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Developmental Pathways in CAVD

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1. Introduction

Calcific Aortic Valve Disease (CAVD) occurs in >2% of the population over 65 years of age and often leads to valvular stenosis that necessitates valve replacement [1]. CAVD is a progressive disease, often manifesting first as aortic valve sclerosis and later developing into stenosis and valve dysfunction [2]. The specific molecular and cellular mechanisms of CAVD initiation and advancement are not well defined, and inhibitors of CAVD progression have not been identified. The current standard of treatment for CAVD is aortic valve replacement [3]. Presently, there are no pharmacologic-based treatments for CAVD, and new therapeutic approaches for CAVD are needed. The majority of aortic valves that are replaced have congenital malformations, such as bicuspid aortic valve (BAV), establishing a link between valve development and disease mechanisms [4].

The molecular mechanisms of CAVD include activation of signaling pathways implicated in both heart valve development (valvulogenesis) and bone development (osteogenesis) [5-8]. These include activation of regulators of progenitor specification, cell proliferation, and differentiation. Heart valves and bone are complex connective tissues with compartmentalized ECM produced by specialized cell types. Over the past several years, extensive progress has been made in defining molecular regulatory mechanisms in heart valve and bone development (Reviewed in [8-10]). Strikingly, regulatory pathways that control development of cartilage, tendon and bone also are active in developing valves [8, 11]. In addition, recent studies have reported induction of molecular regulators of valvulogenesis and osteogenesis in CAVD [7, 12-14]. However, it is not known if these developmental mechanisms have reparative functions or contribute to the progression of CAVD.

Here we review molecular mechanisms of valve and bone development as they relate to molecular mechanisms of CAVD. Recent studies have provided evidence for the involvement of specific regulatory pathways in CAVD as activators or inhibitors of disease progression.

Additional research in animal models and human patient specimens is necessary to determine the detrimental molecular regulatory pathways that promote CAVD progression and also beneficial pathways that potentially inhibit CAVD. In the future, manipulation of these pathways could be exploited therapeutically in the treatment of patients with CAVD or with aortic valve sclerosis that precedes calcification.

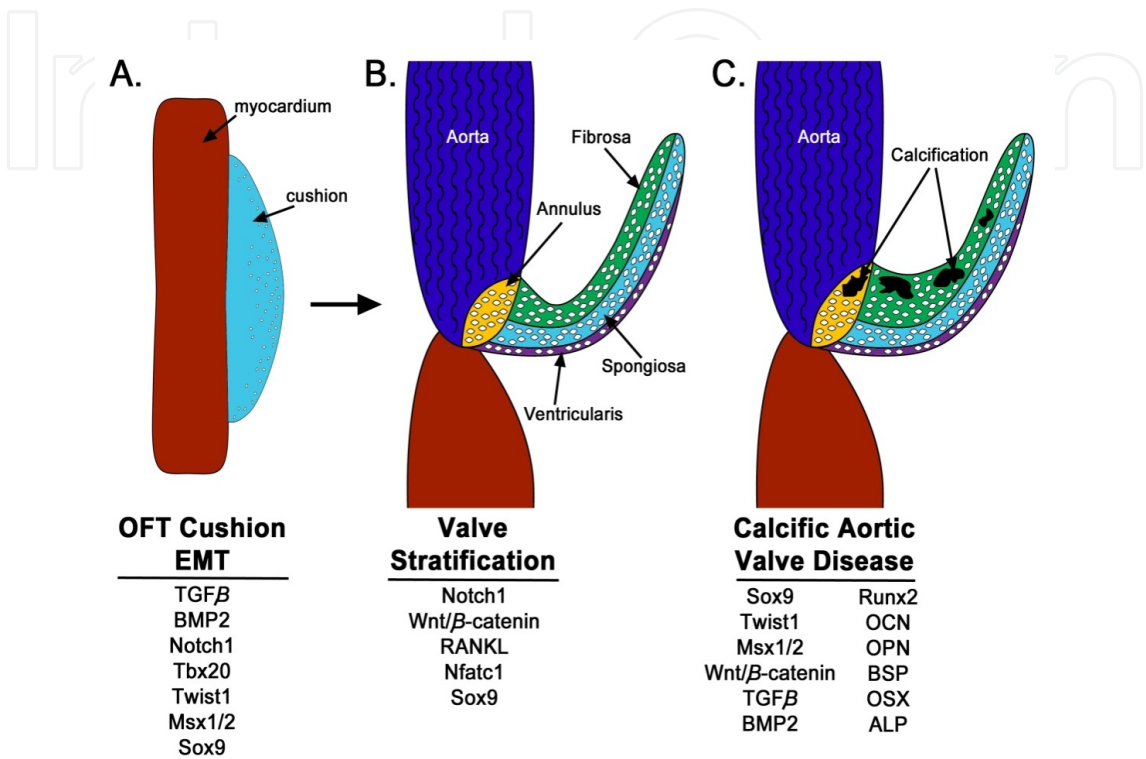


Figure 1. Molecular pathways active during endocardial cushion development and valve stratification are reactivated in CAVD. (A) Early stages of OFT cushion development are marked by ECM deposition, EMT, and neural crest cell infiltration. Factors necessary for EMT and mesenchymal cell function are expressed. (B) During late embryonic development and early postnatal development, the aortic valve becomes stratified and possesses three ECM layers. Factors necessary for ECM remodeling are active at this stage. (C) In CAVD, the ECM remodels and the valve becomes thickened. Calcification (black nodules) is typically observed in the collagen-rich fibrosa layer. Many factors expressed during OFT cushion development and valve stratification are reactivated during disease. Furthermore, osteogenic factors involved in bone development are also observed in CAVD. Please see text for details and references. OFT = outflow tract, EMT = epithelial-to-mesenchymal transition, ECM = extracellular matrix, CAVD = calcific aortic valve disease.

2. The cellular and molecular regulation of valve development

2.1. Overview of valve development

Valve development begins with the formation of endocardial cushions in the atrioventricular (AV) canal and outflow tract (OFT) of the primitive heart tube, which occurs at embryonic day (E)9-10 in mice, E3 in chickens, and E31-35 in humans [8]. The first evidence of endocardial cushion formation is the separation of the endocardium and overlying myocardium in the AV canal by expansion of the cardiac jelly through increased expression of hya-

luronan (Figure 1) [15]. These swellings are invested with mesenchymal cells that arise from endothelial-to-mesenchymal transformation (EMT) of the endocardium [16]. Similar swelling and induction of EMT occur approximately a day later in the cardiac OFT cushions that will contribute to the semilunar valves [17]. Endocardial EMT is induced by signaling molecules, including bone morphogenetic proteins (BMPs), emanating from the adjacent myocardium in the AVC and OFT [8, 18-20]. Once established, the endocardial cushions expand through increased extracellular matrix (ECM) production and cell proliferation of mesenchymal and endothelial cells. The AV cushions subsequently fuse to separate right and left cardiac channels. In addition, lateral cushions are induced in the AV sulcus that will give rise to the mural leaflets of the mitral and tricuspid valves [21]. Neural crest cells (NCCs) migrate into the cushions of the cardiac OFT, contributing to the septum between the aortic and pulmonic roots and also to the morphogenesis of individual semilunar valve leaflets [21, 22]. At this point, distinct primordia of individual valve leaflets become apparent and proliferation of valve interstitial cells (VICs) is reduced [23]. Valve morphogenesis occurs with elongation and thinning of the valve primordia, in addition to ECM remodeling and stratification. In general, the development of the AV and semilunar (SL) valves is similar, but there are some differences in the sources of cells and structure of the resulting leaflets [8, 10, 11, 24]. In mature SL and AV valves, the ECM is stratified into collagen-rich fibrosa, proteoglycan-rich spongiosa, and elastin-rich (atrialis-ventricularis) layers oriented relative to blood flow [24].

2.2. Embryonic origins of valve cell lineages

The primary embryonic source of adult semilunar valve interstitial cells is the endothelial-derived cells of the endocardial cushions, that arise as a result of EMT as determined by Tie2-Cre lineage tracing in mice [23, 25]. Since the cardiac OFT is derived from the secondary heart field (SHF), semilunar VICs derived from OFT endocardium also are SHF-derived [20, 26]. NCC-derived cells are present in adult mouse semilunar valve leaflets as demonstrated by cell lineage tracing with Wnt1-Cre [27]. These cells are predominant throughout the aortic and pulmonic valve leaflets, but are enriched in the leaflets adjacent to the aortopulmonary septum, which also is derived from NCCs [21, 28]. NCCs are required for semilunar valve morphogenesis and remodeling, likely by providing signals necessary for cell lineage differentiation and leaflet maturation [29, 30]. Another potential source of VICs is the epicardium, which contributes cells to the parietal leaflets of AV valves [31]. However, epicardial-derived cells (EPDCs) have not been reported to contribute to the semilunar valves, based on Wt1-Cre fatemapping studies [31, 32]. Recent studies have reported that bone marrow-derived stem cells (BMSCs) are present in the developing and mature semilunar valves [33, 34]. Additional work is necessary to determine if these cells have lineages and functions distinct from the predominant endocardial cushion-derived or neural crest-derived VICs. It is possible that valve cell lineages derived from different developmental sources have distinct functions in normal and diseased aortic valves, but this has not yet been demonstrated. The sources of increased proliferative cells in diseased valves are relatively unknown, but could be any of these embryonic sources or, alternatively, an infiltrating cell type.

2.3. Transcription factors involved in valve development

Several transcription factors have been implicated in various processes of endocardial cushion formation and EMT (reviewed in [8, 35]). Notch pathway function in EMT is dependent on the transcription factor RBPJ, which activates expression of the bHLH transcription factor *snail1* (*Snai1*) in endothelial cells [36]. *Snai1* represses *ve-cadherin* gene expression, and loss of *Snai1* in endothelial cells inhibits endocardial cushion formation [36, 37]. The mesenchymal valve progenitor cells of the endocardial cushions express several transcription factors characteristic of a variety of embryonic mesenchymal progenitor populations. These factors include, *Twist1*, *Msx1/2*, *Tbx20*, and *Sox9* [18, 38–41]. Gain and loss of function studies have demonstrated critical roles for *Tbx20*, *Twist1*, and *Sox9* in endocardial cushion mesenchymal cell proliferation [38–40]. *Twist1* promotes *tbx20* expression directly and also regulates several genes associated with cell proliferation and migration [38, 42]. After endocardial cushion fusion and formation of valve primordia, mesenchymal genes, notably *twist1* [43], are down-regulated and cell proliferation is decreased [23, 24, 44]. In normal adult valves, there is little to no cell proliferation [24, 44], and expression of valve developmental transcription factors including *Twist1*, *Sox9*, and *Msx2* is not detectable [13]. However, all of these factors are predominantly expressed in adult human CAVD (see below).

Additional regulatory pathways control heart valve ECM remodeling and compartmentalization. Loss of NFATc1 results in defective remodeling of the AV and SL valves in mice, with embryonic lethality by E14.5 [45, 46]. EMT occurs with loss of NFATc1, but valve primordia fail to remodel and mature ECM molecules are not expressed in null mice or in cultured VICs with inhibition of receptor activator of nuclear factor κ -B ligand (RANKL) or calcineurin signaling upstream of NFATc1 activation [45, 47]. In addition to being required for endocardial cushion mesenchymal proliferation, *Sox9* also promotes cartilage-like ECM gene expression in valve progenitor cells [48]. In late stage mouse embryos, loss of *Sox9* in remodeling valves results in reduced proteoglycan expression, and *Sox9* haploinsufficiency in adults leads to valve calcification [40, 49]. Conversely, the bHLH transcription factor *Scleraxis*, critical for tendon development, promotes expression of elastic/tendon-like matrix genes in cultured valve progenitor cells [48]. Loss of *Scleraxis* in mice is not lethal, but heart valve defects similar to myxomatous valve disease occur in these animals [50]. Little is known of the transcriptional regulatory mechanisms that control the development of the valve fibrosa layer, which is most critically involved in CAVD.

2.4. Signaling pathways in valve development

Several essential embryonic signaling pathways have been implicated in endocardial cushion formation and EMT (Table 1) (reviewed in [8]). Transforming growth factor (TGF) β signaling was the first pathway implicated in endocardial cushion formation and is required for EMT in chicken and mouse embryonic systems (reviewed in [16]). BMP signaling from the myocardium is required in endothelial cells for the initiation of EMT in the AV canal, and BMP2 and 4 are the predominant ligands involved in endocardial cushion development [18–20]. Notch signaling also is required for EMT as described above. Moreover, Notch signaling is required for expression of TGF β ligands and receptors, in addition to activating

BMP signaling, which promotes mesenchymal cell invasion [36, 51]. Likewise, vascular endothelial growth factor (VEGF) signaling promotes endocardial cushion endothelial cell proliferation and EMT [47, 52]. Furthermore, targeted mutagenesis of β -catenin has implicated Wnt/ β -catenin signaling in endocardial cushion EMT and mesenchymal proliferation [53, 54]. Thus multiple pathways are involved in endocardial cushion EMT and mesenchymal cell proliferation. However, the intersection and specific cellular functions of these pathways have not been fully determined.

A. Signaling pathways			
	Role in valvulogenesis	Role in osteogenesis	Role in CAVD
VEGF	EMT, endothelial proliferation	angiogenesis	angiogenesis
Notch	EMT	Inhibit OB differentiation	represses calcification
TGF β	EMT	bone homeostasis	promotes VIC calcification
FGF	promotes tenascin expression	OB proliferation, differentiation	blocks VIC calcification
BMP	EMT, PG expression	promotes OB specification	active in CAVD
Wnt/ β -catenin	EMT, fibrosa expression	promotes OB differentiation	active in CAVD
RANKL	ECM remodeling	OC differentiation	promotes VIC calcification
B. Transcription factors			
	Role in valvulogenesis	Role in osteogenesis	Role in CAVD
Twist1	ECC proliferation, migration	represses differentiation	active in CAVD
Msx2	EMT, proliferation	present in progenitors, OB	active in CAVD
Sox9	proliferation, PG expression	progenitor proliferation, cartilage differentiation	active in CAVD inhibits calcification
NFATc1	endothelial proliferation, ECM remodeling	promotes OC differentiation promotes OB differentiation	reported in CAVD
Runx2	not present	promotes OB differentiation	active in CAVD
Osterix	not present	promotes OB differentiation	reported in CAVD

^aPlease see text for details and references. ^bAbbreviations used: CAVD=calcific aortic valve disease; ECM=extracellular matrix; EMT=endothelial to mesenchymal transition; OB=osteoblast; OC=osteoclast; PG=proteoglycan; VIC=valve interstitial cell.

Table 1. Signaling pathways and transcription factors involved in valvulogenesis, osteogenesis, and CAVD^{a, b}

Many of the signaling pathways important for endocardial cushion formation also have later functions in valve lineage diversification, remodeling, and stratification. However, these functions have been difficult to elucidate due to limitations of available genetic tools and

critical requirements for these same regulatory pathways in endocardial cushion formation. BMP signaling, as indicated by phosphorylation of the intermediate signaling molecules Smad1/5/8, is active throughout endocardial cushion mesenchymal cells, is associated with mesenchymal cell proliferation [55], and also is active later in valve cell lineage diversification [48]. *BMP Receptor II* mutations and conditional mutagenesis results in thickening of semilunar valve leaflets at late fetal stages [56, 57]. Loss of inhibitory Smad6 leads to increased BMP signaling, in addition to thickening of valve leaflets and CAVD in adult animals [58]. Studies in explanted avian valve progenitors have revealed antagonistic regulatory roles for BMP and fibroblast growth factor (FGF) signaling in promoting diversified ECM gene expression, conserved with mechanisms that control cartilage and tendon lineage development [11, 48, 59]. Wnt pathway activation is evident throughout the remodeling AV and semilunar valve primordia, as indicated in TopGal reporter mice [60]. Multiple Wnt ligands are expressed during valvulogenesis, but the function of Wnt signaling in heart valve remodeling has not yet been determined [60]. Thus, additional in vivo studies are necessary to determine the specific functions and intersecting regulatory mechanisms of these critical signaling pathways in valve leaflet development and also to determine specific contributions to valve degeneration and disease.

The later stages of heart valve development are characterized by leaflet elongation, ECM remodeling, and stratification, all of which are critical for mature valve structure and function [24]. Limited information is available on the regulation of these processes, but several regulatory pathways have been implicated in late valve remodeling and morphogenesis. Strikingly these same pathways have been implicated in adult CAVD (see below). RANKL, expressed by valve endothelial cells, promotes ECM remodeling and Cathepsin K (*Ctsk*) expression by NFATc1 in a mechanism partially conserved with osteoclast differentiation and function [11, 47, 61]. The signaling mechanisms that control stratification and ECM organization of the valve leaflets are relatively unknown. Notch signaling is localized on the ventricularis surface of the remodeling aortic valve in mice [62], and Wnt/ β -catenin signaling is active throughout aortic valve primordia at late gestation and in a subpopulation of VICs after birth [60]. Likewise, Wnt signaling promotes expression of fibrosa genes *periostin* and *matrix gla-protein (mgp)* in cultured chicken embryo aortic VICs, but a role in valve stratification or lineage diversification has not yet been established in vivo [60]. Additional studies are necessary to demonstrate the specific functions and potential biomechanical stimulation of these pathways in an in vivo context. Since, both Notch and Wnt signaling pathways are required for initial stages of endocardial cushion formation, it has been difficult to establish their roles in the later stages of valvulogenesis in vivo using available conditional targeting approaches.

Bicuspid aortic valve is arguably the most common congenital heart malformation with an incidence of 1-2% in the US adult population [63]. BAV often does not often manifest in valve dysfunction in early life, but malformed aortic valves are predisposed to calcification. Strikingly, the majority of stenotic aortic valves that are replaced in adults are congenitally malformed [4]. However, the molecular and cellular mechanisms of BAV are not well defined. In humans, mutations in *NOTCH1* are associated with BAV, but the mechanisms by which valve leaflet number is regulated by Notch signaling have not yet been identified [64]. Likewise, Notch1 haploinsufficiency in mice leads to BAV at very low penetrance and there

are likely to be additional factors necessary for congenital malformation of the aortic valve leaflets [65]. Loss of the zinc finger transcription factor GATA5 in mice [66] and mutations in human *GATA5* [67] are associated with BAV with incomplete penetrance. Likewise eNOS haploinsufficiency also leads to BAV, albeit with incomplete penetrance [68]. The mechanisms by which these genetic lesions lead to BAV in some individuals and not others are not known. However, based on the expression and function of Notch1, GATA5, and eNOS in endothelial cells, it is likely that these cells contribute to development of BAV in these models. The link between BAV and CAVD could be due to similar regulatory mechanisms in development and disease or could, alternatively, result from induction of calcification in a hemodynamically compromised congenitally malformed aortic valve (see other chapters for a more complete discussion of BAV and CAVD).

2.5. Extracellular matrix composition and stratification of the developing valves

The mature valve leaflets are composed of stratified ECM with layered compartments of fibrillar collagen, proteoglycan, and elastin (Figure 1) (reviewed in [10, 69]). During heart valve remodeling, there is little proliferation of VICs, but the cells are highly synthetic and produce multiple ECM proteins of the mature leaflets [24, 44]. The distinct layers of matrix are integral to heart valve function and confer specific biomechanical properties to the valve leaflets [69]. The regulatory mechanisms for ECM remodeling and stratification are not well defined but are relevant to heart valve disease mechanisms. Periostin is required for collagen remodeling, and loss of periostin in mice leads to adult valve malformations and cardiac dysfunction [70, 71]. Likewise, mutations in *Collagen 1a2* or elastin haploinsufficiency also result in aortic valve dysmorphogenesis and adult disease [72, 73]. Gene expression of *CtsK*, a matrix remodeling enzyme expressed during heart valve elongation, is regulated by the RANKL/NFATc1 regulatory pathway [47, 61]. Additional ECM remodeling enzymes, including matrix metalloproteinase (MMP)13, a collagenase, and Adam-TS5 and 9 proteoglycan proteases, also are expressed during late valve morphogenesis and have been implicated in ECM maturation and organization [39, 74, 75]. Several ECM molecules required for normal valve structure/function also are expressed during osteogenesis, and valve progenitors have gene expression profiles similar to bone progenitors [43]. *Osteopontin*, *osteonectin*, and *periostin* gene expression and collagen fiber deposition are increased during heart valve remodeling [24, 43, 60]. However, the regulatory mechanisms for expression of these genes in valve development are not well defined. These proteins also are induced and mislocalized in pediatric and adult heart valve disease [13, 24, 70], but the pathways leading to their dysregulation have not yet been fully characterized.

3. Molecular mechanisms of osteogenesis

3.1. Overview of skeletal development

Many osteogenic regulatory interactions identified in developing bone also are active in CAVD (Table 1). The regulatory hierarchies and ECM composition of the developing valves,

most notably the collagen rich fibrosa layer, are similar to those observed in osteoblast precursor cells [43]. Both the bone substratum and valve ECM are composed primarily of fibrillar collagen. Thus, it is not surprising that there are extensive similarities in their composition and developmental regulation. Normally, heart valves do not progress to mineralization, but striking similarities have been identified between osteogenic pathways that regulate bone mineralization and CAVD mechanisms [7]. Thus the molecular understanding of normal development of bone has clear implications for pathogenic mechanisms of connective tissue mineralization, including CAVD.

The osteogenic precursors of the developing axial skeleton and long bones of the limbs are derived primarily from paraxial mesoderm of the developing somites and also lateral plate mesoderm, the main source of cardiac precursor cells [76, 77]. Additional progenitors of the craniofacial skeleton are derived from cranial neural crest [78]. Most axial skeletal elements develop by endochondral bone formation that occurs through a cartilage intermediate [76, 77]. Alternatively, the craniofacial bones of the skull form through membranous ossification in which condensed osteogenic progenitors differentiate directly into bone and do not go through a cartilage intermediate [76]. The osteochondroprogenitors present in the axial and appendicular skeletal elements develop into both bone and cartilage lineages [77, 79, 80]. Extensive research over the past several years has defined transcriptional regulatory mechanisms and signaling events that control the development of cartilage and bone (Figure 2) [79, 80].

Mature cartilage is composed predominantly of chondroitin sulfate proteoglycans that provides cushioning and flexibility to cartilaginous structures [77, 81]. In addition, the proteoglycan-rich ECM is angiostatic and mature cartilage is avascular [81]. Interestingly, the predominant proteoglycan composition and lack of vasculature also are features of the mature aortic valve leaflet spongiosa layer [82]. Likewise, the cartilage ECM inhibits mineralization, and a similar role has been hypothesized for the proteoglycan-rich matrix of the aortic valve [49]. During normal axial bone development, osteoblasts from the laterally placed periosteum differentiate into trabecular bone, and secondary ossification centers at the ends of the bone displace the growth plate hypertrophic cartilage [79, 80]. During bone differentiation, hypertrophic cartilage cells must die for mineralization to occur in a process of endochondral ossification, which could be related to dystrophic mechanisms of CAVD [79, 80].

Bone cell lineage maturation goes through multiple stages defined by molecular regulatory mechanisms that also are active in valve development and disease processes [80]. Osteochondroprogenitor cells express several mesenchymal transcription factors, including Twist1, Msx2, and Sox9, that also are predominant in valve progenitor cells and diseased aortic valves [79]. Immature pre-osteoblasts express high levels of fibrillar type 1 collagen, in addition to periostin, osteonectin, and osteopontin, similar to normal differentiated VICs [43, 60]. Differentiated osteoblasts are not yet mineralized but express the transcription factor Runx2, in addition to osteocalcin and bone sialoprotein involved in bone mineralization and also in valve calcification, [7, 80]. Later stage osteoblasts and osteocytes express the transcription factor Osterix (Osx), which is regulated by Runx2 and is required for mature bone

formation [83]. Bone mineralization occurs with the deposition of calcium phosphate and hydroxyapatite by osteocytes and is dependent on Runx2 and Osx function [80]. Bone homeostasis is maintained throughout life by the osteogenic activity of osteocytes and bone resorption activity of osteoclasts [80].

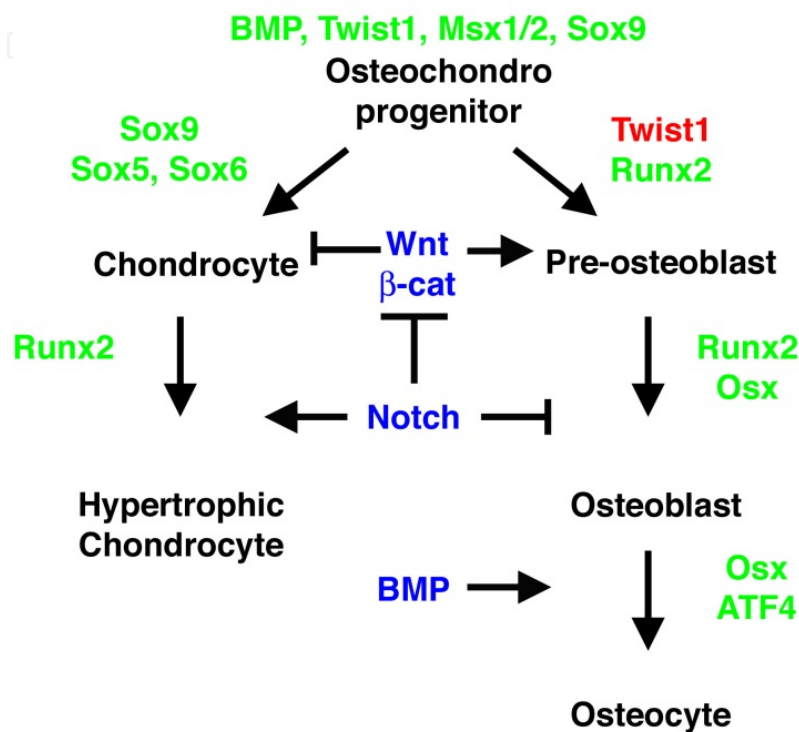


Figure 2. Hierarchies of signaling pathways and transcription factors regulate the differentiation of chondrogenic and osteogenic progenitor cells during skeletal development. Early osteochondrogenic progenitor cells express BMPs, Twist1, Msx1/2, and Sox9. Wnt/β-catenin signaling promotes pre-osteoblast differentiation while inhibiting chondrocyte differentiation. In contrast, Notch signaling promotes cartilage differentiation and inhibits osteoblast differentiation. BMP signaling is further required for osteocyte differentiation in the final stages of bone maturation. Sox5, 6, and 9 are transcription factors crucial for maintaining the chondrogenic lineage, whereas, Runx2, Osx, and ATF4 are transcription factors necessary for osteoblast and osteocyte differentiation and maturation. Many of these factors are also expressed during calcific aortic valve disease and have been implicated in pathologic calcification. Please see text for details and references. Activating factors are shown in green, inhibitory factors are shown in red, and signaling pathways are indicated in blue.

3.2. Transcriptional regulation of osteoblast lineage development and bone differentiation

Twist1 is expressed early in the osteochondroprogenitor lineage and inhibits terminal differentiation of cartilage and bone [84]. In preosteoblasts, Twist1 binds to Runx2 and inhibits its transcriptional activation of bone differentiation genes including *osteocalcin* [84]. Similarly, Twist1 can inhibit cartilage differentiation by binding to Sox9 and preventing activation of cartilage-specific gene expression [85]. Mutations in human *TWIST1* cause Saethre-Chotzen syndrome, characterized by premature bone differentiation evident in premature fusion of

cranial sutures of the skull [86]. *Msx2* also is involved in early mesenchymal stages of osteochondroprogenitor development and is down regulated during osteoblast differentiation [79]. Persistent *Msx2* expression in osteoblasts prevents differentiation and mineralization, while antisense mRNA-mediated loss of *Msx2* accelerates these processes [87]. Thus *Msx2* is expressed in osteoblast progenitor cells but has an inhibitory role in osteogenic differentiation. Together *Twist1* and *Msx2* act to maintain undifferentiated osteochondroprogenitors during development.

Sox9 functions in the expansion of cartilage progenitors and promotes cartilage differentiation, while inhibiting bone differentiation [77, 80]. *Sox9* is required for osteochondroprogenitor lineage specification but is not expressed in differentiated osteoblasts [88]. At early stages of cartilage lineage development, *Sox9* promotes cell proliferation and later is required for cartilage lineage differentiation [88]. BMP signaling induces *Sox9* gene expression in cartilage progenitor cells [89], and *Sox9* regulates expression of cartilage marker genes *Col2a1* and *aggrecan* [90, 91]. *Sox9* transcriptional activity can be inhibited by binding to *Twist1*, thus inhibiting differentiation of early stage osteochondroprogenitor cells [85]. At later stages of cartilage maturation, *Sox9* inhibits *Runx2* transcriptional activity, thus promoting hypertrophic cartilage and inhibiting osteogenic differentiation [92]. Thus downregulation of *Sox9* is required in osteoblasts for differentiation and mineralization of bone.

Runx2, originally called *Cbfa1*, has been defined as a master regulatory gene in bone formation [79, 93]. Gain and loss of function studies in mice demonstrate that *Runx2* is both necessary and sufficient for osteoblast differentiation [93]. During bone development, *Runx2* directly regulates *osteocalcin* gene expression [93]. *Runx2* transcriptional function can be inhibited by interaction with *Twist1* and also by *Hey1*, downstream of Notch signaling [84, 94]. Mice lacking *Runx2* lack mineralized bone, and haploinsufficiency of *Runx2* results in reduced bone formation in mice and humans [80]. Induction of a dominant negative form of *Runx2* in differentiated osteoblasts after birth also leads to reduced bone mineralization, demonstrating a role for *Runx2* in bone homeostasis and mineralization throughout life [95]. *Runx2* has not been implicated in normal heart valve development, and its expression in developing valves has not been reported, consistent with the lack of calcification in normal valves. Likewise, in adult valves *Runx2* is not normally expressed, but its expression is induced in CAVD in both humans and mice [13, 73]. The presence of *Runx2* in diseased aortic valves and association with calcification is consistent with a role in mineralization, as has been established for bone cell lineages.

NFATc1 is a critical transcription factor in osteoclast differentiation and also has been implicated in osteoblast development [80, 96]. Osteoclasts, derived from a macrophage lineage, have bone resorptive activity and are necessary for bone homeostasis [96]. During osteoclast development, RANKL signaling induces activation of NFATc1, which promotes the transcription of bone matrix remodeling genes including *CtsK* and *mmp9* [97, 98]. RANKL activity in bone is antagonized by the receptor decoy osteoprotegerin (OPG) that promotes bone calcification [99, 100]. In osteoblasts, NFATc1 promotes cell proliferation and also enhances differentiation by cooperating with *Osx* to promote *Col1a1* gene expression [101, 102]. Thus, the balance of RANKL and OPG signaling acting on NFATc1 transcriptional function is a

critical mediator of bone calcification and resorption [96]. A similar balance of OPG and RANKL signaling in CAVD has been proposed [103]. While NFATc1 is a critical regulator of heart valve remodeling during development and activates valvular *CtsK* expression [47, 61], its role in CAVD and adult valve homeostasis has not been determined.

Additional transcription factors involved in bone differentiation are not generally found in CAVD, although there are conflicting reports. Most notable is *Osx*, which is required for terminal differentiation of osteoblasts and mineralization of bone [83]. *Osx* promotes expression of *collagen 1a1* and the matrix metalloproteinase *mmp13*, which also are upregulated in aortic valve disease [73, 101, 104, 105]. Studies based on antibody staining demonstrate *Osx* expression in Notch signaling-deficient calcified mouse valves [65] and human CAVD [106]. *ATF4* is an additional transcription factor critical for bone differentiation, mineralization, and homeostasis that has not been found in developing or diseased valves [79]. Further studies are necessary to determine if *ATF4* or *Osx* gene expression is induced or if they contribute to valve mineralization in CAVD.

3.3. Signaling pathways involved in bone development

Multiple signaling pathways control the stages of bone cell lineage determination, differentiation and maturation [80, 107]. These include BMP, Wnt, and Notch pathways, also active in developing and diseased heart valves, as well as FGF, hedgehog, insulin-like growth factor (IGF), and retinoic acid (RA) pathways, not yet characterized in heart valve pathogenesis [80]. BMP, Wnt, and Notch pathways are required at multiple stages of osteogenesis and have distinct regulatory interactions that control transcription factor function and cell type-specific gene expression in cartilage and bone cell lineages (Figure 2). In addition, these pathways crosstalk with each other in synergistic and antagonistic regulatory interactions. Strikingly many of these same regulatory interactions occur in heart valve development and pathogenesis (Table 1) [8].

Bone morphogenetic proteins were originally identified based on their ability to induce ectopic bone formation [108]; however, *in vivo* functions in normal bone development are less clear [109]. In the developing limb buds, BMP signaling has a critical role in mesenchymal condensation, *Sox9* activation, and cartilage lineage differentiation [89]. Thus BMP signaling is an important regulator of the earliest stages of skeletal development. Later in differentiating osteoblasts, BMP signaling through Smad1/5/8 phosphorylation (pSmad1/5/8) promotes osteogenic differentiation and calcification [110]. Runx2 directly binds to activated Smads1 and 5 to cooperatively activate osteoblast gene expression in response to BMP signaling [109]. Conditional loss of BMP2 and BMP4 in the osteoblast lineage in mice inhibits late stage differentiation into *Osx*1-positive osteocytes, and BMP signaling is required for bone homeostasis after birth [80, 109]. Surprisingly, earlier stages of bone lineage development are apparently unaffected with conditional loss of these ligands.

Wnt/ β -catenin signaling is required for osteoblast differentiation as demonstrated by loss of osteoblast differentiation with conditional loss of β -catenin in osteochondroprogenitor cells in mice [80]. In addition, loss of β -catenin in pre-osteoblasts leads to ectopic cartilage formation, thus implicating Wnt signaling in osteogenic versus chondrogenic cell fate determina-

tion. At a molecular level, Wnt/ β -catenin signaling promotes osteoblast lineage differentiation, while inhibiting chondrogenesis, by activating Runx2, while inhibiting Sox9 [77]. In bone lineages, BMP and Wnt signaling act synergistically to promote calcification, although neither pathway alone is sufficient to induce a full osteogenic response [111]. During the initial differentiation of bone progenitor cells, regulatory elements of *Runx2* and *Msx2* genes are bound by Smad1, downstream of BMP signaling, and also by Lef1, activated by Wnt signaling, for cooperative gene activation [112]. Postnatally, Wnt signaling through the Lrp5 receptor is required for bone accrual in mice and humans [80]. In developing bone, osteogenic differentiation and calcification are dependent on sequential activation of BMP, followed by Wnt/ β -catenin, signaling [110]. It is possible that a similar regulatory relationship exists in CAVD, but this has not yet been demonstrated.

Notch activation inhibits osteogenesis through suppression of the Wnt/ β -catenin pathway and Runx2 transcription factor activity [94, 113, 114]. Loss of Notch1 or Notch2 function promotes osteoblast differentiation and leads to increased bone mass in mice [115]. Notch pathway activation inhibits the progression of osteoblast differentiation through direct binding of the activated Notch1 intracellular domain (N1ICD) to β -catenin, thereby counteracting Wnt-mediated induction of osteogenesis [113, 114]. In addition, the Notch target gene *Hey1* encodes a transcriptional repressor that binds and inhibits Runx2 transcriptional function [115]. Precise levels of Notch signaling are required for cell proliferation and chondrogenic differentiation, with defects in these processes occurring with increased or decreased Notch signaling in mice [116]. In early cartilage precursors, Notch signaling is required for cell proliferation, but increased Notch signaling inhibits terminal differentiation of chondrocytes and endochondral ossification [116]. Loss of Notch signaling has been implicated in CAVD [64], but it is not known if this occurs through inhibition of Wnt/ β -catenin signaling, as has been demonstrated for osteoblast differentiation and bone mineralization.

4. Molecular and cellular mechanisms of CAVD

4.1. Overview of CAVD progression

The mature aortic valves are comprised of three ECM layers critical for normal leaflet structure and function [24, 44, 117]. Collagen predominates in the fibrosa layer, which is oriented on the opposite side of blood flow, whereas elastin is enriched in the ventricularis layer on the flow side of the valve. Between the fibrosa and ventricularis layers, is the proteoglycan-rich spongiosa layer [24, 44, 117]. This trilaminar ECM arrangement is preserved among species, and lends both strength and elasticity to the aortic valves [24]. In CAVD, the aortic valve becomes thickened and displays extensive ECM remodeling and mineralization [118-121]. Abnormal thickening (aortic valve sclerosis) and calcification of the aortic valve lead to stiffening of the valve leaflets and can reduce the effective valve opening (aortic valve stenosis), which can impede blood flow and lead to clinical symptoms such as syncope and angina [119, 122, 123]. Histologically, human explanted diseased aortic valves have

extensive ECM remodeling and elastic fiber fragmentation with evidence of both macroscopic calcific nodule formation as well as microscopic mineral deposits [119].

Changes in the resident VICs are apparent in CAVD. Under normal conditions, aortic VICs are quiescent and non-proliferative [13, 24, 104, 124]. However, in disease, a subset of aortic VICs exhibits features of myofibroblast activation, which is characterized by expression of α -smooth muscle actin (α SMA), MMP13, non-muscle myosin heavy chain (SMemb), and markers of proliferation [13, 104, 119, 124, 125]. In vivo, the factors responsible for inducing myofibroblast activation are not well defined. However, in culture, TGF β 1 stimulation and mechanical strain are potent inducers of VIC myofibroblast activation [125, 126]. Activated VICs also exhibit characteristics of valve and bone precursor cells as they induce expression of the common mesenchymal markers Sox9, Twist1, and Msx2 [13]. Currently it is unknown where the mesenchymal-like cells come from and what role these proliferative cells play in the progression of CAVD pathogenesis.

Valve calcification, apparent as hydroxyapatite deposits on the surface of or within the leaflets, is a prominent feature of CAVD [119, 127, 128]. Histologically, valve calcific nodules are primarily acellular [13, 129]. Although traditionally thought to be a completely passive deposition of mineral, in some cases, valve calcification is coincident with endochondral bone-like and cartilaginous-like nodules [129, 130]. Aortic valve calcification is observed primarily in the regions of the valves exposed to the greatest physical strain, specifically at the hinge region of the valve and along the line of leaflet coaptation [120]. Furthermore, calcification is predominantly found in the fibrosa layer of the diseased valve, which is similar to early bone matrix as it contains primarily fibrillar collagen [44]. Expression of other bone matrix molecules, such as osteocalcin and osteopontin, are induced during disease [5]. Furthermore, expression of osteogenic factors, such as Runx2, BMP2, and alkaline phosphatase, also is induced in VICs from calcified valves, suggesting that resident VICs may have the potential to undergo osteogenic transdifferentiation and actively contribute to valve calcification (reviewed in [131]).

Extrinsic factors have been implicated in valve calcification. For example, lipid deposition and immune cell infiltration are common histopathological features of CAVD, and it has been proposed that aortic valve calcification occurs by mechanisms similar to arterial calcification in atherosclerosis [119, 132-135]. In addition, altered external physical forces elicit changes in resident VICs, which play an active role in pathological valve calcification [126]. In contrast to VIC response to immune cell infiltration and altered physical forces, cell intrinsic mechanisms may also contribute to valve calcification, as stimulation with factors such as BMP2 or TGF β 1 in cell culture studies can induce VIC calcification in the absence of inflammatory stimulation or altered physical forces [126, 136-138]. Together, these studies suggest that not only is valve calcification an active cell-regulated process, but that many factors likely contribute to progression of calcification during disease. It is also likely that not all CAVD is created equal. Genetic predisposition, the presence of a malformed aortic valve, and other disease comorbidities, such as coronary artery disease,

hypertension, and kidney disease, likely affect the pathology and underlying cause of CAVD [64, 139-142].

4.2. Activation of progenitor cell and osteogenesis-related molecular pathways in CAVD

4.2.1. Expression of valve and bone progenitor cell genes in CAVD

The mesenchymal markers Twist1, Msx2, and Sox9 are expressed in adult calcific aortic valves in mesenchymal-like activated VICs [12, 13, 106, 143, 144]. As discussed, these genes are expressed in both valve and bone mesenchymal progenitor cells. A recent study has compared gene expression in pediatric versus adult aortic valve disease and shown that the mesenchymal markers Twist1, Msx2, and Sox9 are increased in both [13]. The observation that both pediatric and adult diseased valves have increased expression of the mesenchymal markers suggests that this expression is related to VIC activation and proliferation, which is common to both, and not related to valve calcification, which is found only in advanced adult disease [13]. In both valve and bone progenitors, Twist1, Msx2, and Sox9 induce proliferation and promote a mesenchymal phenotype, thus reactivation in diseased valves is suggestive of a similar role in valve pathogenesis [38, 40-42, 84, 88, 145]. Although it is presumed that resident VICs re-activate these early mesenchymal markers, other possibilities exist. EMT as a mechanism for VIC activation has not been established in CAVD, however, recent studies report EMT-like events in adult valves. Increased cyclic strain and altered hemodynamics, both recognized features of CAVD, can induce EMT in isolated sheep valve endothelial cells [146, 147]. In addition, cultured valve endothelial cells stimulated with TGF β adapt a mesenchymal-like phenotype and express markers of both endothelial and mesenchymal cells, suggesting that they can undergo EMT [148, 149]. Likewise, disruption of Notch signaling in adult mice induces aortic valve thickening with evidence of endocardial EMT, as indicated by endocardial cells with more pseudopodial projections, loose endocardial cell-cell junctions, and α SMA expression [150]. Additional sources of mesenchymal-like cells have been suggested. For example, circulating bone marrow-derived hematopoietic stem cells have been shown to integrate into the valve interstitium, adapt fibroblast-like characteristics, and surround regions of prominent valve calcification in human end stage CAVD [33, 130, 151]. It is uncertain what role reactivation of the mesenchymal markers Twist1, Msx2, and Sox9 have in potential valve repair mechanisms or in the progression of CAVD. It is possible that adult VICs maintain a certain “mesenchymal-plasticity” and are able to revert back to an early progenitor-like mesenchymal cell during disease. Alternatively, they may be indicators of newly derived VICs arising from EMT or circulating progenitor cell populations in response to disease conditions.

4.2.2. Osteogenic factors in CAVD

Molecular mechanisms of endochondral ossification and cartilaginous nodule formation are active in CAVD [7, 13]. Studies in human explanted diseased aortic valve tissues have demonstrated increased expression of the osteogenic factors BMP2, TGF β 1, Runx2, osteocalcin (OCN), osteopontin (OPN), osteoprotegerin (OPG), bone sialoprotein (BSP), alkaline phos-

phatase (ALP), and *Osx* in disease [5, 13, 106, 118, 137, 152]. At a molecular level, BMP2 signaling is a key inducer of VIC calcification, which is thought to act through p-SMAD1/5/8 and phospho-ERK1/2 signaling to stimulate increases in both *Runx2* and *OPN* expression [138]. Induction of VIC calcification by BMP2 stimulation is highly reminiscent of BMP signaling in bone development, suggesting that some parallels exist between osteogenic bone formation and VIC calcification [9]. Histological studies of explanted human valves further support a role for BMP signaling in valve calcification. Comparison of pediatric diseased valves, which do not acquire calcification, and adult calcified valves demonstrates that increased BMP signaling, evident in p-SMAD1/5/8 activation, is exclusive to adult valves with calcification, indicating that BMP signaling may contribute to valve calcification in human disease [13]. Additionally, TGF β 1 is also a potent inducer of osteogenic-like differentiation of VICs in cell culture, as it stimulates VIC activation and calcification, increases ALP activity, and increases expression of ECM remodeling enzymes [126, 136, 137]. Negative regulators of valve calcification have been demonstrated through in vivo studies. One negative regulator of valve calcification is Notch signaling. Animals haploinsufficient for Notch signaling develop aortic valve calcification with increased BMP signaling and increased expression of *Runx2* in the valve leaflets [65, 153]. Studies in isolated aortic VICs further demonstrate that Notch signaling plays an important role in suppressing valve calcification as treatment of VICs with Notch inhibitors induces BMP signaling and subsequent increases in osteogenic gene expression [65, 153]. Another negative regulator of valve calcification is *Sox9*, which potentially acts through induction of proteoglycan expression, similar to what has been observed in developing cartilage [40, 49]. Conditional heterozygous *Sox9* mutant mice develop valve calcification along with increased valve thickness and expression of the osteogenic genes *Runx2*, *osteonectin*, *OPN*, and *OPG* [40, 49]. Based on these studies, it is apparent that many factors involved in endochondral bone formation are active in the process of aortic valve calcification.

CAVD has been linked to chronic kidney disease in human patients and animal models [140, 154-157]. A prominent pathological feature of kidney disease is the inability to regulate calcium and phosphate metabolism [158]. Increased blood phosphate levels (hyperphosphatemia) are highly associated with aortic valve sclerosis and valve calcification in humans [140]. *Klotho*-null mice are a model of accelerated aging that includes development of kidney failure and hyperphosphatemia, along with cardiovascular disease [159-162]. *Klotho*-null mice exhibit extensive valve annulus calcification with increased expression of osteogenic genes, but minimal CD68 positive macrophage infiltration [73]. Thus, valve calcification in the *klotho*-null animals parallels bone formation, where increases in crucial osteogenic genes, such as *Col10a1*, *Runx2*, *OPN*, and *BSP*, are observed [73]. These observations suggest that increased blood phosphate levels could be one stimulus for inducing advanced aortic valve calcification with osteogenic gene expression, but this has not been definitively demonstrated [73, 140].

Atherogenic lipid deposition and inflammation in the valves also has been linked to induction of osteogenic gene expression and disease [163-165]. Rabbit and mouse models of CAVD, induced with hypercholesterolemic or high fat diets, have increased lipid deposition

and macrophage infiltration associated with induction of osteogenic markers such as ALP, OCN, OPN, Runx2, and Osx [165-167]. Although osteogenic gene expression is induced in these models, this type of valve calcification closely mimics vascular calcification observed in atherosclerosis, rather than endochondral bone formation, due to the presence of extensive immune cell infiltration [165, 166]. In support, human aortic VICs stimulated with pro-inflammatory mimetics not only induce the expression of inflammatory cytokines, but also induce the expression of osteogenic factors, such as BMP2 and Runx2, again suggesting that this process may be similar to what is occurring in atherosclerotic disease [164, 168]. Based on this evidence, multiple physiologic factors likely contribute to osteogenic gene induction in calcified diseased aortic valves.

4.2.3. *Valvulogenic- and osteogenic-related signaling pathways in CAVD*

As in both heart valve and endochondral bone development, BMP, TGF β , Notch, and Wnt signaling have been implicated in the progression of CAVD (Figure 2; Table 1). Increased BMP ligand expression, particularly BMP2 and BMP4, has been demonstrated histologically in human explanted calcific aortic valves surrounding and throughout regions of valvular calcification [118, 129]. Furthermore, active BMP signaling, as indicated by pSMAD1/5/8 expression, is present in both human explanted diseased aortic valves and animal models of CAVD [13, 169, 170]. Comparison of pediatric diseased valves void of calcification to heavily calcified adult diseased valves demonstrates extensive ECM remodeling and evidence of VIC activation in both; however, increased pSMAD1/5/8 signaling is exclusive to calcified valves [13]. The observation that pSMAD1/5/8 expression is found only in adult calcified valves is suggestive of a critical role for BMP signaling as an initiating osteogenic factor in CAVD [13]. Furthermore, increased pSMAD1/5/8 expression reportedly localizes to the fibrosa layer of human calcific aortic valves, which is the primary sight of aortic valve calcification [169]. Cell culture studies support this and show that BMP2 stimulation promotes osteogenic-like aortic valve calcification in human aortic VICs by inducing the expression of the osteogenic factors Runx2, OPN, and ALP [138, 171]. Based on this evidence, active BMP signaling may be a potential therapeutic target to treat CAVD, however it has not yet been tested.

TGF β signaling induces α SMA expression and myofibroblast differentiation of porcine aortic VICs, suggesting that TGF β promotes VIC activation, potentially in response to physical strain [125, 172]. Furthermore, TGF β signaling may also have a role in aortic valve calcification, as human explanted calcific aortic valves have increased levels of TGF β 1 expression and ovine aortic VICs in culture calcify in response to TGF β 1 induction [136, 137]. TGF β signaling has also been linked to both Wnt/ β -catenin and FGF signaling pathways in CAVD [173, 174]. Specifically, FGF signals have been shown to induce MAPK signaling, which inhibits aortic VIC α SMA expression and myofibroblast response to TGF β [174]. In addition, TGF β stimulation of aortic VICs induces nuclear localization and activation of β -catenin, which promotes VIC myofibroblast differentiation [173]. Although the role of TGF β in CAVD is not well established in vivo, there is accumulating evidence for a role in VIC activation and calcification from studies in cell culture systems.

Whereas both BMP and TGF β signaling have been found to induce VIC calcification, Notch signaling has been implicated as a negative regulator of valve calcification. Familial studies demonstrated that Notch1 haploinsufficiency is associated with CAVD and aortic stenosis (AS) [64]. During development, Notch1 is expressed in the endothelial cells lining the aortic valve cusps and is also observed at lower levels in the VICs, and this expression pattern is maintained into adulthood [64, 175]. Histological analysis of human explanted aortic valves demonstrates that activated Notch1 intracellular domain (NICD) expression is dramatically reduced in VICs directly adjacent to regions of aortic valve calcification [175]. This observation is consistent with a mechanism whereby Notch signaling inhibits valve calcification and downregulation of Notch expression promotes valve calcification [175]. The idea that Notch signaling functions as a negative regulator of calcification was originally defined in endochondral bone formation, where downstream effectors of Notch signaling, Hes1 and Hey1, repress Runx2 transcriptional function, leading to expansion of hypertrophic cartilage and impaired osteoblast differentiation [115]. Notch1 heterozygous or RBPJ heterozygous mice develop CAVD, as evidenced by increased aortic valve calcification, and also display significant increases in BMP/pSMAD1/5/8 signaling and Runx2 expression in the aortic valves [65, 153]. Likewise, deletion of RBPJ in adult mice results in increased aortic valve thickness with evidence of VIC proliferation and potentially, endothelial EMT [150]. Together these in vivo studies support the idea that Notch signaling represses BMP expression, thereby indirectly repressing other osteogenic factors [65, 153]. Cell culture studies indicate that Notch inhibition promotes calcification of VICs by repressing chondrogenic genes, including *Sox9*, and inducing expression of the osteogenic genes *OPN*, *osteonectin*, *Runx2*, *ALP*, and *BMP2* [65, 153, 175]. Specifically, Notch signaling in the aortic valves is thought to induce expression of *Sox9*, which is a negative regulator of calcification, and to repress the expression of both *Runx2* and *BMP2*, which are known to stimulate osteogenic differentiation [64, 153, 175]. These studies suggest that, in the absence of a negative regulator of calcification, the resident VICs possess an intrinsic calcification mechanism, which becomes activated and subsequently induces valve calcification. Combined, the evidence suggests that Notch signaling is a negative regulator of VIC osteogenic differentiation, and that the absence or dysregulation of Notch signaling can induce valvular calcification.

Wnt/ β -catenin signaling is important for osteoblast maturation during embryonic development and contributes to mineralized bone formation (reviewed in [80]). A number of studies have also shown activation of Wnt/ β -catenin signaling in aortic valve calcification. Canonical Wnt signaling acts through the frizzled receptors and the Wnt co-receptors Lrp5 and Lrp6, resulting in β -catenin nuclear localization and TCF/LEF1 activation [176]. Human explanted calcific AoVs have increased expression of Lrp5, β -catenin, and Wnt3a ligand as compared to control valves [143]. Increased Wnt signaling in diseased aortic valves also has been observed in multiple animal models of CAVD. Pigs and rabbits maintained on an atherogenic diet develop aortic valve disease and display increased expression levels of β -catenin and Lrp5 receptor [173, 177]. Likewise, in a subset of endothelial nitric oxide synthase (eNOS) deficient mice that develop BAV, expression of Wnt3a ligand and Lrp5 receptor is increased when the animals are fed a high cholesterol diet [178]. Cell culture studies also support the idea that Wnt/ β -catenin signaling is important for VIC myofibroblast activation,

proliferation, and chondrogenic gene induction. Studies in porcine aortic VICs show that Wnt3a treatment induces significant VIC proliferation and myofibroblast activation [173, 179]. Furthermore, Wnt3a treatment of embryonic chicken aortic VICs results in increased expression of *periostin* and *mgp*, but does not induce the expression of osteogenic-related genes, suggesting that Wnt3a signaling is not sufficient for VIC osteogenic differentiation [60]. However in adult valves, Wnt signaling can promote the VIC calcification response, as loss of Wnt signaling through the Lrp5 receptor in ApoE knockout mice results in decreased aortic valve calcification [180]. Together these studies demonstrate that Wnt signaling likely contributes to VIC activation, proliferation, and calcification in CAVD.

4.3. Matrix remodeling in CAVD

Diseased aortic valves are characterized by changes in the ECM; in particular, disorganized collagen bundles and extensive elastic fiber fragmentation are observed [181, 182]. Insight into the role of elastin fiber disorganization in the pathogenesis of CAVD has been provided through studies of elastin haploinsufficient mice, which display elastin fiber fragmentation, abnormal ECM remodeling, and increased valve stiffness, suggesting that elastin homeostasis is important for maintaining valve function [72, 183]. Collagen synthesis and remodeling are dramatically increased in CAVD, however, overall collagen content in the valve is actually decreased, suggesting that there is extensive collagen proteolysis during disease [184–186]. In contrast to collagens, expression of proteoglycans, including decorin, biglycan, versican, and hyaluronan, is increased particularly in regions of the diseased valve adjacent to calcific nodules [187]. These changes in ECM composition during CAVD can be compared to matrix remodeling events that occur during valve development and also in bone formation. The decreased collagen content and increased proteoglycan matrix found in CAVD is similar to the primitive ECM characteristic of early valve development [188]. Furthermore, parallels can also be drawn between matrix remodeling in CAVD and bone development. Specifically, matrix remodeling in the immature bone is essential for providing a scaffold upon which the calcified matrix is deposited, and subsequent ECM degradation is essential for expansion of the calcified regions of newly forming bone [189]. The parallels between matrix remodeling in bone development and the disease process of CAVD suggest that valve matrix remodeling may contribute to valvular calcification.

Matrix degradation and remodeling in valvulogenesis, osteogenesis, and CAVD occurs concomitant with increased activity of MMPs and cathepsins, along with increased RANKL signaling. A number of studies have shown significant increases in expression of multiple MMPs, including MMP1, MMP3, MMP7, MMP9, and MMP12, with increased cathepsins B, K, and S in human calcific diseased aortic valves, suggesting that extensive ECM remodeling is a key feature of disease [124, 163, 181, 182, 184, 190]. In bone, RANKL signals through the RANK receptor, which can be inhibited via binding to the soluble receptor OPG, and promotes the expression of proteolytic enzymes, such as MMPs and cathepsin K, through activation of NFATc1 [191, 192]. A similar mechanism has been identified in heart valve remodeling [47, 61]. Comparison of sclerotic diseased aortic valves and advanced stenotic aortic valves determined that OPG levels are significantly higher in sclerotic valves without

calcification, whereas RANKL expression is higher in stenotic calcified valves [103, 193]. This study concluded that OPG may be protective against valve calcification, whereas elevated RANKL expression may promote valve calcification by promoting upregulation of matrix remodeling enzymes [103, 193]. Furthermore, treatment of human aortic VICs with RANKL results in increased MMP1 and MMP2 activity with increased VIC proliferation, concomitant with increased calcification and osteogenic gene expression [103, 191]. In addition, NFATc1 expression is increased in human explanted aortic valve leaflets with CAVD [106]. Together, these studies are consistent with signaling events during bone development, namely RANKL activation of NFATc1, stimulating matrix remodeling enzymes, and promoting calcification [192].

A number of other signaling pathways are likely involved in ECM changes that occur during CAVD. In particular, TGF β 1 stimulation of cultured VICs stimulates myofibroblast differentiation, leading to increased levels of α SMA stress fibers in the VICs [125, 172]. It has been suggested that these myofibroblasts then exert a contractile force on the surrounding valve ECM and stimulate rearrangement of the matrix, particularly in fibronectin fibers [125]. Furthermore, TGF β 1 stimulation also induces increased type I collagen production and expression of the matrix remodeling enzymes MMP9 and MMP2 in cultured aortic VICs [136, 172]. These studies indicate that TGF β 1 signaling may be a key factor in ECM-related changes during CAVD pathogenesis. Moreover, Wnt signaling may work in concert with TGF β 1 to induce changes in ECM during CAVD [173]. TGF β 1 stimulation promotes nuclear localization and activation of β -catenin in cultured VICs, and, when combined, Wnt and TGF β 1 signaling dramatically increases myofibroblast activation [173]. In contrast to TGF β 1 and Wnt signaling, FGF signaling may work to inhibit ECM remodeling during valve disease. FGF signaling has been shown to block TGF β 1 induced myofibroblast differentiation and α SMA expression in porcine aortic VICs through activation of phospho-ERK1/2 signaling [174]. In addition, FGF signaling inhibits myofibroblast contraction of a collagen matrix, supporting the idea that FGF signaling blocks TGF β 1 stimulation of matrix-related changes [174]. Many parallels exist between signaling factors involved in ECM changes in development and disease. In particular, RANKL, TGF β 1, Wnt, and FGF signaling have demonstrated roles in ECM production and regulation in both heart valve and endochondral bone formation [8, 76]. The shared signaling pathways in these tissues, both in development and disease, suggest that developmental pathways may be reactivated in CAVD to induce matrix changes characteristic of the disease.

5. Therapeutic mechanisms in CAVD

Currently, aortic valve replacement surgery is the only effective treatment option for CAVD [122]. There have been numerous studies, which are summarized below, testing the effectiveness of different pharmacotherapies on preventing the progression of AS. Unfortunately, studies on statin therapies, inhibitors of the renin-angiotensin-aldosterone system, and osteoporosis treatments have not been proven to be effective at preventing the symptoms or the progression of CAVD/AS. Following the summary of these studies, additional treatment

options, related to the expression of developmental and osteogenic-related genes in CAVD, are discussed.

5.1. Statins

Lipid deposition and the accumulation of apolipoproteins (Apo) in the aortic valve leaflets have long been associated with CAVD, and many studies have compared the progression of CAVD to atherosclerotic disease [119, 133, 134, 194]. Therefore, it has been hypothesized that cholesterol lowering therapy with statin drugs may be an effective treatment strategy to delay the progression of CAVD. A specialized mouse model called “Reversa” mice develop signs of CAVD when fed a high cholesterol diet, however, when serum cholesterol is lowered via a genetic deletion of the microsomal triglyceride transfer protein (Mttp), reduced levels of aortic valve calcification, as well as decreased expression of the osteogenic markers pSMAD1/5/8, *Msx2*, *Osx*, β -catenin, and *Runx2*, are observed [167, 170]. Thus reducing plasma cholesterol may reduce CAVD pathogenesis, particularly in terms of reducing osteogenic gene expression in the diseased valves. Similarly, statin treatment of human or porcine aortic VICs cultured concomitantly with osteogenic media results in decreased expression of the osteogenic genes ALP, OCN, *Lrp5*, and OPN, and reduced calcific nodule formation [171, 177, 195]. However, when statin treatment of aortic VICs is initiated after osteogenic transformation or calcific nodule formation, it is ineffective at reducing calcification and expression of osteogenic markers, indicating that statin therapy cannot reverse aortic valve calcification and osteogenic differentiation once it has occurred [195, 196]. Results from animal studies are equally contradictory. Rabbits fed a high cholesterol diet supplemented with atorvastatin have decreased aortic valve thickness, reduced VIC proliferation, and reduced expression of *Lrp5*, β -catenin, OPN, *Runx2*, and ALP, compared to those animals fed only a high cholesterol diet [165, 177]. Similarly, endothelial nitric oxide synthase (eNOS) deficient mice, displaying a BAV phenotype and fed a high cholesterol diet, have reduced *Lrp5* and *Wnt3a* expression as well as reduced aortic valve calcification when treated with statins, compared to animals fed only a high cholesterol diet [178]. In contrast, a long term study in rabbits fed a high cholesterol diet showed that atorvastatin therapy initiated after aortic valve disease is established is not effective at reducing the amount of aortic valve calcification present, although some improvements in other histological parameters were noted [197]. Based on both cell culture and animal studies, statin therapy may improve some measures of aortic valve calcification, specifically in terms of reducing osteogenic gene expression, however, firm conclusions as to potential efficacy as a CAVD treatment cannot be drawn.

Clinical studies investigating the use of statin therapy in patients with CAVD are also widely contradictory. An early study investigating the use of statin therapy in patients with moderate to severe aortic stenosis (AS) reported that patients treated with statins had less hemodynamic progression of AS over a 2 year time period than patients who were not on statin therapy [198]. In contrast, three larger prospective clinical studies, SALTIRE (Scottish Aortic Stenosis and Lipid Lowering Trial, Impact on Regression), SEAS (Simvastatin and Ezetimibe in Aortic Stenosis), and ASTRONOMER (Aortic Stenosis Progression Observa-

tion: Measuring Effects of Rosuvastatin), found that statin therapy was not effective at treating the progression of AS [199-201]. In these trials, three different statin therapies were investigated in patients with mild to moderate AS and it was determined that statin therapy did not alter the progression of CAVD/AS nor prevent outcomes such as the necessity to undergo aortic valve replacement surgery [199-201]. In response to the negative outcomes of these large clinical trials, the use of statin therapy was next investigated in patients with the earliest form of CAVD/AS, aortic valve sclerosis, to determine if statin use could prevent, rather than reverse, AS [202]. In this report, statin therapy was significantly associated with a decreased development of AS and a decreased need for aortic valve replacement surgery, suggesting that statin therapy may be an effective treatment if started at the earliest stages of the disease, prior to any indication of valve calcification [202]. Based on these studies, it can be concluded that in humans, statin therapy is ineffective at preventing the progression of AS and reversing aortic valve calcification. However, statin therapy may be useful at preventing the onset of AS in patients with the earliest stages of aortic valve thickening.

5.2. Angiotensin converting enzyme inhibitors/Angiotensin receptor blockers

Another potential therapy to prevent the progression of CAVD/AS is the use of the anti-hypertensive angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB). Currently, ACEIs and ARBs are prescribed to treat hypertension, and function by acting on the renin-angiotensin-aldosterone system to ultimately inhibit the vasoconstrictor effects of angiotensin II [203]. Previous reports have identified the overlapping expression of angiotensin-converting enzyme (ACE) and angiotensin II in calcified human aortic valves surrounding regions of valvular calcification [133]. It has been hypothesized that ACE inhibition may prevent the progression of CAVD by reducing ACE activity in the diseased valve leaflet [204-206]. A study conducted in ApoE knockout mice with induced chronic renal failure concluded that animals treated with the ACEI enalapril had significantly reduced levels of pathologic aortic valve leaflet thickening and valve fibrosis than untreated animals [206]. Similarly, in a rabbit model of CAVD in which the animals were fed a high vitamin D diet, treatment with the ACEI ramipril significantly reduced the progression to AS, improved valve endothelial cell integrity, and reduced aortic valve calcification [205]. It is uncertain how ACE and angiotensin receptor (AR) inhibition would directly affect molecular changes in the valve leaflets. However, in a study of rabbits fed a high cholesterol diet, treatment with the ARB olmesartan decreased the number of α SMA positive myofibroblasts and reduced expression of the osteogenic markers *Runx2* and *OPN*, compared to untreated control animals [204]. These animal studies suggest that ACE and/or AR inhibition may reduce pathologic changes in aortic valve disease by limiting valve fibrosis, reducing myofibroblast activation, and decreasing osteogenic gene expression.

Clinical studies testing the therapeutic benefits of ACEIs and ARBs in CAVD progression have had mixed results. In human explanted aortic valve tissues, ARB therapy is associated with reduced aortic valve remodeling and calcification [207]. As in animal studies, this histological analysis suggests that AR inhibition may limit aortic valve calcification [207]. In a small pilot clinical study (Symptomatic Cardiac Obstruction – Pilot Study of Enalapril in

Aortic Stenosis), use of the ACEI Enalapril was associated with improved clinical symptoms in patients with severe symptomatic AS [208]. The majority of studies investigating the use of ACEIs or ARBs in CAVD/AS with positive outcomes have been retrospective. In three different retrospective studies, ACEI or ARB use in patients with mild to moderate AS was associated with decreased mortality, decreased number of adverse cardiovascular events, slower progression of AS, and less accumulation of valvular calcification [209-211]. Additionally, one prospective study followed a small random population of patients over a 4-year period and reported that the use of ACEIs or ARBs was significantly associated with reduced CAVD/AS disease progression [212]. Together, these studies provide evidence that ACEI and ARB may delay CAVD progression. Conversely, there have also been a number of studies that show no association between ACEI and ARB use and improved outcomes in CAVD progression. The JASS study (Japanese Aortic Stenosis Study) reported that ARB therapy in patients with moderate to severe AS had no beneficial outcomes in CAVD progression, although patients with mild asymptomatic AS had some indication of reduced progression to AS [213]. Furthermore, a large study in patients with very mild asymptomatic AS found that patients on ACEI or ARB therapy had no improvement in the progression of AS compared against a control group [202]. Similarly, a small 2-year study observed no difference in the hemodynamic progression of AS with ACEI use versus non-use [198]. Based on both animal and clinical studies, it is unclear whether ACEI or ARB therapy is an effective treatment option to prevent the progression of CAVD/AS, however there are indications that perhaps this therapy may limit valve calcification [204, 207]. A placebo controlled, blinded trial will be necessary to determine the effectiveness of these therapies in treating CAVD.

5.3. Aldosterone-receptor antagonists

Aldosterone is a component of the renin-angiotensin-aldosterone system that plays a key role in the kidney to regulate water and sodium reabsorption and effectively raise blood pressure [203]. Aldosterone-receptor antagonists (ARA) are commonly prescribed for their diuretic effects [203]. Recently, there have been two studies investigating the use of ARAs in the treatment of CAVD/AS. In an animal study, rabbits fed a high cholesterol diet develop aortic valve sclerosis, with thickening of the valve leaflets and microscopic calcific deposits, which was blocked by treatment with the ARA eplerenone [214]. In addition to reducing valve fibrosis and mineralization, evidence of macrophage infiltration was also reduced [214]. Conversely, in a small placebo-controlled human trial of patients with moderate to severe asymptomatic AS, there was no difference in the progression of AS in those patients receiving the ARA eplerenone versus placebo [215]. To our knowledge, no molecular evidence has been reported in studies on ARA therapy in CAVD and it is unknown whether ARA therapy affects myofibroblast activation or osteogenic gene induction. Additional clinical studies will be necessary to determine if ARA use can prevent AS progression if therapy is started in early disease stages.

5.4. Bisphosphonates

Bisphosphonates (BP) are a class of drugs that mimic inorganic pyrophosphate and prevent ectopic soft tissue calcification and inhibit bone resorption [216]. In adults, especially women, BPs are commonly prescribed to treat excessive bone resorption associated with osteoporosis [216]. Human aortic VICs, grown on collagen gels in the presence of a specialized thiol bisphosphonate, have decreased ALP activity and reduced cellular aggregation, a step that precedes calcific nodule formation, as compared to cells grown on collagen alone [217]. This study suggests that bisphosphonates may inhibit VIC calcification in vitro and could serve as a potential therapeutic strategy to prevent aortic valve calcification [217]. Due to the ability of BPs to prevent ectopic calcification in bone and the availability of patient populations currently using BPs, a number of studies have investigated the use of BPs in the inhibition of aortic valve calcification. Three small retrospective human studies compared measurements of AS progression over a 2-year period in patients with AS taking BPs versus those not taking BPs [218-220]. The results of these studies suggest a modest reduction in the progression of AS in those patients taking BPs [218-220]. The large MESA study (Multi-Ethnic Study of Atherosclerosis) followed women taking BPs, compared to those not taking BPs, and their development of CAVD/AS over time [221]. The results of this study were mixed and showed that, in older women, BP therapy was associated with a slight benefit in terms of aortic valve calcification, whereas, younger women taking BPs had significantly more progression of aortic valve calcification compared to women not taking BPs [221]. Most recently, a large retrospective study investigated the progression of AS in women with mild to moderate AS over a 5-year period and compared the outcomes in patients on BP therapy versus those not taking BPs [222]. The evidence from this study shows that there was no change in survival, or in the number of aortic valve replacement surgeries, in women taking BPs compared to those not taking BPs, suggesting that BP therapy does not suppress the progression of CAVD/AS [222]. Thus far, the outcomes of the human studies investigating the use of bisphosphonate therapy in CAVD demonstrate that this therapy is ineffective at preventing or delaying the progression of CAVD/AS. To definitively determine whether or not BP therapy is effective at suppressing the progression of CAVD/AS, placebo-controlled prospective studies will be necessary.

5.5. Nitric oxide bioavailability

Endothelial nitric oxide synthase (eNOS) produces nitric oxide (NO) from L-arginine, and eNOS expression has been identified in the endothelial cells lining the aortic valves [68]. eNOS deficiency has been linked to defective aortic valve development, as approximately 50% of eNOS deficient mice develop a bicuspid, rather than tricuspid, aortic valve [68]. eNOS deficient mice with a BAV phenotype fed a high cholesterol diet develop hemodynamic symptoms of AS and also display microscopic mineralization in the aortic valve leaflets, indicating that eNOS activity may be important for suppressing aortic valve calcification [178]. Nitric Oxide deficiency is also an indicator of endothelial cell dysfunction, and systemic endothelial cell dysfunction is prevalent in patients with aortic valve sclerosis/stenosis [223-225]. The uncoupling, or dysfunction, of eNOS results in decreased NO pro-

duction and increased generation of reactive oxygen species (ROS) [223]. ROS activity is present in calcific lesions of human stenotic aortic valves, and it has been suggested that ROS activity may speed aortic valve calcification [144, 226]. In animal studies, rabbits fed a high cholesterol/high vitamin D diet develop aortic valve thickening, small deposits of valve calcification, and increased ROS activity in cells surrounding regions of valve calcification [226]. Furthermore, ROS activity was co-localized to clusters of cells expressing Runx2 and OPN, suggesting that ROS activity is associated with VICs displaying an osteogenic-like phenotype [226]. In VIC culture studies, TGF β 1 stimulation induces increased ROS activity, along with calcific nodule formation and ALP activity [227]. Increasing the availability of NO, via NO donors such as sodium nitroprusside, partially blocks both nodule formation and ALP activity, suggesting that NO levels are important for reducing ROS and inhibiting calcification in VICs [227]. There have been a number of small clinical studies investigating the levels of the NOS inhibitor, asymmetric dimethylarginine (ADMA), an indicator of endothelial cell dysfunction, in patients with moderate to severe AS [228-230]. In these studies, plasma levels of ADMA are significantly higher in patients with moderate to severe AS, compared to patients with mild AS or no disease, suggesting that NO production is disrupted in CAVD/AS [228-230]. Combined, these studies suggest that increased ROS production is associated with aortic valve calcification and the induction of osteogenic gene expression. Thus, increasing the bioavailability of NO may be a potential therapeutic avenue to block ROS activity, and thereby disease progression, in CAVD.

5.6. NSAIDs/COX2 inhibitors

Previous reports have demonstrated that immune cell infiltration is common in CAVD [119, 132, 135]. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat pain and inflammation, and act by inhibiting the enzymes COX1 and/or COX2 [231-233]. These enzymes function by converting arachidonic acid to prostaglandins (PGs) [233, 234]. There has been one study conducted in human aortic VICs, demonstrating that stimulation of VICs with pro-inflammatory mimetics induces the expression of COX2 and the release of prostaglandins [235]. This study suggests that COX2 inhibition may be one way to treat the immune response associated with CAVD [235]. Interestingly, COX2 and PG signaling are also involved in bone formation as well as cellular responses to physical stress and strain, processes that also likely contribute to CAVD (reviewed in [131]). In bone, PG signaling has an anabolic effect, and PG treatment of osteoblast cultures results in increased expression of OCN, BMP2, Runx2, OPN, ALP, and BSP [236-244]. In osteoblast cultures, BMP2 stimulation induces COX2 expression through upregulation of Runx2, which binds to and activates the COX2 promoter [245, 246]. Downstream, PG signaling induces the expression of p38 MAPK through the activation of protein kinase A [243, 247]. Furthermore, fluid shear stress and other physical forces induce COX2 expression in osteoblast-like cells, suggesting that increased COX2 and PG signaling is a cellular response to altered mechanical forces [248, 249]. The combined results of these studies suggest that COX2/PG signaling may be an effective therapeutic target to treat CAVD progression, as COX2/PG signaling plays a role in inflammation, osteogenesis, and cellular response to physical strain, all of which are thought to be pathological mechanisms involved in CAVD [131, 243, 244, 248-251]. It would be interesting

to determine whether COX2 inhibition/NSAID use could reduce CAVD progression. However, one caveat is that COX2 inhibitor therapy can be associated with some rare but significant adverse cardiovascular events such as myocardial infarction and stroke [252, 253]. Perhaps therapeutics designed toward downstream targets of PG signaling, such as p38 MAPK, could improve outcomes of CAVD patients without the cardiovascular side effects of selective coxibs [254].

5.7. Development of new therapeutic approaches based on valvulogenic and osteogenic molecular mechanisms.

As reviewed above, Notch, Wnt, and BMP signaling have been implicated in the progression of CAVD. Pharmacotherapies designed to act as Wnt and BMP inhibitors, or Notch agonists, could be a potential avenue for new therapeutics to treat the progression of CAVD. BMP signaling is thought to be a specific indicator of aortic valve calcification as active BMP signaling is observed in adult diseased valves with prominent calcification and is not found in pediatric diseased valves void of calcification [13]. Furthermore, BMP2 signaling stimulates VIC calcific nodule formation and induces osteogenic gene expression [138, 171]. It is possible that therapies designed to inhibit BMP signaling will block osteogenic-like calcification in diseased aortic valves. Likewise, inhibition of the Wnt/ β -catenin signaling pathway may also serve to reduce aortic valve calcification during disease, which is supported by evidence from animal studies in ApoE knockout mice. When fed an atherogenic diet, ApoE knockout mice reportedly develop aortic valve calcification, however, when the Wnt co-receptor Lrp5 is genetically deleted in these mice, the amount of aortic valve calcification is significantly reduced [180]. Therefore, Wnt inhibition may be another potential therapeutic approach for treating CAVD. Lastly, strategies to maintain Notch signaling in the valves may be another potential way to inhibit calcification in CAVD. Notch inhibition of calcification and osteogenic gene expression has been demonstrated in aortic VICs in culture and reduced Notch signaling in vivo leads to CAVD in mice [65, 153, 175]. Furthermore, Notch1 haploinsufficiency in humans is associated with CAVD, indicating that maintaining Notch signaling is important for valve homeostasis [64]. Thus, therapeutic strategies designed to affect one or more of these pathways may serve to prevent valve calcification in CAVD. A potential limitation of this approach is that BMP, Wnt, and Notch signaling pathways are involved in many homeostatic and disease processes. For example, Wnt signaling is increased in many types of cancer, and all three pathways are involved in bone homeostasis. Therefore the development of therapeutics based on these molecular mechanisms must take into account potential effects on multiple organ systems. Nevertheless, targeted approaches based on these pathways could represent a new therapeutic avenue in the development of pharmacologic based approaches to CAVD.

6. Conclusions and future directions

There are numerous examples of shared molecular pathways between valvulogenesis, osteogenesis, and disease pathogenesis of CAVD. In valvulogenesis, signaling factors involved

in early cushion formation, such as BMP, Notch, and Wnt/ β -catenin pathways are active in osteogenesis and in CAVD [7-9, 14]. Furthermore, transcription factors expressed in the early valve mesenchyme, such as Twist1, Msx2, and Sox9, can also be found in the primitive condensed bone mesenchyme and in the mesenchymal-like cells identified in diseased aortic valve tissues [8, 13, 80, 255]. In addition to signaling and transcription factors, molecular pathways governing ECM production and remodeling, such as the RANKL – NFATc1 – CtsK pathway are shared amongst valve progenitor, developing bone, and diseased valve tissues [11, 47, 103, 106, 193]. This commonality suggests that the mesenchymal cells found within these tissues are governed by common molecular pathways and that these developmental pathways are reactivated during disease. Additional parallels can be drawn between calcification of the embryonic bone tissues and calcification observed in diseased aortic valves. For example, the endochondral bone factors Runx2, OCN, and BSP are reactivated during aortic valve disease, suggesting that osteogenic molecular pathways are activated during CAVD and may contribute to pathogenic calcification [5, 13, 76, 80, 106, 152]. Effective pharmacological therapies to treat CAVD remain elusive and identifying potential targets for new pharmacotherapies is a priority, as the only effective treatment for CAVD with AS is valve replacement surgery [256]. Studies testing the effectiveness of statin therapy, inhibitors of the renin-angiotensin-aldosterone, and bisphosphonates in slowing the progression of CAVD have been disappointing (see therapeutic section). New therapeutic strategies are needed and, perhaps, targeted inhibition of BMP and Wnt signaling or maintenance of Notch signaling may provide new avenues for potential CAVD treatments.

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References

- [1] Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics--2011 update: A report from the American Heart Association Circulation. 2011;123:e18-e209.
- [2] Otto CM. Valvular aortic stenosis: disease severity and timing of intervention. J Am Coll Cardiol. 2006;47:2141-2151.
- [3] Bonow RO, Carabello BA, Kanu C, de Leon AC, Jr., Faxon DP, Freed MD, et al. ACC/AHA 2006 guidelines for the management of patients with valvular heart disease: a report of the American College of Cardiology/American Heart Association

Task Force on Practice Guidelines (writing committee to revise the 1998 Guidelines for the Management of Patients With Valvular Heart Disease). *Circulation*. 2006;114:e84-231.

- [4] Roberts WC, Ko JM. Frequency by decades of unicuspid, bicuspid and tricuspid aortic valves in adults having isolated aortic valve replacement for aortic stenosis, with or without associated aortic regurgitation. *Circulation*. 2005;111:920-925.
- [5] Rajamannan NM, Subramaniam M, Rickard DJ, Stock SR, Donovan J, Springett M, et al. Human aortic valve calcification is associated with an osteoblast phenotype. *Circulation*. 2003;107:2181-2184.
- [6] O'Brien KD. Pathogenesis of calcific aortic valve disease: a disease process comes of age (and a good deal more). *Arterioscler Thromb Vasc Biol*. 2006;26:1721-1728.
- [7] Bostrom K, Rajamannan NM, Towler DA. The regulation of valvular and vascular sclerosis by osteogenic morphogens. *Circ Res*. 2011;109:564-577.
- [8] Combs MD, Yutzey KE. Heart valve development: Regulatory networks in development and disease. *Circ Res*. 2009;105:408-421.
- [9] Karsenty G, Kronenberg HM, Settembre C. Genetic control of bone formation. *Annu Rev Cell Dev Biol*. 2009;25:629-648.
- [10] Hinton RB, Yutzey KE. Heart valve structure and function in development and disease. *Annu Rev Physiol*. 2011;73:29-46.
- [11] Lincoln J, Lange AW, Yutzey KE. Hearts and bones: Shared regulatory mechanisms in heart valve, cartilage, tendon, and bone development. *Dev Biol*. 2006;294:292-302.
- [12] Chakraborty S, Wirrig EE, Hinton RB, Merrill WH, Spicer DB, Yutzey KE. Twist1 promotes heart valve cell proliferation and extracellular matrix gene expression during development in vivo and is expressed in human diseased aortic valves. *Dev Biol*. 2010;347:167-179.
- [13] Wirrig EE, Hinton RB, Yutzey KE. Differential expression of cartilage and bone-related proteins in pediatric and adult diseased aortic valves. *J Mol Cell Cardiol*. 2011;50:561-569.
- [14] Miller JD, Weiss RM, Heistad DD. Calcific aortic valve stenosis: Methods, models, and mechanisms. *Circ Res*. 2011;108:1392-1412.
- [15] Schroeder JA, Jackson LF, Lee DC, Camenisch TD. Form and function of developing heart valves: coordination by extracellular matrix growth and signaling. *J Mol Med*. 2003;81:392-403.
- [16] Person AD, Klewer SE, Runyan RB. Cell biology of cardiac cushion development. *Int Rev Cytol*. 2005;243:287-335.

- [17] Camenisch TD, Molin DG, Person A, Runyan RB, Gittenberger-de Groot AC, McDonald JA, et al. Temporal and distinct TGFbeta ligand requirements during mouse and avian endocardial cushion morphogenesis. *Dev Biol.* 2002;248:170-181.
- [18] Ma L, Lu MF, Schwartz RJ, Martin JF. Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. *Development.* 2005;132:5601-5611.
- [19] Rivera-Feliciano J, Tabin CJ. Bmp2 instructs cardiac progenitors to form the heart-valve-inducing field. *Dev Biol.* 2006;295:580-588.
- [20] McCulley DJ, Kang JO, Martin JF, Black BL. BMP4 is required in the anterior heart field and its derivatives for endocardial cushion remodeling, outflow tract septation, and semilunar valve development. *Dev Dyn.* 2008;237:3200-3209.
- [21] Snarr BS, Kern CB, Wessels A. Origin and fate of cardiac mesenchyme. *Dev Dyn.* 2008;237:2804-2819.
- [22] Lin C-J, Lin C-Y, Chen C-H, Zhou B, Chang C-P. Partitioning the heart: mechanisms of cardiac septation and valve development. *Development.* 2012;139:3277-3299.
- [23] Lincoln J, Alfieri CM, Yutzey KE. Development of heart valve leaflets and supporting apparatus in chicken and mouse embryos. *Dev Dyn.* 2004;230:239-250.
- [24] Hinton RB, Lincoln J, Deutsch GH, Osinska H, Manning PB, Benson DW, et al. Extracellular matrix remodeling and organization in developing and diseased aortic valves. *Circ Res.* 2006;98:1431-1438.
- [25] deLange FJ, Moorman AFM, Anderson RH, Manner J, Soufan AT, deGier-deVries C, et al. Lineage and morphogenetic analysis of the cardiac valves. *Circ Res.* 2004;95:645-654.
- [26] Rochais F, Mesbah K, Kelly RG. Signaling pathways controlling second heart field development. *Circ Res.* 2009;104:933-942.
- [27] Nakamura T, Colbert MC, Robbins J. Neural crest cells retain multipotential characteristics in the developing valves and label the cardiac conduction system. *Circ Res.* 2006;98:1547-1554.
- [28] Jiang X, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cardiac neural crest. *Development.* 2000;127:1607-1616.
- [29] Jain R, Engleka KA, Rentschler SL, Manderfield LJ, Li L, Yuan L, et al. Cardiac neural crest orchestrates remodeling and functional maturation of mouse semilunar valves. *J Clin Invest.* 2011;121:422-430.
- [30] Zhang J, Chang JY, Huang Y, Lin X, Schwartz RJ, Martin JF, et al. The FGF-BMP signaling axis regulates outflow tract valve primordium formation by promoting cushion neural crest cell differentiation. *Circ Res.* 2010;107:1209-1219.

- [31] Wessels A, van den Hoff MJ, Adamo RF, Phelps AL, Lockhart MM, Sauls K, et al. Epicardially derived fibroblasts preferentially contribute to the parietal leaflets of the atrioventricular valves in the murine heart. *Dev Biol.* 2012;366:111-124.
- [32] Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, et al. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature.* 2008;454:109-113.
- [33] Visconti RP, Ebihara Y, LaRue AC, Fleming PA, McQuinn TC, Masuya M, et al. An in vivo analysis of hematopoietic stem cell potential: hematopoietic origin of cardiac valve interstitial cells. *Circ Res.* 2006;98:690-696.
- [34] Hajdu Z, Romeo SJ, Fleming PA, Markwald RR, Visconti RP, Drake CJ. Recruitment of bone marrow-derived valve interstitial cells is a normal homeostatic process. *J Mol Cell Cardiol.* 2011;51:955-965.
- [35] Chakraborty S, Combs MD, Yutzey KE. Transcriptional regulation of heart valve progenitor cells. *Pediatr Cardiol.* 2010;31:414-421.
- [36] Timmerman LA, Grego-Bessa J, Raya A, Bertran E, Perez-Pomares JM, Diez J, et al. Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev.* 2004;18:99-115.
- [37] Tao G, Levay AK, Gridley T, Lincoln J. *Mmp15* is a direct target of *Snai1* during endothelial to mesenchymal transformation and endocardial cushion development. *Dev Biol.* 2011;359:209-221.
- [38] Shelton EL, Yutzey KE. *Twist1* function in endocardial cell proliferation, migration, and differentiation during heart valve development. *Dev Biol.* 2008;317:282-295.
- [39] Shelton EL, Yutzey KE. *Tbx20* regulation of endocardial cushion cell proliferation and extracellular matrix gene expression. *Dev Biol.* 2007;302:376-388.
- [40] Lincoln J, Kist R, Scherer G, Yutzey KE. *Sox9* is required for precursor cell expansion and extracellular matrix gene expression. *Dev Biol.* 2007;302:376-388.
- [41] Chen YH, Ishii M, Sukov HM, Maxson RE. *Msx1* and *Msx2* are required for endothelial-mesenchymal transformation of the atrioventricular cushions and patterning of the atrioventricular myocardium. *BMC Dev Biol.* 2008;8:75.
- [42] Lee MP, Yutzey KE. *Twist1* directly regulates genes that promote cell proliferation and migration in developing heart valves. *PLoS One.* 2011;6:e29758.
- [43] Chakraborty S, Cheek J, Sakthivel B, Aronow BJ, Yutzey KE. Shared gene expression profiles in developing heart valves and osteoblasts. *Physiol Genomics.* 2008;35:75-85.
- [44] Aikawa E, Whittaker P, Farber M, Mendelson K, Padera RF, Aikawa M, et al. Human semilunar cardiac valve remodeling by activated cells from fetus to adult. *Circulation.* 2006;113:1344-1352.

- [45] de la Pompa JL, Timmerman LA, Takimoto H, Yoshida H, Elia AJ, Samper E, et al. Role of NF-ATc transcription factor in morphogenesis of cardiac valves and septum. *Nature*. 1998;392:182-186.
- [46] Ranger AM, Grusby MJ, Gravallesse EM, de la Brousse FC, Hoey T, Mickanin C, et al. The transcription factor NF-ATc is essential for cardiac valve formation. *Nature*. 1998;392:186-190.
- [47] Combs MD, Yutzey KE. VEGF and RANKL regulation of NFATc1 in heart valve development. *Circ Res*. 2009;105:565-574.
- [48] Lincoln J, Alfieri CM, Yutzey KE. BMP and FGF regulatory pathways control cell lineage diversification of heart valve precursor cells. *Dev Biol*. 2006;292:290-302.
- [49] Peacock JD, Levay AK, Gillaspie DB, Tao G, Lincoln J. Reduced Sox9 function promotes heart valve calcification phenotypes in vivo. *Circ Res*. 2010;106:712-719.
- [50] Levay AK, Peacock JD, Lu Y, Koch M, Hinton RB, Kadler KE, et al. Scleraxis is required for cell lineage differentiation and extracellular matrix remodeling during murine heart formation in vivo. *Circ Res*. 2008;103:948-956.
- [51] Luna-Zurita L, Prados B, Grego-Bessa J, Luxan G, del Monte G, Benguria A, et al. Integration of a Notch-dependent mesenchymal gene program and BMP2-driven cell invasiveness regulates murine cardiac valve formation. *J Clin Invest*. 2010;120:3493-3507.
- [52] Dor Y, Camenisch TD, Itin A, Fishman GI, McDonald JA, Carmeliet P, et al. A novel role for VEGF in endocardial cushion formation and its potential contribution to congenital heart defects. *Development*. 2001;128:1531-1538.
- [53] Liebner S, Cattelino A, Gallini R, Rudini N, Iurlaro M, Piccolo S, et al. β -catenin is required for endothelial-mesenchymal transformation during heart cushion development in the mouse. *J Cell Biol*. 2004;166:359-367.
- [54] Person AD, Garriock RJ, Krieg PA, Runyan RB, Klewer SE. Frzb modulates Wnt-9a-mediated β -catenin signaling during avian atrioventricular cardiac cushion development. *Dev Biol*. 2005;278:35-48.
- [55] Jackson LF, Qiu TH, Sunnarborg SW, Chang A, Zhang C, Patterson C, et al. Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. *EMBO J*. 2003;22:2704-2716.
- [56] Delot EC, Bahamonde ME, Zhao M, Lyons KM. BMP signaling is required for septation of the outflow tract of the mammalian heart. *Development*. 2003;130:209-220.
- [57] Beppu H, Malhotra RR, Beppu Y, Lepore JJ, Parmacek MS, Bloch KD. BMP type II receptor regulates positioning of the outflow tract and remodeling of the atrioventricular cushion during cardiogenesis. *Dev Biol*. 2009;331:167-175.

- [58] Galvin KM, Donovan MJ, Lynch CA, Meyer RI, Paul RJ, Lorenz JN, et al. A role for smad6 in development and homeostasis of the cardiovascular system. *Nat Genet.* 2000;24:171-174.
- [59] Zhao B, Etter L, Hinton RB, Benson DW. BMP and FGF regulatory pathways in semilunar valve precursor cells. *Dev Dyn.* 2007;236:971-980.
- [60] Alfieri CM, Cheek J, Chakraborty S, Yutzey KE. Wnt signaling in heart valve development and osteogenic gene induction. *Dev Biol.* 2010;338:127-135.
- [61] Lange AW, Yutzey KE. NFATc1 expression in the developing heart valves is responsive to the RANKL pathway and is required for endocardial expression of cathepsin K. *Dev Biol.* 2006;292:407-417.
- [62] del Monte G, Grego-Bessa J, Gozalez-Rajal A, Bolos V, delaPompa JL. Monitoring Notch1 activity in development: evidence for a feedback regulatory loop. *Dev Dyn.* 2007;236:2594-2614.
- [63] Cripe L, Andelfinger G, Martin LJ, Shooner K, Benson DW. Bicuspid aortic valve is heritable. *J Am Coll Cardiol.* 2004;44:138-143.
- [64] Garg V, Muth AN, Ransom JF, Schluterman MK, Barnes R, King IN, et al. Mutations in NOTCH1 cause aortic valve disease. *Nature.* 2005;437:270-274.
- [65] Nus M, MacGrogan D, Martinez-Poveda B, Benito Y, Casanova JC, Fernandez-Aviles F, et al. Diet-induced aortic valve disease in mice haploinsufficient for the Notch pathway effector RBPJK/CSL. *Arterioscler Thromb Vasc Biol.* 2011;31:1580-1588.
- [66] Laforest B, Andelfinger G, Nemer M. Loss of Gata5 in mice leads to bicuspid aortic valve. *J Clin Invest.* 2011;121:2876-2887.
- [67] Padang R, Bagnall RD, Richmond DR, Bannon PG, Semsarian C. Rare non-synonymous variations in the transcriptional activation domains of GATA5 in bicuspid aortic valve disease. *J Mol Cell Cardiol.* 2012;53:277-291.
- [68] Lee TC, Zhao YD, Courtman DW, Stewart DJ. Abnormal aortic valve development in mice lacking endothelial nitric oxide synthase. *Circulation.* 2000;101:2345-2348.
- [69] Schoen FJ. Evolving concepts of cardiac valve dynamics. *Circulation.* 2008;118:1864-1880.
- [70] Snider P, Hinton RB, Moreno-Rodriguez R, Wang J, Rogers R, Lindsley A, et al. Periostin is required for maturation and extracellular matrix stabilization of noncardiomyocyte lineages of the heart. *Circ Res.* 2008;102:752-760.
- [71] Norris RA, Moreno-Rodriguez RA, Sugi Y, Hoffman S, Amos J, Hart MM, et al. Periostin regulates atrioventricular valve maturation. *Dev Biol.* 2008;316:200-213.
- [72] Hinton RB, Adelman-Brown J, Witt S, Krishnamurthy VK, Osinska H, Sakthivel B, et al. Elastin haploinsufficiency results in progressive aortic valve malformation and latent valve disease in a mouse model. *Circ Res.* 2010;107:549-557.

- [73] Cheek JD, Wirrig EE, Alfieri CM, James JF, Yutzey KE. Differential activation of valvulogenic, chondrogenic, and osteogenic pathways in mouse models of myxomatous and calcific aortic valve disease. *J Mol Cell Cardiol.* 2012;52:689-700.
- [74] Kern CB, Wessels A, McGarity J, Dixon LJ, Alston E, Argraves WS, et al. Reduced versican cleavage due to Adamts9 haploinsufficiency is associated with cardiac and aortic anomalies. *Matrix Biol.* 2010;29:304-316.
- [75] Dupuis LE, McCulloch DR, McGarity JD, Bahan A, Wessels A, Weber D, et al. Altered versican cleavage in ADAMTS5 deficient mice; a novel etiology of myxomatous valve disease. *Dev Biol.* 2011;357:152-164.
- [76] Karsenty G, Wagner EF. Reaching a genetic and molecular understanding of skeletal development. *Dev Cell.* 2002;2:389-406.
- [77] Lefebvre V, Bhattaram P. Vertebrate skeletogenesis. *Curr Top Dev Biol.* 2010;90:291-317.
- [78] Chai Y, Jiang X, Ito Y, Bringas P, Jr., Han J, Rowitch DH, et al. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development.* 2000;127:1671-1679.
- [79] Karsenty G. Transcriptional control of skeletogenesis. *Annu Rev Genomics Hum Genet.* 2008;9:183-196.
- [80] Long F. Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol.* 2012;13:27-38.
- [81] Goldring MB, Tsuchimochi K, Ijiri K. The control of chondrogenesis. *J Cell Biochem.* 2006;97:33-44.
- [82] Yoshioka M, Yuasa S, Matsumura K, Kimura K, Shiomi T, Kimura N, et al. Chondromodulin-I maintains cardiac valvular function by preventing angiogenesis. *Nat Med.* 2006;12:1151-1159.
- [83] Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell.* 2002;108:17-29.
- [84] Bialek P, Kern B, Yang X, Schrock M, Sosoc D, Hong N, et al. A Twist code determines the onset of osteoblast differentiation. *Dev Cell.* 2004;6:423-435.
- [85] Gu S, Boyer TG, Naski M. Basic helix-loop-helix transcription factor Twist1 inhibits transactivator function of master chondrogenic regulator Sox9. *J Biol Chem.* 2012;287:21082-21092.
- [86] Yousfi M, Lasmoles F, Lomri A, Delannoy P, Marie PJ. Increased bone formation and decreased osteocalcin expression induced by reduced Twist dosage in Saethre-Chotzen syndrome. *J Clin Invest.* 2001;107:1153-1161.

- [87] Dodig M, Tadic T, Kronenberg MS, Dacic S, Liu YH, Maxson RE, et al. Ectopic Msx2 overexpression inhibits and Msx2 antisense stimulates calvarial osteoblast differentiation. *Dev Biol.* 1999;209:298-307.
- [88] Akiyama H, Chaboissier M-C, Martin JF, Schedl A, de Crombrughe B. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev.* 2002;16:2813-2828.
- [89] Chimal-Monroy J, Rodriguez-Leon J, Montero JA, Ganan Y, Macias D, Merino R, et al. Analysis of the molecular cascade responsible for mesodermal limb chondrogenesis: Sox genes and BMP signaling. *Dev Biol.* 2003;257:292-301.
- [90] Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrughe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. *Mol Cell Biol.* 1997;17:2336-2346.
- [91] Sekiya I, Tsuji K, Koopman P, Watanabe T, Yamada Y, Shinomiya K, et al. SOX9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acids in a cartilage-derived cell line, TC6. *J Biol Chem.* 2000;275:10738-10744.
- [92] Dy P, Wang W, Bhattaram P, Wang Q, Wang L, Ballock RT, et al. Sox9 directs hypertrophic maturation and blocks osteoblast differentiation of growth plate chondrocytes. *Dev Cell.* 2012;22:597-609.
- [93] Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell.* 1997;89:747-754.
- [94] Zamurovic N, Cappellen D, Rohner D, Susa M. Coordinated activation of Notch, Wnt, and transforming growth factor-beta signaling pathways in bone morphogenetic 2-induced osteogenesis. *J Biol Chem.* 2004;279:37704-37715.
- [95] Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, et al. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev.* 1999;13:1025-1036.
- [96] Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature.* 2003;423:337-342.
- [97] Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling and terminal differentiation of osteoclasts. *Dev Cell.* 2002;8:889-901.
- [98] Ishida N, Hayashi K, Hoshijima M, Ogawa T, Koga S, Miyatake Y, et al. Large scale gene expression analysis of osteoclastogenesis in vitro and elucidation of NFAT2 as a key regulator. *J Biol Chem.* 2002;277:41147-41156.
- [99] Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, et al. Osteopontin: a novel secreted protein involved in the regulation of bone density. *Cell.* 1997;89:309-319.

- [100] Yasuda H, Shima N, Nakagawa N, Mochizuki SA, Yano K, Fujise N, et al. Identity of osteoclastogenesis inhibitor factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. *Endocrinology*. 1998;139:1329-1337.
- [101] Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrugge B, Nakashima K, et al. NFAT and Osterix cooperatively regulate bone formation. *Nat Med*. 2005;11:880-885.
- [102] Winslow MM, Pan M, Starbuck M, Gallo EM, Deng L, Karsenty G, et al. Calcineurin/NFAT signaling in osteoblasts regulates bone mass. *Dev Cell*. 2006;10:771-782.
- [103] Kaden JJ, Bickelhaupt S, Grobholz R, Haase KK, Sarikoc A, Kilic R, et al. Receptor activator of nuclear factor κ B ligand and osteoprotegerin regulate aortic valve calcification. *J Mol Cell Cardiol*. 2004;36:57-66.
- [104] Rabkin-Aikawa E, Farber M, Aikawa M, Schoen FJ. Dynamic and reversible changes in interstitial cell phenotype during remodeling of cardiac valves. *J Heart Valve Disease*. 2004;13:841-847.
- [105] Nishimura R, Wakabayashi M, Hata K, Matsubara T, Honma S, Wakisaka S, et al. Osterix regulates calcification and degradation of chondrogenic matrices through matrix metalloproteinase (MMP13) expression in association with transcription factor Runx2 during endochondral ossification. *J Biol Chem*. 2012;287:33179-33190.
- [106] Alexopoulos A, Bravou V, Peroukides S, Kaklamanis L, Varakis J, Alexopoulos D, et al. Bone regulatory factors NFATc1 and Osterix in human calcific aortic valves. *Int J Cardiol*. 2010;139:142-149.
- [107] Kobayashi T, Kronenberg H. Transcriptional regulation in development of bone. *Endocrinology*. 2005;146:1012-1017.
- [108] Wozney JM, Rosen V, A.J. C, Mitsock LM, Whitters MJ, Kriz RW, et al. Novel regulators of bone formation: molecular clones and activities. *Science*. 1988;242:1528-1534.
- [109] Nishimura R, Hata K, Matsubara T, Wakabayashi M, Yoneda T. Regulation of bone and cartilage development by network between BMP signaling and transcription factors. *J Biochem*. 2012;151:247-254.
- [110] Lin GL, Hakenson KD. Integration of BMP, Wnt, and Notch signaling pathways in osteoblast differentiation. *J Cell Biochem*. 2011;112:3491-3501.
- [111] Rawadi G, Vayssiere B, Dunn F, Baron R, Roman-Roman S. BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. *J Bone Min Res*. 2003;18:1842-1853.
- [112] Rodriguez-Carballo E, Ulsamer A, Susperregui AR, Manzanares-Cespedes C, Sanchez-Garcia E, Bartrons R, et al. Conserved regulatory motifs in osteogenic gene promoters integrate cooperative effects of canonical Wnt and BMP pathways. *J Bone Miner Res*. 2011;26:718-729.

- [113] Sciaudone M, Gazzero E, Priest L, Delanty AM, Canalis E. Notch1 impairs osteoblastic cell differentiation. *Endocrinology*. 2003;144:5631-5639.
- [114] Deregowski V, Gazzero E, Priest L, Rydzial S, Canalis E. Notch 1 overexpression inhibits osteoblastogenesis by suppressing Wnt/ β -catenin but not bone morphogenetic protein signaling. *J Biol Chem*. 2006;281:6203-6210.
- [115] Hilton MJ, Tu X, Wu X, Bai S, Zhao H, Kobayashi T, et al. Notch signaling maintains bone marrow mesenchyme progenitors by suppressing osteoblast differentiation. *Nat Med*. 2008;14:306-314.
- [116] Mead TJ, Yutzey KE. Notch pathway regulation of chondrocyte differentiation and proliferation during appendicular and axial skeleton development. *Proc Natl Acad Sci USA*. 2009;106:14420-14425.
- [117] Gross L, Kugel MA. Topographic Anatomy and Histology of the Valves in the Human Heart. *Am J Pathol*. 1931;7:445-474.
- [118] Kaden JJ, Bickelhaupt S, Grobholz R, Vahl CF, Hagl S, Brueckmann M, et al. Expression of bone sialoprotein and bone morphogenetic protein-2 in calcific aortic stenosis. *J Heart Valve Dis*. 2004;13:560-566.
- [119] Otto CM, Kuusisto J, Reichenbach DD, Gown AM, O'Brien KD. Characterization of the early lesion of 'degenerative' valvular aortic stenosis. Histological and immunohistochemical studies. *Circulation*. 1994;90:844-853.
- [120] Thubrikar MJ, Jaffar A, Nolan SP. Patterns of calcific deposits in operatively excised stenotic or purely regurgitant aortic valves and their relation to mechanical stress. *Am J Cardiol*. 1986;58:304-308.
- [121] Turri M, Thiene G, Bortolotti U, Milano A, Mazzucco A, Gallucci V. Surgical pathology of aortic valve disease. A study based on 602 specimens. *Eur J Cardiothorac Surg*. 1990;4:556-560.
- [122] Bonow RO, Carabello BA, Chatterjee K, de Leon AC, Jr., Faxon DP, Freed MD, et al. 2008 Focused update incorporated into the ACC/AHA 2006 guidelines for the management of patients with valvular heart disease: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 1998 Guidelines for the Management of Patients With Valvular Heart Disease). *Circulation*. 2008;118:e523-661.
- [123] Cosmi JE, Kort S, Tunick PA, Rosenzweig BP, Freedberg RS, Katz ES, et al. The risk of the development of aortic stenosis in patients with "benign" aortic valve thickening. *Arch Intern Med*. 2002;162:2345-2347.
- [124] Rabkin E, Aikawa M, Stone JR, Fukumoto Y, Libby P, Schoen FJ. Activated interstitial myofibroblasts express catabolic enzymes and mediate matrix remodeling in myxomatous heart valves. *Circulation*. 2001;104:2525-2532.

- [125] Walker GA, Masters KS, Shah DN, Anseth KS, Leinwand LA. Valvular myofibroblast activation by transforming growth factor β . *Circ Res*. 2004;95:253-260.
- [126] Fisher CI, Chen J, Merryman WD. Calcific nodule morphogenesis by heart valve interstitial cells is strain dependent. *Biomech Model Mechanobiol*. 2012; In Press.
- [127] Ortlepp JR, Schmitz F, Mevissen V, Weiss S, Huster J, Dronskowski R, et al. The amount of calcium-deficient hexagonal hydroxyapatite in aortic valves is influenced by gender and associated with genetic polymorphisms in patients with severe calcific aortic stenosis. *Eur Heart J*. 2004;25:514-522.
- [128] Weska RF, Aimoli CG, Nogueira GM, Leirner AA, Maizato MJ, Higa OZ, et al. Natural and prosthetic heart valve calcification: morphology and chemical composition characterization. *Artif Organs*. 2010;34:311-318.
- [129] Mohler ER, Gannon F, Reynolds C, Zimmerman R, Keane MG, Kaplan FS. Bone formation and inflammation in cardiac valves. *Circulation*. 2001;103:1522-1528.
- [130] Egan KP, Kim JH, Mohler ER, 3rd, Pignolo RJ. Role for circulating osteogenic precursor cells in aortic valvular disease. *Arterioscler Thromb Vasc Biol*. 2011;31:2965-2971.
- [131] Li C, Xu S, Gotlieb AI. The progression of calcific aortic valve disease through injury, cell dysfunction, and disruptive biologic and physical force feedback loops. *Cardiovasc Pathol*. 2012; In Press.
- [132] Mazzone A, Epistolato MC, De Caterina R, Storti S, Vittorini S, Sbrana S, et al. Neo-angiogenesis, T-lymphocyte infiltration, and heat shock protein-60 are biological hallmarks of an immunomediated inflammatory process in end-stage calcified aortic valve stenosis. *J Am Coll Cardiol*. 2004;43:1670-1676.
- [133] O'Brien KD, Shavelle DM, Caulfield MT, McDonald TO, Olin-Lewis K, Otto CM, et al. Association of angiotensin-converting enzyme with low-density lipoprotein in aortic valvular lesions and in human plasma. *Circulation*. 2002;106:2224-2230.
- [134] Olsson M, Thyberg J, Nilsson J. Presence of oxidized low density lipoprotein in non-rheumatic stenotic aortic valves. *Arterioscler Thromb Vasc Biol*. 1999;19:1218-1222.
- [135] Steiner I, Krbal L, Rozkos T, Harrer J, Laco J. Calcific aortic valve stenosis: Immunohistochemical analysis of inflammatory infiltrate. *Pathol Res Pract*. 2012;208:231-234.
- [136] Clark-Greuel JN, Connolly JM, Sorichillo E, Narula NR, Rapoport HS, Mohler ER, et al. Transforming growth factor- β 1 mechanisms in aortic valve calcification: increased alkaline phosphatase and related events. *Ann Thorac Surg*. 2007;83:946-953.
- [137] ian B, Narula N, Li QY, Mohler ER, 3rd, Levy RJ. Progression of aortic valve stenosis: TGF-beta1 is present in calcified aortic valve cusps and promotes aortic valve interstitial cell calcification via apoptosis. *Ann Thorac Surg*. 2003;75:457-465.
- [138] Yang X, Meng X, Su X, Mauchley DC, Ao L, Cleveland JC, et al. Bone morphogenetic protein 2 induces Runx2 and osteopontin expression in human aortic valve intersti-

tial cells: Role of Smad1 and extracellular signal-regulated kinase 1/2. *J Thorac Cardiovasc Surg.* 2009;138:1008-1015.

- [139] Beppu S, Suzuki S, Matsuda H, Ohmori F, Nagata S, Miyatake K. Rapidity of progression of aortic stenosis in patients with congenital bicuspid aortic valves. *Am J Cardiol.* 1993;71:322-327.
- [140] Linefsky JP, O'Brien KD, Katz R, de Boer IH, Barasch E, Jenny NS, et al. Association of serum phosphate levels with aortic valve sclerosis and annular calcification: the cardiovascular health study. *J Am Coll Cardiol.* 2011;58:291-297.
- [141] Rosenhek R, Klaar U, Schemper M, Scholten C, Heger M, Gabriel H, et al. Mild and moderate aortic stenosis. Natural history and risk stratification by echocardiography. *Eur Heart J.* 2004;25:199-205.
- [142] Varadarajan P, Kapoor N, Bansal RC, Pai RG. Clinical profile and natural history of 453 nonsurgically managed patients with severe aortic stenosis. *Ann Thorac Surg.* 2006;82:2111-2115.
- [143] Caira FC, Stock SR, Gleason TG, McGee EC, Huang J, Bonow RO, et al. Human degenerative valve disease is associated with up-regulation of low-density lipoprotein-related protein 5 receptor-mediated bone formation. *J Am Coll Cardiol.* 2006;47:1707-1712.
- [144] Miller JD, Chu Y, Brooks RM, Richenbacher WE, Pena-Silva R, Heistad DD. Dysregulation of antioxidant mechanisms contributes to increased oxidative stress in calcific aortic valvular stenosis in humans. *J Am Coll Cardiol.* 2008;52:843-850.
- [145] Satokata I, Ma L, Ohshima H, Bei M, Woo I, Nishizawa K, et al. Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. *Nat Genet.* 2000;24:391-395.
- [146] Balachandran K, Alford PW, Wylie-Sears J, Goss JA, Bischoff J, Aikawa E, et al. Cyclic strain induces dual-mode endothelial-mesenchymal transformation of the cardiac valve. *Proc Natl Acad Sci USA.* 2011;108:19943-19948.
- [147] Dal-Bianco JP, Aikawa E, Bischoff J, Guerrero JL, Handschumacher MD, Sullivan S, et al. Active adaptation of the tethered mitral valve: Insights into a compensatory mechanism for functional mitral regurgitation. *Circulation.* 2009;120:334-342.
- [148] Paranya G, Vineberg S, Dvorin E, Kaushal S, Roth SJ, Rabkin E, et al. Aortic valve endothelial cells undergo transforming growth factor-beta-mediated and non-transforming growth factor-beta-mediated transdifferentiation in vitro. *Am J Pathol.* 2001;159:1335-1343.
- [149] Paruchuri S, Yang JH, Aikawa E, Melero-Martin JM, Khan ZA, Loukogeorgakis S, et al. Human pulmonary valve progenitor cells exhibit endothelial/mesenchymal plasticity in response to vascular endothelial growth factor-A and transforming growth factor-beta2. *Circ Res.* 2006;99:861-869.

- [150] Li Z, Feng L, Wang CM, Zheng QJ, Zhao BJ, Yi W, et al. Deletion of RBP-J in adult mice leads to the onset of aortic valve degenerative diseases. *Mol Biol Rep.* 2012;39:3837-3845.
- [151] Hajdu Z, Romeo SJ, Fleming PA, Markwald RR, Visconti RP, Drake CJ. Recruitment of bone marrow-derived valve interstitial cells is a normal homeostatic process. *J Mol Cell Cardiol.* 2011;51:955-965.
- [152] Pohjolainen V, Taskinen P, Soini Y, Rysa J, Ilves M, Juvonen T, et al. Noncollagenous bone matrix proteins as a part of calcific aortic valve disease regulation. *Hum Pathol.* 2008;39:1695-1701.
- [153] Nigam V, Srivastava D. Notch1 represses osteogenic pathways in aortic valve cells. *J Mol Cell Cardiol.* 2009;47:828-834.
- [154] Aikawa E, Aikawa M, Libby P, Figueiredo J-L, Rusanescu G, Iwamoto Y, et al. Arterial and aortic valve calcification abolished by elastolytic cathepsin S deficiency in chronic renal disease. *Circulation.* 2009;119:1785-1794.
- [155] Hjortnaes J, Butcher J, Figueiredo J-L, Riccio M, Kohler RH, Kozloff KM, et al. Arterial and aortic valve calcification inversely correlates with osteoporotic bone remodeling: a role for inflammation. *Eur Heart J.* 2010;31:1975-1984.
- [156] Ix JH, Shlipak MG, Katz R, Budoff MJ, Shavelle DM, Probstfield JL, et al. Kidney function and aortic valve and mitral annular calcification in the Multi-Ethnic Study of Atherosclerosis (MESA). *Am J Kidney Dis.* 2007;50:412-420.
- [157] Shuvy M, Abedat S, Beerli R, Danenberg HD, Planer D, Ben-Dov IZ, et al. Uraemic hyperparathyroidism causes a reversible inflammatory process of aortic valve calcification in rats. *Cardiovasc Res.* 2008;79:492-499.
- [158] Shanahan CM, Crouthamel MH, Kapustin A, Giachelli C. Arterial calcification in chronic kidney disease: key roles for calcium and phosphate. *Circ Res.* 2011;109:697-711.
- [159] Hu MC, Kuro-o M, Moe OW. Klotho and kidney disease. *J Nephrol.* 2010;23 Suppl 16:S136-144.
- [160] Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature.* 1997;390:45-51.
- [161] Segawa H, Yamanaka S, Ohno Y, Onitsuka A, Shiozawa K, Aranami F, et al. Correlation between hyperphosphatemia and type II Na-Pi cotransporter activity in klotho mice. *Am J Physiol Renal Physiol.* 2007;292:F769-779.
- [162] Takeshita K, Fujimori T, Kurotaki Y, Honjo T, Tsujikawa H, Yasui K, et al. Sinoatrial node dysfunction and early unexpected death of mice with a defect of klotho gene expression. *Circulation.* 2004;109:1776-1782.

- [163] Aikawa E, Nahrendorf M, Sosnovik D, Lok VM, Jaffer FA, Aikawa M, et al. Multimodality molecular imaging identifies proteolytic and osteogenic activities in early aortic valve disease. *Circulation*. 2007;115:377-386.
- [164] Meng X, Ao L, Song Y, Babu A, Yang X, Wang M, et al. Expression of functional Toll-like receptors 2 and 4 in human aortic valve interstitial cells: potential roles in aortic valve inflammation and stenosis. *Am J Physiol Cell Physiol*. 2008;294:C29-35.
- [165] Rajamannan NM, Subramaniam M, Springett M, Sebo TC, Niekrasz M, McConnell JP, et al. Atorvastatin inhibits hypercholesterolemia-induced cellular proliferation and bone matrix production in the rabbit aortic valve. *Circulation*. 2002;105:2660-2665.
- [166] Aikawa E, Nahrendorf M, Figueiredo JL, Swirski FK, Shtatland T, Kohler RH, et al. Osteogenesis associates with inflammation in early-stage atherosclerosis evaluated by molecular imaging in vivo. *Circulation*. 2007;116:2841-2850.
- [167] Miller JD, Weiss RM, Serrano KM, Brooks RM, Berry CJ, Zimmerman K, et al. Lowering plasma cholesterol levels halts progression of aortic valve disease in mice. *Circulation*. 2009;119:2693-2701.
- [168] Babu AN, Meng X, Zou N, Yang X, Wang M, Song Y, et al. Lipopolysaccharide stimulation of human aortic valve interstitial cells activates inflammation and osteogenesis. *Ann Thorac Surg*. 2008;86:71-76.
- [169] Ankeny RF, Thourani VH, Weiss D, Vega JD, Taylor WR, Nerem RM, et al. Preferential activation of SMAD1/5/8 on the fibrosa endothelium in calcified human aortic valves - Association with low BMP antagonists and SMAD6. *PLoS One*. 2011;6:e20969.
- [170] Miller JD, Weiss RM, Serrano KM, Casteneda LE, Brooks RM, Zimmerman K, et al. Evidence for active regulation of pro-osteogenic signaling in advanced aortic valve disease. *Arterioscler Thromb Vasc Biol*. 2010;30:2482-2486.
- [171] Osman L, Yacoub MH, Latif N, Amrani M, Chester AH. Role of human valve interstitial cells in valve calcification and their response to atorvastatin. *Circulation*. 2006;114:I-547-I-552.
- [172] Merryman WD, Lukoff HD, Long RA, Engelmayr GC, Jr., Hopkins RA, Sacks MS. Synergistic effects of cyclic tension and transforming growth factor-beta1 on the aortic valve myofibroblast. *Cardiovasc Pathol*. 2007;16:268-276.
- [173] Chen JH, Chen WLK, Sider KL, Yip CY, Simmons CA. beta-catenin mediated mechanically regulated, transforming growth factor-beta1-induced myofibroblast differentiation of aortic valve interstitial cells. *Arterioscler Thromb Vasc Biol*. 2011;31:590-597.
- [174] Cushing MC, Mariner PD, Liao JT, Sims EA, Anseth KS. Fibroblast growth factor represses Smad-mediated myofibroblast activation in aortic valvular interstitial cells. *FASEB J*. 2008;22:1769-1777.

- [175] Acharya A, Hans CP, Keonig SN, Nichols HA, Galindo CL, Garner HR, et al. Inhibitory role for Notch1 in calcific aortic valve disease. *PLoS One*. 2011;6:e27743.
- [176] Tamai K, Zeng X, Liu C, Zhang X, Harada Y, Chang Z, et al. A mechanism for Wnt coreceptor activation. *Mol Cell*. 2004;13:149-156.
- [177] Rajamannan NM, Subramaniam M, Caira F, Stock SR, Spelsberg TC. Atorvastatin inhibits hypercholesterolemia-induced calcification in the aortic valves via the Lrp5 receptor pathway. *Circulation*. 2005;112:I229-I234.
- [178] Rajamannan NM. Oxidative-mechanical stress signals stem cell niche mediated Lrp5 osteogenesis in eNOS(-/-) null mice. *J Cell Biochem*. 2012;113:1623-1634.
- [179] Xu S, Gotlieb AI. Wnt3a/beta-catenin increases proliferation in heart valve interstitial cells. *Cardiovasc Pathol*. 2012; In Press.
- [180] Rajamannan NM. The role of Lrp5/6 in cardiac valve disease: experimental hypercholesterolemia in the ApoE-/- /Lrp5-/- mice. *J Cell Biochem*. 2011;112:2987-2991.
- [181] Fondard O, Detaint D, Iung B, Choqueux C, Adle-Biasette H, Jarraya M, et al. Extracellular matrix remodelling in human aortic valve disease: the role of matrix metalloproteinases and their tissue inhibitors. *Eur Heart J*. 2005;26:1333-1341.
- [182] Perrotta I, Russo E, Camastra C, Filice G, Di Mizio G, Colosimo F, et al. New evidence for a critical role of elastin in calcification of native heart valves: immunohistochemical and ultrastructural study with literature review. *Histopathology*. 2011;59:504-513.
- [183] Krishnamurthy VK, Opoka AM, Kern CB, Guilak F, Narmoneva DA, Hinton RB. Maladaptive matrix remodeling and regional biomechanical dysfunction in a mouse model of aortic valve disease. *Matrix Biol*. 2012;31:197-205.
- [184] Bosse Y, Miqdad A, Fournier D, Pepin A, Pibarot P, Mathieu P. Refining molecular pathways leading to calcific aortic valve stenosis by studying gene expression profile of normal and calcified stenotic human aortic valves. *Circ Cardiovasc Genet*. 2009;2:489-498.
- [185] Eriksen HA, Satta J, Risteli J, Veijola M, Vare P, Soini Y. Type I and type III collagen synthesis and composition in the valve matrix in aortic valve stenosis. *Atherosclerosis*. 2006;189:91-98.
- [186] Matsumoto KI, Satoh K, Maniwa T, Araki A, Maruyama R, Oda T. Noticeable Decreased Expression of Tenascin-X in Calcific Aortic Valves. *Connect Tissue Res*. 2012; In Press.
- [187] Stephens EH, Shangkuan J, Kuo JJ, Carroll JL, Kearney DL, Carberry KE, et al. Extracellular matrix remodeling and cell phenotypic changes in dysplastic and hemodynamically altered semilunar human cardiac valves. *Cardiovasc Pathol*. 2011;20:e157-167.

- [188] Lockhart M, Wirrig E, Phelps A, Wessels A. Extracellular matrix and heart development. *Birth Defects Res A Clin Mol Teratol*. 2011;91:535-550.
- [189] Mackie EJ, Tatarczuch L, Mirams M. The skeleton: a multi-functional complex organ: the growth plate chondrocyte and endochondral ossification. *J Endocrinol*. 2011;211:109-121.
- [190] Balachandran K, Sucosky P, Jo H, Yoganathan AP. Elevated cyclic stretch alters matrix remodeling in aortic valve cusps: implications for degenerative aortic valve disease. *Am J Physiol Heart Circ Physiol*. 2009;296:H756-764.
- [191] Kaden JJ, Dempfle CE, Kilic R, Sarikoc A, Hagl S, Lang S, et al. Influence of receptor activator of nuclear factor kappa B on human aortic valve myofibroblasts. *Exp Mol Pathol*. 2005;78:36-40.
- [192] Takayanagi H. Mechanistic insight into osteoclast differentiation in osteoimmunology. *J Mol Med (Berl)*. 2005;83:170-179.
- [193] Steinmetz M, Skowasch D, Wernert N, Welsch U, Preusse CJ, Welz A, et al. Differential profile of the OPG/RANKL/RANK-system in degenerative aortic native and bio-prosthetic valves. *J Heart Valve Dis*. 2008;17:187-193.
- [194] O'Brien KD, Reichenbach DD, Marcovina SM, Kuusisto J, Alpers CE, Otto CM. Apolipoproteins B, (a), and E accumulate in the morphologically early lesion of 'degenerative' valvular aortic stenosis. *Arterioscler Thromb Vasc Biol*. 1996;16:523-532.
- [195] Benton JA, Kern HB, Leinwand LA, Mariner PD, Anseth KS. Statins block calcific nodule formation of valvular interstitial cells by inhibiting alpha-smooth muscle actin expression. *Arterioscler Thromb Vasc Biol*. 2009;29:1950-1957.
- [196] Monzack EL, Masters KS. A time-course investigation of the statin paradox among valvular interstitial cell phenotypes. *Am J Physiol Heart Circ Physiol*. 2012;303:H903-9.
- [197] Hamilton AM, Boughner DR, Drangova M, Rogers KA. Statin treatment of hypercholesterolemic-induced aortic valve sclerosis. *Cardiovasc Pathol*. 2011;20:84-92.
- [198] Rosenhek R, Rader F, Loho N, Gabriel H, Heger M, Klaar U, et al. Statins but not angiotensin-converting enzyme inhibitors delay progression of aortic stenosis. *Circulation*. 2004;110:1291-1295.
- [199] Chan KL, Teo K, Dumesnil JG, Ni A, Tam J. Effect of Lipid lowering with rosuvastatin on progression of aortic stenosis: results of the aortic stenosis progression observation: measuring effects of rosuvastatin (ASTRONOMER) trial. *Circulation*. 2010;121:306-314.
- [200] Cowell SJ, Newby DE, Prescott RJ, Bloomfield P, Reid J, Northridge DB, et al. A randomized trial of intensive lipid-lowering therapy in calcific aortic stenosis. *N Engl J Med*. 2005;352:2389-2397.

- [201] Rossebø AB, Pedersen TR, Boman K, Brudi P, Chambers JB, Egstrup K, et al. Intensive lipid lowering with simvastatin and ezetimibe in aortic stenosis. *N Engl J Med*. 2008;359:1343-1356.
- [202] Ardehali R, Leeper NJ, Wilson AM, Heidenreich PA. The effect of angiotensin-converting enzyme inhibitors and statins on the progression of aortic sclerosis and mortality. *J Heart Valve Dis*. 2012;21:337-343.
- [203] Powers B, Greene L, Balfe LM. Updates on the treatment of essential hypertension: a summary of AHRQ's comparative effectiveness review of angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and direct renin inhibitors. *J Manag Care Pharm*. 2011;17(8 Suppl):S1-14.
- [204] Arishiro K, Hoshiga M, Negoro N, Jin D, Takai S, Miyazaki M, et al. Angiotensin receptor-1 blocker inhibits atherosclerotic changes and endothelial disruption of the aortic valve in hypercholesterolemic rabbits. *J Am Coll Cardiol*. 2007;49:1482-1489.
- [205] Ngo DT, Stafford I, Sverdlov AL, Qi W, Wuttke RD, Zhang Y, et al. Ramipril retards development of aortic valve stenosis in a rabbit model: mechanistic considerations. *Br J Pharmacol*. 2011;162:722-732.
- [206] Simolin MA, Pedersen TX, Bro S, Mayranpää MI, Helske S, Nielsen LB, et al. ACE inhibition attenuates uremia-induced aortic valve thickening in a novel mouse model. *BMC Cardiovasc Disord*. 2009;9:10.
- [207] Cote N, Couture C, Pibarot P, Despres JP, Mathieu P. Angiotensin receptor blockers are associated with a lower remodelling score of stenotic aortic valves. *Eur J Clin Invest*. 2011;41:1172-1179.
- [208] Chockalingam A, Venkatesan S, Subramaniam T, Jagannathan V, Elangovan S, Alagesan R, et al. Safety and efficacy of angiotensin-converting enzyme inhibitors in symptomatic severe aortic stenosis: Symptomatic Cardiac Obstruction-Pilot Study of Enalapril in Aortic Stenosis (SCOPE-AS). *Am Heart J*. 2004;147:E19.
- [209] Nadir MA, Wei L, Elder DH, Libianto R, Lim TK, Pauriah M, et al. Impact of renin-angiotensin system blockade therapy on outcome in aortic stenosis. *J Am Coll Cardiol*. 2011;58:570-576.
- [210] O'Brien KD, Probstfield JL, Caulfield MT, Nasir K, Takasu J, Shavelle DM, et al. Angiotensin-converting enzyme inhibitors and change in aortic valve calcium. *Arch Intern Med*. 2005;165:858-862.
- [211] Wakabayashi K, Tsujino T, Naito Y, Ezumi A, Lee-Kawabata M, Nakao S, et al. Administration of angiotensin-converting enzyme inhibitors is associated with slow progression of mild aortic stenosis in Japanese patients. *Heart Vessels*. 2011;26:252-257.
- [212] Sverdlov AL, Ngo DT, Chan WP, Chirkov YY, Gersh BJ, McNeil JJ, et al. Determinants of aortic sclerosis progression: implications regarding impairment of nitric oxide signaling and potential therapeutics. *Eur Heart J*. 2012; 33:2419-2425.

- [213] Yamamoto K, Yamamoto H, Yoshida K, Kisanuki A, Hirano Y, Ohte N, et al. Prognostic factors for progression of early- and late-stage calcific aortic valve disease in Japanese: the Japanese Aortic Stenosis Study (JASS) Retrospective Analysis. *Hypertens Res.* 2010;33:269-274.
- [214] Gkizas S, Koumoundourou D, Sirinian X, Rokidi S, Mavrilas D, Koutsoukos P, et al. Aldosterone receptor blockade inhibits degenerative processes in the early stage of calcific aortic stenosis. *Eur J Pharmacol.* 2010;642:107-112.
- [215] Stewart RA, Kerr AJ, Cowan BR, Young AA, Occleshaw C, Richards AM, et al. A randomized trial of the aldosterone-receptor antagonist eplerenone in asymptomatic moderate-severe aortic stenosis. *Am Heart J.* 2008;156:348-355.
- [216] Russell RG. Bisphosphonates: mode of action and pharmacology. *Pediatrics.* 2007;119 Suppl 2:S150-162.
- [217] Rapoport HS, Connolly JM, Fulmer J, Dai N, Murti BH, Gorman RC, et al. Mechanisms of the in vivo inhibition of calcification of bioprosthetic porcine aortic valve cusps and aortic wall with triglycidylamine/mercapto bisphosphonate. *Biomaterials.* 2007;28:690-699.
- [218] Innasimuthu AL, Katz WE. Effect of bisphosphonates on the progression of degenerative aortic stenosis. *Echocardiography.* 2011;28:1-7.
- [219] Skolnick AH, Osranek M, Formica P, Kronzon I. Osteoporosis treatment and progression of aortic stenosis. *Am J Cardiol.* 2009;104:122-124.
- [220] Sterbakova G, Vyskocil V, Linhartova K. Bisphosphonates in calcific aortic stenosis: association with slower progression in mild disease--a pilot retrospective study. *Cardiology.* 2010;117:184-189.
- [221] Elmariah S, Delaney JA, O'Brien KD, Budoff MJ, Vogel-Claussen J, Fuster V, et al. Bisphosphonate Use and Prevalence of Valvular and Vascular Calcification in Women MESA (The Multi-Ethnic Study of Atherosclerosis). *J Am Coll Cardiol.* 2010;56:1752-1759.
- [222] Aksoy O, Cam A, Goel SS, Houghtaling PL, Williams S, Ruiz-Rodriguez E, et al. Do bisphosphonates slow the progression of aortic stenosis? *J Am Coll Cardiol.* 2012;59:1452-1459.
- [223] Kietadisorn R, Juni RP, Moens AL. Tackling endothelial dysfunction by modulating NOS uncoupling: new insights into its pathogenesis and therapeutic possibilities. *Am J Physiol Endocrinol Metab.* 2012;302:E481-495.
- [224] Matsumoto Y, Adams V, Walther C, Kleinecke C, Brugger P, Linke A, et al. Reduced number and function of endothelial progenitor cells in patients with aortic valve stenosis: a novel concept for valvular endothelial cell repair. *Eur Heart J.* 2009;30:346-355.

- [225] Poggianti E, Venneri L, Chubuchny V, Jambrik Z, Baroncini LA, Picano E. Aortic valve sclerosis is associated with systemic endothelial dysfunction. *J Am Coll Cardiol*. 2003;41:136-141.
- [226] Liberman M, Bassi E, Martinatti MK, Lario FC, Wosniak J, Jr., Pomerantzeff PM, et al. Oxidant generation predominates around calcifying foci and enhances progression of aortic valve calcification. *Arterioscler Thromb Vasc Biol*. 2008;28:463-470.
- [227] Kennedy JA, Hua X, Mishra K, Murphy GA, Rosenkranz AC, Horowitz JD. Inhibition of calcifying nodule formation in cultured porcine aortic valve cells by nitric oxide donors. *Eur J Pharmacol*. 2009;602:28-35.
- [228] Cagirci G, Cay S, Canga A, Karakurt O, Yazihan N, Kilic H, et al. Association between plasma asymmetrical dimethylarginine activity and severity of aortic valve stenosis. *J Cardiovasc Med*. 2011;12:96-101.
- [229] Ngo DT, Heresztyn T, Mishra K, Marwick TH, Horowitz JD. Aortic stenosis is associated with elevated plasma levels of asymmetric dimethylarginine (ADMA). *Nitric Oxide*. 2007;16:197-201.
- [230] Schumm J, Luetzkendorf S, Rademacher W, Franz M, Schmidt-Winter C, Kiehn topf M, et al. In patients with aortic stenosis increased flow-mediated dilation is independently associated with higher peak jet velocity and lower asymmetric dimethylarginine levels. *Am Heart J*. 2011;161:893-899.
- [231] Boursinos LA, Karachalios T, Poultsides L, Malizos KN. Do steroids, conventional non-steroidal anti-inflammatory drugs and selective Cox-2 inhibitors adversely affect fracture healing? *J Musculoskelet Neuronal Interact*. 2009;9:44-52.
- [232] Grosser T. Variability in the response to cyclooxygenase inhibitors: toward the individualization of nonsteroidal anti-inflammatory drug therapy. *J Investig Med*. 2009;57:709-716.
- [233] Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem*. 2000;69:145-182.
- [234] Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Rev*. 1999;79:1193-1226.
- [235] Lopez J, Fernandez-Pisonero I, Duenas AI, Maeso P, Roman JA, Crespo MS, et al. Viral and bacterial patterns induce TLR-mediated sustained inflammation and calcification in aortic valve interstitial cells. *Int J Cardiol*. 2012;158:18-25.
- [236] Alander CB, Raisz LG. Effects of selective prostaglandins E2 receptor agonists on cultured calvarial murine osteoblastic cells. *Prostaglandins Other Lipid Mediat*. 2006;81:178-183.
- [237] Choudhary S, Alander C, Zhan P, Gao Q, Pilbeam C, Raisz L. Effect of deletion of the prostaglandin EP2 receptor on the anabolic response to prostaglandin E2 and a selective EP2 receptor agonist. *Prostaglandins Other Lipid Mediat*. 2008;86:35-40.

- [238] Chyun YS, Raisz LG. Stimulation of bone formation by prostaglandin E2. *Prostaglandins*. 1984;27:97-103.
- [239] Gao Q, Xu M, Alander CB, Choudhary S, Pilbeam CC, Raisz LG. Effects of prostaglandin E2 on bone in mice in vivo. *Prostaglandins Other Lipid Mediat*. 2009;89:20-25.
- [240] Jee WS, Ueno K, Kimmel DB, Woodbury DM, Price P, Woodbury LA. The role of bone cells in increasing metaphyseal hard tissue in rapidly growing rats treated with prostaglandin E2. *Bone*. 1987;8:171-178.
- [241] Kaneki H, Takasugi I, Fujieda M, Kiri M, Mizuochi S, Ide H. Prostaglandin E2 stimulates the formation of mineralized bone nodules by a cAMP-independent mechanism in the culture of adult rat calvarial osteoblasts. *J Cell Biochem*. 1999;73:36-48.
- [242] Li M, Healy DR, Li Y, Simmons HA, Crawford DT, Ke HZ, et al. Osteopenia and impaired fracture healing in aged EP4 receptor knockout mice. *Bone*. 2005;37:46-54.
- [243] Minamizaki T, Yoshiko Y, Kozai K, Aubin JE, Maeda N. EP2 and EP4 receptors differentially mediate MAPK pathways underlying anabolic actions of prostaglandin E2 on bone formation in rat calvaria cell cultures. *Bone*. 2009;44:1177-1185.
- [244] Zhang X, Schwarz EM, Young DA, Puzas JE, Rosier RN, O'Keefe RJ. Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. *J Clin Invest*. 2002;109:1405-1415.
- [245] Chikazu D, Li X, Kawaguchi H, Sakuma Y, Voznesensky OS, Adams DJ, et al. Bone morphogenetic protein 2 induces cyclo-oxygenase 2 in osteoblasts via a Cbfa1 binding site: role in effects of bone morphogenetic protein 2 in vitro and in vivo. *J Bone Miner Res*. 2002;17:1430-1440.
- [246] Susperregui AR, Gamell C, Rodriguez-Carballo E, Ortuno MJ, Bartrons R, Rosa JL, et al. Noncanonical BMP signaling regulates cyclooxygenase-2 transcription. *Mol Endocrinol*. 2011;25:1006-1017.
- [247] Kakita A, Suzuki A, Ono Y, Miura Y, Itoh M, Oiso Y. Possible involvement of p38 MAP kinase in prostaglandin E1-induced ALP activity in osteoblast-like cells. *Prostaglandins Leukot Essent Fatty Acids*. 2004;70:469-474.
- [248] Celil Aydemir AB, Minematsu H, Gardner TR, Kim KO, Ahn JM, Lee FY. Nuclear factor of activated T cells mediates fluid shear stress- and tensile strain-induced Cox2 in human and murine bone cells. *Bone*. 2010;46:167-175.
- [249] Mehrotra M, Saegusa M, Voznesensky O, Pilbeam C. Role of Cbfa1/Runx2 in the fluid shear stress induction of COX-2 in osteoblasts. *Biochem Biophys Res Commun*. 2006;341:1225-1230.
- [250] Boniface K, Bak-Jensen KS, Li Y, Blumenschein WM, McGeachy MJ, McClanahan TK, et al. Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *J Exp Med*. 2009;206:535-548.

- [251] Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, et al. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. *Nat Med.* 2009;15:633-640.
- [252] Garcia Rodriguez LA, Gonzalez-Perez A, Bueno H, Hwa J. NSAID use selectively increases the risk of non-fatal myocardial infarction: a systematic review of randomised trials and observational studies. *PLoS One.* 2011;6:e16780.
- [253] Salvo F, Fourrier-Reglat A, Bazin F, Robinson P, Riera-Guardia N, Haag M, et al. Cardiovascular and gastrointestinal safety of NSAIDs: a systematic review of meta-analyses of randomized clinical trials. *Clin Pharmacol Ther.* 2011;89:855-866.
- [254] Willette RN, Eybye ME, Olzinski AR, Behm DJ, Aiyar N, Maniscalco K, et al. Differential effects of p38 mitogen-activated protein kinase and cyclooxygenase 2 inhibitors in a model of cardiovascular disease. *J Pharmacol Exp Ther.* 2009;330:964-970.
- [255] Wirrig EE, Yutzey KE. Transcriptional regulation of heart valve development and disease. *Cardiovasc Pathol.* 2011;20:162-167.
- [256] Rajamannan NM, Evans FJ, Aikawa E, Grande-Allen KJ, Demer LL, Heistad DD, et al. Calcific aortic valve disease: not simply a degenerative process: A review and agenda for research from the National Heart and Lung and Blood Institute Aortic Stenosis Working Group. Executive summary: Calcific aortic valve disease-2011 update. *Circulation.* 2011;124:1783-1791.