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Extracellular Matrix and Heart Development

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Abstract

The extracellular matrix (ECM) of the developing heart contains numerous molecules that together form a dynamic environment that plays an active and crucial role in the regulation of cellular events. ECM molecules found in the heart include hyaluronan, fibronectin, fibrillin, proteoglycans, and collagens. Tight regulation of the spatiotemporal expression, and the proteolytic processing of ECM components by proteases including members of the ADAMTS family, is essential for normal cardiac development. Perturbation of expression of genes involved in matrix composition and remodeling can interfere with a myriad of events involved in the formation of the four-chambered heart and result in prenatal lethality and/or cardiac malformations as seen in humans with congenital heart disease (CHD). In this review we will briefly summarize what is known about the specific importance of some of the components of the ECM in relation to the cardiovascular development.

Introduction

While in the past the extracellular matrix (ECM) was commonly viewed as a rather inert scaffold, merely providing structural support for the cells embedded in its environment, it is now well recognized that the ECM forms in fact a very dynamic and plastic milieu which plays an active and crucial role in the regulation of a wide variety of cellular events. In recent years it has been convincingly demonstrated that the ECM is involved in cell-cell signaling, the regulation of cell proliferation, cell differentiation, and cell migration (Bowers and others, 2010; Daley and others, 2008; Frantz and others, 2010).

The ECM is of particular importance during embryogenesis, as the spatiotemporal regulation of cell migration, reorganization, and differentiation must be tightly orchestrated during the formation of the embryo and its respective complex anatomical structures such as the heart. There is a growing body of evidence that shows that perturbation of expression of ECM components, and/or alterations in the signaling pathways that regulate their production, can lead to cardiac malformations that in many cases resemble those found in patients with congenital heart disease (CHD).

In this review we will describe what is currently known regarding the role of the ECM in heart development, focusing on those molecules implicated in the pathogenesis of heart defects. Because of the wealth of information available, we will in this review focus on the role of the ECM in cardiac development in the mouse. In the first part of the paper we will briefly discuss a few key features of heart formation to set the stage for the discussion on

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how ECM components play a role in the respective steps involved in the morphogenesis of the 4-chambered heart. It is obviously beyond the scope of this review to discuss all aspects of cardiac development in detail. If and where needed we will elaborate on some of these aspects in the text below. In the second part of our review we will discuss a number of ECM molecules that have been demonstrated to play important roles in these developmental events. We acknowledge that this review does not provide a complete comprehensive summary of all the information available on the role of the ECM in heart development. In addition, we realize that some of the data presented in this review is open to alternative interpretations. However, we hope that we will be able to convey the message that the ECM plays a very important and diverse role in the regulation of developmental events in the 4-chambered heart and that perturbation of expression and/or processing can lead to cardiac abnormalities as seen in patients suffering from congenital and acquired heart disease.

Heart Development; a short introduction

The Early Steps

During gastrulation, cardiac precursor cells emerge from the primitive streak and migrate laterally and cranially into the lateral plate mesoderm to form bilateral fields of cells that will eventually form the myocardial and endocardial layers of the primary heart tube. These cells are specified by endodermal cardiac-inducing signals and form the primary or first heart field (PHF or FHF). Anterior migration of these cells, combined with the fusion of the lateral walls of the developing embryo, will bring the two areas of cardiogenic cells together, resulting in the formation of a tubular heart (Abu-Issa and Kirby, 2007; Stainier, 2001; Yutzey and Kirby, 2002). At this point, the wall of the heart tube is composed of two layers of cells. The inner cell layer forms the endocardium which lines the lumen of the heart tube and the outer layer of cells forms the myocardium. The endocardium and myocardium are separated by an ECM-rich, acellular space which is commonly referred to as the cardiac jelly. This cardiac jelly is critically important for the proper formation of the endocardial cushions at the atrioventricular (AV) junction. The cells in the tubular heart at this stage are largely derived from the PHF and will primarily contribute to the developing atria, atrioventricular canal, and the left ventricle (Kelly and others, 2001; Meilhac and others, 2004; Zaffran and others, 2004).

After formation of the tubular heart, the newly formed organ hangs suspended in the future pericardial space, connected to the rest of the embryo by the dorsal mesocardium (Snarr and others, 2008). As the heart tube loops and expands, the dorsal mesocardium disintegrates, with the exception of its caudal-most part at the venous pole of the heart, by a yet unknown mechanism. This persisting dorsal mesocardium plays a crucial role in a number of events, including formation of the atrioventricular mesenchymal complex and the development of the pulmonary veins. (Snarr and others, 2008; Snarr and others, 2007a; Snarr and others, 2007b)

The expansion/elongation of the heart tube at this stage is a result of the addition, at either end of the heart, of a second wave of cardiac progenitor cells derived from the pharyngeal and splanchnic mesoderm. This population of highly proliferative cardiac precursor cells is commonly referred to as the second heart field (SHF) (Dyer and Kirby, 2009). At the arterial pole, these cells will ultimately give rise to the outflow tract (OFT), right ventricle, and the interventricular septum (Verzi and others, 2005). At the venous pole, the SHF adds cells to the developing atria and the atrial septum, but also forms a wedge of mesenchymal cells that protrude into the dorsal aspect of the atrial cavity using the dorsal mesocardium as its portal of entry. This mesenchymal cell mass is known as the dorsal mesenchymal protrusion (DMP) and will contribute to the formation of the AV mesenchymal complex (Snarr and others, 2008; Snarr and others, 2007a; Snarr and others, 2007b).

The Mesenchymal Tissues of the Heart—Extracellular matrix is found in all components of the heart. However, it is particularly abundant in the developing mesenchymal structures which play a crucial role in valvuloseptal morphogenesis. Cardiac mesenchyme derives from a variety of sources (Snarr and others, 2008). As will be demonstrated later in this review, the role of the ECM in the cardiac mesenchyme is of particular importance. Perturbation of the development of these structures often leads to defects in the derived valvuloseptal structures as seen in patients with congenital heart disease. Below, we provide a brief overview of the mesenchymal tissues seen in the developing heart.

The Atrioventricular Cushions: As mentioned earlier, the primary heart tube consists of an endocardial and myocardial layer in between which we find the cardiac jelly (Markwald and others, 1977). Accumulation of ECM in the region of the developing AV junction results in localized “swelling” of the cardiac jelly. This process is the first step in the formation of the AV cushions which will play an important role in AV septation and formation of the AV valves. Surprisingly little is known about the mechanisms that regulate this localized accumulation of ECM. The formation of these, initially acellular, AV cushions is followed by an epithelial-to-mesenchymal transformation (EMT) of the endocardial cells that line the cushions. The endocardially-derived mesenchymal cells (ENDCs) that result from this endocardial EMT populate the AV cushions (Eisenberg and Markwald, 1995; Markwald and others, 1996). Insight into the regulation of endocardial EMT has grown rapidly over the last few decennia. Thus, it is now well-established that EMT is critically dependent on proper expression of members of the family of growth factors, including BMP2 and TGF β 2 (Inai and others, 2008; Mercado-Pimentel and Runyan, 2007; Tian and others). It is also becoming increasingly clear that the ECM plays an important role in this event. In the second part of this review we will briefly address what is known about the involvement of ECM macromolecules in the regulation of EMT.

The Outflow Tract Cushions: The development of the OFT is a relatively late event in cardiac morphogenesis; the cells that form the OFT are largely derived from the SHF (Dyer and Kirby, 2009; Verzi and others, 2005). It is important to realize that the development and growth of the heart at the early stages is one continuous process (Moorman and others, 2007) and that, even though the cells of the SHF can be distinguished from the PHF-derived cells by a different molecular and “transgenic” signature, the SHF cells are gradually added to the growing and remodeling heart and that there are no sharp anatomical/morphological boundaries between tissues derived from the PHF and SHF. As the SHF is added to the growing heart tube, the cardiac jelly at the luminal side of the OFT develops endocardial cushions which are, in many ways, reminiscent to those seen in the AV junction. In this review we will refer to these ECM-rich spaces as the OFT cushions, recognizing that these structures may be called endocardial OFT ridges, conal swellings and/or truncal ridges in other publications. Just like in the AV junction, endocardial EMT is playing a role in the generation of mesenchymal cells that populate the OFT cushions. Around ED 9.5–10, cardiac neural crest derived cells (NCDCs) start to migrate into the distal end of the OFT cushions and form two “prongs” of mesenchyme which eventually will occupy a significant portion of the cushions. The ENDC and NCDC populations do not intermingle and as a result they occupy distinct parts of the developing OFT cushions. This can be nicely demonstrated using cre-recombinase mice that allow the identification of ENDCs (e.g. Tie2-cre) and NCDCs (e.g. Wnt1-cre) in the developing heart (Snarr and others, 2008). In addition, the ECM in which the NCDCs are found and the ECM surrounding the ENDCs are characterized by the expression of different extracellular components.

The Dorsal Mesenchymal Protrusion: The dorsal mesenchymal protrusion (DMP) is a SHF-derived mesenchymal structure which, around ED 9.5–10.5, grows into the unseptated atrial cavity at the venous pole of the heart using the dorsal mesocardium as a portal of entry (Snarr and others, 2007a; Snarr and others, 2007b). It can be distinguished from the endocardially derived AV cushions by the expression of the SHF-specific transcription factor *Isl1* (Snarr and others, 2007a), but it is also identified as a SHF derivative when using the SHF transgenic AHF-mef2C-cre mouse (Verzi and others, 2005) in combination with ROSA26 reporter mice (Goddeeris and others, 2008). The DMP develops in close association with the a mesenchymal cap on the leading edge of the developing primary atrial septum and fuses with the AV cushions to form the AV mesenchymal complex, an essential process in atrioventricular septation (Snarr and others, 2007a; Snarr and others, 2007b). A series of independent publications have demonstrated that perturbation of DMP development is associated with the pathogenesis of atrioventricular septal defects (Goddeeris and others, 2008; Snarr and others, 2007a; Tian and others, 2010; Wirrig and others, 2007).

The Epicardium: Another source of cardiac mesenchyme is the proepicardium which develops around ED9.5 in the mouse and Hamburger/Hamilton (H/H)16 in the chick. (Wessels and Perez-Pomares, 2004). Cells derived from the proepicardium attach to the outer surface of the myocardium and subsequently spread out over the heart to form the epicardial epithelium. Epicardial cells then undergo an epicardial EMT. In this process the Wilms' tumor gene *WT1* plays an important role through direct transcriptional regulation of *Snail* (*Snail1*) and *E-cadherin* (*Cdh1*) which are both mediators of EMT (Martinez-Estrada and others, 2010). Epicardial EMT is also regulated by *TGFβ2*, through the regulation of hyaluronan (Craig and others, 2010a). This epicardial EMT generates a population of epicardially-derived mesenchyme. A subset of the epicardially-derived cells (EPDCs) subsequently migrate into the ventricular myocardial walls where they either remain and reside as interstitial fibroblast, differentiate into coronary smooth muscle cells, or possibly, become coronary endothelium or cardiomyocytes (Cai and others, 2008; Perez-Pomares and others, 2002a; Perez-Pomares and others, 2002b; Zhou and others, 2008). In addition, relatively large numbers of EPDCs are also found within the developing endocardial cushions (Manner, 1999; Perez-Pomares and others, 2002b). The significance of EPDCs for valvuloseptal morphogenesis is illustrated by studies in which the development of the epicardium is perturbed, showing that this results in valvuloseptal defects (Wessels and Perez-Pomares, 2004).

The ECM and Cardiac Morphogenesis—As described above, respective cardiac tissues have different origins, functions, and fate. It is therefore not surprising that we see important spatial and temporal changes in the composition of the ECM in the individual structures of the heart as they develop, remodel, and mature. In the developing cardiac tissues we find representatives of all classes of ECM macromolecules. This includes hyaluronan, proteoglycans, collagens, elastin, fibrillin, tenascin, fibronectins, and laminins. While the specific roles of many of these molecules have not (yet) been fully elucidated, the importance of others has been revealed by genetic linkage to congenital disease in the human and/or by studies in animal models. Below we will, in the context of heart development and the etiology of cardiac malformations, discuss a number of these ECM molecules focusing on the mouse as the animal model of choice. An overview of the information provided is summarized in Table 1. It is important to note that while we can learn a lot from studying how genetic mutations lead to perturbation of heart development, the embryonic lethality often observed in animal models, and studied in developmental series in these models, may never manifest in humans as the severity of these defects may

not be compatible with life and human embryos with the severe abnormalities seen in the animal models would not survive until birth.

I: Hyaluronan, Proteoglycans, and Hyaluron and Proteoglycan Link

Proteins

Hyaluronan

Glycosaminoglycans (GAGs) are polysaccharide chains consisting of repeating amino sugar and uronic acid disaccharide units. There are four classes of GAGs. The most abundant GAG in the developing heart is hyaluronan (HA) which consists of alternating UDP glucuronic acid and UDP-N-acetylglucosamine molecules which alternate to combine in a chain-like fashion (Toole, 2004). The enzymes UDP N-acetylglucosamine-1-phosphate uridylyltransferase and UDP glucose dehydrogenase are necessary for the synthesis of glucuronic acid and N-acetylglucosamine, forming the building blocks of HA (Mengin-Lecreulx and van Heijenoort, 1994; Spicer and McDonald, 1998). Mutation of UDP glucose dehydrogenase in zebrafish results in malformation of the AV cushions, and gene patterning defects in the AV myocardium (Stainier, 2001; Walsh and Stainier, 2001). The other three classes of GAGs are chondroitin sulfate/dermatan sulfate (CS/DS), heparan sulfate/heparin (HS), and keratan sulfate (KS). These GAGs are found in the ECM of the heart linked to proteoglycan core proteins as discussed elsewhere in this review. Unlike the other GAGs, HA is synthesized at the plasma membrane and does not become linked to a core protein. Three different HA synthase enzymes (Has1, Has2, and Has3) are known to be involved in the synthesis of HA (Kramer and others, 2010). HA is part of a pericellular matrix that provides a hydrated environment that facilitates cellular proliferation and motility (Toole, 2001). In the developing heart, HA is found in the cardiac jelly and, as development progresses, in the derived AV and OFT cushion tissues and forming leaflets (Figures 1 and 2). In addition, HA is also present in epicardial mesenchyme and in the interstitial space surrounding the cardiomyocytes of the myocardial structures in the heart (Figure 2).

The importance of HA for proper heart development has been known for several decades. In 1979, Bernanke and Markwald showed that HA treatment of avian AV cushion explants resulted in an increase of mesenchymal cells invading a 3D collagen matrix (Bernanke and Markwald, 1979). Around the same time, Nakamura and Manasek showed the significance of HA in cardiac development by demonstrating that injection of hyaluronidase into the cardiac jelly of embryonic chick hearts caused severe reduction of cushion material and associated cardiac defects (Nakamura and Manasek, 1981). That HA is also important for the development of the mammalian heart was first demonstrated by Baldwin and colleagues who showed that treatment of cultured rat embryos with hyaluronidase, resulted in a loss of cardiac jelly, abnormal formation of the endocardial cushions, thinning of the ventricular myocardial wall, and altered ventricular performance (Baldwin and others, 1994). These results were similar to those found using the hyaluronan-synthase 2 (Has2) knock-out mouse (Camenisch and others, 2002; Camenisch and others, 2000). Has2 mRNA is normally expressed by the myocardial and endocardial cells at ED8, but as the AV junction develops, expression becomes virtually restricted to the endocardium and mesenchyme of the AV canal and OFT endocardial cushions. Has2 knockout mice are unable to produce HA which leads to embryonic lethality at approximately ED10.5. Has2 null embryos are characterized by severe cardiac malformations including thin myocardium and impaired AV cushion development (Camenisch and others, 2000). Using the 3D collagen gel assay, Camenisch and co-workers showed that the AV cushion defects are most likely caused by failure of the cushion endocardium to undergo EMT. They determined that the specific role of HA in this step of cushion development is to activate endocardial ErbB2-ErbB3 receptors and that the

EMT defect can be rescued by either adding HA to the assay or by adding Heregulin (HRG) that activates the ErbB receptors (Camenisch and others, 2002; Schroeder and others, 2003).

There are also indications that HA plays a role in epicardial development. In an immortalized mouse epicardial cell line, stimulation with high molecular weight hyaluronan (HMW-HA) results in binding of MEKK1 to the CD44 receptor and in MEKK1-dependent activation of ERK1/2 and NF κ B signaling pathways. Treatment of epicardial cells with HMW-HA also results in increased invasion in a collagen gel matrix assay, yet whether HA is able to affect epicardial cell migration in vivo has yet to be determined (Craig and others, 2010b).

The recent development of a Has2-flox mouse (Matsumoto and others, 2009), which in combination with tissue-specific cre mice will allow tissue-specific deletion of Has2, will undoubtedly be useful in unraveling in more detail the importance of HA in specific tissues and populations of cells within the developing heart.

Despite the fact that studies in the avian and murine heart clearly show the importance for HA in heart development, there are, to the best of our knowledge, no published studies that show that HA insufficiency is associated with congenital heart disease (CHD) in the human.

Proteoglycans

Proteoglycans are composed of a core protein and associated glycosaminoglycan (GAG) sidechains. The four classes of GAGs are hyaluronan (see above), chondroitin sulfate/dermatan sulfate (CS/DS), heparan sulfate/heparin (HS), and keratin sulfate (KS). During their synthesis, HS, CS/DS, and KS are covalently linked to the core proteins and sulfated before being secreted into the ECM as proteoglycans.

Chondroitin Sulfate Proteoglycans—The chondroitin sulfate proteoglycans versican, aggrecan, and neurocan have each been identified as being expressed in the developing heart. However, only versican deficiency is associated with heart malformations in animal models. While versican is expressed in the avian, murine and human heart, its family members aggrecan and neurocan have only been observed in the avian heart. Interestingly, there are two reported human mutations in chondroitin sulfate proteoglycan processing enzymes that result in cardiovascular defects. Human patients carrying mutations in CHST3 (the gene encoding for the chondroitin 6-O-sulfotransferase-1 (C6ST-1)) suffer from ventricular septal defects while patients carrying a mutation in CHST14 (carbohydrate (N-acetyl)galactosamine 4-O) sulfotransferase 14) have atrial septal defects (Dundar and others, 2009; Unger and others, 2010).

Versican: Versican is a chondroitin sulfate proteoglycan. Its core protein, a product of the CSPG2 gene, has four splice variants. The longest (full length) form of versican is named V0. It contains a G1 domain, which can bind HA and cartilage link protein (Crtl1), two GAG domains (GAG-alpha and GAG-beta) where the GAG chains attach, and a G3 domain, which is able to interact with other extracellular matrix proteins such as fibulin and ECM cell surface receptors such as integrins (Kern and others, 2007; Kern and others, 2006). The V1 splice variant of versican contains the G1, G3, and GAG-beta domain, the V2 isoform contains the G1, G3, and GAG-alpha, while the V3 isoform only contains the G1 and G3 domains (Kern and others, 2007; Kern and others, 2006; Sheng and others, 2005). The relative amounts of the respective splice variants can be analysed by reverse transcriptase-polymerase chain reaction (Kern and others, 2006) while, using antibodies that specifically recognize the GAG-alpha and GAG-beta domains, the spatial distributions of the versican variants can be determined by immunohistochemistry (Kern and others, 2006; Wirrig and others, 2007). Following these approaches, it has been established that the respective

versican variants are expressed in very specific spatiotemporal patterns during heart development. The V0 and V1 isoforms are typically found in the cardiac jelly, in the AV and OFT cushions (Figure 1), in the developing leaflets of the AV (Figure 2) and semilunar valves, and OFT and in the mesenchymal cap of the primary atrial septum (Kern and others, 2007; Kern and others, 2006; Wirrig and others, 2007), whereas the V2 isoform is found in the myocardium and cardiac neural crest. As the AV valves mature, the expression of versican slowly decreases. It is interesting to note that this process occurs first in the leaflets of the mitral valve and only later in the leaflets of the developing tricuspid valve.

Versican can be cleaved by matrix metalloproteinases (MMPs) and members of the ADAMTS (“a disintegrin and metalloproteinase with thrombospondin motifs”) family (reviewed in (Kern and others, 2006). Cleavage by ADAMTS family takes place in the GAG-beta domain and the neopeptide sequence DPEAAE resulting from this process can be detected by specific antibodies that recognize the site of cleavage. Using these V0/V1-Neo antibodies in the developing mouse embryo, it has been determined that the versican cleavage products are primarily present in the densely packed mesenchyme adjacent to the endocardium of the AV cushions while intact V0 and/or V1 versican isoforms are predominantly observed in the “core” of the AV cushions where the EMT derived mesenchyme is loosely packed (Kern and others, 2006). The cleavage of versican is thought to play a significant role in maturation of the AV cushions and remodeling of the outflow tract (Kern and others, 2007; Kern and others, 2006). Specifically it has been suggested that this process facilitates the dense packing of the mesenchymal cells in the elongating valves (Kern and others, 2006). Furthermore, it has been hypothesized that in the outflow tract, cleavage of versican may promote the disaggregation of cardiomyocytes and a thinning of the outflow tract myocardium, thus promoting remodeling of the outflow tract (Barbosky and others, 2006; Kern and others, 2007; Sheng and others, 2005).

Additional insight into the role of versican in heart development comes from a number of studies using a versican mutant mouse (the hdf mutant) in which a lacZ transgene is inserted into the intronic sequences between exon 7 and exon 8 of the CSPG2 gene (Yamamura and others, 1997). Heterozygous hdf mice (hdf/+) are viable, although some display isolated ventricular septal defects (Wirrig and others, 2007). Embryos homozygous for the hdf insertional mutation (hdf/hdf) do not express versican and die between ED10.5 and 11.0 (Yamamura and others, 1997). Embryos isolated right before they die, are characterized by severe cardiac defects including an underdeveloped right ventricle and outflow tract, absent AV and OFT cushions, thinning of the ventricular myocardium, virtual absence of trabeculations, and a flattening of the endocardium against the myocardium (Mjaatvedt and others, 1998; Yamamura and others, 1997). It is interesting to note that these defects are very similar to those seen in mice that do not express HA, a binding partner of versican (Camenisch and others, 2000).

Aggrecan: Aggrecan is a chondroitin sulfate proteoglycan which, across species, is predominantly expressed in cartilaginous structures. Similar to versican, the aggrecan core protein is comprised of three globular domains, G1, G2, and G3 and two GAG attachment domains. Like the other chondroitin sulfate proteoglycan family members versican, neurocan, and brevican, aggrecan can interact with cartilage link protein (Crtl1) and HA to form large aggregates that contribute to the hydration of the extracellular matrix.

In the chick heart, aggrecan is expressed in the epicardium and the mesenchyme of the AV and OFT cushions (Zanin and others, 1999). As the AV cushions mature, aggrecan expression becomes restricted to the spongiosa and fibrosa layers (Hinton and others, 2006). Nanomelic chickens carry an autosomal recessive aggrecan gene mutation and display

chondroplasia and are characterized by a parrot-like beak but cardiac abnormalities have not been documented (Wong and others, 1992).

While aggrecan is strongly expressed in the murine cartilage, it does, in contrast to what was reported in the avian heart, not appear to be expressed in the murine heart. Heterozygous aggrecan-deficient mice (cartilage matrix deficiency, *cmd*) are viable, but develop spinal abnormalities in adulthood (Watanabe and others, 1998). Homozygous cartilage matrix deficiency (*cmd/cmd*) mice die perinatally from respiratory failure and are characterized by dwarfism, short snout, and a cleft-palate. Heart defects have not been reported in the *cmd* mouse which is, given the expression pattern of aggrecan in the murine embryo, not surprising.

Heparin Sulfate Proteoglycans—Heparan sulfate proteoglycans (HSPGs) are found at the cell surface and in the ECM of most tissues. They play a crucial role in development and homeostasis, and are implicated in disease processes. Via their heparan sulfate chains they are able to bind to multiple growth factors and cytokines that have been demonstrated to be important for cardiac development, including VEGF, BMPs, FGFs, and TGFβs.

Perlecan: Perlecan is expressed in pericellular matrices and basement membranes during development (Handler and others, 1997) and is known to interact with other ECM proteins and ECM cell surface receptors, such as integrins and laminins, as well as with important signaling ligands such as fibroblast growth factor 2 (FGF2) and neuregulins (Handler and others, 1997; Li and Loeb, 2001). There are 16 different isoforms of laminin glycoproteins, which are derived from multiple forms of α , β , and γ chains, the three primary components of laminins (reviewed in Kruegel and Miosge, 2010). Laminins self assemble into ECM networks and form the major framework for basement membrane assembly (reviewed in Kruegel and Miosge, 2010). Laminins interact with perlecan via binding to the heparan sulfate chains (Battaglia and others, 1992; Sasaki and others, 1998).

Expression of perlecan is found in the cardiac jelly surrounding the developing trabeculae, in a diffuse staining pattern throughout the pericellular matrix of the ventricular myocardial cells, and, albeit at lower levels, in the AV cushions (Costell and others, 2002; Handler and others, 1997; Stankunas and others, 2008). Immunohistochemical staining for perlecan in the OFT indicates that the highest level of expression is in the distal part of the cushions, with lower levels of expression found in the proximal cushions (Costell and others, 2002). Perlecan is also expressed in the smooth muscle walls of the developing aorta and pulmonary artery (Handler and others, 1997).

Perlecan mutant mice show a spectrum of cardiac malformations, including defects in rotation of the outflow tract and abnormal development of the ventricular myocardial wall. Intriguingly, there are two developmental windows in which prenatal lethality is observed (Costell and others, 2002). Specimens with more severe phenotypes die between E10.5 and E12.0, while those with lesser malformations die perinatally. In the more severely affected specimens, hemorrhaging of blood into the pericardial cavity is observed, a defect caused by fenestrations in a thin and discontinuous ventricular myocardial wall, (Costell and others, 2002; Sasse and others, 2008). This hemorrhaging is associated with a virtual absence of the ECM basement membranes that typically surrounds the developing cardiomyocytes (Sasse and others, 2008). In perlecan mutants with a less severe phenotype, the primary defect is malrotation of the outflow tract. This appears to be caused by an overabundance of highly disorganized cardiac neural crest derived cells (CNDCs) that reach the proximal OFT cushions leading to abnormal development of these cushions and malrotation of the OFT (Costell and others, 2002). This remodeling defect results in complete transposition of the aorta and pulmonary artery, a situation in which the aorta connects to the right ventricle and

the pulmonary trunk arises from the left ventricle (Costell and others, 2002). Thus, perlecan is important in the formation of the cardiomyocyte basement membrane, in maintaining the integrity of the ventricular wall, and in supporting the organized migration of cardiac neural crest into the OFT cushions (Costell and others, 2002; Sasse and others, 2008).

Agrin: Agrin is an ECM protein localized at the neuromuscular junction where it signals through MuSK, a muscle-specific receptor tyrosine kinase, to mediate the motor neuron-induced accumulation of acetylcholine receptors in the postsynaptic muscle fiber membrane (Ngo and others, 2007). Recent studies have shown that in the fetal and adult murine heart, agrin is expressed in the myocardium (Hilgenberg and others, 2009), where, by interacting with the $\alpha 3$ subunit of the Na, K-ATPase it modulates the basal frequency of myocyte contraction (Hilgenberg and others, 2009). Myocytes isolated from agrin mutant mice show a significantly higher spontaneous contraction frequency, a phenotype that is also achieved by treating wildtype cardiomyocytes with an agrin antagonist (Hilgenberg and others, 2009). The role of agrin in cardiac development has not been studied yet.

Glypicans: Of the 6 members in the Glypican family (Gpc1-6), Gpc3 is the most thoroughly studied. Gpc3 is a membrane-bound HSPG expressed during vertebrate development. In early studies of avian glypican it was demonstrated that it is expressed in the endocardial cushions at Hamburger/Hamilton stages 20–25 (Niu and others, 1998). In cultured rat neonatal cardiomyocytes, Gpc3 was reported to be expressed at the lateral regions of myocyte plasma membrane that contact the basement membrane, but also at sites of myocyte adhesion junctions on the cell surface (Asundi and others, 1997). In humans, mutations in the *GPC3* gene, localized at Xq26, are associated with Simpson–Golabi–Behmel syndrome (SGBS), a rare disorder associated with predisposition to certain childhood cancers, both pre- and postnatal overgrowth, and multiple congenital abnormalities including congenital heart disease, rhythm defects and cardiomyopathy (DeBaun and others, 2001; Lin and others, 1999; Neri and others, 1998). Gpc3 null mice have a high incidence of heart defects (69%) including ventricular septal defect (VSD), coronary artery fistula, double outlet right ventricle (DORV), atrioventricular septal defect (AVSD), and patent ductus arteriosus (PDA). Gpc3 deficient mice display a delayed development of the coronary vascular plexus and an increased number of coronary arteries relative to the number of veins (Ng and others, 2009).

Syndecans: Syndecans are type 1 transmembrane heparan sulfate proteoglycans (HSPGs) that play important roles during development and in events like wound healing and tumour progression. They are involved in controlling cell proliferation, differentiation, adhesion and migration. In mammals, four syndecan members can be distinguished (Syndecan1–4). The heparin sulfate (HS) chain on the extracellular domain of syndecan can interact with numerous ligands found in the ECM, including glycoproteins, collagens, and growth factors. Proteolytic cleavage of the extracellular domain, including the HS chain, is known as shedding (Manon-Jensen and others, 2010). Enzymes that participate in this process are called sheddases and include members of the matrix metalloproteinases family (MMPs) and members of the ADAMTS family (disintegrin and metalloproteinase with thrombospondin motifs). Shedding serves two functions; on one hand, it may downregulate signal transduction, on the other, it converts the extracellular receptor domain into a soluble effector or antagonist. Studies on the developing rat heart (Asundi and others, 1997) show that high levels of syndecan 3, moderate levels of syndecan 2, and low levels of syndecan 1 are found in the adult heart. Furthermore, it was demonstrated using primary cultures of cardiomyocytes and non-cardiomyocytes, that syndecan 3 is not expressed by the myocardium but instead by the cardiac fibroblasts of the postnatal heart.

Hyaluronan and Proteoglycan Link Proteins

Hyaluronan and Proteoglycan Link Proteins (Haplns) form a family of ECM proteins consisting of four members; Hapln1 (better known as Cartilage link protein 1/Crtl1), Hapln2 (or Brain link protein 1), Hapln3 (or Lp3/Osteoblast-Habp), and Hapln4 (or Brain link protein 2) (Spicer and others, 2003). Each Hapln isoform contains two proteoglycan tandem repeats that are conserved among other hyaluronan (HA) binding proteins such as chondroitin sulfate proteoglycans (CSPGs). Interestingly, in the vertebrate genome every single HAPLN gene is found to be located immediately adjacent to one of the four CSPG genes, resulting in the following HAPLN/CSPG pairings: HAPLN 1/CSP G2(versican), HAPLN2/BCAN(brevican), HAPLN3/CSPG1(aggrecan), and HAPLN4/CSPG3(neurocan).

Of the four Hapln isoforms, only Crtl1 (Hapln1) and Hapln3 have been reported to be expressed in the developing heart (Lincoln and others, 2007; Ogawa and others, 2004; Wirrig and others, 2007). Moreover, only Crtl1 has been demonstrated to play an essential role in cardiac development (Wirrig and others, 2007). Crtl1 is an ECM protein that functions to stabilize the interaction between hyaluronan and proteoglycans such as aggrecan, versican, and neurocan (Binette and others, 1994; Matsumoto and others, 2003; Rauch and others, 2004; Shi and others, 2004). While Crtl1 is best known for its role in cartilage formation, where it stabilizes aggregates of aggrecan and hyaluronan to give cartilage its tensile strength and elasticity (Hardingham, 1979), it also mediates and stabilizes the binding between versican and HA (Matsumoto and others, 2003; Shi and others, 2004).

In the developing heart, Crtl1 expression is first seen in the mesenchyme of the AV and OFT cushions and in the endocardial lining of the developing heart between ED9.5 and ED10.5 where it is co-expressed with its putative cardiac binding partners hyaluronan and versican (Wirrig and others, 2007). By ED13, the expression of Crtl1 becomes largely restricted to the endocardially-derived mesenchymal tissues of the AV complex (Figure 1), the endocardially-derived cells in the distal-most part of the OFT cushions, and to the endocardially-derived cells in the most proximal part of the OFT cushions. At late fetal stages, Crtl1 expression is virtually restricted to the leaflets of tricuspid valve (Figure 2). The leaflets of the mitral valve show, however, very little expression and virtually no expression is seen in any other part of the heart (Wirrig and others, 2007).

The importance of Crtl1 in development was initially demonstrated in publications focusing on the role of Crtl1 in cartilage and bone formation. In these papers it was demonstrated that homozygous Crtl1 knock-out animals are characterized by dwarfism and craniofacial abnormalities. Reportedly, most homozygotes that survive the pre-natal period die shortly after birth (Czipri and others, 2003; Watanabe and Yamada, 1999).

Analysis of the cardiac phenotype of Crtl1-deficient mouse embryos revealed a spectrum of cardiac malformations, including atrial and atrioventricular septal defects due to hypoplasia of the mesenchymal-derived structures. Myocardial defects, including thinning of the ventricular walls and septum and muscular ventricular septal defects, were also observed (Wirrig and others, 2007).

While the abnormalities observed in the endocardially-derived cushion tissues are probably resulting from perturbation of one of the processes involved in cushion formation (e.g. EMT, migration, proliferation, and differentiation), the myocardial proliferation-defects found in the ventricular myocardial walls of the Crtl1 knockout mouse are probably associated with the absence of expression of Crtl1 in the endocardium lining of the developing chamber myocardium at early stages of development. This suggests that Crtl1, in

all likelihood through interactions with its putative cardiac binding partners HA and versican, is involved in the regulation of myocardial proliferation.

II: Collagens

In vertebrates, there are at least 28 different types of collagen (Gordon and Hahn, 2010) and in the developing heart several of them are expressed. Collagen types I, III, V, and VI are found in the ventricular myocardium, the developing AV valves and the chordae tendineae. In contrast, collagen II, IV, XI, and XIII appear to be essentially restricted to the developing valve structures and the tendonous apparatus (Favor and others, 2007; Hinton and others, 2006; Lincoln and others, 2004; Lincoln and others, 2006; Liu and others, 1997; Metsaranta and others, 1992; Poschl and others, 2004). Collagen is primarily produced by the developing mesenchyme and fibroblasts. Within the AV valves, collagens form a dense, ordered fibrous network on the ventricularis side of the leaflet, while on the atrialis side of the leaflet, collagens are more loosely packed, creating a spongy ECM (Icardo and Colvee, 1995). In addition, two non-fibrillar collagens, types XV and XVIII, are expressed in the developing and adult heart (Halfter and others, 1998; Saarela and others, 1998). Below we have summarized some of the heart development-related data published on collagen as relate to cardiac development. It is noteworthy that relatively little work has been done to elucidate the roles of collagens in early stages of development.

Collagen type I, III, V (fibrillar collagens)

In the mouse, collagen type I is initially expressed throughout the developing leaflets of the AV valves, gradually becoming restricted to the ventricular side of the leaflets after birth (Kruithof and others, 2007; Lincoln and others, 2004). Collagen I is also expressed in the aortic wall and important for maintenance of this tissue's elasticity and integrity. Mice that lack a large portion intron 1 of the *Col1a1* gene survive to adulthood but are more susceptible to aortic dissection and rupture. Mice with an insertional mutation in intron 1, *Mov13* mice, are embryonic lethal at E12-14 due to rupturing of developing vessels (Lohler and others, 1984; Rahkonen and others, 2004).

In the chick, collagen type III is co-expressed with aggrecan in the fibrosa layer of the maturing cushion until the juvenile stage (Hinton and others, 2006). Collagen type III is also expressed in the vasculature of mice. The *Col3a1* knockout mouse shows a phenotype similar to that seen in the *Col1a1* Intron 1 deletion mouse, as they die late in adult life from rupturing of blood vessels (Liu and others, 1997).

Collagen type V is seen throughout the mural leaflet at E18.5 but becomes restricted to the ventricular side of the leaflet by 8 weeks of age (Kruithof and others, 2007). At E18.5, Collagen V is one of the first collagens to appear in the annulus fibrosus and it remains expressed within this tissue until adulthood (Kruithof and others, 2007). *Col5a1* knockout mice have significantly decreased collagen fibril formation and are embryonic lethal at ED10 due to cardiovascular insufficiency (Wenstrup and others, 2004).

Collagen type VI (a network-forming collagen)

Collagen type VI expression has been documented in the developing AV cushions and the adult AV valves of a number of species (Bonaldi and others, 1998; Gittenberger-de Groot and others, 2003; Klewer and others, 1998; Kruithof and others, 2007). In addition, collagen type VI is seen in the myocardium and epicardium of the developing heart as well as in the aorta and pulmonary vasculature where its expression is thought to be necessary for providing tensile strength to the surrounding tissue (Klewer and others, 1998). It is noteworthy that the genes for the alpha 1 and alpha 2 chains of collagen type VI are located on chromosome 21 within a region associated with Down Syndrome related CHD. Although

there is increased expression of collagen type VI within the AV valves in the hearts of human fetuses with Down Syndrome, there is no evidence for the involvement of collagen type VI in the pathogenesis of the congenital defects found in Down Syndrome patients (Gittenberger-de Groot and others, 2003).

Collagen types XV and XVIII (MULTIPLEXIN collagens)

Type XV and XVIII collagens are nonfibrillar collagens and belong to the MULTIPLEXIN (“multiple triple-helix domains with interruptions”) subgroup of the collagen family that is unique in its multi-domain structure. Both collagen XV and XVIII have associated GAG chains (which also classifies them as proteoglycans), C-terminal regions that can be cleaved to generate endostatin or restin, and are localized in basement membranes.

Collagen XV is expressed in basement membranes and the capillaries of the myocardium during development and adulthood. Collagen XV deficient mice have defects in vessel architecture and are more susceptible to cardiomyopathy under conditions of increased cardiac load (Eklund and others, 2001; Muona and others, 2002; Rasi and others).

Collagen XVIII can be alternatively spliced to form multiple variants. It has a c-terminal non-collagenous domain that can undergo proteolytic cleavage to produce the biologically active fragment endostatin (Saarela and others, 1998). Collagen XVIII is expressed in the endocardial and myocardial basement membrane during early AV cushion development and later becomes localized to the AV and semilunar valves (Carvalhaes and others, 2006). Coll18a1 knockout mice are characterized by a thickening of the endothelial basement membrane surrounding the AV valves (Utriainen and others, 2004).

Collagen type XI

Collagen XI is thought to be involved in the regulation of collagen fibril initiation and assembly. In the developing heart it is expressed in the developing AV valves as well as in the myocardial component of the trabeculae of the ventricles. It is, however, not found in the compact layer of the ventricular myocardium (Lincoln and others, 2006). Collagen XI deficient mice die at birth, presumably resulting from respiratory failure. Analysis of late fetal collagen XI null specimens reveals an increase in the thickness of the interventricular septum, thickening of the AV valve leaflets, and a pronounced change in the overall shape of the heart. The thickened leaflets show a prominent increase in the expression of collagen I, pointing to a compensatory mechanism in collagen production in the hearts of these animals (Lincoln and others, 2006).

III. Periostin

Periostin is a 90kDa secreted ECM protein belonging to the fascilin gene family (Horiuchi 1999). Periostin can interact with fibronectin, tenascin-C, collagen I, collagen V, and itself (Conway and Molkentin, 2008; Kii and others, 2006; Norris and others, 2007). In the chick, periostin expression is first observed at stage 17 in the endothelial lining of the right ventricle and then extends to the OFT cushions at stage 18. By stage 21, the periostin expression domain includes the endothelium of the trabeculae, the AV and the OFT cushions. As the chick heart matures, periostin expression is observed in the left AV valve, the chordae tendineae, and the semilunar valves but not in the right AV valve (Norris and others, 2004). It is noteworthy to mention that in the chick the right AV valve is predominantly muscular in nature. The function of periostin, a TGFbeta-3 responsive gene, appears to be to mediate the maturation of the AV valve in the chick by promoting mesenchymal-to-fibroblast differentiation while at the same time blocking the differentiation of other cell types, in particular cardiomyocytes (Norris and others, 2009). The expression pattern of periostin in the mouse is reminiscent to that seen in the chick. Initially, periostin is

observed in the AV and OFT cushions, in the endocardium, and in the epicardium. As the heart matures, periostin becomes localized to both the mitral and tricuspid valves, the chordae tendineae, and the semilunar valves (Kruzynska-Frejtag and others, 2001; Norris and others, 2008).

Periostin knockout mice (Kii and others, 2006) and *Peri^{lacZ}*-null mice (Rios and others, 2005) exhibit shortened and thickened mitral and tricuspid leaflets (Norris and others, 2008; Snider and others, 2008). The leaflets show reduced collagen expression (Snider and others, 2008) and ectopically express cardiac muscle markers indicating abnormal muscularization of the valvular tissues (Norris and others, 2008; Snider and others, 2008). The chordae tendineae of periostin null mice are also shorter and thicker than that of wildtype mice and, in addition, some periostin knockout mice exhibit atrial septal defects (Norris and others, 2008).

IV. Proteases

ADAMTS isoforms

The ADAMTS (a disintegrin and metalloprotease with thrombospondin repeats) family of ECM metalloproteases consists of 19 related genes which play important roles in the regulation of many different processes in cells and tissues. Three family members, ADAMTS1, ADAMTS5, and ADAMTS9 have been reported to be expressed in the developing heart, each having a unique expression domain. Degradation of ECM molecules by proteases, including members of the ADAMTS family, has been demonstrated to be necessary for maturation of the AV cushions, trabeculation, and outflow tract remodeling (Kern and others, 2007; Kern and others, 2006; Stankunas and others, 2008).

ADAMTS1—Cleavage of versican by ADAMTS1 has been demonstrated to be necessary for AV cushion, OFT, and trabecular development (Kern and others, 2007; Kern and others, 2006; Stankunas and others, 2008). Cardiac expression of ADAMTS1 is found as early as ED9.5. The expression of ADAMTS1 is initially observed in the AV endocardium and AV cushion mesenchyme where it overlaps with that of its co-factor Fibulin1, its substrate versican V0/V1, and its versican cleavage product (Kern and others, 2006). Additionally, ADAMTS1 is expressed at relatively low levels in the endocardium and myocardium of the ventricles up to ED12.5-14.5, when it becomes robustly expressed in the ventricular endocardium (Stankunas and others, 2008). At later stages, ADAMTS1 is also found in the smooth muscle cells of the tunica media of the dorsal aortae and the pulmonary artery (Thai and Iruela-Arispe, 2002). Based on a series of observations it has been suggested that proteolytic cleavage of versican by ADAMTS1 during AV cushion development may reduce cell-cell adhesion of the AV endocardium and newly formed mesenchyme and promote proliferation of the AV mesenchymal cells (Kern and others, 2006). Proteolytic cleavage of intact versican V1 isoform by ADAMTS1 is also thought to be responsible for the loss of cell-cell association of the outflow tract cardiomyocytes, resulting in a thinning of the OFT myocardium, and allowing for the normal process of OFT remodeling to occur (Kern and others, 2007). In the developing ventricles, ADAMTS1 transcription is believed to be mediated by Brg1, which represses ADAMTS1 expression in the ventricular endocardium until approximately ED12.5-14.5 when repression is lifted (Stankunas and others, 2008). High levels of ADAMTS1 expression in the ventricular endocardium between ED12.5-14.5 are thought to contribute to cessation of trabeculation and compaction of the ventricular myocardium through degradation of versican in the ventricular cardiac jelly (Stankunas and others, 2008). In fact, abnormalities in myocardial trabeculation are observed in ADAMTS1 knockout mice. The ventricular chambers of these mice are characterized by hypertrabeculation. It is suggested that this is the result of persisting expression of versican in the ventricular cardiac jelly surrounding the trabeculae (Stankunas and others, 2008).

ADAMTS5—A detailed analysis of ADAMTS5 expression in the developing heart has to date not been reported. However, published data using an ADAMTS5^{lacZ} mice show that at 16.5ED ADAMTS5 is expressed in the aortic wall of the OFT as well as in the developing semilunar valve leaflets. Furthermore, expression is also seen in the endocardial lining of the cardiac chambers and in scattered cells throughout the myocardium (McCulloch and others, 2009). Despite this cardiac expression profile ADAMTS5 knockout mice are reportedly phenotypically normal (Majumdar and others, 2007; McCulloch and others, 2009).

ADAMTS9—ADAMTS9 is expressed as early as ED9.5 in the developing heart tube where it is expressed in SHF-derived cells of the developing OFT (Kern and others, 2010). At ED11.5 ADAMTS9 is found in the mesenchyme surrounding the dorsal aortae and in the myocardium and right-sided endocardial cushion of the OFT (Jungers and others, 2005; Kern and others). As cardiac development progresses ADAMTS9 continues to be expressed in the aortic wall and is in the leaflets of the aortic valve, aortic valvulosis, interstitial cells of the mitral valve, and the mitral valve hinge region that all develop as a result of OFT remodeling. In these tissues, ADAMTS9 is co-expressed with the cleaved versican isoform recognized by the anti-DPEAAE antibody, indicating that ADAMTS9 is playing an active role in remodeling of these tissues. To study the importance of ADAMTS9 for heart development a mouse model carrying a lacZ insertion in exon 12 was used. Mice homozygous for this null allele, ADAMTS9^{lacZ/lacZ} die by ED7.5. Haploinsufficient animals, however, survive into adulthood but show a spectrum of cardiac anomalies believed to be related to an accumulation of versican within the myocardium and valves. The valvular abnormalities include cartilage-like nodules within the valvular sinus, malformed semi-lunar valves, enlarged aortic leaflets, and myxomatous mitral valves. The myocardium of ADAMTS9^{+/lacZ} mice exhibits defects in compaction. This results in a “spongy” myocardium that features abnormal projections into the ventricular space (Kern and others, 2010). This “spongy myocardium” is phenotypically reminiscent of a condition known in humans as “left ventricular noncompaction LVNC” which is a rare and unclassified cardiomyopathy (Sarma and others, 2010).

V: Fibulins

The fibulin gene family consists of 7 members. Each fibulin molecule has a common structural signature consisting of a series of repeated EGFLike domains followed by a C-terminal element referred to as a ‘fibulin module’ (Argaves and others, 2003). During murine cardiac development In mouse embryos, both bulin-1 and fibulin-2 are expressed at sites of EMT and cell migration.

Fibulin 1

Fibulin-1 can bind a variety of ECM molecules, including aggrecan and versican, which are known targets for cleavage by ECM metalloproteases (Cooley and others, 2008; Kern and others, 2006; Lee and others, 2005). The alleged role for Fibulin 1 is to simultaneously interact with the carboxy-terminal of ADAMTS1 as well as with ADAMTS1 substrates, thereby bringing the molecules closer together and enhancing proteolytic cleavage of the substrate (Cooley and others, 2008; Kern and others, 2006; Lee and others, 2005). At ED9.0-9.5 in the mouse, fibulin 1 is expressed in the dorsal neural tube and associated with the developing cardiac neural crest cells. By ED10.5, fibulin 1 is found in pharyngeal arches 3–4 and the distal OFT cushions (Cooley and others, 2008; Spence and others, 1992). Fibulin 1 is also expressed throughout the extracellular matrix of the AV cushions (ED9.5-ED14.5), eventually becoming restricted to the elongating portions of the AV valves (Kern and others, 2006). Fibulin 1 has been implicated in the regulation of cell migration of the

AV mesenchyme. Addition of fibulin 1 to collagen gels containing fibronectin resulted in a significant reduction in the number of invading mesenchymal cells and the distance over which the mesenchymal cells migrated (Twal and others, 2001). It is believed that this effect of fibulin 1 on the migratory response of AV mesenchymal cells is related to its role, as a co-factor to ADAMTS1, in the cleavage of ECM molecules known to promote migration, such as versican V0/V1 (Kern and others, 2006; Twal and others, 2001). Additional evidence for the role of Fibulin 1 in heart development comes from studies on the Fibulin 1 knockout mouse (Cooley and others, 2008). Fibulin deficient mice die perinatally and are characterized by a number of cardiac malformations; approximately half of fibulin 1 knockout animals present with atrial septal defects and a thinning of the ventricular myocardium. Additionally, there is a high incidence of abnormalities typically associated with defects in cardiac neural crest, such as abnormal remodeling of the aortic arch arteries, double outlet right ventricle, overriding aorta, and ventricular septal defects (Cooley and others, 2008). In most cases, the ventricular septal defects are secondary to malrotation of the outflow tract as both outflow vessels are connected to the right ventricle, thus preventing blood flow from the left ventricle except through an abnormal opening in the interventricular septum. These data demonstrate that fibulin 1 is essential for migration of the cardiac neural crest into the aortic arches and the outflow tract cushions, for proper rotation of the great vessels, and for promoting versican cleavage during AV cushion morphogenesis by acting as a cofactor to ADAMTS1 (Cooley and others, 2008; Kern and others, 2006; Twal and others, 2001).

VI: Fibronectin

Fibronectin is a multi-domain ECM protein that interacts with multiple integrins, heparan sulfate proteoglycans, collagens, and fibrins to mediate cellular behaviors (Pankov and Yamada, 2002). Fibronectin can be alternatively spliced at its EIIIA, EIIIB, and V exons to yield a multitude of splice variants that are differentially expressed in development and adulthood. The EIIIA and EIIIB exons are especially significant in development as they are included in the majority of embryonic fibronectin mRNAs, but have been detected in very few adult tissues (Ffrench-Constant, 1995). Fibronectin is expressed early in embryonic development in the mesoderm and in the space between the embryonic germ layers (Ffrench-Constant and Hynes, 1989; George and others, 1993). As cardiac development progresses, EIIIA- and EIIIB-containing fibronectins are expressed in the dorsal aortae, pharyngeal arch arteries and the endocardium, (Astrof and others, 2007b; Ffrench-Constant and Hynes, 1989). Fibronectin is also expressed in the mesenchyme of the endocardial cushions where it is required for EMT-mediated development (Icardo and others, 1992; Mjaatvedt and others, 1987).

In fibronectin deficient embryos, developmental abnormalities are first seen at ED8.0 with the most severely affected mutants failing to undergo fusion of the PHF into a primitive heart tube. Embryos that do form a heart tube have a thickened myocardium, lack of cardiac jelly, and abnormal endocardium. Fibronectin deficient embryos do not survive beyond ED10 due to cardiovascular and vascular defects (George and others, 1993; Georges-Labouesse and others, 1996). Because of the importance of alternative splicing of fibronectin during development, mice lacking both the fibronectin EIIIA and EIIIB exons were generated. While individually fibronectin EIIIA and fibronectin EIIIB nulls are phenotypically normal, the combined fibronectin EIIIA/EIIIB knockout mouse is embryonic lethal at ED9.5-10.5 (Astrof and others, 2004; Tan and others, 2004). Cardiovascular defects become apparent in fibronectin EIIIA/EIIIB double knockouts where the embryos fail to form normal endocardial cushions, have a thin outflow tract, and are prone to vascular hemorrhage (Astrof and others, 2007a).

VII: Fibrillin

Fibrillin is an elastic-type ECM molecule which forms microfibrils along with other ECM molecules such as elastin (Hurle and others, 1994; Sakai and others, 1986; Visconti and others, 2003). Fibrillin is expressed in the developing heart, associated with the basal surface of the early endocardium of both the AV and OFT cushions. Prior to EMT, fibrillin appears to be concentrated under the endocardial surface and, subsequent to EMT, fine fibrillar tracts can be found associated with the migrating cells (Hurle and others, 1994). Fibrillin expression persists in the AV valve leaflets until late stages of development (Hurle and others, 1994). Following septation of the OFT, intense fibrillin staining is observed in the walls of both the pulmonary artery and the aorta (Hurle and others, 1994; Sakai and others, 1986).

Mutations in fibrillin 1 gene (FBN1) are associated with a systemic disorder of connective tissue known as Marfan's syndrome (Judge and others, 2004). The incidence of Marfan's is about 2–3 per 10000 individuals (Judge and Dietz, 2005). The cardiac abnormality found most frequently in Marfan patients is thickening of the AV valves which is often associated with mitral and/or tricuspid valve prolapse. Aortic aneurysm and dissection is the most serious and life-threatening cardiovascular abnormality in Marfan's syndrome. Mutant mice harboring a missense mutation in the fibrillin 1 gene are characterized by a phenotype that recapitulates the Marfan syndrome (Judge and others, 2004; Ng and others, 2004). Fibrillin 1 knockout mice die shortly after birth due to rupture (dissection) of aortic aneurysms caused by a severe weakening of the aortic wall (Ng and others, 2004). Heterozygote fibrillin 1 mutants have reduced microfibrils in the aortic wall, which leads to the disorganization of the aortic smooth muscle cells and an abnormal deposition of extracellular matrix molecules (Judge and others, 2004). Both Fibrillin 1 knockout and heterozygote specimens are characterized by mitral valve prolapse (Ng and others, 2004). Histologically, the mitral valves are thickened and elongated in comparison to wild type valves (Ng and others, 2004). There is a high degree of homology between fibrillin 1 and latent transforming growth factor β (TGF β) binding proteins (Pereira and others, 1993). Based on this homology and on some of the histological features of the Fibrillin deficient mouse, the hypothesis was developed that dysregulation of TGF β signaling could be involved in the etiology of the abnormalities seen in the knockout mouse and in patients with Marfan's syndrome. Indeed, in a series of studies on the fibrillin 1 knockout mouse it was found that in the abnormally developing lungs and valves of this mouse, TGF β signaling was upregulated and that this dysmorphogenesis could be rescued with TGF β -neutralising antibodies (Neptune and others, 2003; Ng and others, 2004). In addition, it was demonstrated that systematic administration of TGF β neutralizing antibody also prevented the pathological changes in the aortic root leading to aortic dilatation (Habashi and others, 2006). Based on these results, studies were initiated with the angiotensin II type1 receptor blocker, losartan, known to antagonize TGF β signaling. Using a heterozygous fibrillin-1 mutant mouse, *Fbn1*^{C1039G/+}, which develops aortic dilatation reminiscent of those seen in human Marfan patients, it was demonstrated that administration of losartan resulted in normalization of aortic growth in the mutant mouse (Habashi and others, 2006). These and other results have prompted trials to study the effect of losartan in the treatment of patients with Marfan's syndrome (Detaint and others, 2010; Moberg and others, 2011; Radonic and others, 2010).

Conclusion and Perspectives

The extracellular matrix (ECM) is involved in all aspects of heart development and, although not emphasized in this review, plays a critical role in important post-natal remodeling events in health and disease. The mechanisms through which ECM molecules

regulate cardiac morphogenesis are in many cases still poorly understood. Increasing our fundamental knowledge of ECM-controlled steps in early heart development may be very valuable in elucidating pathways and mechanisms involved in the pathology of the hearts of patients suffering from cardiac disease.

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Figure 1. Expression of Crt11, versican, and hyaluronan in the developing atrioventricular cushions of the mouse at ED 13.5

This figure shows transverse serial sections through the AV junction of an ED13.5 wildtype mouse heart. The section in panel A is stained for hematoxylin-eosin. The immunofluorescent stainings in panels B–D demonstrate the overlapping expression of Crt11 (B), versican (C), and hyaluronan (D) in the AV cushions but also the fact that the mesenchyme of the dorsal mesenchymal protrusion (white arrows) is expressing versican and hyaluronan but not Crt11. The black arrows in panels C and D point to the muscular component of the interventricular septum in which expression of versican and hyaluronan is also, albeit it at a low level, expressed. iAVC=inferior AV cushion; sAVC=superior AV cushion; llAVC=left lateral AV cushion; rAVC=right lateral AV cushion; DMP=dorsal mesenchymal protrusion; LA=left atrium; LV=left ventricle; RA=right atrium; RV=right ventricle.

Figure 2. Expression of versican, hyaluronan, and Crtl1 in the developing leaflets of the AV valves at ED 17

This figure shows serial sections through the developing AV valves of a mouse heart at an ED 17 stained for versican (with an antibody recognizing the GAG β epitope), hyaluronan (using an hyaluronan binding protein HABP approach) and Crtl1. The staining procedures used are essentially as described in Wirrig and others, 2007). Note that the staining for versican is more intense in the leaflets of the developing tricuspid valve when compared to the staining in the aortic and mural leaflet of the mitral valve (A). While versican (A,B) and hyaluronan (C) are still expressed at considerable levels in the tricuspid leaflets, the expression of Crtl1 has significantly decreased at this stage.

Table 1
Animal models of extracellular matrix and cardiovascular development

Numerous animal models have been described that have proven useful for the study of the role of extracellular matrix in congenital heart disease.

Gene Affected	Animal Model	Normal Cardiovascular Expression	Lethality	Cardiovascular Phenotype	Reference
I. Hyaluronan, Proteoglycans, and Hyaluronan and Proteoglycan Link Proteins					
Aggrecan	Cartilage Matrix Deficiency (<i>cmd</i>) mouse	None	Perinatal	None reported	Wong and others, 1992
Aggrecan	Nanomelic Chicken	Epicardium, mesenchyme of the OFT and AVC	Neonatal	None reported	Wong and others, 1992; Zannin and others, 1999; Hinton and others, 2006
Cartilage Link Protein (Hapla1)	Crtl1 Knockout	AV and OFT cushions, endocardium	Perinatal	ASD, VSD, AVSD, TMP	Watanabe and Yamada, 1999; Wirrig and others, 2007
Glypican-3	Gpc3 knockout	AV cushions	Perinatal	ASD, VSD, Coronary Artery Fistula, PDA, DORV	Ng and others, 2009
Hyaluronan	Has2 Knockout	Myocardium and endocardium of the heart tube, endocardium and mesenchyme of the AV and OFT cushions, AV valves, CT	ED9.5-10	Defects in vasculogenesis, fail to form AVC	Camenish and others, 2000
Perlecan	Perlecan Knockout	BM, Cardiac Jelly, OFT Cushions, SMCs of Ao and PA	ED10.5-12 or perinatally	TMP, defects in OFT rotation	Costell and others, 2002
Versican	Hdf	Cardiac jelly, AV and OFT cushions, mesenchyme of the PAS	ED10.5	Fail to form RV, OFT, AVC, and TMP; Haploinsufficiency results in VSD	Yamamura and others, 1997; Mjaatvedt and others, 1998; Wirrig and others, 2007
II. Collagens					
Collagen Type I	Mov13 mouse (insertional mutant)	AV valves, Vasculature	ED12-14	Vascular Defects	Lohler and others, 1984; Lincoln and others, 2004; Kruithof and others, 2007
Collagen Type I	Coll1a1 Int1Δ	AV valves, Vasculature	Late Adulthood	Aortic dissection and rupture	Rahkonen and others, 2004; Lincoln and others, 2004

Gene Affected	Animal Model	Normal Cardiovascular Expression	Lethality	Cardiovascular Phenotype	Reference
Collagen Type II	Col2a1 Knockout	CT, AV valves	Perinatal	None reported	Metsaranta and others, 1992; Lincoln and others, 2004
Collagen Type III	Col3a1 Knockout	AV valves, Vasculature	Late Adulthood	Blood Vessel Rupture	Liu and others, 1997; Hinton and others, 2006; Kruithof and others, 2007
Collagen Type IV	Col4a1/2 Knockout	BM, AV valves	ED10.5-11.5	Vascular Defects	Poschl and others, 2004; Kruithof and others, 2007
Collagen Type IV	Col4a1 and Col4a2 missense mutations	BM, AV valves	ED15	Vascular Defects	Favor and others, 2006; Kruithof and others, 2007
Collagen Type V	Col5a1 Knockout	Ao, Annular Fibrosis, Mural Leaflet	ED10	Undescribed cardiovascular defects; Haploinsufficiency results in decreased Ao stiffness	Wenstrup and others, 2004; Roulet and others, 2006; Kruithof and others, 2007
Collagen Type VI	Col6a1 Knockout	AV valves, myocardium, epicardium, Ao, and PA	None	None reported	Bonaldo and others, 1998; Klewer and others, 1998; Kruithof and others, 2007
Collagen Type XV	Coll5a1 Knockout	BM, Myocardial Capillaries	None	Vascular defects, cardiomyopathy	Eklund and others, 2001; Muona and others, 2002
Collagen Type XVIII	Coll18a1 Knockout	BM of myocardium and endocardium, Semilunar Valves	None	Broadened AV Valves	Carvalhaes and others, 2006; Utriainen and others, 2004
III. Periostin					
Periostin	Periostin Knockout and Periostin ^{lacZ}	Endothelium, OFT and AV cushions, Mitral and Tricuspid valves	Some neonatal lethality	Leaflet abnormalities and valve disease	Rios and others, 2005; Kii and others, 2006; Snider et al., 2008; Norris and others, 2008
IV. Proteases					

Gene Affected	Animal Model	Normal Cardiovascular Expression	Lethality	Cardiovascular Phenotype	Reference
ADAMTS1	ADAMTS1 Knockout	Endocardium, myocardium, AV cushion	Embryonic Lethality in 50%	Hypertrabeculation	Thai and Irule-Arispe, 2002; Kern and others, 2006; Stakunas and others, 2008
ADAMTS5	ADAMTS5 Knockout	Endocardium, myocardium, pericardium, Ao, aortic valves	None	Normal	Majumdar and others, 2007; McCulloch and others, 2009
ADAMTS9	ADAMTS9 +/-lacZ	Myocardium, right-sided OFT cushions, SLV, Ao, mitral valve	ED7.5 in Homozygous	Haploinsufficiency results in enlarged SLV, myxomatous mitral valve, "spongy" myocardium, cartilage nodules	Jungers and others, 2005; Kern and others, 2010
V. Fibulins					
Fibulin 1	Fibulin1 Knockout	AV and OFT cushions, CNDCs	Perinatal	ASD, TMP, abnormal AAA, DORV, overriding aorta, VSD, hemorrhage	Kern and others, 2006; Cooley and others, 2008
VI. Fibronectin					
Fibronectin	Fibronectin Knockout	Embryonic Mesoderm, AVC, endocardium, dorsal Ao	ED8.5 – 10.5	Fail to form heart tube in most severely affected, vascular defects	Ffrench-Constant and Hynes, 1989; George and others, 1993; Georges-Labouesse and others, 1996
FibronectinEIIIA andEIIIB	Fibronectin EIIIA/EIIIB Knockout	Embryonic Mesoderm, AVC, endocardium, dorsal Ao, pharyngeal arch arteries	ED9.5 – 10.5	Fail to form AVC, thin OFT, vascular hemorrhage	Tan and others, 2004; Astrof and others, 2007a,b
VII. Fibrillin					
Fibrillin 1	Fibrillin 1 Knockout	Endocardium, AV and OFT cushions, AV valves, PA and Ao	Postnatal	Aortic Aneurism, MVP	Judge and others, 2004; Ng and others, 2004

AAA= Aortic Arch Artery

Ao= Aorta

ASD= Atrial septal defect

AV= atrioventricular

CNDCs= cardiac neural crest derived cells

CT= Chordea Tendineae

DORV= Double Outlet Right Ventricle

MVP= Mitral Valve Prolapse

OFT= Outflow Tract

PDA= Persistent ductus arteriosus

RV= Right Ventricle

SLV= Semi-lunar Valves

TMP= Thin Myocardium Phenotype

VSD= Ventricular septal defect