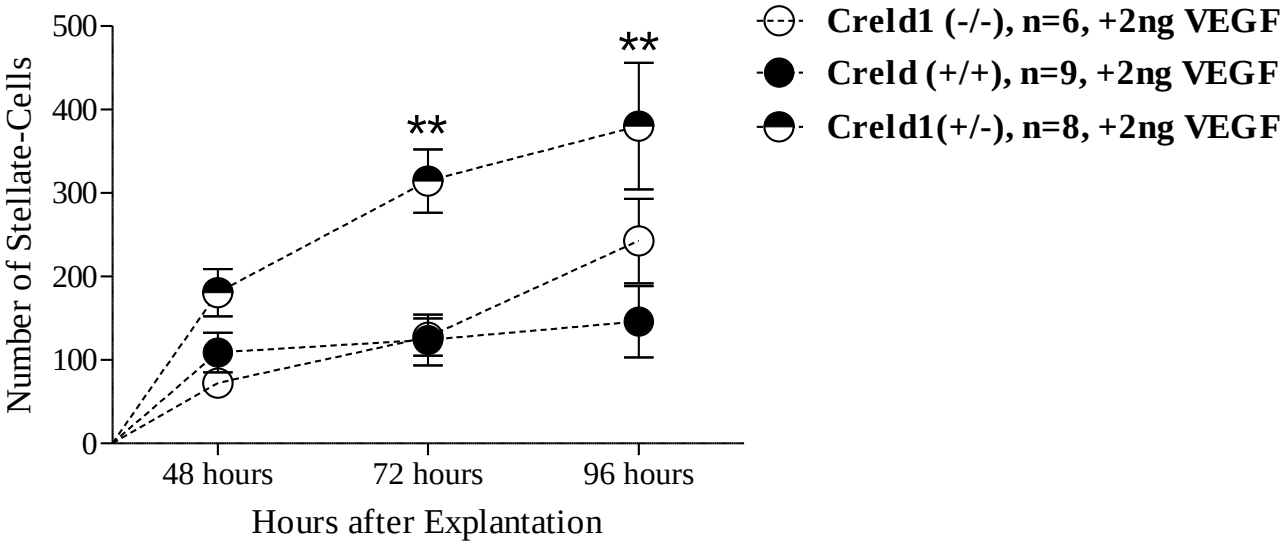
A. Addition of 200 pg/ml of VEGF resulted in increased EMT from the *Creld1*$^{(-/-)}$ and *Creld1*$^{(+/-)}$ explants compared to the *Creld1*$^{(+/+)}$ explants at both 72 hours (* = p value ≤ 0.1) and at 96 hours (* = p value ≤ 0.05). **B.** Addition of 200 pg/ml of VEGF has no effect on *Creld1*$^{(+/+)}$ EMT, as can be seen when compared to the EMT that occurred under endogenous VEGF conditions .

It is especially important to note that the concentration of VEGF tested, 200 pg/ml of VEGF, is a biologically relevant but elevated level of VEGF. Past studies have reported that E9.5 heart explants under normoxic conditions produce 3.4 pg/ml of VEGF, while hypoxic hearts (10% O2) increase VEGF expression to 45 pg/ml (Dor et al. 2003), which is similar to the in vivo levels observed in embryonic mouse hearts post EMT (15 pg/ml) (Miquerol et al. 2000). Although, the exact amount of over-expression caused by the -634C allele in the atrioventricular canal is unknown. It would appear that the 200 pg/ml is elevated but not absurdly so, as it was within the normal VEGF level found in adult human serum, which is 229 ± 147 pg/ml in men and 182 ± 112 pg/ml in women (Kimura et al. 2007).

After finding an interaction at 200 pg/ml of VEGF, I wanted to test other doses of VEGF, as the response of endocardial cushions to VEGF appears to be sensitive to dose. Specifically, there is evidence suggesting that while low levels of VEGF are needed to stimulate cushion EMT, higher levels can be inhibitory (Lambrechts and Carmeliet 2004). Consistent with this, when I tested a ten-fold higher dose of VEGF (2ng/ml), I found that the explanted cushions had very different responses (Figure 34). While the *Creld1*$^{(+/-)}$ cushions still had increased EMT, the *Creld1*$^{(-/-)}$ cushions' EMT was inhibited by the addition of 2 ng/ml of VEGF.

Thus, depending on the *Creld1* background and levels of VEGF addition, the explanted cushions either had no response, a proliferative response or an inhibitory response. These various responses can be explained, if the explanted cushions are assumed to be subject to increased VEGF from two sources, first by my direct addition of purified VEGF-protein to the media and second by the *CRELD1*-deficiency. If this is the case, then a mild increase in VEGF should stimulate cell proliferation/migration, while extreme increases in VEGF levels inhibit cell proliferation/migration. This is summarized in Figure 35.

A. The response of explanted endocardial cushions to 2 ng/ml of VEGF.



B. The response of explanted endocardial cushions to 2ng of VEGF.

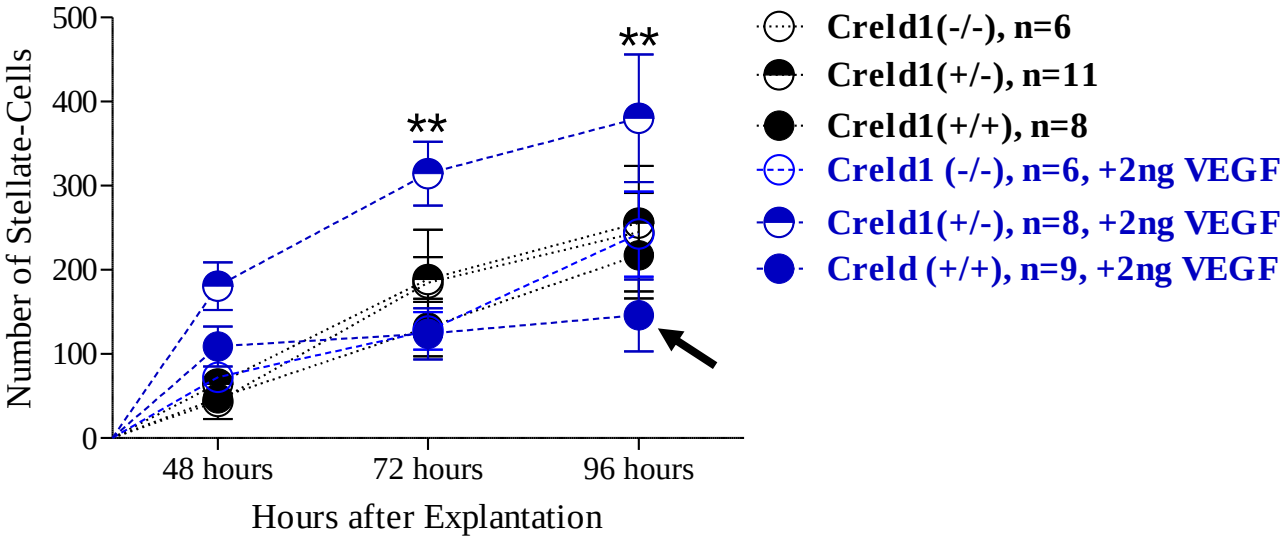


Figure 34: Explant response depends on VEGF dose.

A. Addition of 2ng/ml of *VEGF* resulted in the *Creld1*$^{(+/-)}$ explants having higher levels of stellate-cell counts than the *Creld1*$^{(+/+)}$ explants at both 72 hours and at 96 hours (** = p value ≤ 0.01). However, now the *Creld1*$^{(-/-)}$ cushions are inhibited at this increased VEGF dose, although not significantly (arrow). **B.** Addition of 2ng/ml of *VEGF* had no effect on the *Creld1*$^{(+/+)}$ EMT, as compared to EMT under endogenous VEGF levels.

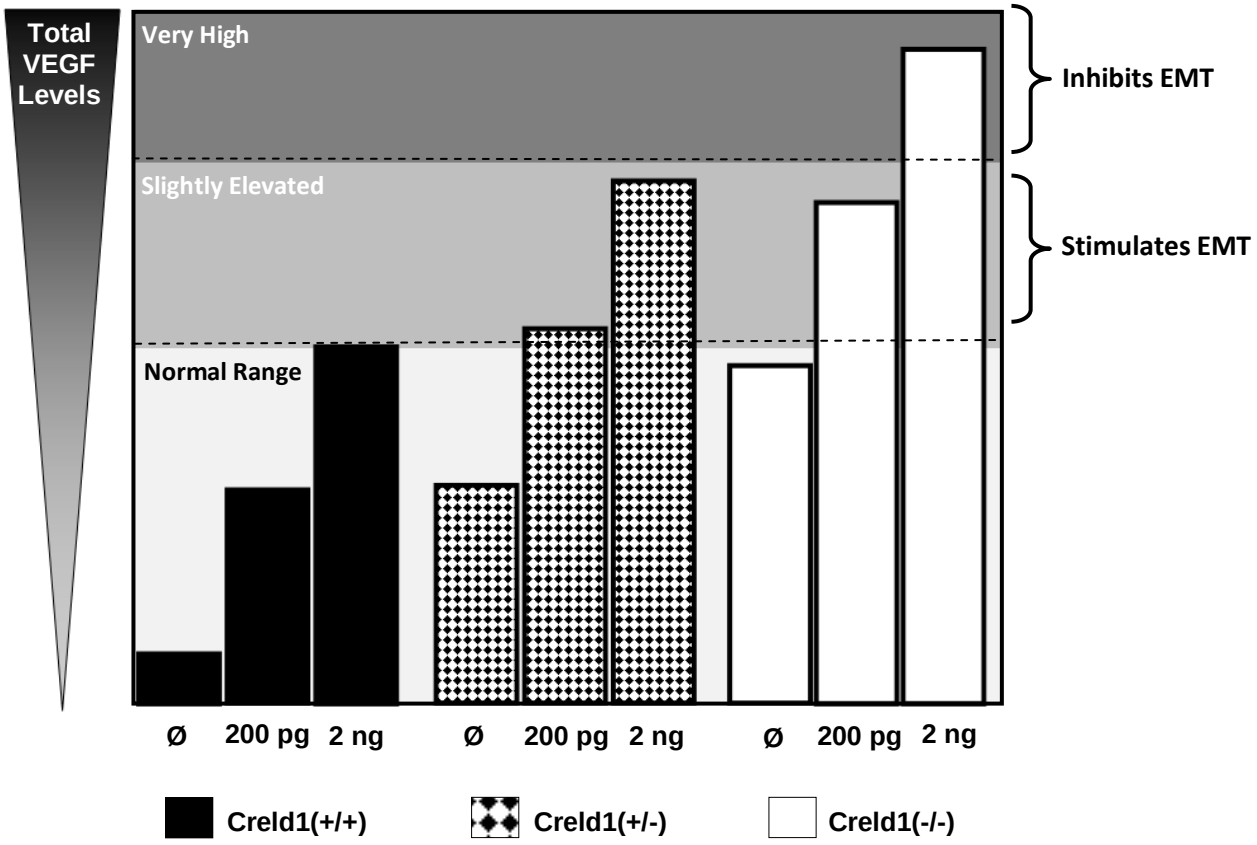

Total VEGF Levels
Very High
Slightly Elevated
Normal Range
Inhibits EMT
Stimulates EMT
Ø
200 pg
2 ng
Ø
200 pg
2 ng
Ø
200 pg
2 ng
Creld1(+/+)
Creld1(+/-)
Creld1(-/-)

Figure 35: The developing endocardial cushions are sensitive to VEGF dosing. This schematic illustrates how the explanted endocardial cushions responded to various total VEGF levels. Explanted endocardial cushion were subjected to increased VEGF from two sources, first by my direct addition of purified VEGF-protein to the media and second by CRELD1-deficiency. These two sources of VEGF contribute to the total VEGF present, which depending on the total dose has varying affects on EMT. Illustrated above is how the addition of 200 pg/ml or 2ng/ml of VEGF to *Creld1*$^{(+/+)}$ cushions resulted in total VEGF levels still within the "normal range" of VEGF and thus caused no abnormal phenotype. However, addition of 200 pg/ml or 2ng/ml of VEGF to the *Creld1*$^{(+/-)}$ cushions pushed the total VEGF levels into the "slightly elevated" range and caused increased EMT. This was also the result for *Creld1*$^{(-/-)}$ cushions exposed to 200 pg/ml of VEGF. Conversely, addition of 10X more VEGF to the *Creld1*$^{(-/-)}$ cushions (2ng/ml) resulted in "very high" VEGF levels and thus EMT inhibition because the null cushions were already more sensitized. Creld1$^{(+/+)}$, Creld1-wild-type explanted cushions; Creld1$^{(+/-)}$, Creld1-heterozygotic explanted cushions Creld1$^{(-/-)}$,Creld1-null explanted cushions; Ø, no purified VEGF protein added; 200 pg, 200 pg/ml of purified VEGF protein added; 2ng/ml, 2ng/ml of purified VEGF protein added.

CHAPTER 7:

DISCUSSION

It is believed that a combination of genetic risk factors can contribute to AVSD risk. However, until now no causative AVSD-risk profiles have been described. Here I identified an AVSD-risk profile that appears to explain 10% of idiopathic AVSD cases. The ability to define and characterize this risk profile was made possible by the numerous unique studies performed in this thesis.

CRELD1 biology

CRELD1 was essential for normal cardiovascular development

The work presented here is the first characterization of CRELD1 during development. My finding of CRELD1 expression in the endocardial cushions supports our premise that mutation of *CRELD1* can directly affect septal and valve formation. Additionally, my characterization of a constitutive *Creld1*-knockout mouse found that CRELD1 is essential for viability and normal cardiovascular development, including atrioventricular cushion morphogenesis.

This supports our hypothesis that *CRELD1*-missense mutations can increase the risk of incomplete valvuloseptal formation and thus cause AVSD.

However, I also found that CRELD1 was ubiquitously expressed and that it was necessary for numerous other developmental processes. This is consistent with the developmental roles of previously described congenital heart risk factors. For example, the CITED2 knockout mouse has numerous abnormalities, including cardiac malformations, adrenal agenesis, neural crest defects and exencephaly (Bamforth et al. 2001), while *CITED2* mutations are associated with isolated human congenital heart defects. And the TBX5 knockout mouse has both limb and heart abnormalities, even though *TBX5* mutations can be a risk factor for some isolated human congenital heart defects cases (Bruneau et al. 2001). Therefore, the fact that CRELD1 has widespread effects is consistent with the widespread effects of other isolated congenital heart defect risk genes. However, CRELD1 may also act as a risk factor for other developmental diseases.

CRELD1 affected apoptosis

The existence of abnormal extraembryonic vascularization made me consider the possibility that poor tissue perfusion and nutrient delivery might also be

responsible for some of the defects seen in the *Creld1*$^{(-/-)}$ embryos. Therefore, I decided to assess TUNEL staining in the *Creld1*$^{(-/-)}$ embryos because TUNEL is specific for physiologically regulated cell death (apoptosis) rather than stress factor induced cell death (hypoxia) and while hypoxia can inhibit development of E10.5 embryos, hypoxia is not known to increase TUNEL-positive cell incidence in E10.5 mouse embryos (Wendler et al. 2007). Therefore, I concluded that the increased apoptosis, seen as increased TUNEL positive cells, in the E10.5 embryos was primarily due to the absence of CRELD1 rather than necrosis due to hypoxia caused by extraembryonic vascular insufficiencies.

There was increased and widespread apoptosis in the *Creld1*$^{(-/-)}$ mice. Locations of highest TUNEL staining correlated with higher CRELD1 expression at E10.5, such as the brain, glossopharyngeal arches and heart, including the endocardial cushions. This suggests that CRELD1 can influence apoptosis susceptibility, a conclusion that is consistent with previous work showing that CRELD1 can control apoptosis by direct interaction with the pro-apoptotic factor RTN3 in cell culture (Xiang and Zhao 2009). My discovery of increased apoptosis during the critical period of atrioventricular valvuloseptal morphogenesis suggests that a CRELD1-deficiency can contribute to AVSD by increasing valve and septal primordium susceptibility to apoptosis. However, as mentioned there was also widespread apoptosis throughout the embryo. This widespread apoptosis is also

consistent with other congenital heart defect risk factors, such as *NOTCH1*, whose mutations are associated with familial valve defects but whose complete knockout in a mouse model causes widespread and severe abnormalities, including widespread apoptosis (Swiatek et al. 1994).

CRELD1 may contribute to a heart specific defect

How then could a gene, like CRELD1, that is necessary for global development, cause a heart specific defect? There are a few possible explanations: 1) Human AVSD are associated with heterozygous mutations in *CRELD1*, not complete gene knockdown. Accordingly, CRELD1 mutations found in AVSD cases may only disrupt heart specific genetic interaction, i.e. AVSD-associated CRELD1 mutations may be silent in other tissues. 2) Alternatively, CRELD1 mutations may provide a systemic genetic hit but the heart specificity is caused by a second heart specific hit from another gene. This would be consistent with AVSD as complex diseases. 3) Then again, there are some who theorize that congenital heart defects can be caused by postzygotic errors, specifically in cardiac progenitors cells, i.e., somatic mutations in the heart. In support of this, one group reports that they found *NKX2-5* mutations in defective heart tissue but not in the neighboring normal heart tissue of individuals with septal defects (Reamon-Buettner and Borlak 2004). Accordingly, it is possible that the CRELD1 mutation detected in peripheral blood and associated with AVSD arose from somatic mutations in the

hemangioblast lineage and that CRELD1 mutations found in humans are only affecting the vascular endothelium and blood-forming cells.

CRELD1 is possibly involved in the neural crest

My finding of both cardiac and craniofacial abnormalities in the *Creld1*$^{(-/-)}$ mouse is interesting considering that these defects often occur concordantly in human syndromes such as trisomy 21, DiGeorge, velo-cardio-facial and conotruncal anomaly face syndromes. This concordance implies a shared developmental origin. Accordingly, I proposed that the TUNEL-positive cells found in the pharyngeal arches of the *Creld1*$^{(-/-)}$ mouse are migrating neural crest cells, since neural crest cells are known to contribute to both the forebrain and the heart.

Neural crest cells arise between the superficial and neural ectoderm from a bulging of cells called the neural fold. As the neural tube forms, these cells delaminate, adopt a mesenchymal morphology and migrate throughout the embryo, where they will differentiate into neurons, endocrine, glial cells, pigment cells and components of the heart, specifically the outflow tract septum. More specifically, the cranial neural crest cells delaminate from the posterior diencephalon, spread over the telencephalic vesicles and contribute to the naso-frontal and naso-lateral buds (Creuzet 2009). Accordingly, ablation of

the cephalic neural crest can cause a loss of forebrain tissue, a reduction in the interocular distances and sometimes cyclopia (Etchevers et al. 1999). The presence of cyclopia is a feature found in extreme cases of holoprosencephaly, a structural anomaly of the brain caused by incomplete forebrain separation (Dubourg et al. 2007), a phenotype that I found in the *Creld1*$^{(-/-)}$ mouse. This caused us to speculate that knockout of CRELD1 might be disturbing neural crest migration or development.

Neural crest cells can also contribute to the heart and are thus called cardiac neural crest cells. Cardiac neural crest cells normally migrate through pharyngeal arch III and IV and populate the developing heart, most notably the outflow tract endocardial cushion mesenchyme, which will contribute to the aortopulmonary septum (Jain et al. 2011). Accordingly, I found that the outflow tract mesenchyme was deficient in the *Creld1*$^{(-/-)}$ embryos. However, the endocardium-derived mesenchyme and neural-crest-derived mesenchyme is difficult if not impossible to distinguish by routine histology, so whether the outflow tract was deficient because the cardiac neural crest was inhibited, or because secondary heart field derived outflow tract endothelial cells are abnormal still needs to be determined. Also, whether the outflow tract cushion defects found in the *Creld1*$^{(-/-)}$ embryos actually cause outflow tract

abnormalities remains unknown because the *Creld1*$^{(-/-)}$ embryos do not live long enough for us to observe this septation.

Whether CRELD1 is important in the neural crest only remains speculation at this time. While I found that there is a subset CRELD1-positive cells in the pharyngeal arches, I am unable to determine in what arch. This is because the CRELD1 immunohistochemistry was performed on wild type mouse sections purchased from Zyagen, thus I did not have the serial sections needed to conclusively determine what arch was affected. Similarly, while it is apparent the pharyngeal arches do have increased apoptosis, the sections processed were optimized for their cardiac views, thus what arch or arches containing this increased apoptosis remains to be determined.

CRELD1 was necessary for angiogenesis

The formation of the vascular network during development is called neovascularization. This is a complex process that includes both vasculogenesis (the in situ differentiation of endothelial cell progenitors, or angioblasts, into blood vessels) and angiogenesis (the development and remodeling of new blood vessels from an already existing vasculature). The ability of new capillaries to

sprout in an organized fashion from previously existing blood vessels during angiogenesis involves specialized “tip cells.” These tip cells are responsible for extending filopodia, sensing environmental cues and guiding capillary growth along VEGF gradients (Gridley 2010). Tip cell formation relies on NOTCH signaling, which suppresses branching at the tip of these developing angiogenic sprouts (Gridley 2010, Jakobsson et al. 2009, Bentley et al. 2009). Accordingly, without NOTCH signaling numerous tip cells can form and abnormal angiogenesis results, characterized by an immature, disorganized capillary network.

The vascular abnormalities associated with a knockout of NOTCH are strikingly similar to the *Creld1*$^{(-/-)}$ embryos (Gale et al. 2004, Huppert et al. 2000). Especially notable was the primitive vascular network in the *Creld1*$^{(-/-)}$ yolk sack, which was similar to the “orange peel” phenotype seen in the *delta-like 4* (a NOTCH ligand) knockout mice (Gale et al. 2004). These results prompted me to propose that CRELD1 could be involved in NOTCH signaling; particularly since CRELD1, which is predicted to be a cell surface protein with extracellular tandem EGF domains, is superficially reminiscent of the NOTCH family of proteins. However, without functional data this remains only speculation.

Regardless of the mechanism, however, the necessity of CRELD1 for vascularization has implications for carcinogenesis. CRELD1 is located in the interval for loss of heterozygosity for nasopharyngeal carcinoma (Deng et al. 1998) and lung cancer (Wistuba et al. 2000). Altered *CRELD1* expression has been reported in various malignancies including non-small cell lung cancer (Dehan et al. 2007), multiple endocrine neoplasia (Dilley et al. 2005) and colon cancer (Saaf et al. 2007). In addition, microRNAs that can control CRELD1 expression have been related to esophageal squamous cell carcinoma survival (Guo et al. 2008). Furthermore, the response of cancerous cell lines to radiotherapy (Xu et al. 2008) and parathyroid hormone (Tsigelny et al. 2005) is associated with altered *CRELD1* expression. Lastly, *CRELD1* in combination with three other genes is a specific marker for endometrial cancer (Koga et al. 2011). Therefore based on my findings, we now hypothesize that the alteration in CRELD1 expression found regularly in cancer might be explained by its role as a regulator of angiogenesis or apoptosis.

The *Creld1*-heterozygote mouse should be a powerful genetic tool

In contrast to the *Creld1*$^{(-/-)}$ embryos, the *Creld1*$^{(+/-)}$ embryonic and adult mice were indistinguishable from wild-type littermates. This was expected because in humans *CRELD1*-missense mutations are incompletely penetrant, suggesting that additional modifiers are needed in combination with a CRELD1-deficiency to

cause AVSD. Therefore, it was not surprising that the loss of one *Creld1* allele was not sufficient to cause AVSD in our genetically homogeneous, highly inbred, mouse population.

The phenotypically normal *Creld1*$^{(+/-)}$ mouse is a powerful tool for investigating the penetrance of other genetic modifiers of AVSD. This was recently demonstrated when the *Creld1*$^{(+/-)}$ mouse was crossed with another AVSD susceptible mouse, the Down syndrome mouse model, Ts65Dn. This Ts65Dn mouse model is trisomic for the Down syndrome 'critical region' and thus like the human Down syndrome population is sensitized to developing AVSD (18% of Ts65Dn mice have AVSD). AVSD penetrance was increased, however, by crossing the Ts65Dn and *Creld1*$^{(+/-)}$ mouse models (50% of these mice have an AVSD) (Cherry et al. 2008). This supports our hypothesis that a CRELD1 haploinsufficiency can increase AVSD susceptibility.

VEGF/CRELD1 interaction as an AVSD risk profile

Our study population was unique

This work concerns the role of VEGF in AVSD etiology. VEGF has long been appreciated as an important morphogen in early valve development. Interestingly, only one other study has ever found a significant association between the *VEGF*-634C polymorphism and septal heart defects (Vannay et al. 2006). We believe that the discrepancy between our results and other past studies (Xie et al. 2007, Smedts et al. 2010) is due to variations in study populations compositions. Contradictory past studies have included very heterogeneous study populations, which included both idiopathic and Down-syndrome-associated AVSD cases, as well as other congenital heart defect cases that likely lowered the statistical power. Therefore, in our study, in order to ensure the highest possible power, we imposed stringent inclusion/exclusion criteria to create a homogenous congenital heart defect population, composed of only idiopathic AVSD cases and purposefully absent of Down-syndrome-associated AVSD cases, which are seemingly caused by different combinations of risk factors. I propose that our strict population selection allowed us to identify a significant association between the *VEGF*-634C polymorphism and AVSD. Contradictory past studies have also focused on different racial backgrounds than we did (Chinese versus Caucasian) (Xie et al. 2007). Considering this, our results may be specific to the Northern European population.

VEGF both inhibited and promoted EMT depending on the dose

It has been speculated, based on the convoluted VEGF literature, that VEGF can promote EMT (Dor et al. 2001, Ferrara et al. 1996) but that elevated or premature VEGF expression can inhibit EMT (Dor et al. 2001, Dor et al. 2003, Lambrechts and Carmeliet 2004). However, the exact correlation between VEGF dose and cellular response was unknown. This is largely because no single study has ever shown both the inhibitory and stimulatory effects of VEGF in the heart; and the only past study to address VEGF's effects on explanted endocardial cushion used very high levels of VEGF (100 ng/ml) (Dor et al. 2003). My experiment is unique, however, in that I choose to explore lower, more biologically relevant VEGF doses (200 pg – 2 ng/ml) than past studies (1 μg) (Dor et al. 2001)(Dor et al. 2001). By doing this I found conditions (total VEGF levels) that were within the "normal range", which caused no phenotype; conditions that caused increased EMT; and conditions that inhibited EMT (Figure 35).

I speculate that the ability of low levels of VEGF to cause increased cell proliferation/migration and high levels of VEGF to inhibit cell proliferation/migration is due to the affinity of VEGF for its receptors. Both VEGFR1 and VEGFR2 are expressed in the atrioventricular endocardial cushions

by E10.5 (Dor et al. 2001), so VEGF may signal through both receptors after EMT initiation. However, VEGF has a higher affinity for VEGFR1 (Mustonen and Alitalo 1995), whose signaling can induce endothelial migration (Lesslie et al. 2006) and thus VEGF should preferentially bind to VEGFR1 at low concentrations. This receptor specificity may be responsible for the increased proliferation I observed in explants under slightly elevated VEGF conditions. In contrast, VEGF has a lower affinity for VEGFR2 (Mustonen and Alitalo 1995), whose signaling appears to be able to inhibit cellular proliferation, migration and invasion in cell culture (Silva et al. 2011). Thus VEGFR2 signaling and its inhibitory effects may only be observed at higher VEGF concentrations (>2ng/ml).

CRELD1 and VEGF as an AVSD risk profile

We found that the combination of the *VEGF*-634C over-expression polymorphism and *CRELD1*-missense mutation is exclusive to idiopathic AVSD cases. This suggests that the combination of a CRELD1-functional deficiency and VEGF over-expression can cause AVSD. I was then able to functionally validate a CRELD1/VEGF interaction in the developing heart by demonstrating that mouse endocardial cushions deficient in CRELD1 [*Creld1*$^{(+/-)}$ and *Creld1*$^{(-/-)}$] respond abnormally to increased VEGF expression, which mimics the human genetic VEGF-634C allele over-expression. This CRELD1/VEGF functional interaction

within the endocardial cushions is important because abnormalities in cushion development are an accepted developmental basis for AVSD.

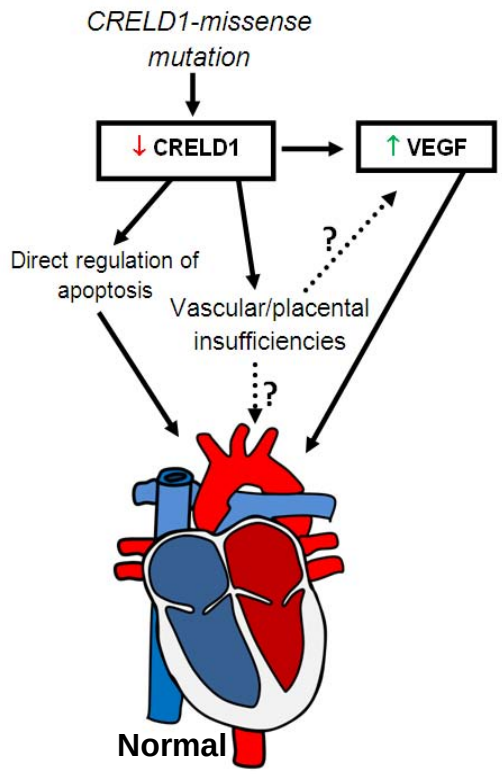
I propose that a CRELD1-deficiency plus VEGF over-expression contributes to AVSD, not by preventing the development of the atrioventricular endocardial cushions, as these cushions can still undergo EMT; but by causing abnormal migration or differentiation of the cushion mesenchyme, as an overabundance of mesenchymal cells can be seen in the explants. In vivo this abnormal mesenchymal migration could result in an AVSD by causing a misalignment of the cushion mesenchyme with other septal structures; and thereby preventing the closure of the atrioventricular septum and normal valve formation. Additionally or alternatively, improper location the cushion mesenchyme might lead to apoptosis because of inappropriate environmental signals and a complete loss of cushion tissues.

The combination of a CRELD1 deficiency plus the VEGF over-expression appears to be enough to breach the AVSD disease threshold. Ex vivo, the abnormal endocardial cushion development may be as simple as the additive accumulation of VEGF over-expression (from both the *VEGF*-634C polymorphism and the *CRELD1*-missense mutation). How *CRELD1*-missense mutations and the *VEGF*-634C polymorphism impinge upon the heart in vivo may be more complicated.

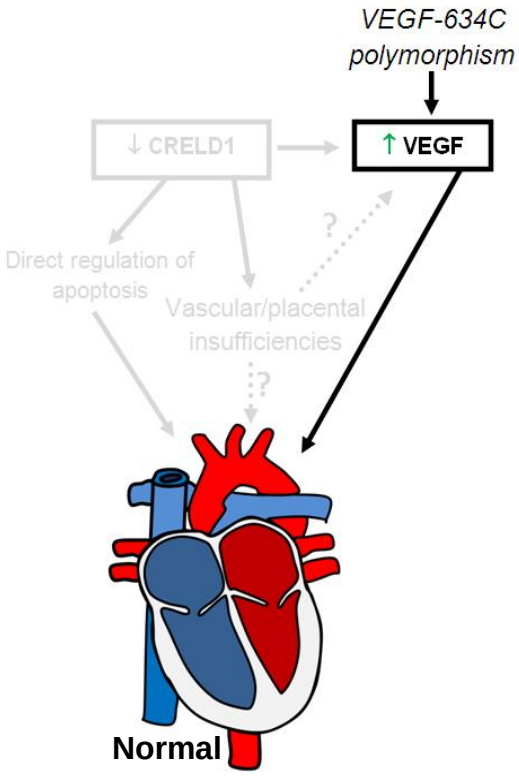
The cumulative addition of VEGF over-expression (from both the *VEGF*-634C polymorphism and *CRELD1*-missense mutations) is likely still a major contributor but in vivo there may also be contributions from placental or vascular abnormalities (Figure 36).

The clinical implications of a CRELD1/VEGF AVSD risk profile include advances in disease prediction, prognoses and more personalized management. However, before this profile can be used diagnostically, the specificity of the CRELD1/VEGF risk profile needs to be confirmed in other AVSD study populations, as the number of AVSD cases examined in this study was limited. Meanwhile, the work presented here is a proof-of-principle that a combination of rare genetic variants and common polymorphisms can work together to contribute to disease risk; that some AVSD cases can be caused by a digenic risk profile; and that there may be a CRELD1/VEGF signaling axis in heart development.

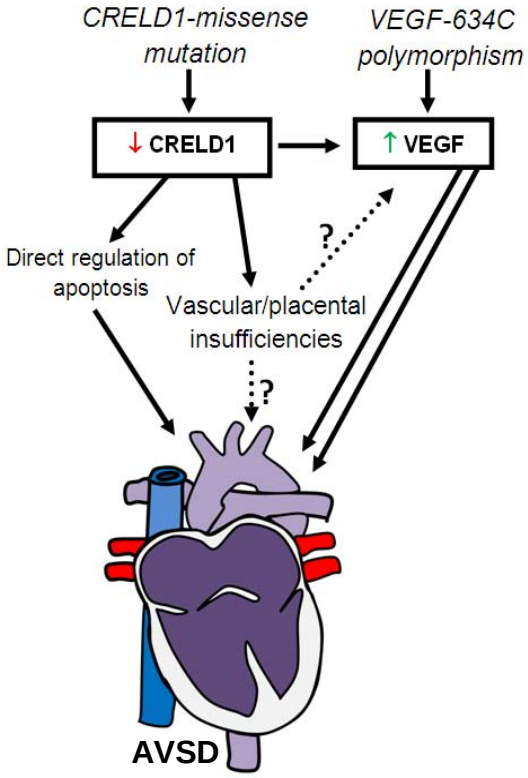
A. **Just a *CRELD1*-missense mutation is not enough to cause an AVSD.**



B. **Just the *VEGF*-634C polymorphism is not enough to cause an AVSD.**



C. **A *CRELD1*-missense mutation plus the *VEGF*-634C polymorphism *is* enough to cause an AVSD.**



D. **Mitigation of VEGF over-expression may prevent an AVSD caused by the CRELD1/VEGF profile.**

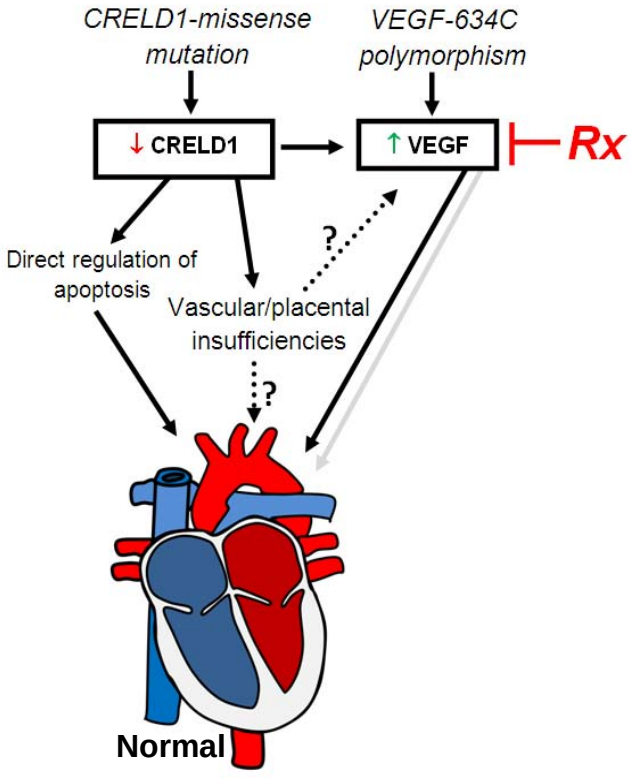


Figure 36: CRELD1/VEGF disease model.

Across is my model to explain how CRELD1 and VEGF may additively affect the developing heart and cause an AVSD. **A**. A CRELD1 deficiency can cause increased apoptosis in the endocardial cushions; and vascular and placental deficiencies, all of which may contribute to AVSD. A CRELD1 deficiency is also associated with increased VEGF expression, which is also known to cause endocardial cushion defects. But while *CRELD1*-missense mutations are strongly associated with AVSD, a *CRELD1*-missense mutation by itself is not completely penetrant for AVSD. **B.** VEGF over-expression is known to cause endocardial cushion abnormalities and the *VEGF*-634C polymorphism, which causes VEGF over-expression, is associated with AVSD. But without a CRELD1-missense mutation, the *VEGF*-634C polymorphism is not completely penetrant for AVSD. **C.** However, the combination of a *CRELD1*-missense mutations plus the *VEGF*-634C polymorphism provides the multiple hits needed to reliably cause an AVSD, likely in large part due to the repetitive hits to the VEGF pathway. **D.** I propose that therapies addressing the VEGF over-expression may be used to prevent AVSD by bringing the VEGF levels back into the acceptable normal range.

CHAPTER 8:

CONCLUSIONS & FUTURE DIRECTION

The novelty of this dissertation exposed numerous new scientific questions. Below I will discuss a few of the most important questions and some immediate next steps that should be undertaken. First, I propose further characterizations of CRELD1 during development, including a study to determine if the vascular and placental deficiencies observed in the *Creld1*$^{(-/-)}$ mouse contribute to the endocardial cushion defects observed. Second, I propose the creation of mouse models to determine the role of the secondary heart field and cardiac neural crest in the *Creld1*$^{(-/-)}$ phenotype. Third, I propose an experiment to test whether the observed extracellular matrix changes seen in the *Creld1*$^{(-/-)}$ mouse can affect EMT. Fourth, I propose an experiment to test if a CRELD1 deficiency plus VEGF over-expression can cause an AVSD in vivo. Lastly, I will speak to how the CRELD1/VEGF profile may be used clinically.

"Science is always wrong, it never solves a problem without creating ten more."

-*George Bernard* Shaw

Create a tissue-specific *Creld1*-knockout mouse

Our initial decision to create a global *Creld1*-knockout mouse model proved advantageous because it revealed multiple novel roles for CRELD1. These roles included the ability of CRELD1 to modulate apoptosis in the developing cardiac and neural crest derived tissues; the necessity of CRELD1 in vascular maturation; and the need of CRELD1 for normal placental development and placental gene expression. And while I concluded that the cardiac defects are largely due to CRELD1's direct regulation of apoptosis in the heart, I cannot rule out poor circulation and perfusion damaging the developing heart. Understanding how a CRELD1-deficiency causes heart defects is important for the development of any future, targeted therapeutics. To directly test if vascular or placental deficiencies (and therefore poor circulation and perfusion) are contributing to the *Creld1*$^{(-/-)}$ heart phenotype, I proposed the creation of a heart-specific *Creld1*-knockout mouse, driven by the heart specific marker Nkx2.5, which would knock out CRELD1 expression throughout the heart tube, pharyngeal endoderm, secondary heart field and first arch ectoderm. This should result in a loss of CRELD1 in the majority of tissues that contribute to the heart, including the atrioventricular endocardial cushions. If there is mitigation of the endocardial cushion phenotype in this heart-specific CRELD1 knockout then we can conclude that the placental and vascular abnormalities are an important contributor to AVSD risk. Conversely if the hearts appears equally damaged then we can conclude that the vascular and placental abnormalities are only nominally involved in the AVSD etiology associated with a CRELD1 deficiency.

The traditional view that endocardial cushion errors are all that underlie an AVSD is too simplistic. AVSD may also be the result of abnormal right-left patterning, as AVSD are often associated with abnormal patterning syndromes like heterotaxy. Additionally, AVSD may also result from abnormal myocardial structures, as the majority of the primary atrial septum and ventricular septum are of myocardial in origin. Conceivably, misalignment of the myocardial septal structures or improper myocardial signaling could prevent the proper development of the membranous endocardial cushion-derived septal structures. Finally, both endothelium-derived mesenchyme (from the tip of the atrial septum and from the developing AV cushions) and non-endothelium-derived mesenchyme (found in the dorsal mesenchymal protrusion) are necessary for normal atrioventricular septal formation. This was recently demonstrated by Goddeeris et al. who showed that if sonic hedgehog (Shh) signaling is ablated in the secondary heart field (including the dorsal mesenchymal protrusion) an AVSD results (Goddeeris et al. 2008). However, we were unable to assess the dorsal mesenchymal protrusion contribution in the *Creld1*-knockout mouse because it did not live long enough.

If we find that the *Nkx2-5* driven CRELD1 knockout lives long enough (E13.5) we may be able to assess if the dorsal mesenchymal protrusion is involved in CRELD1-induced AVSD risk. Alternatively, to directly assess the role of the second heart field and the dorsal mesenchymal protrusion in CRELD1-induced AVSD, a secondary

heart-field specific CRELD1 knockout should be created. To do this CRELD1 knockout could be driven by the transcription factor myocyte-specific enhancer factor 2C, which is expressed only in the splanchnic and core arch mesoderm but not in the pharyngeal endoderm (Verzi et al. 2005).

While fate-mapping has determined that *Pax3*- and *Wnt1*-derived neural crest precursors do not contribute to the atrioventricular endocardial cushions, there appears to be some *Wnt1*-Cre derivatives in the myocardial and epicardial layers of the heart (Li et al. 1999, Brown et al. 2001, Stottmann et al. 2004). Considering then the intertwined nature of heart development, it is possible that these neural crest derivatives could affect signaling to the endocardial cushions and thus contribute to AVSD. Therefore, in order to determine if CRELD1 is involved in the cardiac neural crest and whether this can contribute to AVSD, a neural crest specific knockout mouse should be created that is driven by *Wnt1*. Additionally, such a *Wnt*-specific CRELD1 knockout could tell us if a lack of CRELD1 in the neural crest is responsible for the cranial defects we observed in the constitutive *Creld1*$^{(-/-)}$.

Test whether changes in matrix composition can affect EMT

I observed fewer mesenchymal cells and less extracellular matrix in the *Creld1*$^{(-/-)}$ endocardial cushions. At this time I can only speculate whether the reduced

mesenchymal cell count in the cushions caused the reduction in matrix or whether a change in the matrix hindered cell migration. Specifically, I am interested in exploring how the matrix protein vitronectin affects endocardial cushion EMT. This is because I found, using microarray assays, that *vitronectin* was up-regulated in the E9.5 *Creld1*-deficient heart. It would be interesting to test if the up-regulation of vitronectin contributes to the reduction of mesenchymal cells migration. To do this, I propose to explant and culture endocardial cushions on a 3-D collagen matrix spiked with purified vitronectin. If changes in the extracellular matrix composition (specifically an increase in vitronectin) can impede EMT, I would expect less migration of mesenchymal cell from the cushions cultured on vitronectin-spiked matrix, opposed to those cultured on the normal 100% collagen matrix.

Test the CRELD1/VEGF interaction in vivo

This work identified a previously unrecognized interaction between CRELD1 and VEGF in human AVSD cases and in explanted mouse endocardial cushion. Next, I would like to directly test whether a CRELD1 deficiency and VEGF over-expression can interact in vivo. To do this I will cross a well-characterized *Vegf-A* hypermorphic mouse model (*Vegf-A*$^{(+/KI)}$) (Miquerol et al. 2000) with the *Creld1*-knockout mouse (*Creld1*$^{(+/-)}$) model. Past characterizations have found that both of these mouse models when homozygous for their respective knock-in alleles [*Vegf*$^{(kI/kI)}$ or *Creld1*$^{(-/-}$

$^{)}$] die in utero and have endocardial cushions defects. This supports the notion that both CRELD1 and VEGF are necessary for normal valve and septa formation. However, in contrast to the homozygous knock-ins, the heterozygous knock-ins [*Vegf*$^{(kl/+)}$ or *Creld1*$^{(+/-)}$] survive to adulthood and appear normal with no appreciable heart defects. From this we conclude that a single hit, either half the normal CRELD1 levels or twice the normal VEGF expression, is not sufficient to cause AVSD. This is consistent with the incomplete penetrance observed in humans who carry a *CRELD1*-missense mutation or the *VEGF*-634C over-expression polymorphism. Knowing this I now want to test whether a combination of a CRELD1 deficiency with VEGF over-expression could increase the penetrance of AVSD in these mice. To do this I will cross the *Vegf*$^{(+/kl)}$ mouse with the *Creld1*$^{(+/-)}$ mouse and assess the incidence of heart defects in the F1 double heterozygotes [*Vegf*$^{(+/kl)}$*;Creld1*$^{(+/-)}$ mice]. I will only assess the F1 generation to control for any intrastrain variations. If I find any septal defects in the compound heterozygotes it will be a confirmation that VEGF over-expression combined with a CRELD1 deficiency can cause AVSD in vivo.

Translating the CRELD1/VEGF interaction to the bedside

AVSD risk profiles, such as the CRELD1/VEGF risk profile, could be used to identify carrier parents and therefore high risk pregnancies. Such early identification of high risk pregnancies is critical to the successful implementation of any AVSD interventions because of how early the heart develops (14 weeks of gestation).

Interventions could conceivably include modest lifestyle changes, such as avoiding high altitudes and following good nutrition regimens during early pregnancy to mitigate the embryo's exposure to hypoxia or hypoglycemia, respectively. This might reduce any additional VEGF expression caused by hypoxia or hypoglycemia (Figure 36). Furthermore, there are numerous drugs that can attenuate VEGF activity, some of which have already proven beneficial in treating other diseases including age-related macular degeneration (Bopp 2007); and pancreatic and breast tumors (Di Domenico et al. 2011). So it is conceivable that VEGF modulation by pharmaceuticals could prevent heart defects caused by VEGF over-expression. However, considering the ubiquitous nature of VEGF and how sensitive development is to VEGF dosing, the use of drug therapies to prevent AVSD must be approached with extreme caution.

The identification of the CRELD1/VEGF AVSD risk profile is a proof of principle that AVSD-risk genes identified from animal models are relevant to human genetics and that AVSD have risk profiles. This has encouraged our continued search for other AVSD risk factors and risk profiles, which is critical to the completion of our long term goal: To identify correlations between specific AVSD-risk profiles and the long-term prognoses of AVSD patients. We are interested in doing this because the identification of such correlations could help identify high-risk AVSD patients, which could improve both the effectiveness and efficiency of care offered to the ever growing population of adults living with repaired congenital heart defects.

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