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Heart Valve Development:

Regulatory networks in development and disease

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Abstract

Rationale—In recent years, significant advances have been made in the definition of regulatory pathways that control normal and abnormal cardiac valve development.

Objective—Here, we review the cellular and molecular mechanisms underlying the early development of valve progenitors and establishment of normal valve structure and function.

Methods and Results—Regulatory hierarchies consisting of a variety of signaling pathways, transcription factors, and downstream structural genes are conserved during vertebrate valvulogenesis. Complex intersecting regulatory pathways are required for endocardial cushion formation, valve progenitor cell proliferation, valve cell lineage development, and establishment of extracellular matrix (ECM) compartments in the stratified valve leaflets. There is increasing evidence that the regulatory mechanisms governing normal valve development also contribute to human valve pathology. In addition, congenital valve malformations are predominant among diseased valves replaced late in life.

Conclusion—The understanding of valve developmental mechanisms has important implications in the diagnosis and management of congenital and adult valve disease.

Keywords

valvulogenesis; developmental biology; cell signaling; extracellular matrix; transcriptional regulation

Introduction

Defective development of the heart valves occurs in 20–30% of congenital cardiovascular malformations, and the incidence of congenital valve malformations has been estimated as high as 5% of live births.[1, 2] Heart valve replacement is the second most common cardiac surgery in the United States, and the majority of replaced aortic valves have congenital malformations.[3, 4] Developmental defects in valve structure and function occur in several syndromes with identified genetic lesions, including trisomy 21, Noonan, Marfan, Williams, and Holt-Oram syndromes.[5] Additional isolated gene mutations have been associated with valve development and disease.[6–8] However, in many cases, the underlying causes of valve developmental anomalies and associated dysfunction have not been identified. Here, we review studies of heart valve development and related disease mechanisms in animal models and in

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tissue culture. These research efforts provide extensive information on the molecular mechanisms and cellular events that govern the initial formation, maturation and function of heart valves with implications for development of new therapies for valve disease.

Overview of valve development

The four-chambered vertebrate heart has aortic and pulmonic semilunar (SL) valves at the arterial pole as well as mitral and tricuspid valves separating the atria and ventricles. The coordinated opening and closing of the heart valves occurs approximately three billion times in an average human lifespan and is required for unidirectional blood flow.[9] The three cusps of each SL valve and the two (mitral) or three (tricuspid) leaflets of the atrioventricular (AV) valves consist of complex stratified connective tissue.[9, 10] The valve leaflets are ensheathed in endocardial endothelial cells with intervening valve interstitial cells (VIC) that function in homeostasis and disease.[11, 12] The valves are stratified into extracellular matrix (ECM) layers rich in elastin (ventricularis of SL/atrialis of AV), proteoglycan (spongiosa) and collagen (fibrosa), oriented relative to blood flow (Figure 1).[12] The most obvious difference between the AV and SL valves is the presence of supporting chordae tendineae on the ventricular aspect of the tricuspid and mitral valves. However, comparable supporting connective tissue is present in the aortic and pulmonic roots and hinge regions of the SL valves.[12, 13] Morphogenetic and structural differences also exist among the individual mural and septal AV valve leaflets, but, in general, the molecular mechanisms of valve development are conserved among AV and SL valve leaflets. Extensive conservation of valve developmental mechanisms also has been observed among vertebrate species including chicken, mouse, and human.

The first evidence of valvulogenesis during embryonic development is the formation of endocardial cushions in the AV canal (AVC) and outflow tract (OFT) of the primitive looped heart tube.[14, 15] Valve primordia corresponding to individual leaflets and cusps are derived from the endocardial cushions, although the precise cushion origins of specific valve components are not well-defined. For the AV valves, the septal valve leaflets are derived from the fused inferior and superior endocardial cushions that form in the AVC of the primitive heart tube, whereas the mural leaflets are derived from mesenchymal cushions that arise laterally in the AVC after cushion fusion.[16] Less is known of how the SL valves arise from the complex arrangement of proximal and distal cushions that form in the OFT. The valve progenitor cells of the endocardial cushions are highly proliferative, whereas little or no cell cycling is apparent later in remodeling and mature valves.[11, 12, 17] The valve primordia continue to grow and elongate into thin fibrous leaflets of the AV valves and cusps of the SL valves, with increased ECM deposition and remodeling.[12] This process differs somewhat for individual valve leaflets. For example, the septal leaflet of the tricuspid valve delaminates from the closely apposed muscular ventricular septum, in contrast to the corresponding mitral valve leaflet that protrudes into the ventricular lumen much earlier in its development.[16–19] During late gestation and soon after birth, the valve leaflets become stratified into highly organized collagen-, proteoglycan- and elastin-rich ECM compartments.[12, 19] In mammals, valve maturation and remodeling continues into juvenile stages.[11–13]

Cell lineage studies in mice, based on examination of *Tie2-Cre* expressing cells and their derivatives, demonstrate that the vast majority of the cells present in the valves after birth are of endothelial endocardial cushion origin.[16, 17] These studies demonstrate that few, if any, cells of myocardial origin are present in the valve leaflets.[16] Likewise, in avians, myocytes are absent from the mature heart valves, with the exception of the mural aspect of the tricuspid valve, which is almost entirely muscle.[17, 20] Although neural crest and secondary heart field cells are in close proximity to the SL valves, the leaflets themselves are predominantly of endothelial endocardial cushion origin.[16, 21] However, there are neural crest-derived melanocytes and dendritic cells of unknown function on the surface of the mature SL and AV valves.[13, 22–24]

Epicardium-derived cells also have been identified as a source of valve progenitor cells, based on quail-chick transplantation studies.[25] While cell lineage analysis of the chicken proepicardium does not show valve cell investment[26], Cre-positive cells are apparent in the developing valves of *Tbx18-Cre* and *WT-1Cre* mice.[27, 28] However, studies by de Lange et al. demonstrate no investment of epicardial cells in the mature avian valves and conclude that all four valves in mice are almost entirely of endothelial origin.[16] Overall, multiple lines of evidence support the conclusion that the mature valves are derived from endothelial endocardial cushion progenitors with little or no contribution from other cell types.

Comparison of adult aortic valve leaflet structure and composition demonstrates similar stratification in humans, sheep, chickens, rabbits, and mice.[12] While hearts with multiple chambers and valves evolved in response to the demands of separate systemic and pulmonary circulation required for terrestrial life, the molecular pathways and cellular processes of valve formation have their origins in simpler hearts that also drive unidirectional fluid flow. Conserved valve cell regulatory mechanisms consisting of signaling pathways and transcription factors have been reported in ostia cells of the *Drosophila* dorsal vessel.[29, 30] In zebrafish, endocardial cushions form in the primitive heart tube, although there is some debate regarding whether the cellular events of early endocardial cushion formation are conserved.[31, 32] Recently, high-speed imaging of zebrafish heart valve development demonstrated that the endocardial cushions form initially by invagination of the endocardium, and not an epithelial-to-mesenchymal transition (EMT) of endocardium at the AVC, as is observed in avians and mammals.[32] However, the mature AV valve of the adult zebrafish two-chambered heart is structurally similar to the mammalian AV valves with stratified ECM and supporting chordae tendineae.[33] Therefore, the major cellular and molecular events of valve development are largely conserved among animals with hearts composed of multiple chambers.

Since the initial reports of endocardial cushion composition by Markwald et al. in the late-1970s[34, 35], the study of heart valve development has expanded to include investigation of signaling and transcriptional mechanisms that control many aspects of valve development and function. These studies encompass a broad spectrum of approaches and animal model systems with relevance to human congenital and postnatal valve abnormalities. Here we focus on the molecular regulation of valve development in hearts with four chambers, based on human disease mutation analysis, genetic studies in mice, and embryological manipulations in avians.

Endocardial cushion formation and EMT

The first evidence of endocardial cushion formation is swellings that appear in the AVC and OFT regions of the looping heart (E3 chick, E9.5 mouse, E31–35 human).[36–38] Endocardial cushion formation is induced by myocardial production of signaling molecules that inhibit expression of chamber-specific genes in the AVC and OFT, while increasing synthesis of ECM components (Figure 2A).[39–42] This increased ECM or ‘cardiac jelly’ deposition between the myocardium and endocardium, along with the hydrophilic nature of the ECM proteoglycans, causes the tissue to protrude or swell into the interior lumen of the heart forming the endocardial cushions.[35, 43, 44] Even at this early stage, endocardial cushions act as physical barriers that prevent the backflow of blood through the primitive heart tube.[15] Signaling molecules originating from both the myocardium and endocardium of the AVC and OFT are necessary for proper endocardial cushion formation and EMT of endocardial endothelial cells (Figure 2B).[45] EMT occurs as a subset of endocardial cushion endothelial cells break connections with neighboring cells and migrate into the cardiac jelly to populate the endocardial cushions with mesenchymal cells. The processes of endocardial cushion formation and EMT have been extensively studied using *in vitro* cell culture as well as *in vivo* model systems.

In general, the regulatory interactions and cellular events of valvulogenesis are conserved in AVC and OFT cushion development. The AVC cushions develop approximately a day earlier than the OFT cushions, and the examination of the OFT cushions is complicated by the presence of neural crest-derived progenitors that form the aorticopulmonary septum. [46, 47] Defects in secondary heart field development also preferentially compromise SL, but not AV, valve development related to defects in outflow tract morphogenesis (reviewed in Rochais et al.). [48–50] Many of the molecular regulatory hierarchies that control early stages of valvulogenesis have been defined using AVC explants from mouse or chick embryos due to the larger size and accessibility of cushion tissue. *In vivo* studies confirm that these interactions also occur in the developing OFT cushions with the exceptions noted below.

Bone Morphogenic Proteins (BMPs) are members of the Transforming Growth Factor Beta (TGFβ) superfamily and signal predominantly through activation of SMAD1/5/8.[51] Data collected using both *in vitro* primary cell culture and *in vivo* model systems suggests that BMPs act as the major myocardially-derived signals for initiation of endocardial cushion formation and EMT. BMP2 and 4 are expressed in the AVC and OFT myocardium during endocardial cushion morphogenesis in chick and mouse.[52, 53] Mice lacking myocardial *BMP2* expression fail to express *Tbx2* in AVC myocardium, which is necessary for suppression of chamber-specific gene expression and for increased ECM deposition in this region.[42, 54] Studies using mouse AVC explants demonstrated that BMP2 is sufficient to increase TGFβ2 expression and initiate EMT in AVC endothelial cells.[55] The role for BMPs in initiation of EMT is further supported by *in vivo* analysis of mice lacking myocardial *BMP2* expression, which show no AVC endocardial cushion mesenchymal cell formation.[42, 54] Mice lacking expression of *BMP-receptor1a* in the endocardium also exhibit decreased phospho-SMAD1/5/8 activity and defective EMT in the AVC, further substantiating the requirement for BMP receptor signaling in endocardial cushion endothelial cells during EMT. Aberrant BMP signaling results in downregulation of multiple EMT-related pathways in AVC endocardial cushions, including TGFβ and Notch1, as well as decreased expression of transcription factors such as *Twist1* and *Msx2*.[42] Taken together, these studies demonstrate a role for BMPs as important myocardially-derived signals that initiate endocardial cushion formation and EMT.

TGFβs were among the first signaling molecules to be implicated in initiation of endocardial cushion EMT.[56] TGFβ ligands and receptors are expressed in the AVC and OFT during endocardial cushion formation and EMT in avian and murine embryos. In both chick and mouse model systems, TGFβ ligands and receptors are required for EMT, however, species-specific differences have been noted.[14] TGFβ signaling through SMADs 2/3 induces expression of the transcription factor Slug, which promotes AVC endocardial cushion endothelial cell activation and invasion during EMT.[57, 58] TGFβ activity has also been associated with increasedβ-catenin signaling during AVC endocardial cushion EMT in mice.[59] Mice harboring genetic deletion of *β-catenin* in cells of the *Tie-2* lineage fail to populate AVC endocardial cushions with mesenchymal cells due to defective EMT. [59] In zebrafish, overexpression of Wnt inhibitors Adenomatous polyposis coli (APC) or Dickkopf1 blocks AVC endocardial cushion formation, that likely occurs through invagination and not EMT. [32, 60] Together, these studies suggest that TGFβ and Wnt/β-catenin signaling are important inducers of endocardial cushion formation, but the regulatory relationships of these pathways have not been defined.

Notch signaling also plays an indispensable role in endocardial cushion EMT. The Notch signaling ligand Delta4 and receptors Notch1–4 are expressed by endocardial cushion endothelial cells of the AVC and OFT prior to and throughout EMT.[61] In mice that lack expression of *Notch1* or the interacting transcription factor *RBPJK*, the AVC and OFT endocardial cushion swellings are present, but are devoid of mesenchymal cells due to a failure of EMT. In Notch signaling mutants, endocardial cushion endothelial cells extend processes into the cardiac jelly, but they are unable to delaminate and migrate into the ECM. In addition,

Notch signaling induces expression of the pro-migratory transcription factor Snail in AVC and OFT endocardial cushion endothelial cells undergoing EMT. Snail directly represses *VE-cadherin* promoter activity, thereby allowing activated mesenchymal cells to break contact with neighboring cells and migrate into the endocardial cushion interior. Notch signaling also is required for expression of *TGFβ2* and multiple TGFβ receptors in AVC and OFT endocardial cushion endothelial cells, serving to further promote EMT. Mutations in Notch signaling components in humans are associated with a spectrum of cardiac abnormalities, including defects in tissues derived from AVC and OFT endocardial cushions.[6, 62] These observations suggest that Notch signaling is dispensable for initial ECM deposition during formation of endocardial cushion swellings, but is required for endocardial cushion endothelial cell EMT.

During endocardial cushion formation, the AVC myocardium secretes biologically active adheron-like protein complexes containing ES1, fibronectin, transferrin, ES130, hLAMP1 and other extracellular components to activate adjacent endothelial cells and induce EMT.[45, 63–67] Proper function of these and other signaling components during AVC endocardial cushion formation and EMT requires the appropriate ECM environment. The endocardial cushion ECM is a hydrated matrix that provides physical support for mechanical function, promotes the invasive phenotype mesenchymal cells, and serves as a scaffold for cell migration.[15, 43] Disruption of *hyaluronan synthase-2* (*has2*) or *versican* gene expression in mice prevents AVC endocardial cushion formation, and hyaluronan also is required for mesenchymal cell migration associated with EMT.[43, 68] ErbB receptor activation is coupled to hyaluronan function in endocardial cushion EMT, as addition of heregulin to *has2*−/− AVC explants rescues EMT.[47, 69] Furthermore, *ErbB3*−/− null mice exhibit lethality at E13.5 with hypoplastic AVC endocardial cushions due to lack of adequate EMT. Because of its diverse functions, ECM synthesis must be properly regulated to ensure that the resulting extracellular environment has the appropriate physical and molecular characteristics to support endocardial cushion formation and EMT.

Growth of endocardial cushions and valve primordia

After EMT, the endocardial cushions and subsequent valve primordia undergo growth via cell proliferation and continued ECM synthesis.[12, 36, 70] The AVC valve primordia are part of a larger mass of tissue called the septum intermedium that is formed via fusion of the endocardial cushions at E4.5 in chicks, E11.5 in mice, and E37–42 in humans.[36, 38, 71, 72] Septum intermedium tissue contributes to the membranous ventricular septum and fibrous continuity overlying the ventricular septum adjacent to the valve primordia that form the septal tricuspid and mitral valve leaflets.[36] The OFT endocardial cushions also fuse and contribute to the formation of the aortic and pulmonary valve leaflets and supporting structures.[73] Molecular mechanisms regulating growth of post-EMT endocardial cushions and valve primordia are reviewed below.

Endocardial cushion and valve primordia mesenchymal cell proliferation is both positively and negatively regulated during growth of these structures (Figure 3). BMPs promote growth, as *BMP4* mouse mutants display hypocellular AVC and OFT valve primordia that remain unremodeled.[74, 75] Double mutants for *BMP6/7* and those harboring *BMPRII* mutations have hypoplastic OFT valve primordia with grossly normal AVC, demonstrating an important difference in the local requirements for BMP6/7 signaling.[76, 77] Conversely, genetic deletion of the BMP inhibitory SMAD, *SMAD6*, in mice results in AVC and OFT valve primordia hyperplasia, further illustrating the need for proper levels of BMP signaling to achieve normal valvulogenesis.[78] FGFs also promote post-EMT endocardial cushion/valve primordia mesenchymal cell proliferation. FGF4 is expressed throughout the AVC and OFT of chick embryos during growth of endocardial cushions and valve primordia, while FGF receptors 1, 2, and 3 expression is restricted to endocardial cushion/valve primordia cells.[79] FGF4 treatment

of chick AVC endocardial cushion explants or injection of replication defective retrovirus containing FGF4 coding sequence into chick hearts *in ovo* increases mesenchymal cell proliferation and results in hyperplastic AVC valve primordia. Conversely, Epidermal Growth Factor (EGF) signaling inhibits endocardial cushion and valve primordia mesenchymal cell proliferation through antagonism of BMP-mediated activation of SMAD1/5/8 in the AVC and OFT.[14] Therefore, mutations causing reduced EGF signaling result in hypercellular AVC and OFT valve primordia.[80, 81] These data demonstrate complex regulation of mesenchymal cell proliferation during endocardial cushion/valve primordia growth.

In humans, mutations in several genes impinging on the Ras/MAPK pathway, including *Protein Tyrosine Phosphatase Non-receptor type 11 (PTPN11)*, which encodes the protein tyrosine phosphatase SHP2, cause Noonan syndrome.[82] Noonan syndrome is associated with multiple congenital defects including cardiac abnormalities in tissues derived from AVC and OFT endocardial cushions.[83] SHP2 promotes Ras/MAPK activation and also acts downstream of EGF and other growth factor receptors.[83, 84] Mice bearing activating SHP2 mutations in *Tie-2* expressing cells have increased ERK1/2 activation and increased proliferation of AVC and OFT endocardial cushion/valve primordia endothelial and mesenchymal cells.[84] This phenotype is rescued by genetic deletion of ERK1. It is hoped that further studies of mouse models such as these will lead to better understanding of, and therapies for, Noonan syndrome.

Canonical Wnt signaling is active in growing AVC and OFT endocardial cushions and valve primordia.[59, 60, 85] *Wnt4* and *Wnt9b* are expressed by endothelial cells of the mouse AVC and OFT endocardial cushion and valve primordia, while *Wnt2*, *Lef1* and the Wnt receptor *Fzd2* are expressed in corresponding mesenchymal cells (Alfieri et al., unpublished). *Wnt9a* is expressed in chick AVC endocardium, and introduction of replication competent retrovirus expression Wnt9a leads to hypercellular valve primordia.[86] The reverse phenotype is seen when avian AVC explants are treated with the Wnt inhibitor Frzb.[86] Similarly, mutation of the Wnt signaling inhibitor *APC* in zebrafish causes increased AVC endocardial cushion and valve primordia mesenchymal cell proliferation.[59, 60, 85] These data suggest that Wnt signaling must be tightly regulated during endocardial cushion/valve primordia growth to maintain proper levels of mesenchymal cell proliferation.

During growth of valve primordia and in cellularized endocardial cushions, mesenchymal cells are distributed throughout the ECM.[12] This ECM is rich in hyaluronan, versican, and other basement membrane components, however, differentiating mesenchymal cells also begin to produce collagens 1, 2, 3, 4 and 9 as well as cartilage and tendon-related ECM components such as aggrecan and tenascin.[12, 87–90] AVC endocardial cushion explant experiments as well as mouse models demonstrate a role for BMP-regulated transcription factors in maintaining a balance between endocardial cushion/valve primordia mesenchymal cell proliferation and differentiation. Tb×20 and Twist1 are expressed by AVC endocardial cushion/valve primordia cells during growth of these structures and are associated with high levels of valve cell proliferation as well as expression of pro-migratory genes such as *periostin*, *cadherin-11* and *matrix-metalloproteinase(MMP)-2*.[91, 92] Sox9, another BMP-regulated transcription factor, also promotes cell proliferation and maintenance of proper ECM architecture during endocardial cushion/valve primordia growth.[93, 94] *Sox9* mutant embryos have hypocellular AVC and OFT endocardial cushions due to defective proliferation of mesenchymal cells and display dysmorphic valve primordia ECM. Further, expression of transcription factors Msx1/2 in OFT myocardium and endocardial cushion/valve primordia cells induces expression of *BMP4*, which negatively regulates OFT endocardial cushion and valve primordia mesenchymal cell proliferation.[95] Therefore, *Msx1/2* double mutants exhibit hypercellular SL valve primordia. It is clear that a complex network of transcription factors is necessary to promote proper levels of endocardial cushion/valve primordia mesenchymal cell proliferation

and maintain the appropriate ECM architecture during endocardial cushion/valve primordia growth.

Endocardial cushion initiation, EMT, and growth of endocardial cushions and valve primordia are associated with high levels of endothelial cell proliferation.[12, 35, 36] Vascular Endothelial Growth Factor A (VEGF) is a potent cytokine that promotes endothelial cell proliferation as well as survival.[96] *VEGF* is highly expressed by myocardium and endocardium prior to endocardial cushion formation, however, endocardial *VEGF* expression becomes restricted to endothelial cells of the AVC and OFT during endocardial cushion initiation, EMT, and growth of valve primordia.[97, 98] VEGF-receptors 1 and 2 (VEGFR1, 2) are expressed throughout the endocardium; however, VEGF and VEGFR expression is absent in mesenchymal endocardial cushion/valve primordia cells. Studies in chick, mouse, and zebrafish demonstrate that VEGF signaling contributes to AVC and OFT endocardial cushion cell proliferation.[96, 98, 99] VEGF signaling also inhibits AVC endocardial cushion EMT by promoting maintenance of an endothelial cell phenotype, thereby maintaining a proliferative population of endothelial cells throughout endocardial cushion formation, EMT, and endocardial cushion/valve primordia growth. VEGF expression must be strictly controlled during endocardial cushion EMT and endocardial cushion/valve primordia growth, as overexpression inhibits EMT, while underexpression of VEGF results in failure to maintain a proliferative endothelial cell population.

Nuclear Factor of Activated T-cells cytoplasmic 1 (NFATc1) is an NFAT family transcription factor expressed by AV and SL endocardial cushion/valve primordia endothelial cells throughout growth and remodeling.[100–102] *NFATc1*−/− mouse embryos have normal initiation of endocardial cushion formation and EMT. However, established AVC endocardial cushions have a reduced proliferative index, and AV and SL valve primordia fail to undergo remodeling, with embryonic lethality by E14.5 (Combs and Yutzey, unpublished).[100, 101] In cultured chicken AVC endocardial cushion cells, VEGF promotes endothelial cell proliferation through NFATc1 activation (Combs and Yutzey, unpublished). Likewise, VEGF treatment of human pulmonary valve endothelial cells induces NFATc1-dependent proliferation demonstrating a role for this interaction in adult valve homeostasis.[103] VEGF and NFATc1 expression are extinguished in AVC and OFT endocardial cushion mesenchymal cells upon EMT, but are maintained in the overlying endothelial cell layer.[97, 100, 101] Concomitant with valve remodeling, VEGF expression in AVC valve primordia endothelial cells is downregulated and valve endothelial cell proliferation is greatly diminished, while AV and SL valve endothelial cell NFATc1 expression is maintained.[12, 97, 102]

Receptor Activator of NFκB Ligand (RANKL), an upstream activator of NFATc1, is expressed in AV and SL valve endothelial cells during the transition from valve primordia growth to remodeling (Combs and Yutzey, unpublished).[102] RANKL treatment of primary chicken AVC endocardial cushion cells activates NFATc1 to induce expression of ECM remodeling enzymes, such as *Cathepsin K*(*CtsK*), while inhibiting cell proliferation (Combs and Yutzey, unpublished). Likewise, RANKL treatment of cultured mouse hearts increases *NFATc1* and *CtsK* transcription.[102] *CtsK* is normally expressed in AV and SL valve endothelial cells during remodeling, however, *NFATc1*−/− mice lack expression of this proteinase and their valves remain unremodeled.[100–102] These data suggest NFATc1 serves as a nodal point in the transition from growth of valve primordia via endothelial cell proliferation to valve remodeling.

Diversification of valve cell types

During fetal stages of the chicken (E14), mouse (E16.5–17.5) and human (20–39 weeks), the valve primordia elongate into thin valve leaflets. Valve patterning is evident in differential gene expression on the surface of the valve exposed to unidirectional pulsatile blood flow

versus the side of the valve away from flow (Figure 1). Elastin expression is localized to the flow side of the valves, whereas organized collagen fibrils are apparent in the fibrosa layer away from blood flow.[12, 19, 104] Additional specialized ECM compartments are the proteoglycan-rich spongiosa layer as well as the tenascin-rich chordae tendineae and supporting structures.[17, 89] Together, these ECM compartments are required for normal valve structure and function, with dysregulation leading to disease (see below). The developmental and molecular mechanisms regulating valve stratification currently are not known. Hemodynamics is often evoked as a driving force in valve development, and there is evidence that blood flow is required for valve maturation in zebrafish.[105, 106] However, it has been particularly difficult to manipulate blood flow in the four-chambered heart in order to determine specific effects on the developing valves, distinct from compromised myocardial function or embryonic viability.

One of the first indicators of valve polarity in mouse and chicken embryos that distinguishes the flow side versus fibrosa side is localized Notch pathway activation and expression of downstream effectors *Hey/Hrt/Hesr1* and *2* on the flow side (Mead and Yutzey unpublished).[6, 107] Mice lacking *Hesr2* exhibit AV valve thickening and regurgitation after birth, providing evidence for Notch pathway activation in valve leaflet maturation.[108] The role of Notch signaling in establishing polarity of the valves has not been established, but this signaling pathway appears to have multiple roles in valve development and disease.[6, 61] An attractive hypothesis is that shear stress on the flow side of the valve promotes localized Notch signaling, thereby initiating valve polarity and stratification, but this has not yet been demonstrated.

There is emerging evidence for diversified cell types in the developing valves that give rise to distinct gene expression profiles associated with ECM compartments (Figure 4). However, the commitment of VIC to fixed lineages has not been unequivocally demonstrated. Likewise, the specific origin of VIC in distinct valve compartments has not been defined by fate mapping or cell lineage analysis of subpopulations or individual valve progenitors *in vivo*. The examination of the regulatory hierarchies controlling specialized cell types in the valves has been aided by studies of corresponding connective tissue types in other organ systems. Signaling pathways required for cell lineage development in cartilage, tendon and bone are active during valve remodeling.[109] For example, transcription factors involved in cartilage and tendon development are localized to subsets of valve progenitor cells and are required for valve differentiation and patterning.[89, 94, 110] In addition, the upstream regulators and downstream targets of these transcription factors also are expressed together in the developing valves. Overall, there is increasing evidence that development of distinct ECM compartments with specific biomechanical properties in the valves shares molecular regulatory mechanisms with other connective tissue types of similar ECM composition.

The spongiosa layer of the valve leaflets is rich in chondroitin sulfate proteoglycans that provide a compressible ECM similar to cartilage.[9] In addition, the valve leaflets express the transcription factor Sox9 and structural proteins aggrecan, collagen2a1 and cartilage link protein, characteristic of cartilaginous structures.[89, 94, 111] In contrast, valve supporting structures, including the chordae tendineae, are composed of elastic matrix similar to that observed in tendons, and both express the bHLH transcription factor *scleraxis* as well as tenascin and *collagen14*.[88, 89, 110] In cultured AVC or OFT valve progenitor cells, BMP2 treatment promotes expression of *Sox9* and *aggrecan*, whereas FGF4 treatment promotes expression of *scleraxis* and *tenascin*.[89, 112] These two pathways antagonize each other in induction of lineage-specific gene expression in the developing valve progenitor cells, as was also observed in the developing limb buds. [113, 114] *In vivo*, Sox9 is required early in proliferation of the AVC and OFT endocardial cushion mesenchyme and later in expression of collagen2a1 and cartilage link protein in the differentiated AV valves.[94] Likewise loss of *scleraxis* results in decreased *collagen14* expression as well as increased expression of cartilage

marker genes and abnormal valve ECM organization.[110] Together these studies provide evidence that multipotential valve progenitors of the endocardial cushions differentiate into cells of the valve spongiosa layer or supporting apparatus depending on exposure to BMP or FGF signaling, respectively.

Less is known of development of the valve fibrosa layer. During heart valve remodeling, ECM proteins characteristic of fibroblasts and preosteoblast lineages are restricted to the fibrosa layer, oriented away from blood flow (Alfieri et al., unpublished).[12, 19] These ECM proteins include osteonectin, periostin, collagens 1 and 3, and fibronectin that contribute to the highly organized collagen matrix, conferring stiffness necessary for valvular sufficiency.[115–117] The mature aortic valve fibrosa layer is the usual site of pathologic calcification, and the coexpression of collagen1, osteonectin, and periostin, is characteristic of fibrous connective tissues with the potential to mineralize, such as bone or dermal fibroblasts.[118, 119] Likewise, cultured aortic VIC express fibrosa markers and can be induced to express osteogenic markers under conditions that also promote mineralization of bone (Alfieri et al., unpublished).[120, 121] Wnt signaling has been implicated in bone lineage development as well as aortic valve calcification.[122–124] Multiple Wnt ligands, including *Wnt3a* and *Wnt7b* involved in bone development, are expressed together with the Wnt pathway reporter TOPGAL in remodeling mouse AV and SL valve leaflets (Alfieri et al., unpublished).[85] In addition, Wnt treatment of avian embryo aortic VIC in culture promotes expression of *periostin* (Alfieri et al., unpublished). Together these analyses provide initial evidence for Wnt regulation of fibrosa layer maturation as well as conserved regulatory pathways with osteogenic cell lineages. Further studies are necessary to determine the requirements for Wnt signaling in heart valve stratification and disease mechanisms.

Heart valve ECM maturation and organization

Heart valve development is characterized by increasing complexity and organization of the ECM. The ECM of endocardial cushions prior to EMT is rich in hyaluronan, and the mesenchymal cells in the cushions after EMT express network collagens and MMPs 1, 2, and 13, that promote cell migration.[88, 91, 92, 125] Electron microscopy studies show high cellularity and relatively unstructured ECM in endocardial cushions and valve primordia.[12, 35] Biomechanical studies of avian AVC endocardial cushions demonstrate increased rigidity of the tissue with increased cellularity and collagen deposition over time.[126] Selective degradation of ECM components of endocardial cushions demonstrated that glycosaminoglycans in the cellularized cushions confer elasticity, whereas collagen provides rigidity.[126] In the stratified valves, the structurally distinct layers of ECM provide specific biomechanical properties. Elastin fibers, of the ventricularis layer of SL and atrialis layer of the AV valves, confer elasticity to the valve, extending when the valve is open and recoiling when the valve is closed.[9] The relatively unstructured proteoglycans of the spongiosa layer absorb compressible forces on the leaflets and mediate movements between the highly structured elastin fibrils of the ventricularis/atrialis and fibrous collagen of the fibrosa layer.[9] The collagen-rich fibrosa layer provides stiffness and strength to the valve leaflet and is the major structural component of the valves. The collagen composition of the valves changes during maturation of the valve leaflets, with increased mature collagen fibrils at later stages, corresponding to increased structural and functional demands.[88, 90, 104] Fibrous collagen is the most abundant protein in the mature valves, and the fibrosa layer is predominantly collagen1 fibrils, but collagen3 fibrils also are present.[9, 12, 19] Overall, the precise regulation and organization of the complex layers of the valve ECM is critical for normal valve development, structure and function.

Abnormal expression and distribution of ECM proteins expressed in the valves is associated with developmental valve abnormalities and disease (Tables 1–2). *Elastin* mutations are associated with Williams syndrome, which includes supravalvular aortic stenosis as well as

SL valve disease.[127] Loss of *elastin* in mice leads to arterial abnormalities and perinatal death prior to significant investment in the stratified valves.[128] However, *elastin* heterozygous mutant mice exhibit aortic valve anomalies, thus demonstrating the importance of elastin in normal valve structure and function (Hinton, unpublished). Marfan syndrome, which includes aortic valve anomalies in addition to aortic dilation, is caused by mutations in *fibrillin-1*, also present in aortic valve elastic fibrils.[129] Likewise, mice lacking the associated elastic fibril ECM protein *fibulin-4* exhibit thickening and calcification of the aortic valve.[130] Proteoglycan gene mutants have not yet been associated with defects in the stratified valves, but increased synthesis and disorganization of proteoglycans is predominant in pediatric aortic valve disease and adult myxomatous mitral valves.[12, 131] It seems likely that additional ECM gene mutations contribute to isolated and familial congenital valve malformations, and ECM genes expressed in the valves are certainly strong candidates in ongoing human genetic analyses.

Complex regulation of collagen composition is an important feature of valve maturation and homeostasis. Mutations in multiple collagen genes are associated with connective tissue disorders that include valve dysfunction and disease. Osteogenesis imperfecta is caused by *collagen1a1* mutations that can lead to mitral and/or aortic valve insufficiency necessitating replacement, in addition to prevalent skeletal and vascular anomalies.[132, 133] Ehlers-Danlos syndrome is associated with mutations in *collagens 3, 5, 11* or *tenascin X*, and Stickler syndrome is caused by *collagen 2* or *11* mutations.[134–136] Both of these syndromes include widespread connective tissue disease, as well as heart valve dysfunction, that can be severe enough to necessitate replacement.[137, 138] Dysregulation of the expression and distribution of fibrous collagen in the valves occurs in valve disease, with increased collagen3 relative to collagen1 fibrils in myxomatous mitral valves.[139] In mice, targeted mutagenesis of facit collagen genes *collagen5a1* and *11a1* results in thickening of SL and AV valves with increased expression of fibrous collagens 1 and 3 evident at birth.[140] Similarly, loss of *periostin*, which regulates collagen fibrillogenesis, also leads to congenital AV and SL valve anomalies that compromise heart valve structure and function.[116, 117] Overall, a variety of lesions related collagen dysregulation are linked to defects in valve development and also in valve disease.

Heart valve development and disease

There is increasing evidence for a link between congenital valve malformations and late-onset valve disease. The most common valve malformation is bicuspid aortic valve (BAV), which often goes undetected until the valve becomes stenotic and requires replacement late in life.[141] Prenatally, there is increasing evidence that aortic valve malformations can lead to more severe congenital heart anomalies, including hypoplastic left heart.[142, 143] BAV is heritable, and mutations in the *NOTCH1* gene have been associated both with BAV and aortic valve calcification.[6, 141] Aortic valve calcification has been characterized as an osteogenic process with activation of several genes involved in bone mineralization, including *Runx2* and *osteocalcin*.[144–146] In developing bone progenitors, Notch1 signaling inhibits mineralization by repressing the transcriptional activity of Runx2, and a related mechanism has been evoked as a protective mechanism in aortic valve disease.[6, 147] Increased Wnt signaling, also implicated in valve and bone development and antagonized by Notch signaling, is associated with aortic valve disease.[122, 147] Therefore, signaling pathways involved in normal valve development likely have both positive and negative effects in valve pathogenesis that could be exploited in the treatment of these common conditions.

In the normal adult valve, the VIC are relatively quiescent with little or no synthetic activity or cell proliferation.[12, 104] The most common types of valve disease are myxomatous, characterized by insufficiency and inappropriate ECM production, and stenotic, with leaflets that are thickened, stiff and mineralized.[10, 118] Activation of VIC with increased synthetic activity is observed with both types of valve pathogenesis.[11, 12, 146] It is not known if VIC can

reenter the cell cycle under pathologic conditions. Recent studies have begun to define distinct types of VIC, that may have specific roles in valve pathogenesis, and these may be related to diversified cell types seen during development.[121] In addition, bone marrow-derived hematopoietic stem cells have been reported to be present in adult valves, but the function of these cells in valve homeostasis and pathogenesis has not been defined.[148] There is initial evidence that the increased ECM production and VIC activation in valve pathogenesis is related to developmental pathways, but further studies are necessary to rigorously test this hypothesis.[6, 122, 146, 149]

Conclusions and perspectives

Complex regulatory mechanisms that govern normal and abnormal valve development have been defined as a result of the work of many laboratories using a variety of experimental systems. This work has identified conserved regulatory hierarchies involving signaling pathways and transcriptional mechanisms active during both early and late valve development, as well as in other related types of connective tissue. Still, there are many remaining questions to be addressed in the study of valve development. Although the vast majority of cells in the mature valve are of endothelial cushion origin, specific contributions of epicardial-and neural crest-derived cells have yet to be fully defined. In addition, further studies are necessary to map the specific fates of individual endocardial cushion cells in the stratified valves and to determine the plasticity of mature VIC. Likewise little is known of how the common endocardial cushions contribute to specific valve leaflets, especially for the SL valves. In general, individual reports on valvulogenesis have focused on regulatory interactions acting in isolation at specific times and in specific cells of the developing valves. Further studies are necessary to fully define the interactions of these many regulatory pathways in order to have a more complete understanding of how valves form during prenatal development and how alterations in these processes lead to valve dysfunction and disease.

The emerging evidence for activation of valve developmental pathways during adult valve disease pathogenesis has potentially important implications in the treatment of human cardiovascular disease.[10, 11, 131, 144] It is not known if VIC that express valve developmental genes represent a dedifferentiated cell type or if there is a relatively undifferentiated cell population in normal adult valves. Alternatively, cells from extra-cardiac origins, such as mesenchymal or hematopoietic stem cells, may populate the adult valves and could contribute to disease pathology or have valve regenerative potential.[148] A valve stem cell population has not been identified. The detailed analysis of regulatory pathways that control valve development also has implications in valve tissue engineering. In general, current efforts directed towards generating engineered valves do not take into account the diversity of VIC or their abilities to generate ECM with distinct structural characteristics.[150] The application of recent research into valve developmental mechanisms to the generation of engineered valves will likely improve the long-term function of these tissue constructs and could lead to improved therapeutics or replacement strategies. Likewise, manipulation of known valve developmental mechanisms could be applied to the treatment and management of the most common types of valve disease.

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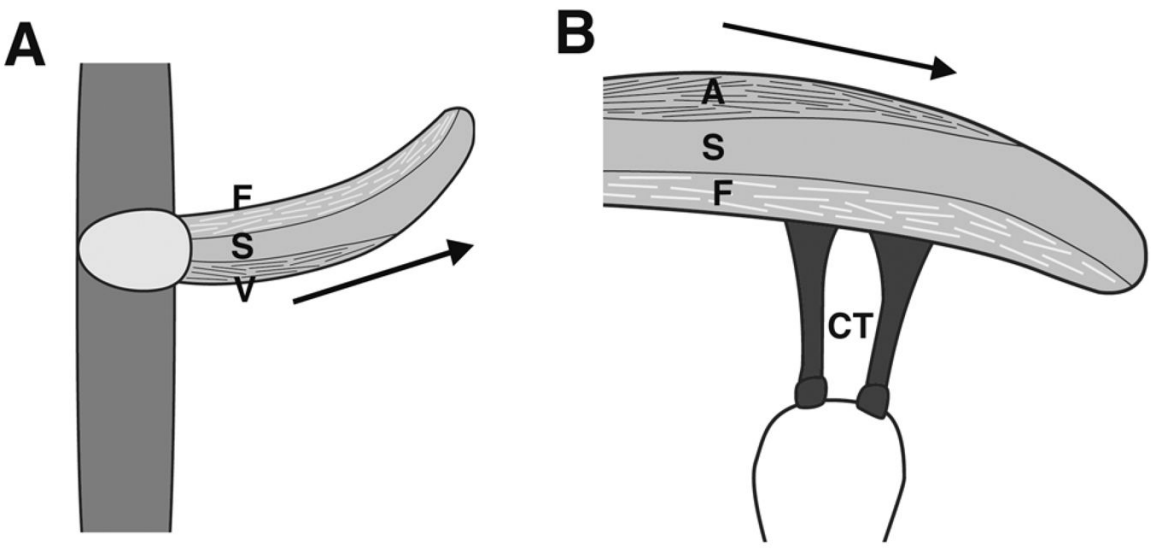

Figure 1. Stratified ECM compartments are evident in mature SL and AV valves

A) Schematic representation of one of three valve cusps of the aortic or pulmonic SL valve with fibrosa (F), spongiosa (S) and ventricularis (V) layers indicated. B) Schematic representation of one AV valve leaflet with atrialis (A), spongiosa (S), and fibrosa (F) layers indicated. The mitral valve has two leaflets, whereas the tricuspid valve has three leaflets, all of which are supported by chordae tendineae (CT). The direction of pulsatile blood flow is indicated for both SL and AV valves (arrow).

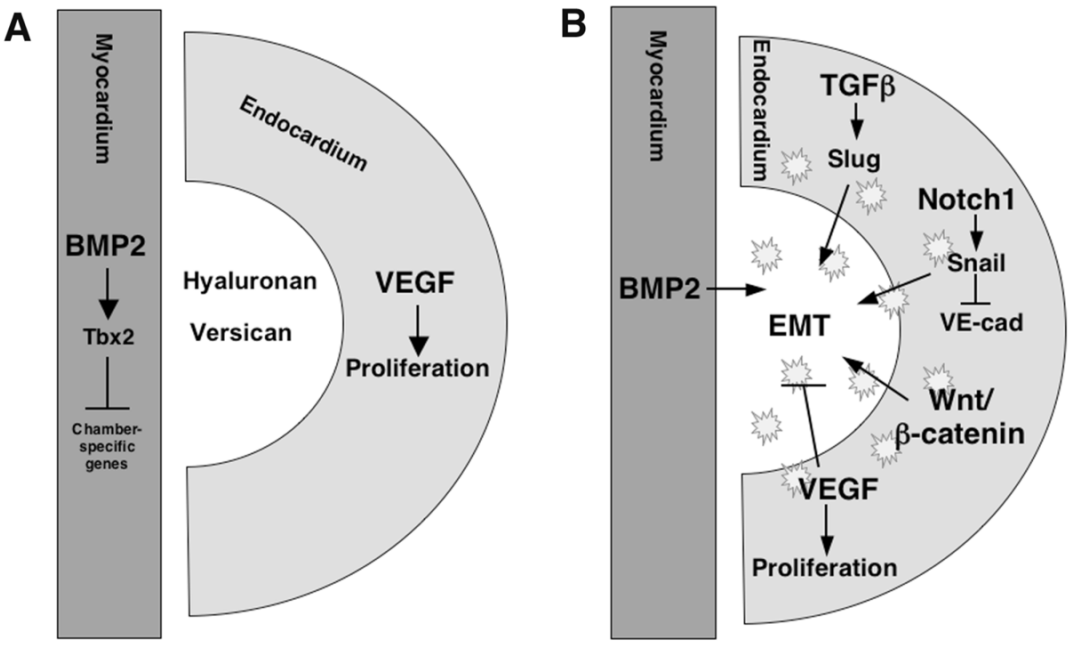

Figure 2. Model for regulatory interactions that control endocardial cushion formation (A) and EMT (B)

A) Myocardial BMP2 expression increases hyaluronan and versican deposition in cushion-forming regions of the AVC and OFT. BMP2 induces *Tbx2* transcription in the myocardium, inhibiting chamber-specific gene expression. VEGF, expressed in endothelial cells, promotes endocardial cushion endothelial cell proliferation. B) Myocardial expression of BMP2 promotes endocardial cushion EMT. Multiple endocardially-derived signals promote endocardial cushion EMT (delaminating and mesenchymal cells are indicated by white stars). TGFβ signals through Slug to promote EMT, while Notch1 signals through Snail to suppress *VE-cadherin* (VE-cad) expression and promote EMT. Wnt/β-catenin signaling increases endocardial cushion EMT. Once the cushions are established, endocardial VEGF expression maintains endothelial cell proliferation and inhibits EMT.

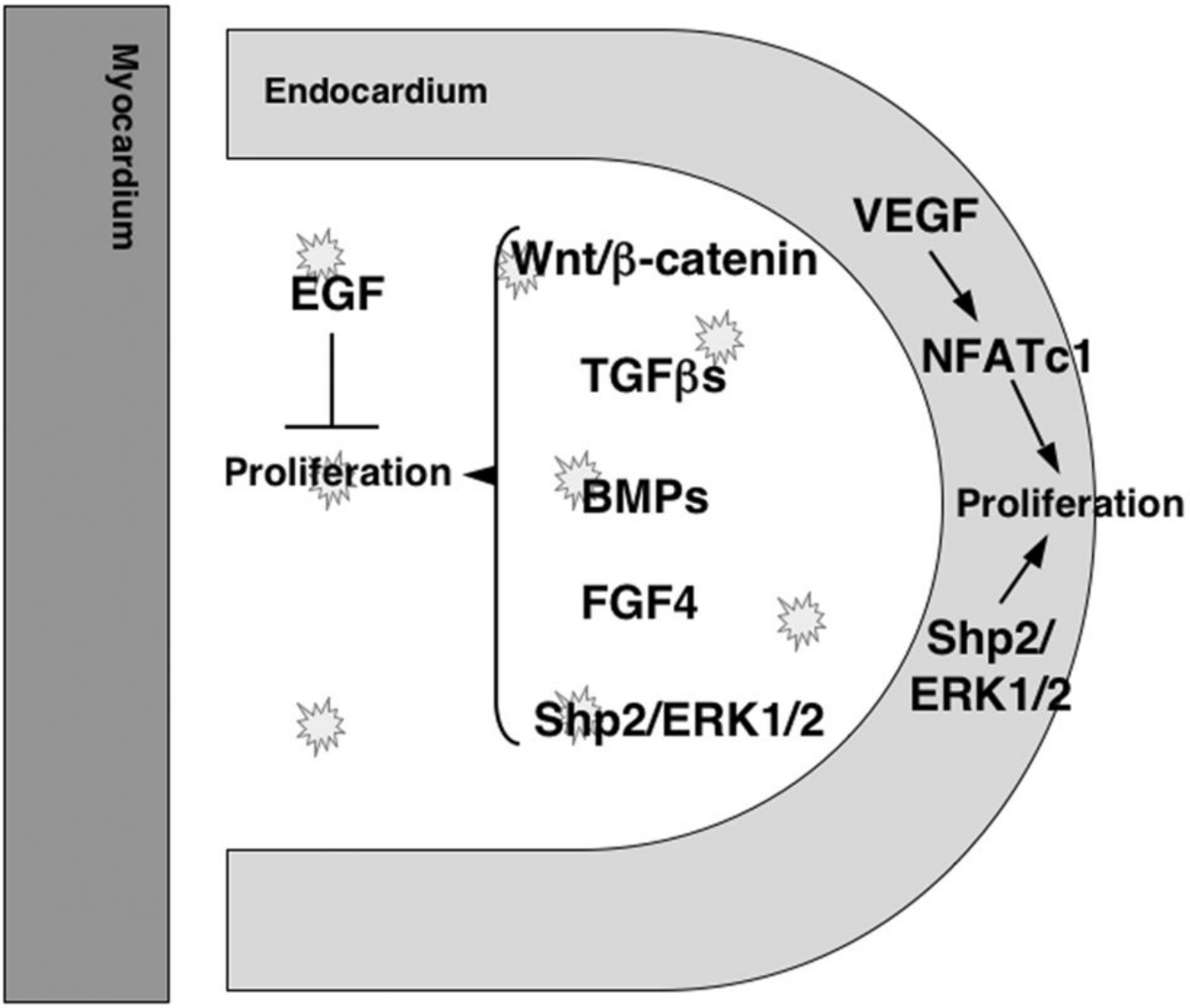

Figure 3. Model for regulatory interactions that control growth of endocardial cushions/valve primordia

Cell proliferation in the endothelial cells in the endocardial cushions is induced by VEGF/NFATc1 and Shp2/ERK1/2 signaling. Mesenchymal cell proliferation is induced by multiple signaling mechanisms including Wnt/β-catenin, TGFβs, BMPs, FGF4 and Shp2/ERK1/2. EGF signaling inhibits mesenchymal cell proliferation.

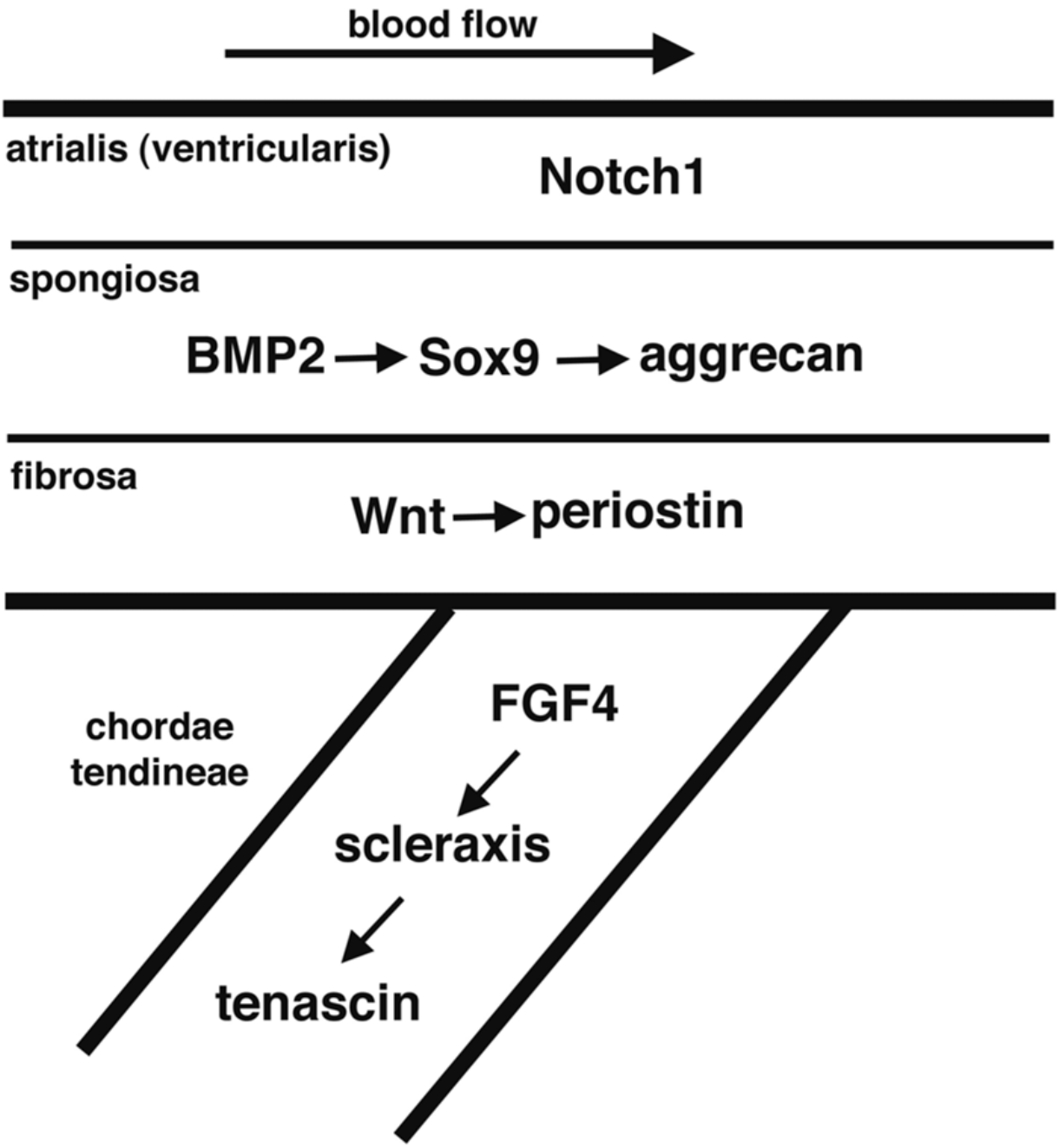

Figure 4. Model for regulatory interactions controlling AV valve stratification and lineage diversification

Notch1 expression is localized to the flow side of the stratifying valve. In the spongiosa, BMP2 signaling promotes Sox9 expression and deposition of cartilage-related ECM components, such as aggrecan. Wnt signaling in the fibrosa promotes expression of fibroblast/pre-osteoblast-related ECM components, such as *periostin*. Maturation of valve supporting structures (chordae tendineae) is associated with FGF4 signaling, which induces expression of the tendon-related transcription factor *scleraxis* and the ECM component tenascin. Although the SL valves do not have chordae tendineae, these signaling pathways also are active in the corresponding regions of the stratified aortic valve cusps and supporting structures.

Table 1

Genetic lesions in ECM components of endocardial cushions/valve primordia

ECM	Animal Model	Phenotype	Associated Human Disease	Refs
Hyaluronan	*Has2*−/− mouse	Embryonic lethal E9.5. Lack of EC formation and other defects.		43
	Jekyll zebrafish	Lack of EC formation and AVC specification defect.		151
Versican	*hdf* mouse	Embryonic lethal E10.5. Lack of EC formation and other heart defects.		152
Fibronectin	*FN*−/− mouse	Embryonic lethal by E10.5. Defects in mesodermally-derived tissues including failure of EC formation.		153
Laminin	Various mouse mutants exits	Most display embryonic or perinatal lethality.	Assoc. with a wide spectrum of diseases, many of which include compromised cardiovascular function.	154
Periostin	*Peri*-lacz mouse	Spectrum of lethal and non-lethal valve defects.		116
Cartilage Link Protein	*Crtl1*−/− mouse	P0 lethality due to EC-derived structures and/or other abnormalities.		111
Perlecan	*Perlecan*−/− mouse	Embryonic lethal E10-P0 with OFT cushion and other heart and non-cardiac defects.	Dyssegmental Dysplasia, Silverman-Handmaker type; Schwartz-Jampel syndrome	155, 156

Table 2

Genetic lesions in ECM components of cardiac valves

ECM	Animal Model	Phenotype	Associated Human Disease	Refs
Collagen 1	*Mov13* mouse	Embryonic lethal E12–14 due to vascular and other defects. Heart defects are reported.	Ehlers-Danlos syndrome; Osteogenesis imperfecta with valve abnormalities prevalent.	136, 157
Collagen 2	*Col2a1*−/− mouse	Perinatal lethality with skeletal abnormalities.	Stickler syndrome; Wide spectrum of cartilage and bone diseases.	136
Collagen 3	*Col3a1*−/− mouse	Most die by P2 from unknown causes. Survivors have decreased lifespan with cardiovascular and other defects.	Ehlers-Danlos syndrome	136, 158
Collagen 5	*Col5a1*−/− mouse	Embryonic lethal E10.5 with cardiac insufficiency.	Ehlers-Danlos syndrome	140, 159
Collagen 11	*Col11a1*−/− mouse	Thickened heart valves. Perinatal lethality due to respiratory failure.	Ehlers-Danlos syndrome; Stickler syndrome; Marshall syndrome	136, 140
Tenascin	*TNX*−/− mouse	Mice are viable and fertile.	Ehlers-Danlos syndrome with increased incidence of mitral valve prolapse.	160
Elastin	*Eln*−/− mouse	P0 death from vascular obstruction.	Williams syndrome; Cutis laxa	161
Fibrillin-1	*Fbn1*−/− mouse	Die by P14 from vascular complications.	Marfan syndrome; Weill-Marchesani syndrome; MASS syndrome	162
Fibulin-4	*Fibulin4-R/R* hypomorph mouse	Adult mice have thickened aortic valves and other vascular abnormalities.	Cutis laxa and other connective tissue diseases.	130