Part IV

Valve Development and Diseases

Perspective

Roger R. Markwald

Cardiac valve diseases are common clinical problems that affect from 4 to 10 % of the human population, increasing with age. For many valve problems, there are few options other than surgery, adding to the more than 16,000 surgical cases occurring each year. While surgical techniques continue to improve, the number of surgical cases and associated mortality rates still continue to increase. Secondary complications such as arrhythmias, heart failure, aortic dissection, myocardial hypertrophy, and sudden cardiac death exacerbate primary effects upon blood flow and hemodynamics, indicating the importance of identifying remedial etiologies which remain poorly understood.

Discovery of mutations in patients with congenital heart defects or valve diseases which display progressive (age related) forms of matrix degeneration (e.g., a myxomatous or a calcification phenotype) is currently providing a useful approach for uncovering mechanisms that impact heart valve development and provide candidate targets for therapy. One example of this was the discovery in 2007 by Profs. Herve Le Marec and Jean-Jacques Schott (Nantes, France) of a point mutation in patients with a non-syndromic form of mitral valve prolapse (MVP) that is characterized by progressive, degenerative changes in the extracellular matrix. The mutation occurred in the actin-binding region of a multifunctional cytoskeletal protein, filamin A (FLNA). In mice models, loss of cytoskeletal FLNA function revealed a similar phenotype to MVP resulting in hypertrophied valves with reduced mechanical properties. Further studies indicated that

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downstream kinase pathways associated with increased canonical and noncanonical TGFβ signaling were associated with the FLNA mutation. How this information might be used to help patients with valve diseases like MVP is an opportunity for basic scientists and clinicians to come together to achieve remedial therapies. In this instance, the finding of altered TGFβ signaling in MVP patients evokes the question of whether similar pharmacological approaches currently being used to treat Marfan patients who also have elevated TGFβ signaling could be used to treat patients with a myxomatous valve phenotype.

The FLNA gene is normally expressed in the mesenchymal progenitors of valve or ventricular interstitial fibroblasts and is downregulated during neonatal life as shown by Russell Norris and his colleagues (Developmental Dynamics 2010). This has two important implications: (i) the roots of adult valve defects and diseases can extend back into embryonic life and (ii) heart valve development does not end at birth but is completed postnatally in neonatal life. The expression of the FLNA gene only in mesenchyme progenitors of valve or ventricular fibroblasts points to yet another important implication for valve development: lineage.

In addition to endothelial-derived, progenitor cells, extracardiac mesenchymal cells of neural crest origin or epicardial origin migrate into the heart adding to the mesenchymal population of future valve cells in the outflow track and AV inlets, respectively. Why multiple lineages of progenitor cells are needed for valvulogenesis, especially those derived from outside the heart, is an important unresolved question in heart development and a subject touched upon by three papers presented in Part IV. Dr. Andy Wessels and his collaborators indicate in their chapter that epicardial-derived cells specifically migrate into the lateral (future parietal) AV cushions and uniquely contribute to the posterior leaflets of the left and right AV valve. He then discusses the role of Bmp signaling, through the Bmp receptor BmpR1A/Alk3, in regulating the migration of epicardial-derived cells or EPDCs initially as an epithelial sheet under the endocardium of the AV mitral valve and later as free cells within the matrix. In another paper presented in Part IV, Dr. Scott Baldwin utilizes tissue-specific Cre lineage drivers in mice to differentially assess whether Tie1 is expressed in the endocardium vs. the prevalvular mesenchyme of the AV junction or outflow tract. Dr. Baldwin's lineage-tracing studies revealed that a non-cell autonomous form of cross talk occurs between the endocardial epithelium and subjacent valve interstitial cells that affects the secretion of extracellular matrix and the normal formation and the differential remodeling of the valves of the AV junction and outflow tract in late gestation and postnatal life. His work also provides in vivo support for the in vitro observations presented in the chapter by Drs. Kei Inai and Yukiko Sugi and their collaborators that valvular endocardial cells can regulate the migration and differentiation of valve interstitial cells. Similarly, in another chapter in Part IV, Mizuta et al. demonstrate that if Tmem100, a novel, endothelial-specific, *membrane* protein that is a downstream of BMP9/BMP10-ALK1 signaling, is genetically deleted, the resulting Tmem100 null embryos exhibit an atrioventricular defect. The authors suggest that the atrioventricular septal defect is the result of disrupting an active

dialogue or cross talk between the AV endocardial endothelial cells and the subjacent AV prevalvular mesenchyme.

Whether this putative cross talk between endocardium and prevalvular mesenchyme involves the secretion of a paracrine signal into the extracellular matrix, transport of developmental cues within membrane-enclosed exosomes (or adherons) or direct cell contact (e.g., gap junctions) remains another important developmental heart question to be resolved. The answer to this question could provide more candidate targets for developing or engineering remedial therapies, especially if the signal requires extracellular processing or specific receptors and downstream signaling pathways.

Cross talk does not have to be unidirectional from the endocardium to the mesenchyme but may also originate from the mesenchyme and induce changes in the endocardium. One frequently suggested function for the EPDCs is that they somehow signal the termination of the transformation of AV endothelial cells into new valve progenitor mesenchyme. If correct, this could serve as a mechanism for regulating the normal size or shape of lateral AV cushions and their future parietal leaflets. Whether the neural crest plays a similar instructive "cross-talk" role in determining the size, shape, or fate of the semilunar valves derived from the outflow tract cushions also remains to be determined. Understanding how lineage and "cross talk" may intersect to shape and model inlet and outlet valve development is likely to be one of the more exciting and promising future directions for research into the mechanisms of cardiac valvuloseptal morphogenesis. Already, there are emerging clues and new insights that point to primary (non-motile) cilia as specialized structures on the surfaces of embryonic epithelial and/or mesenchymal cells that may potentially act as sites for sending or receiving developmental signals between interactive cells.

Finally, valve development – be it AV or semilunar – is largely a story about the extracellular matrix and the progenitor cells that normally differentiate into valve interstitial fibroblasts. Like all fibroblasts, they can be "friend or foe." During normal development, they secrete a collagenous matrix which they organize and compact into mature cusps and leaflets. In disease states, they may transdifferentiate into myofibroblasts that secrete metalloproteinases and proteoglycans that disrupt the matrix resulting in a myxomatous phenotype in the AV valves, or, in the outflow tract, they may abnormally enter osteogenic lineages resulting in calcified aortic valves. In her chapter, Dr. Katherine Yutzey and her colleagues review positive and negative changes in the extracellular matrix that precede normal or pathological remodeling and dissect some of the candidate transcriptional regulatory mechanisms that regulate lineage progression and organization of valve extracellular matrix.

Atrioventricular Valve Abnormalities: From Molecular Mechanisms Underlying Morphogenesis to Clinical Perspective

17

Kei Inai

Abstract

Malformation of the atrioventricular (AV) cushion is a common congenital heart defect. Ebstein's anomaly, characterized by a heart defect related to the AV cushion, involves not only a valve defect but also a myocardial abnormality such as Uhl's anomaly. The morphogenetic features of the heart in the case of these diseases can be used as a reference for investigating valvuloseptal and myocardial formations in the human heart.

The AV endocardium transforms into the cushion mesenchyme through epithelial–mesenchymal transition (EMT). After the EMT, distal outgrowth and maturation of endocardial cushions are important morphogenetic steps for AV valvuloseptal morphogenesis. While bone morphogenetic protein (BMP)-2 is known to be critical for AV EMT, little is known about the functional relationship between BMP and ECM and their roles in cushion mesenchymal cell (CMC) migration after EMT. In our previous study, we showed that BMP-2 and BMP signaling induced AV CMC migration. We have been exploring the role of BMP-2 in the regulation of valvulogenic extracellular matrix (ECM) components, periostin, versican, and hyaluronic acid (HA), and cell migration during post-EMT AV cushion expansion and maturation.

We further examined whether BMP-2-promoted cell migration is associated with expression of periostin, versican, and HA. BMP-2-promoted cell migration is significantly impaired by treatment with versican siRNA and HA oligomer. We also found that transcription of *Twist and Id1*, implicated in cell migration in embryogenesis and activation of the periostin promoter, was induced by BMP-2 but repressed by noggin in CMC cultures.

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_17

Taken together, we provide evidence that BMP-2 induces expression and deposition of three major ECM proteins, periostin, versican, and HA, and that these ECM components contribute to BMP-2-induced CMC migration during post-EMT AV cushion expansion and maturation.

Based on these findings, we discuss the morphogenetic process of AV valve abnormalities and crosstalk between valve and cardiomyocytes morphogenesis.

Keywords

Bone morphogenetic protein (BMP) • Cardiac cushions • Periostin • Extracellular matrix protein • Valvulogenesis

17.1 Introduction

Our institute houses a huge amount of congenital heart disease specimens, of which approximately 4,000 are referred to as Prof. Ando's collections, and the late Dr. Ando, former professor at our department, wrote 30 books describing the anatomical features of the specimens.

In one of the books, I read the following text at the edge of a page: "I think there is a right ventricle (RV)–tricuspid valve (TV) dysplastic syndrome as a new clinical entity, which comprises three abnormalities, Ebstein's anomaly, Uhl's anomaly, and tricuspid absence." This note can be likened to the one written by Fermat, on the basis of which Fermat's son Clément-Samuel deduced Fermat's last theorem (Fig. 17.1).

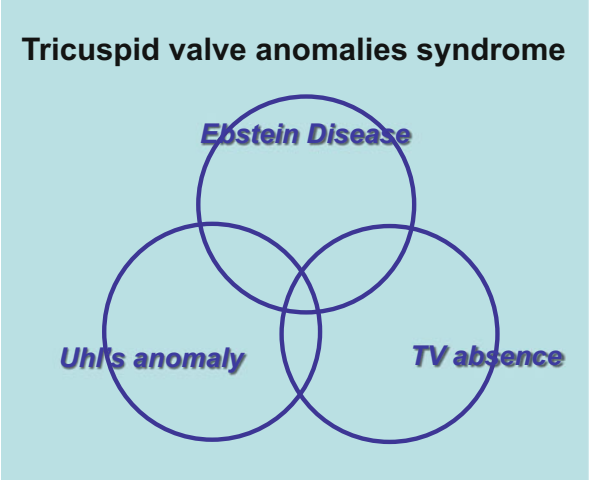

Fig. 17.1 Tricuspid valve anomalies syndrome or RV-TV dysplastic syndrome: Ebstein's anomaly, Uhl's anomaly and tricuspid valve absence sometimes coexist in a heart

17.2 RV–TV Dysplastic Syndrome

17.2.1 Anatomic Features of the Heart in Ebstein's Anomaly Patients

Ebstein's anomaly is a rare congenital cardiac disease in which the TV leaflets are displaced to the right ventricular cavity, resulting in atrialization of the RV [1]. Marked right atrial enlargement caused by severe tricuspid insufficiency, cardiomegaly, and increased right atrial pressure; cyanosis caused by right–left shunt by way of interatrial communication, if present; and right ventricular volume overload are often observed. Figure 17.2 illustrates Ebstein's anomaly with severe TV insufficiency involving two-thirds of the RV.

17.2.2 Morphogenetic Features of the Heart in Patients with Uhl's Anomaly

In the case of patients with Ebstein's anomaly, the RV of the heart often shows partial or total loss of myocardium; in other words, the features of Ebstein's anomaly overlap those of Uhl's anomaly. Uhl's anomaly, a rare congenital heart disease characterized by the absence of apical trabeculations in the right ventricular cavity with a thin hypokinetic ventricular wall, was first described in 1952 [2]. In Fig. 17.3, almost complete absence of the right ventricular myocardium, preserved septum, and left ventricular myocardium is seen. These clinical and anatomic findings strongly indicate that AV valve formation and ventricular myocardium formation may play a role during cardiac morphogenesis.

Fig. 17.2 Ebstein's anomaly with severe TV plastering down into two thirds of right ventricle (RV). The cushion tissue plastered almost throughout RV with a something like plaster and it looks very smooth surface

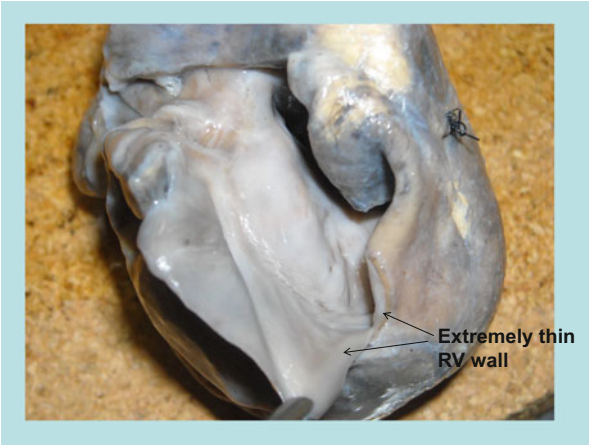

Fig. 17.3 Ebstein's anomaly with Uhl's anomaly. Note the complete absence of the right ventricular myocardium and preserved septal and left ventricular myocardium

17.2.3 Absence of the TV

Unguarded TV orifice is an unusual cardiac malformation that involves partial or total agenesis of the TV tissue (absence of the TV). Kanjuh et al. described the anatomic features of the heart in this disease: the wall of the RV is formed by slightly trabeculated muscle and lined by thickened endocardium [3]; the free wall of the RV is only 2 mm thick, and the ventricular septum is intact. In Fig. 17.4, the specimen shows TV absence and partial loss of the right ventricular myocardium.

TV–RV dysplastic syndrome revealed the important link between TV morphogenesis and right ventricular myocardium formation during the biological process of cardiac morphogenesis. Therefore, it is vital to determine the biological key factors that link the myocardium and cushion tissue.

17.3 Bone Morphogenetic Proteins (BMPs) and Their Important Role in Cushion Formation

17.3.1 Role of BMP2 in Cushion Mesenchymal Cell (CMC) Migration

Anomalies related to valvuloseptal formation are some of the most common congenital heart anomalies. Two segments of the endocardium, the atrioventricular (AV) and outflow tract (OT) endocardium, transform into the cushion mesenchyme—the primordia of the valves and membranous septa—through epithelial–mesenchymal transformation (EMT). Transformed endocardial cells subsequently migrate to the underlying extracellular matrix (ECM), called "cardiac jelly," and remodel the ECM into cardiac cushions [4]. Distal outgrowth and maturation of the cardiac cushion are the initial and critical morphogenetic steps in post-EMT valve formation.

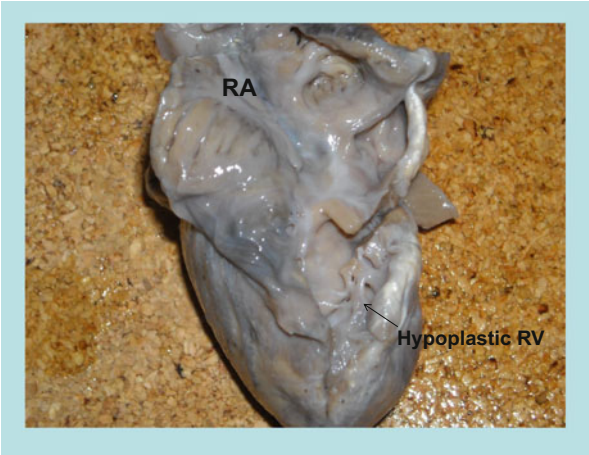

Fig. 17.4 Ebstein's anomaly with tricuspid valve absence and Uhl's anomaly. Unguarded tricuspid valve orifice and partial absence of the right ventricular myocardium concomitant with Ebstein's anomaly. Note the absence of the rudimentum of the tricuspid valve and tension apparatus

BMP is a member of the TGF-ß superfamily proteins, one of many molecules related to AV EMT. BMP signaling was found to be crucial for AV EMT in studies involving explant cultures in mice and chicks and BMP-2 conditional knockout (CKO) experiments in mice [5]. BMP-2 conditional KO at the EMT stage, however, causes subsequent lethality, thereby hindering the examination of the role of BMP-2 in post-EMT valve formation. BMPs bind to the cell surface of BMP receptors (BMPRs)—Type I and Type II receptors. Type II receptors transphosphorylate the glycine–serine-rich domain of Type I receptors and transduce intracellular signals. The Type II BMP receptor, BMPRII, is reported to be expressed ubiquitously throughout the embryonic period [6].

We previously showed the expression patterns of Type I BMPRs by localizing *BMPR-1A* (*ALK3*), *BMPR-1B* (*ALK6*), and *ALK2* in the post-EMT AV cushion mesenchyme in a chick. These findings indicate that BMP signaling regulates the biological processes necessary for distal outgrowth and maturation of post-EMT cushion mesenchyme during early valvulogenesis [4].

17.3.2 BMP2 Induces CMC Migration and Id and Twist Expression

BMP-2 is localized in the AV cushion mesenchyme during post-EMT valve formation, suggesting autocrine signaling by BMP-2 in CMCs during post-EMT valve formation. Therefore, to determine the roles of BMP-2 and BMP signaling in post-EMT AV CMCs, we established a hanging-drop culture system and spatiotemporal viral gene-transfer technique using chick AV CMC cultures both in vitro and

whole embryo cultures in vivo (*ovo*) [5]. Although most BMP receptors have ligand-binding affinity with other TGF-ß superfamily proteins, BMPR-1A and BMPR-1B bind specifically with BMP-2 and BMP-4 (bind with BMP-7 at low affinity) but do not bind with other TGF-ß superfamily proteins. We showed that *dnBMPR-1B* infection as well as treatment with a BMP antagonist, noggin, significantly inhibited endogenous phospho-Smad 1/5/8 expression in AV CMCs, indicating that intracellular BMP signaling in the CMCs is effectively inhibited by treatment with dnBMPR-1B or noggin. On the other hand, BMP-2 or caBMPR-1B-virus treatment induced expression of phospho-Smad 1/5/8, indicating that intracellular BMP signaling was

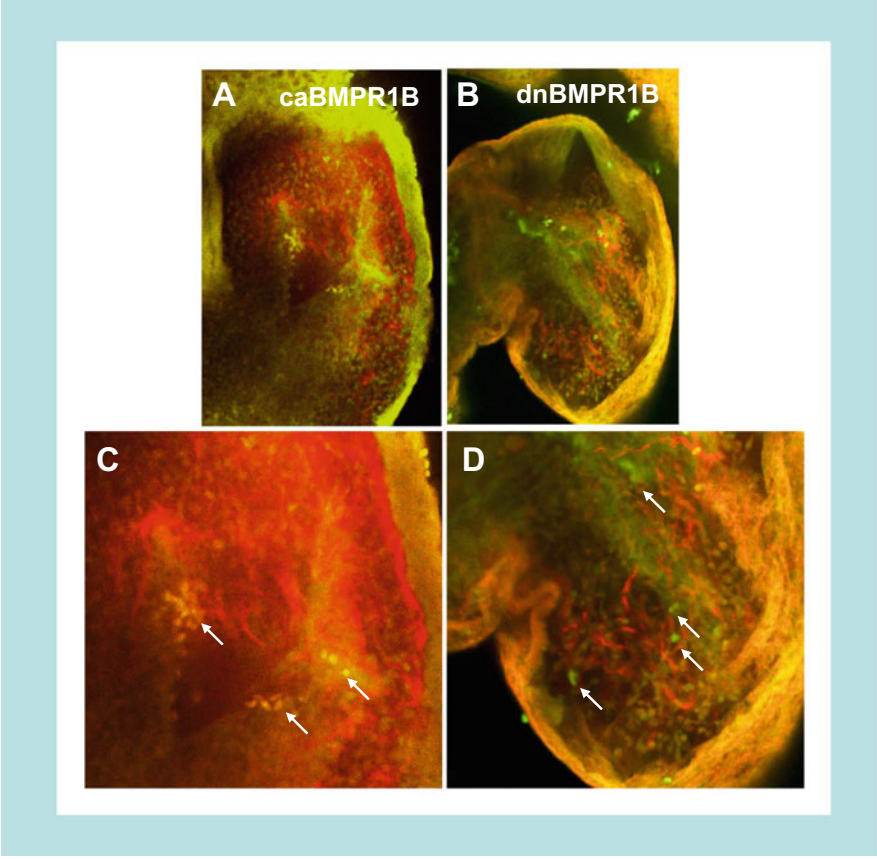

Fig. 17.5 AV cushions were microinjected with caBMPR-1B virus (**a**) or dnBMPR-1B virus. Cushion mesenchyme microinjected with dnBMPR-1B virus showed a significant decrease in periostin immunostaining (*red colour* in **b**). Conversely, infection with caBMPR-1B virus showed significant increase periostin expression in cushion mesencyme (*red colour* in **a**). High magnification view of virus-infected cushion mesenchyme (**c** and **d**). In the caBMPR-1B infected cushion, there was extensive overlapping of viral marker expression (*green*) and periostin expression (*red*) (*yellow arrows* in **c**). Although periostin staining (*red*) was detected in the dnBMPR-1B infected cushion, there was little overlapping of periostin expression (*red*) and viral marker expression (*green arrows* in **d**)

effectively intensified by BMP-2 or caBMPR-1B. Based on these findings, it can be said that the effects of dnBMPR-1B treatment on CMC migration and phosphor-Smad1/5/8 expression are as profound as the effects of noggin treatment. Moreover, caBMPR-1B treatment enhances CMC migration as much as BMP2 treatment does (Fig. 17.5). These facts strongly suggest that BMP signaling plays an important role in mesenchymal cell migration after EMT. We also showed that expressions of Id and Twist-1, which are important transcriptional factors involved in cell migration, are enhanced by BMP signaling [4].

17.3.3 BMP2 Induces Expression of ECM Proteins in the Post-EMT Cushion

We showed the expression patterns of three major ECM components, periostin, versican, and hyaluronic acid (HA), during AV cushion expansion and maturation. We also elucidated the role of BMP2 in the production of periostin, versican, and HA. Using a well-defined 3D CMC aggregate by hanging-drop culture on hydrated collagen gels, we found that BMP2 induces production of periostin, versican, and HA and that these ECM components contribute to BMP2-supported CMC migration during post-EMT cushion expansion and maturation [7].

Taken together, BMP signaling plays a critical role for AV cushion formation and AV valve maturation.

17.4 The Role of BMP2 for Cardiomyocytes Formation

It is well known that the BMP signaling pathway plays a central role in cardiomyogenesis [8–10]. In the past, it was found that administration of BMP-2 counteracts apoptosis of neonatal cardiomyocytes in culture, induced by serum starvation [9]. Moreover, BMP10 expression is restricted to cardiomyocytes in the developing and postnatal heart. Ligands of BMP10 have specificity for ALK1, ALK6, and BMPR2 receptors. Global disruption of BMP10 is embryonic lethal with severely impaired cardiomyocyte proliferative capacity [11]. BMP2 and BMP10 appear to control cardiomyocyte differentiation through activation of transcription factors NKX2.5, MEF2C, and TBX20 [12].

Chakraborty et al. reported that cardiomyocyte-specific Tbx20 overexpression beginning in the fetal period is sufficient for promoting cardiomyocyte proliferation [12].

Therefore, BMP signaling plays special role in linking AV cushion and cardiomyocyte formation.

17.5 Future Direction

At present, many aspects of the molecular mechanisms underlying RV–TV dysplastic syndrome remain to be elucidated. To fully describe the morphogenetic mechanisms underlying TV–RV dysplastic syndrome, we need to determine the molecular mechanisms underlying the interaction between TV and RV myocardium through BMP signaling. Moreover, we need to outline the steps involved in cardiac valve and myocardium morphogenesis.

What is the next target molecule? Here, I would like to discuss about the potential of the "Notch" signaling pathway. The Notch signaling pathway plays multiple roles in cardiac morphogenesis, including regulation of valve formation, outflow tract development, and cardiac chamber maturation [13]. Notch activation is upstream of ephrin- and neuregulin-based modulation of trabeculation and BMP-2 and BMP-10 modulation of cardiomyocyte proliferation. Consistent with the involvement of Notch signaling in multiple aspects of cardiac development, components of the Notch pathway show dynamic spatial and temporal expression patterns in the developing vertebrate heart, and both endocardial and myocardial expression have been observed. Furthermore, the cross reaction between NOTCH and BMP signaling is a well-known fact [14]. Therefore, understanding the complicity of these signaling pathways is imperative for elucidating the relationship between cardiac valve and myocardium formation.

Acknowledgments Special thanks to Dr. Yukiko Sugi and Prof. Roger R. Markwald for their generous support in the experiments and excellent mentorship.

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Molecular Mechanisms of Heart Valve Development and Disease

18

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Abstract

The mature heart valves consist of stratified extracellular matrix (ECM) layers, and heart valve disease is characterized by ECM dysregulation and mineralization. There is increasing evidence that regulatory pathways that control heart valve development also are active in disease. In human diseased valves and mouse models, the expression of valve progenitor markers, including Twist1, Msx1/2 and Snail1/2, is induced. Additional markers of osteogenesis, including Runx2, osteocalcin and bone sialoprotein, also are expressed in calcific aortic valve disease (CAVD) in humans and mice. New mouse models have been developed for studies of valve disease mechanisms. Klotho-null mice are a model for premature aging and exhibit calcified nodules in aortic valves with osteogenic gene induction. Osteogenesis Imperfecta mice, bearing a collagen1a2 mutation, develop features of myxomatous valve disease, including thickening, increased proteoglycan deposition and chondrogenic gene induction. Together, these findings demonstrate specific molecular indicators of valve disease progression, including the identification of early disease markers, which represent potential targets for therapeutic intervention.

Keywords

Heart valve • Embryo • Mouse model • Aortic valve disease

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_18

18.1 Introduction

The semilunar and atrioventricular (AV) valves of the heart are made up of highly organized extracellular matrix (ECM) layers populated by quiescent valve interstitial cells (VICs) [1]. In healthy valves, the ECM is compartmentalized into layers composed of collagens, proteoglycans, and elastin, which are maintained by the VICs for proper valve function throughout life (Reviewed in [2]). In diseased valves, the leaflets are thickened as a result of ECM dysregulation and VIC activation. Calcific aortic valve disease (CAVD) includes calcification of the cusps [3], whereas mitral valve prolapse (MVP) is accompanied by increased proteoglycans and myxomatous changes in the leaflets [4]. Currently, the standard treatment for severe heart valve disease is surgical replacement, and new therapies based on molecular mechanisms are needed.

Molecular mechanisms associated with heart valve disease include activation of signaling pathways involved in progenitor specification, cell proliferation, and differentiation of heart valve and bone precursors [5, 6]. We have reported that pediatric and adult diseased valves are characterized by expression of markers of valve mesenchymal and chondrogenic progenitor cells, while adult diseased aortic valves express markers of osteogenic calcification [7]. We also have identified novel mouse models of calcific and myxomatous valve disease [8] that will be useful in determination of the underlying mechanisms driving disease and in development of pharmacologic-based therapies.

18.2 Heart Valve Development

Heart valve development in vertebrate embryos begins with the formation of endocardial cushions in the AV canal and outflow tract of the primitive heart tube [5]. The mesenchymal cells of the endocardial cushions originate from the endocardium after an endothelial-to-mesenchymal transition (EMT). Valve progenitors are highly proliferative and migratory and express transcription factors Twist1, Tbx20, Sox9, Msx1/2, and Snai1. The endocardial cushion cells diversify into lineages that express distinct ECM profiles regulated by BMP and FGF signaling [9]. Wnt/β-catenin signaling also is active during endocardial cushion maturation, but the specific role for this pathway in valve lineage differentiation is yet to be determined [10]. Valve development continues with the remodeling of the endocardial cushions into thin elongated leaflets composed of stratified ECM, which occurs soon after birth in mice and humans [2]. The ECM layers consist of the collagen-rich fibrosa, proteoglycan-rich spongiosa, and elastin-rich atrialis/ventricularis [2]. These layers are oriented in the semilunar and AV valves with the elastin layer adjacent to blood flow. While it is likely that hemodynamics has a role in leaflet stratification, the regulatory pathways that control ECM organization and compartmentalization during valve maturation are largely unknown.

18.3 Heart Valve Disease

Heart valve disease can result from congenital malformation or gene mutations, or it may be acquired later in life [1]. The prevalence of heart valve disease increases significantly with age, such that ~10 % of people >75 years old have moderate aortic or mitral valve disease [11]. However, the pathogenic mechanisms that drive the development of heart valve disease and that could serve as potential therapeutic targets are not well understood. There is increasing evidence that regulatory pathways that control heart valve and bone development also are active in disease. However, the roles of these pathways in valve pathogenesis and/or repair are not well defined.

18.3.1 Calcific Aortic Valve Disease (CAVD)

CAVD is a progressive disease, initially presenting with aortic valve (AoV) thickening (sclerosis) and resulting in valve stenosis and insufficiency later in life [12]. End-stage disease is characterized by the presence of calcific nodules at the hinge region of the AoV, underlying the pathology of CAVD [3]. In an effort to draw parallels between the progression of disease and the underlying molecular mechanisms, pediatric and adult diseased AoV were analyzed for markers of valve development and endochondral bone formation [7]. Activated VICs in both pediatric and adult valves have increased expression of valvulogenic markers Twist1, Msx2, and Sox9. Strikingly, the formation of calcific nodules was found to be an exclusive feature of adult calcified AoV. Furthermore, phosphorylation of Smads1/5/8, indicative of active BMP signaling, in addition to expression of osteogenic genes, such as Runx2, was observed only in adult calcified AoV. These findings demonstrate that both pediatric and adult diseased AoV express valvulogenic markers, while adult calcified AoV also express markers of osteogenic calcification. Differential expression of these markers suggests that an osteogenic regulatory mechanism contributes specifically to CAVD.

The incidence of human CAVD strongly correlates with aging, which is an independent risk factor for AoV disease. We have recently identified *Klotho*$^{-/-}$ mice, a model of premature aging, as a novel mouse model of CAVD [8]. Notably, *Klotho*$^{-/-}$ mice develop calcific nodules at the hinge region of the fibrosa side of the AoV (Fig. 18.1a, b), similar to human CAVD. In these mice, calcification occurs independent of inflammation and cusp thickening, providing initial evidence for a valve-intrinsic molecular mechanism for age-related calcification common in elderly patients. *Klotho*$^{-/-}$ AoV have increased expression of osteogenic factors *Runx2* and *Osteopontin*, in addition to increased expression of chondrogenic factors *Sox9* and *Col10a1*, consistent with an osteochondrogenic-like mechanism of disease (Fig. 18.1e). Increased activation of pSmad1/5/8 also precedes calcification in the *Klotho*$^{-/-}$ mice, and inhibition of BMP signaling represents an attractive new therapeutic approach for CAVD.

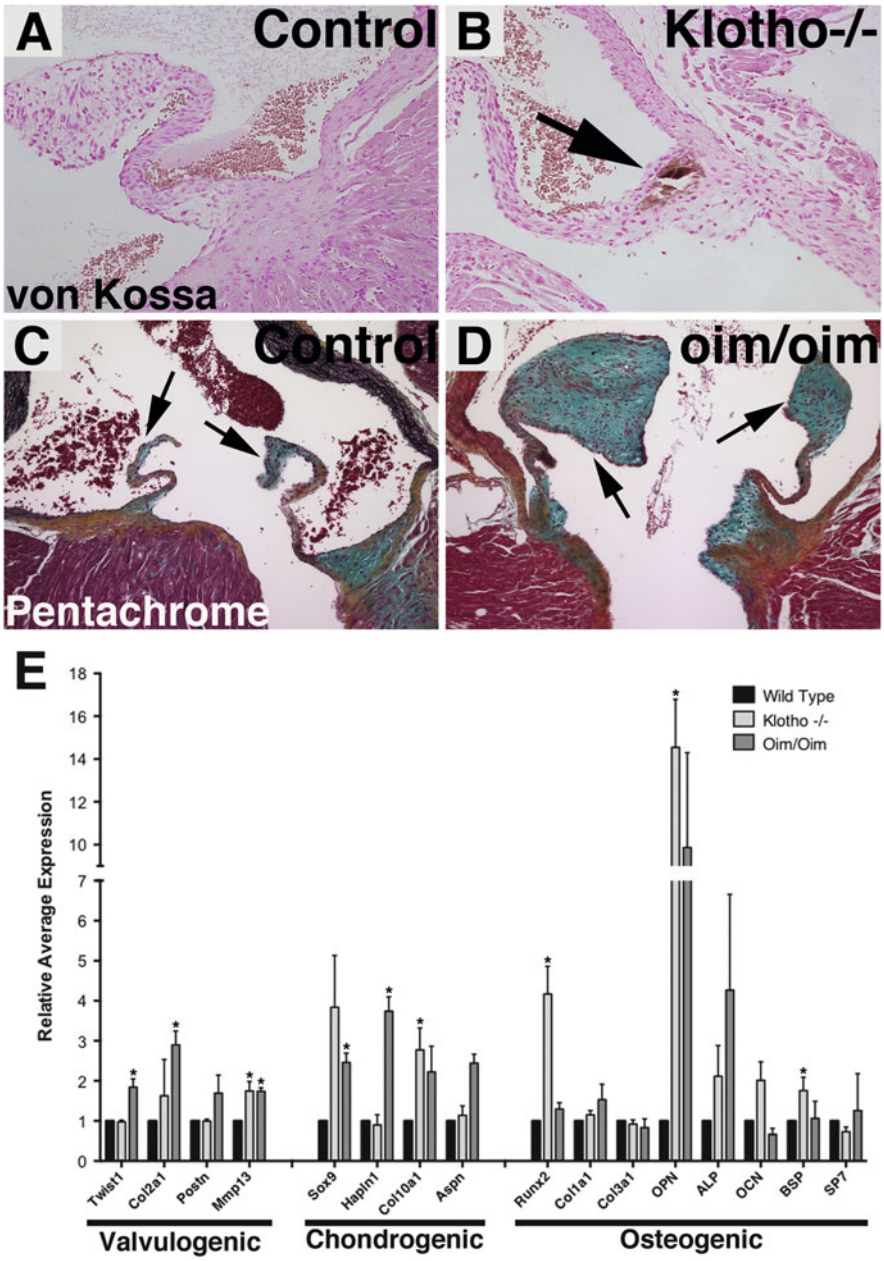

Fig. 18.1 Valvulogenic, chondrogenic, and osteogenic programs are induced in mouse models of calcific (*Klotho*$^{-/-}$) and myxomatous (*Oim*/*Oim*) valve disease. *Klotho*$^{-/-}$ mice (**b**) exhibit AoV nodular calcification (*arrows*), as compared to wild-type littermate controls (**a**) at 6 weeks of age, as detected by von Kossa staining. *Oim*/*Oim* AoV cusps (**d**) exhibit distal thickening and increased proteoglycan deposition (aqua, *arrows*), compared to WT littermates (**c**) at 9 months of age as observed by Movat's pentachrome staining. Expression of genes involved in valvulogenesis, chondrogenesis, and osteogenesis was examined by qRT-PCR of RNA isolated from *Klotho*$^{-/-}$

18.3.2 Myxomatous Valve Disease

The most common cause of MVP is myxomatous valve disease, which is defined by pathological thickening of the valve leaflets, primarily due to accumulation of proteoglycans [4]. This is accompanied by alterations in the distribution of ECM components, such as disrupted collagen fiber organization and elastic fiber fragmentation. The pathogenesis of MVP is not well understood; however, MVP is often linked to connective tissue disorders or specific mutations in ECM genes, supporting the concept that defects originating during valve development could underlie adult disease.

While myxomatous disease most commonly affects the mitral valves, myxoid AoV have been reported. *Osteogenesis imperfecta murine* (*Oim*) have a spontaneous mutation in the *Col1a2* gene and display bone fragility characteristic of human osteogenesis imperfecta (OI) [13]. Interestingly, humans with OI or *Col1a2* mutations have a predisposition to AoV disease [14]. Likewise, the AoV of *Oim*/*Oim* mice exhibit distal cusp thickening and increased proteoglycan accumulation, characteristic of myxomatous valve disease (Fig. 18.1c, d) [8]. Furthermore, the *Oim*/*Oim* mice have increased expression of valve progenitor markers *Twist1*, *Col2a1*, *Mmp13*, *Sox9*, and *Hapln1*, in addition to increased *Col10a1* and *Asporin* expression (Fig. 18.1e). These changes in gene expression are consistent with increased proteoglycan accumulation and cartilage gene induction, which are key features of myxomatous disease.

18.4 Signaling Pathways in Heart Valve Development and Disease

Similar to heart valve and bone development, studies of human explanted valves implicate BMP, Notch, and Wnt signaling pathways in the progression of CAVD. Thus, heart valve disease shares signaling networks with valve and bone developmental pathways. Together, these studies demonstrate that activation of both BMP and Wnt signaling correlates with progression of CAVD [15, 16]. On the other hand, loss-of-function mutations in *NOTCH1* are associated with bicuspid aortic valve (BAV) and CAVD, in humans and mice, suggesting an inhibitory function for the Notch pathway in valve calcification [17]. Human genetic conditions including Marfan syndrome and Loeys-Dietz syndrome lead to myxomatous mitral valve disease and are associated with increased TGF-β signaling [18, 19]. However, the specific mechanisms by which these different pathways contribute to the development and progression of heart valve disease remain unknown.

Fig. 18.1 (continued) and *Oim*/*Oim* mice aortic valves relative to wild-type littermate controls (**e**). Normalized values are shown as average fold changes compared to wild-type group set at 1.0. * is *p*-value ≤ 0.05 calculated by paired student's *t*-test

18.5 Future Directions and Clinical Implications

Klotho$^{-/-}$ and *Oim*/*Oim* mice are novel mouse models of CAVD and myxomatous valve disease that will be useful for determination of the underlying pathogenic mechanisms driving valve disease. Understanding how signaling networks contribute to disease will likely have a significant impact on clinical outcomes, since knowledge gained from these studies will allow for the development and design of new drugs/treatments for patients with valve disease.

Acknowledgments This work was supported by funding from NIH HL082716, HL094319, HL114682 (K.E.Y), F32HL110390 (E.E.W.), and the American Heart Association-Great Rivers Affiliate (M.V.G. and M.F.).

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A Novel Role for Endocardium in Perinatal Valve Development: Lessons Learned from Tissue-Specific Gene Deletion of the Tie1 Receptor Tyrosine Kinase

19

Xianghu Qu and H. Scott Baldwin

Abstract

The mechanisms regulating late-gestational and early postnatal semilunar valve remodeling and maturation are poorly understood. Tie1 is a receptor tyrosine kinase with broad expression in embryonic endothelium. During semilunar valve development, Tie1 expression becomes restricted to the turbulent, arterial surfaces of the valves in the perinatal period. Previous studies in our laboratory have demonstrated that Tie1 can regulate cellular responses to blood flow and shear stress. We hypothesized that Tie1 signaling would regulate the flow-dependent remodeling of the semilunar valves associated with the conversion from maternal/placental to independent neonatal circulation. To circumvent the embryonic lethality of the Tie1 null mutation, we developed a floxed Tie1 allele and crossed it with an *Nfactc1*^{en}*Cre* line that mediates gene excision exclusively in the endocardial cushion endothelium. Excision of Tie1 resulted in aortic valve leaflets displaying hypertrophy with perturbed matrix deposition. The valves demonstrated insufficiency and stenosis by ultrasound, and atomic force microscopy documented decreased stiffness in the mutant aortic valve consistent with an increased glycosaminoglycan to collagen ratio. These data suggest that active endocardial to mesenchymal signaling, at least partially mediated by Tie1, is uniquely required for normal remodeling of the aortic but not pulmonary valve in the late gestation and postnatal animal.

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_19

Keywords
Semilunar valves • Endocardium • Extracellular matrix • Tie1

19.1 Introduction

Cardiovascular defects are the most common congenital abnormality in the human population, affecting approximately 1 out of every 100 live births worldwide [1]. Defects in valve development account for 25–30 % of these malformations. Therefore, there is significant interest in understanding the mechanisms that underlie the complex process of heart valve development. During heart valve formation, a subset of endothelial cells overlying the future valve site are specified to delaminate, differentiate, and migrate into the cardiac jelly, a process referred to as endothelial-mesenchymal transformation or transdifferentiation (EMT). Locally expanded swellings of cardiac jelly and mesenchymal cells are referred to as cardiac cushions. In a poorly understood process, these cardiac cushions undergo extensive remodeling from bulbous swellings to eventual thinly tapered heart valve leaflets [2]. Numerous studies have focused on identifying the major regulators of valve development using murine and avian embryos and have particularly focused on the early stages of valve development; however, the pathways regulating the final events of remodeling and maturation have not been well defined.

The orphan receptor tyrosine kinase Tie1 is primarily expressed in endothelial cells and is closely related to Tie2, the receptor for the angiopoietins. Both Tie1 and Tie2 are essential for developmental vascularization where they appear to have roles in promoting microvessel maturation and stability. Targeted disruption of Tie1 gene in mice results in a lethal phenotype between E14.5 and P0 characterized by extensive edema, hemorrhage, and defective microvessel integrity [3–5]. Expression of Tie1 is first detected in the endothelium of mice at E8.0, and by E12.5, Tie1 is robustly expressed in the valvular endothelium, vasculature, heart, and lungs [6–9]. Tie1 expression subsides in the postnatal period, although low levels of Tie1 persist into adulthood on the arterial side of the valve leaflet as well as branch points of the descending aorta [10]. Thus, Tie1 is expressed in the vasculature, valves, and other endothelial cell populations in the developing heart during critical periods of valve morphogenesis. However, its potential roles in heart valve development are totally unknown. Here, using a conditional knockout mouse model, we show that lack of Tie1 in endocardial cells leads to hypertrophic semilunar valves in the postnatal and adult heart.

19.2 Model for Valvar Endocardial-Specific Gene Deletion

To specifically determine the potential role of Tie1 in valve morphogenesis, we have developed a novel valve endocardial cell-specific Cre mouse line (*Nfatc1*^{en}*Cre*). We previously identified a transcriptional enhancer that regulates the sustained expression of *Nfatc1* exclusively in the endocardium overlying the

endocardial cushions [11]. We then utilized this enhancer to develop a transgenic mouse line, *Nfactc1^{en}Cre*, which has recently been described by our lab [12]. To determine the utility and efficiency of this model for deletion at latter stages of cardiac development, we have analyzed Cre expression in the developing valves by *lacZ* staining and qRT-PCR of *Nfactc1^{en}Cre* transgenic mice bred with R26R reporter mice (Fig. 19.1a–d). *lacZ* staining confirmed that Cre activation is first detected at E9.5 in the developing AVC region and in OFT region by E10.5 (data not shown). Cre activation is localized to endocardial cushion endocardium of the outflow track at E12.5 and at E14.5 (Fig. 19.1a, b) and remains restricted to the endocardium of overlying the developing valves throughout gestation into the perinatal period (Fig. 19.1c) and in the adult (data not shown). To determine the utility of *Nfactc1^{en}Cre* for tissue-specific deletion, we crossed these mice with our mice harboring a floxed Tie1 allele previously described [5]. When Tie1 expression as determined by QT-PCR was normalized to expression detected in the adult animal, we observed the normal attenuation of Tie1 previously described by our group [5]. In addition we observed significant attenuation of Tie1 expression as a result of Cre-mediated deletion in the heart that was not detected in other vascular beds (data not shown). Thus our Cre transgenic line *Nfactc1^{en}Cre* mediates *loxP* excision exclusively in the endocardium overlying the endocardial cushions and not in mesenchyme derived from EMT or other endothelial populations.

19.3 Tie1 Is Required for Late-Gestational and Early Postnatal Aortic Valve Remodeling

To investigate the potential role of Tie1 in valve morphogenesis, we generated endocardial-specific *Tie1* knockout (*Tie1$^{fl/lz}$;Nfactc1^{en}Cre*) mice by breeding mice homozygous for a floxed Tie1 allele [5] with mice heterozygous for a null mutation in *Tie1$^{fl/lz}$* (*Tie1$^{+/lz}$* is a "knock-in"/knockout" insertion of lacZ into the Tie 1 locus [3]) as well as the *Nfactc1^{en}Cre* transgene. Analysis of timed matings revealed that no embryos died in utero during early gestation. However, within a few days after birth, *Tie1$^{fl/lz}$;Nfactc1^{en}Cre* pups could be distinguished from *Tie1$^{fl/fl}$* or *Tie1$^{fl/lz}$* littermates as the mutants were often growth retarded (Fig. 19.2) and lethargic, both of which are characteristics of human patients with heart defects. Most of mutants did not survive to adulthood. Histological analysis revealed no significant differences in valve formation between mutants and littermates until E16.5 (Fig. 19.3a, b), indicating early events of valvulogenesis occur normally in *Tie1* mutant mice. However, an increase in semilunar valve size was noted in *Tie1$^{fl/lz}$;Nfactc1^{en}Cre mice* from E18.5 (Fig. 19.3c, d) and early perinatal periods with the discrepancy in valve size continuing into the adult (Fig. 19.3e, f). Interestingly, the valve abnormalities were not only limited to the late gestation and postnatal period, but they were also primarily only detected in the aortic valves. The aortic valve areas of adult *Tie1$^{fl/lz}$;Nfactc1^{en}Cre* mice were nearly twice as large as *Tie1$^{fl/fl}$* aortic valves (increased by 88.3 %). The pulmonary valves of *Tie1$^{fl/lz}$;Nfactc1^{en}Cre* mice were somewhat thicker than the controls, but the difference was not significant. There were no differences in cell proliferation or apoptosis between mutant mice

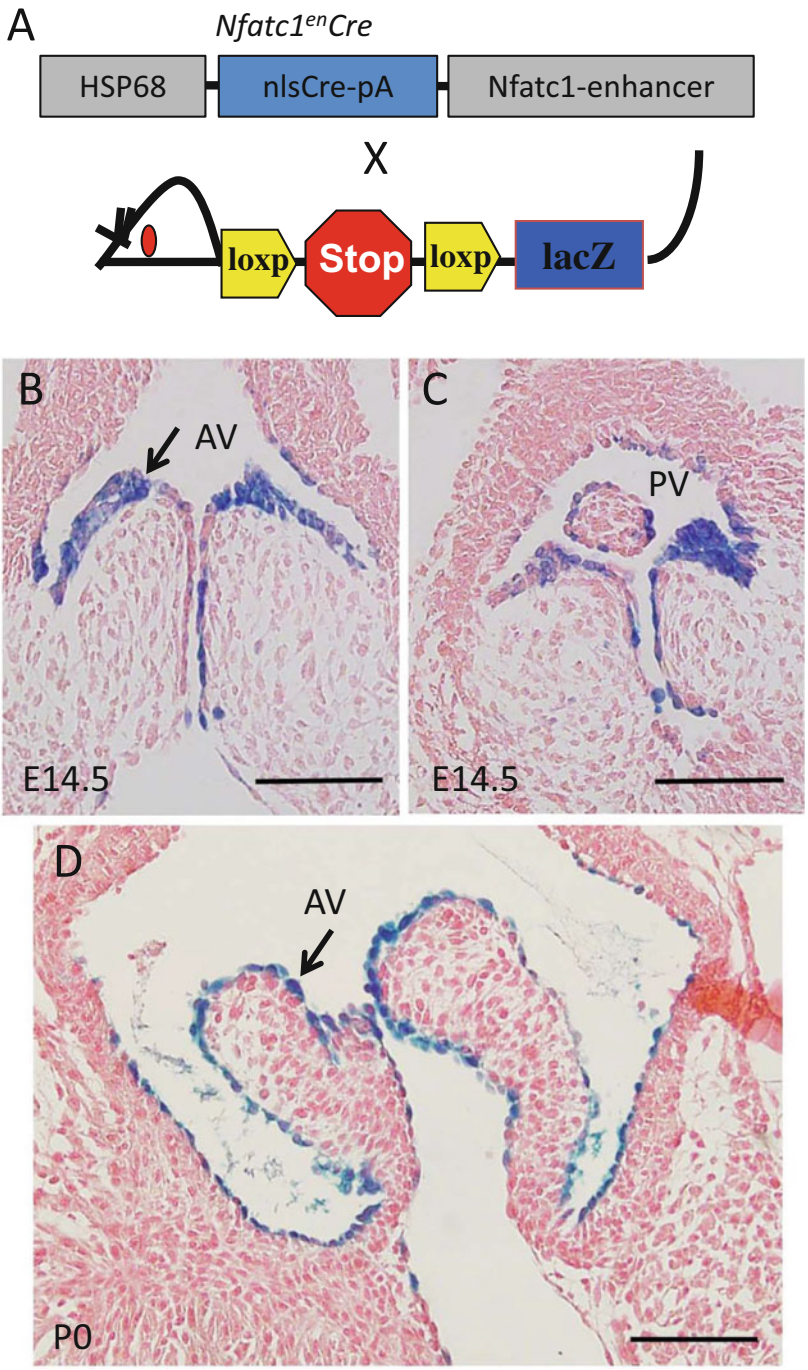

Fig. 19.1 *Nfactc1^{en}Cre* mediates loxP excision exclusively in the valvular endocardium. (**a**) Schematic of breeding of *Nfatc1enCre* and *R26fslz* mice. X-gal staining of *R26^{fslz}*;*Nfactc1^{en}Cre* heart sections shows that expression of the Cre is restricted to the endothelium of the developing

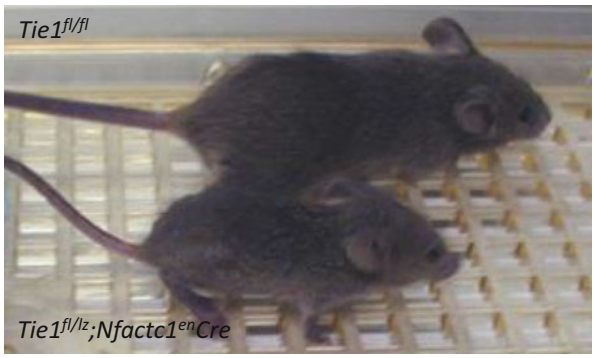

Fig. 19.2 *Tie1*$^{fl/lz}$; *Nfactc1*^{en}*Cre* animals are smaller than the controls

and littermates suggesting a difference in extracellular matrix (ECM) composition or production. Consistent with this, Movat's pentachrome stain revealed an increase in proteoglycan/glycosaminoglycan (GAGs) production and/or degradation in the valve interstitium of postnatal *Tie1*$^{fl/lz}$;*Nfactc1*^{en}*Cre* mice. As compared to *Tie1*$^{fl/fl}$, GAGs content (Fig. 19.3e, f) was increased and total collagen content (data not shown) was decreased in aortic valve leaflets of *Tie1*$^{fl/lz}$;*Nfactc1*^{en}*Cre* animals. Thus, attenuation of Tie1 results in severe abnormalities in ECM remodeling that are characteristic of critical events in late gestation and postnatal valve development in chicken, mouse, and humans [13, 14]. This work suggests that there is a non-cell autonomous defect that results from endocardial-specific Tie1 deletion as the ECM is produced primarily from the valvular interstitial cells (VICS), not the endocardium. This work also provides in vivo support for the in vitro observations that valvular endocardial cells regulate the phenotype of VICS. In addition, Tie1 mutant aortic valves demonstrated a decrease in rigidity as measured by atomic force microscopy and valvular insufficiency, as determined by high-resolution ultrasound (data not shown).

19.4 Future Directions

This study describes a unique dosage-dependant role for Tie1 during later stages of valve remodeling. Tie1 is essential for valve remodeling, and abnormalities within late-gestational valve remodeling lead to flaccid valve leaflets, which in turn do not function correctly in the animal. It is likely that the mortality observed in *Tie1*$^{fl/lz}$; *Nfactc1*^{en}*Cre* mice can be attributed to aortic insufficiency and ultimate heart failure. However, the mechanisms regulating late-gestational and early postnatal semilunar valve remodeling and maturation are still poorly understood.

Fig. 19.1 (continued) valves (*arrowhead*) at E14.5 (**b**, **c**) and at P0 (**d**), and weak *lacZ* expression remains in the majority of the valvular endothelial cells through adulthood (**d**). HSP68 indicates heat shock protein 68; *AV* aortic valve, *PV* pulmonary and aortic valve. Scale bars: 50 μm

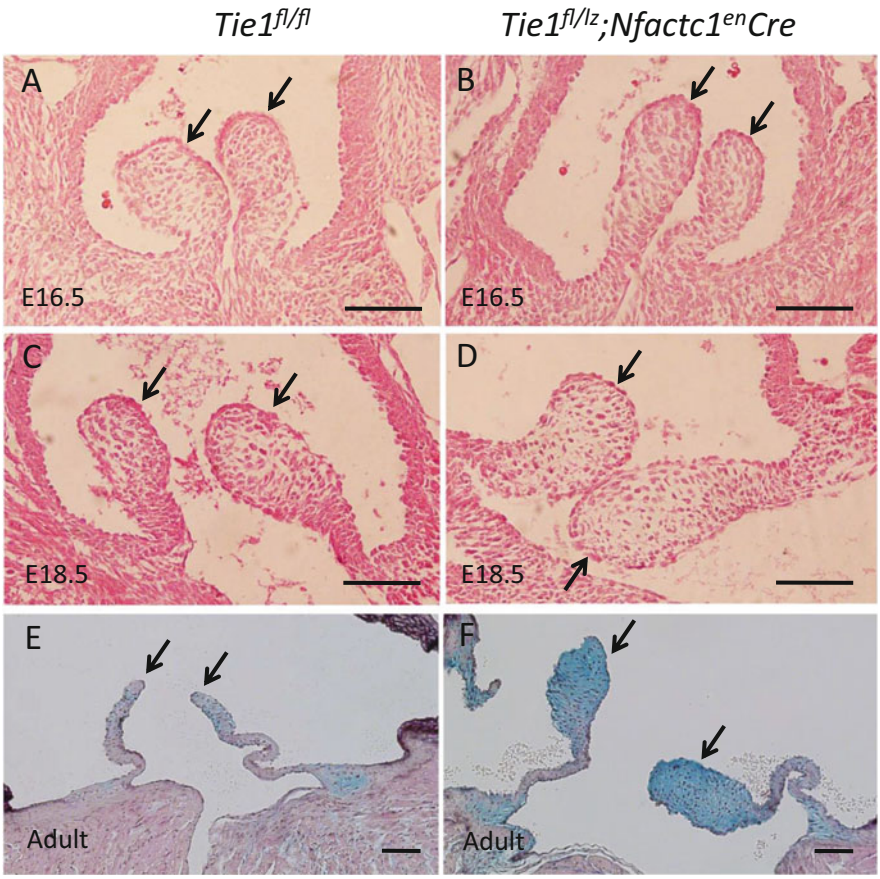

Fig. 19.3 Tie1 attenuation leads to enlarged aortic valves and abnormal ECM deposition and organization. H&E-stained sections of valves of *Tie1*$^{fl/fl}$ and *Tie1*$^{fl/lz}$;*Nfactc1*^{en}*Cre* mice at E16.5 (**a**, **b**) and at E18.5 (**c**, **d**). Compared to *Tie1*$^{fl/fl}$ mice, *Tie1*$^{fl/lz}$;*Nfactc1*^{en}*Cre* mice have enlarged aortic valves. Movat's pentachrome stain shows enlarged aortic valves with increased GAGs deposition (*blue*) in adult (9 weeks) *Tie1*$^{fl/lz}$;*Nfactc1*^{en}*Cre* mice (**f**) as compared to the controls (**e**). *Arrows* indicate valve leaflets. Scale bars: 50 μm

Tie1, although a close sequence homologue of Tie2, does not interact directly with the angiopoietins, and its in vivo ligands are yet to be identified. Nevertheless, growing reports based on in vitro studies suggest that a primary function of Tie1 might be to modulate Tie2 signaling and function [15–17]. Furthermore, both Tie1 and Tie2 are co-expressed in endocardial cells from very early endocardial cushion stage to the mature valve stage. So, we hypothesize that Tie1 signals independent of Tie2 and also acts an inhibitory co-receptor for Tie2 activation. Further investigation on this project using double (Tie1 and Tie2) conditional knockouts is currently on the way. Additional animal models are being developed to characterize the mechanism of Tie1-Tie2 interactions in modulating critical events in cardiac ontogeny in vivo, and expression profiling is being implemented to delineate

downstream signaling cascades that are activated by Tie1 and Tie1-Tie2 interactions. These studies will be essential for understanding the role of the endocardium in modulation valve matrix deposition and remodeling in an effort to unravel basic mechanisms of congenital heart disease.

Acknowledgements This work was supported by grant from NHLB/NIH: RL1HL0952551 (H.S.B.)

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The Role of the Epicardium in the Formation of the Cardiac Valves in the Mouse

20

Marie M. Lockhart, Maurice van den Hoff, and Andy Wessels

Abstract

In recent years, insights into the role of the epicardium in cardiac development have significantly changed. An important contribution to this increasing knowledge comes from the availability of mouse models that facilitate the study of the fate of the epicardial cell lineage and that allow epicardial-specific manipulation of expression of genes involved in regulation of epicardial cell behavior. In this contribution we will discuss our growing understanding of the role of the epicardium and epicardially derived cells in the formation of the atrioventricular valve leaflets. We will illustrate how epicardially derived cells specifically contribute to the development of the leaflets that derive from the lateral atrioventricular cushions, and we will discuss the role of Bmp signaling, through the Bmp receptor BmpR1A/Alk3, in the regulation of the preferentially migration of EPDCs into the parietal AV valve leaflets.

Keywords

Epicardium • Valves • Bmp2 • Bmp4 • Alk3

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_20

20.1 Introduction

20.1.1 The AV Valves and Their Leaflets

The atrioventricular (AV) valves are important cardiac components that, when properly formed, prevent the retrograde flow of blood through the AV junction during ventricular systole. The precursor tissues of the AV valves are the endocardial AV cushions, formed by (1) the accumulation of cardiac jelly in the AV canal followed by (2) the population of these cushions by endocardially derived cells (ENDCs) resulting from an endocardial epithelial-to-mesenchymal transformation (or endoEMT) [1]. Not all AV cushions develop at the same time. The two "major" AV cushions (the inferior and superior cushion) form first, while the two "lateral" cushions form later [2, 3]. The major AV cushions will eventually fuse together. They will also fuse with the mesenchymal cap of the primary atrial septum and the dorsal mesenchymal protrusion (DMP) to form the AV mesenchymal complex [4]. This process is essential in the partition of the left and right atrial and ventricular components. The AV mesenchymal complex is also involved in the formation of the septal leaflet of the right AV valve and the aortic (or anterior) leaflet of the left AV valve [4, 5]. The lateral AV cushions are significantly smaller than the major AV cushions and develop at the lateral myocardial AV junctions. Just like the major AV cushions, the lateral AV cushions become populated with ENDCs. The right lateral cushion eventually forms the parietal leaflet of the right AV valve, while the left lateral cushion forms the parietal (or mural/posterior) leaflet of the left AV valve (Fig. 20.1).

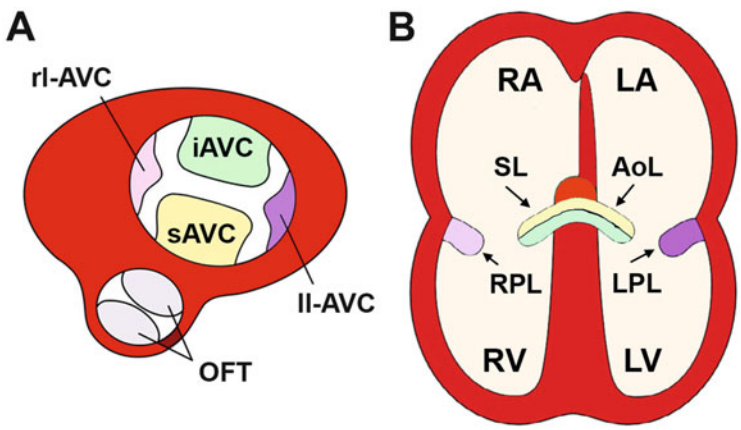

Fig. 20.1 This cartoon shows the fate of the individual AV cushions. The superior and inferior AV cushion (sAVC and iAVC) fuse at the midline and give rise to the septal leaflet of the right AV valve (SL) and the aortic leaflet (AoL) of the left AV valve. The right lateral AV cushion (rlAVC) forms the right parietal leaflet of the right AV valve (RPL), whereas the left lateral AV cushion forms the left parietal leaflet of the left AV valve (LPL)

20.1.2 The Epicardium and Epicardially Derived Cells (EPDCs)

In the mouse, epicardial development starts around embryonic day 9.5 (ED9.5) when the proepicardium, a heterogeneous cluster of cells, can be seen as a "cauliflower-like" mesothelial structure at the inferior margin of the cardiac sinus venosus at the venous pole of the heart. Around ED10, proepicardial cells reach and attach to the myocardium. Subsequently, they migrate out over the surface of the developing heart, ultimately covering the heart entirely. As a result of an epicardial epithelial-to-mesenchymal transition (epiEMT), epicardially derived cells (EPDC) are generated that migrate into the sub-epicardial space and into the underlying myocardium. Over the last 15 years or so, a series of cell fate studies in avian and murine models have been published reporting that EPDCs can, or have the potential to, differentiate in multiple cell types including coronary smooth muscle cells, interstitial fibroblasts, coronary endothelium, and potentially cardiomyocytes [6–9]. It is important to note that the contribution of EPDCs to the coronary endothelium and myocardium remains controversial.

20.1.3 The Contribution of EPDCs to the Developing AV Valves

In recent papers we have discussed the cascade of epicardial-related events involved in the formation and development of the AV junction [7, 10]. This process starts with the establishment of the AV-epicardium. After the formation of the AV-epicardium, subsequent AV-epiEMT leads to the generation of AV-EPDCs, a process regulated by a variety of factors. These AV-EPDCs eventually form the AV sulcus, which can be seen as a mesenchymal wedge between the expanding atrial and ventricular myocardial walls. As AV-EPDCs from the sulcus start to penetrate the AV myocardial junction, the formation of the annulus fibrosus is initiated. The annulus fibrosus is a fibrous tissue plane that physically and electrically separates the atrial from the ventricular working myocardium. From the annulus fibrosus, a subset of AV-EPDCs continues to migrate into the parietal AV valve leaflets, where they eventually comprise a large percentage of the mesenchymal cells found of the leaflet (Fig. 20.2). Just like the ENDCs found in the developing leaflets, the AV-EPDCs in the valves eventually become valve interstitial cells (VICs). It is important to note that very few AV-EPDCs are found in the leaflets that are developing from the fused major AV cushions [7].

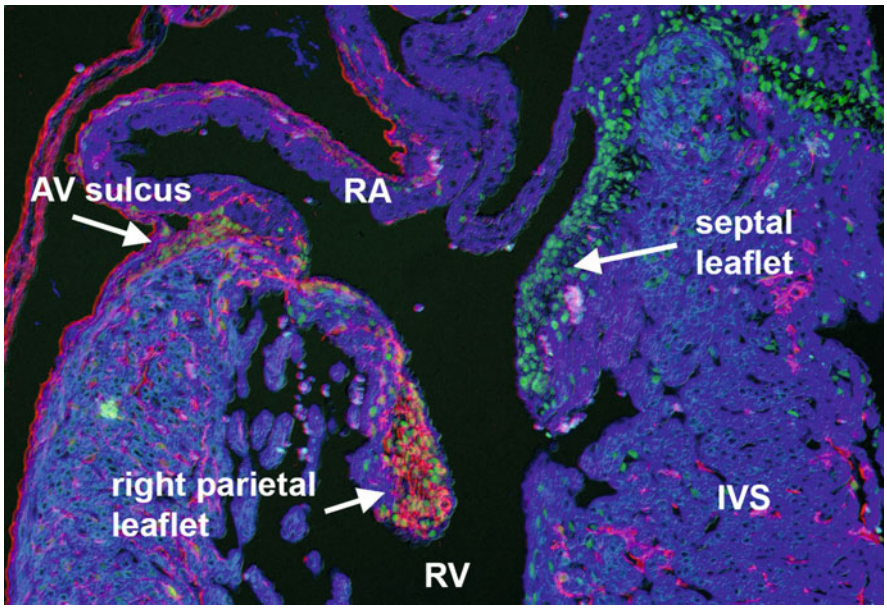

Fig. 20.2 A section of a 17ED Wt1-cre mouse crossed with a ROSA26$^{mT/mG}$ reporter mouse was stained for the presence of epicardial and epicardially derived cells (*red*), the transcription factor Sox9 (*green*), and cardiac myosin heavy chain (*blue*). The section shows the abundant presence of EPDCs in the right parietal leaflets and the virtual absence of EPDCs in the septal leaflet of the right AV valve

20.2 The Role of Bmp Signaling in Regulating the Contribution of EPDC to the AV Valves

20.2.1 Epicardial-Specific Deletion of the Bmp Receptor BmpR1A/Alk3 Leads to Disruption of AV Junction Development

To elucidate the mechanisms that regulate the events that are responsible for the contribution of AV-EPDCs to the tissues of the AV junction, we investigated the importance of bone morphogenetic protein (Bmp) signaling in the epicardium. Bmp isoforms are known to be involved in a variety of developmental steps in heart development. In particular Bmp2, found at high levels in the AV myocardium, has been shown to be important for the development of the AV cushions by promoting endoEMT [11]. In our study, we determined that Bmp4 is highly expressed in the AV-epicardium and in the AV-EPDCs that form the AV sulcus. Furthermore, strong phospho-Smad-1/5/8 labeling of the AV-EPDCs indicates that canonical Bmp signaling is active in these cells, strongly suggesting that this signaling pathway is important in regulating the events associated with the cascade of epicardial-related events at the AV junction [12]. To test the hypothesis that Bmp

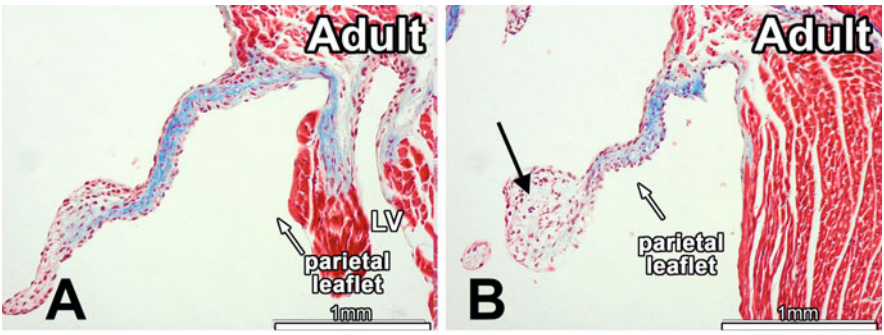

Fig. 20.3 Masson's trichrome staining of adult wild-type (**a**) and Wt1-cre;Alk3 mouse hearts. Panel (**b**) demonstrates the myxomatous phenotype of the left parietal leaflet of the epicardial Alk3 knockout mouse (*arrow* in **b**)

signaling is important for the development of the structures that rely on the contribution of AV-EPDCs, we deleted the Bmp receptor Alk3 from the epicardial and epicardially derived cells using an epicardial-specific cre-mouse (the Wt1-cre mouse [7, 12]). This approach resulted in a decrease in the size of the AV sulcus, an inhibition of the formation of the annulus fibrosus, and a reduction of the number of EPDCs that migrate into the parietal AV valve leaflets. Remarkably, electrophysiological analysis of postnatal Wt1-cre;Alk3 mice did not show, despite the defect in the formation of the annulus fibrosus, ventricular pre-excitation [12]. In addition, and also quite unexpectedly, postnatal Wt1-Cre;Alk3 showed a myxomatous mitral valve phenotype, particularly of the left parietal leaflet (Fig. 20.3).

20.2.2 Discussion

Our studies convincingly show that Bmp signaling plays a critical role in the establishment of the mesenchymal and fibrous tissues at the AV junction. We propose that during normal development, AV-epiEMT is a crucial process as it is responsible for generating the critical amount of AV-EPDCs needed for the subsequent formation of the annulus fibrosus and the associated migration of AV-EPDCs into the parietal leaflets. We believe that perturbation of Bmp signaling by conditionally deleting Alk3 from the epicardial cell population inhibits AV-epiEMT resulting in a domino effect leading to (1) inhibition of AV sulcus formation, (2) failure of the annulus fibrosus to form, and (3) reduction of the number of EPDCs that migrate into the AV valve leaflets (Fig. 20.4).

20.2.3 Future Direction and Clinical Implications

Myxomatous valve disease is generally considered an acquired disease, often observed in patients with mitral valve prolapse. The observed myxomatous changes

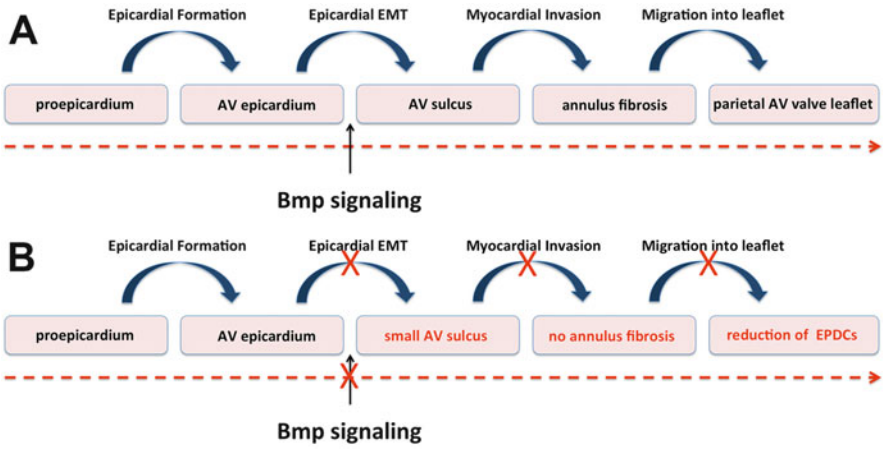

Fig. 20.4 Simplified hypothetical model showing the cascade of epicardial-related events at the AV junction. (**a**) In normal development, Bmp signaling positively regulates epiEMT resulting in the formation of the AV sulcus and subsequent development of annulus fibrosus, followed by the migration of EPDCs into the parietal leaflets. (**b**) When Bmp signaling is perturbed, AV sulcus formation and all AV-epicardial events downstream of this are inhibited

in the leaflets of the postnatal Wt1-cre;Alk3 mice, however, are associated with a reduced influx of EPDCs within those leaflets during embryonic development. We therefore propose that perturbation in the normal development of EPDCs in the AV junction might play the role in the etiology of myxomatous valve degeneration in human heart disease.

Acknowledgments The authors would like to acknowledge the financial support by NIH grants P30 GM103342 (A.W.), UL1RR029882 and UL1TR000062 (A.W.), R01HL033756 (A.W.) American Heart Association Grant-in-Aid 13GRNT16220004 (A.W.), American Heart Association Predoctoral grant 12PRE11340000 (M.M.L.), European Community's Sixth Framework Program Grant LSHM-CT-2005-018630 (MJBvdH), and Netherlands Heart Foundation Grant 1996M002 (MJBvdH).

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TMEM100: A Novel Intracellular Transmembrane Protein Essential for Vascular Development and Cardiac Morphogenesis

21

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Keywords
TMEM100 • BMP • ALK1 • Cardiovascular development

Among members of the TGFβ superfamily, bone morphogenetic protein (BMP) 9 and BMP10 regulate vascular endothelium differentiation and morphogenesis by activating the specific receptor complex, which consists of ALK1 (or ACVRL1), BMPR2, and endoglin. Mutations in *ACVRL1*, *BMPR2*, or *ENG* are associated with hereditary hemorrhagic telangiectasia and pulmonary arterial hypertension in humans [1, 2]. We previously identified *TMEM100* as a downstream target gene of BMP9/BMP10-ALK1 signaling pathway [3]. TMEM100 is a novel intracellular protein with two putative transmembrane domains, and its amino acid sequence is highly conserved from fish to humans.

To clarify the physiological significance of TMEM100, we generated *Tmem100*-deficient mice and found that all mutant embryos died in utero around embryonic day 10.5 (E10.5). LacZ reporter driven by the *Tmem100* locus was predominantly expressed in endothelial cells of developing arteries and endocardium. *Tmem100* null embryos showed severe vascular dysmorphogenesis and cardiac enlargement at E9.5 and massive pericardial effusion and growth retardation at E10.5 (Fig. 21.1). These phenotypic abnormalities were virtually identical to those observed in *Alk1*/*Acvrl1*-deficient mice, suggesting that *Tmem100* is an important downstream gene of BMP9/BMP10-ALK1 signaling during cardiovascular

Note: This Chapter is also related to Part VI.

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_21

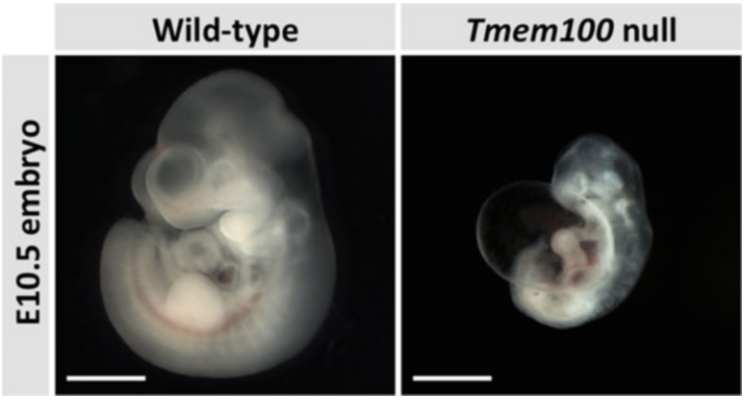

Fig. 21.1 *Tmem100* null embryos at E10.5 show severe cardiovascular dysmorphogenesis, massive pericardial effusion, and growth retardation (*scale bar*, 1 mm)

development. We also demonstrated that *Tmem100* null embryos showed atrioventricular canal cushion formation defect, indicating Tmem100 works also as an important factor for valve and septum morphogenesis.

Taken together, our studies indicate that TMEM100 is a novel endothelial-specific protein for cardiovascular morphogenesis downstream of BMP9/BMP10-ALK1 signaling. Clarifying the function of TMEM100 will lead to a better understanding of the mechanisms of cardiovascular morphogenesis and the etiologies of human congenital diseases.

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The Role of Cell Autonomous Signaling by BMP in Endocardial Cushion Cells in AV Valvuloseptal Morphogenesis

22

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Keywords
Atrioventricular (AV) canal • Endocardial cushion • BMP-2 • BMP receptors • Valvuloseptal morphogenesis

Distal outgrowth and fusion of the mesenchymalized AV endocardial cushions are essential morphogenetic events in AV valvuloseptal morphogenesis. BMP-2 myocardial conditional knockout (cKO) mice die by embryonic day (ED) 10.5 [1] at the initial stage for the formation of endocardial cushions, hampering investigation of the role of BMP-2 in AV valvuloseptal morphogenesis at the later stages. In our previous study, we localized BMP-2 and type I BMP receptors, *BMPR1A* and *Alk2*, in AV endocardial cushions [2, 3]. Based on their expression patterns, we hypothesize that autocrine signaling by BMP-2 within mesenchymalized AV cushions plays a critical role during AV valvuloseptal morphogenesis. To test this hypothesis, we employed recently generated endocardial/endocardial cushion-specific cre-driver line *Nfact1*^{Cre}. Unlike a previously generated *Nfatc1*^{enCre} line whose cre-mediated recombination is restricted to AV

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_22

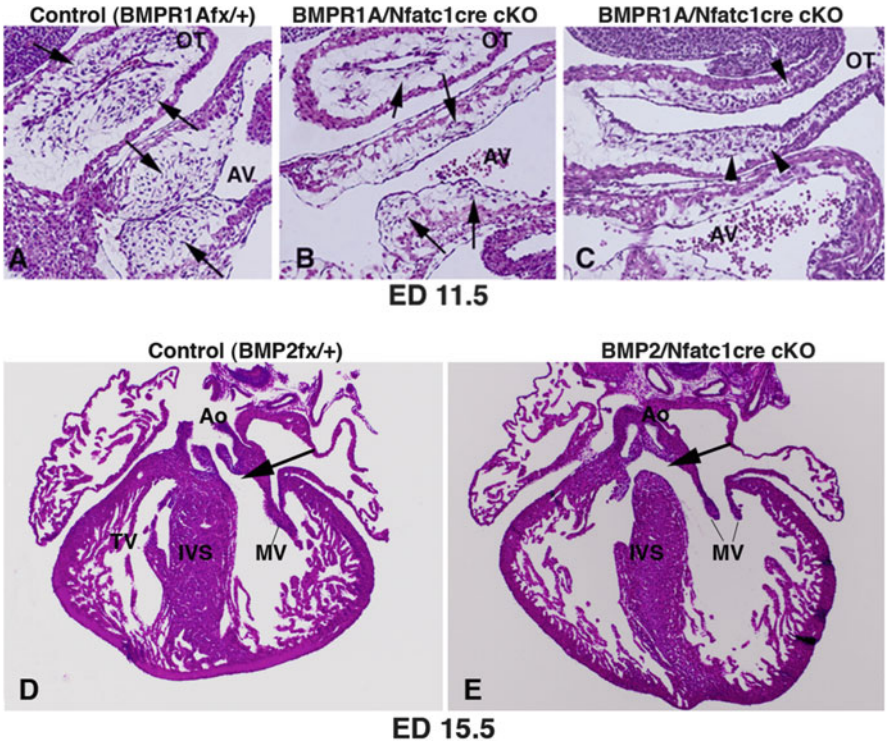

Fig. 22.1 (**a**–**c**) *BMPR1A cKO*^{Endo} mouse embryos exhibit failure of cellularization in AV cushions. *Arrows* indicate abundant mesenchymal cells in AV and outflow tract (OT) cushions in a control heart (**A**). *Arrows* show a few mesenchymal cells in AV and OT in a mutant heart (**B**). *Arrowheads* indicate abundant cells at the distal part of the mutant OT, which appear to be neural crest-derived and not reduced by *BMPR1A* endocardial/cushion-specific inactivation (**C**). (**d**, **e**) Endocardial/cushion-specific deletion of BMP-2 results in perimembranous septal defects (*arrow* in **E**), whereas control mouse heart shows well-formed ventricular septum (*arrow* in **D**). *Ao* aorta, *IVS* interventricular septum, *TV* tricuspid valves, *MV* mitral valves

and OT endocardium, this *Nfatc1*^{Cre} line confers cre-mediated recombination within the endocardial cells as well as their mesenchymal progeny. Using the *Nfactc1*^{Cre} driver line, we disrupted BMPR1A (Alk3) and BMP-2 specifically from AV endocardium and endocardial cushions. *BMPR1A* endocardial cushion cKO (*cKO*^{Endo}) mouse embryos died by ED 12.5 and exhibited failure of cellularization of AV cushions (Fig. 22.1a–c) and disruption of extracellular matrix (ECM) protein deposition in the cushion mesenchyme. On the other hand, AV cushion formation occurred in the *BMP-2 cKO*^{Endo} mice that survived beyond the AV cushion formation stage because BMP-2 expression remained intact in the AV myocardium during AV cushion formation. *BMP-2 cKO*^{Endo} mice exhibited perimembranous ventricular septal defects (VSDs) (Fig. 22.1d, e), defective deposition of ECMs in the membranous septum, and AV mitral valve dysplasia, suggesting the cell autonomous requirement of BMP-2 in AV endocardial cushions.

BMP-2 cKO^{Endo} did not exhibit muscular VSDs. These data strongly support our hypothesis that cell autonomous signaling by BMP-2 in the endocardial lineage plays a critical role in mesenchymalized AV cushions during AV valvuloseptal morphogenesis.

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