Calcific aortic valve disease (CAVD) increasingly afflicts our aging population. One third of our elderly have echocardiographic or radiological evidence of calcific aortic valve sclerosis (CAVS), an early and subclinical form of CAVD. Age, sex, tobacco use, hypercholesterolemia, hypertension, and type II diabetes mellitus all contribute to the risk of disease that has worldwide distribution. On progression to its most severe form, calcific aortic stenosis, CAVD becomes debilitating and devastating, and 2% of individuals >60 years are affected by calcific aortic stenosis to the extent that surgical intervention is required. No effective pharmacotherapies exist for treating those at risk for clinical progression. It is becoming increasingly apparent that a diverse spectrum of cellular and molecular mechanisms converge to regulate valvular calcium load; this is evidenced not only in histopathologic heterogeneity of CAVD, but also from the multiplicity of cell types that can participate in valve biomineralization. In this review, we highlight our current understanding of CAVD disease biology, emphasizing molecular and cellular aspects of its regulation. We end by pointing to important biological and clinical questions that must be answered to enable sophisticated disease staging and the development of new strategies to treat CAVD medically. (Circ Res. 2013;113:198-208.)

Key Words: aortic valve, calcification of vascular calcification vascular senescence
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
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<td>BMP</td>
<td>bone morphogenetic protein</td>
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<td>CAS</td>
<td>calcific aortic stenosis</td>
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<td>CAVD</td>
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<td>CAVS</td>
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<td>COP</td>
<td>circulating osteogenic precursor cell</td>
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<tr>
<td>CNP</td>
<td>C-type natriuretic peptide</td>
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<td>EC</td>
<td>endothelial cell</td>
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<td>EnMT</td>
<td>endothelial-mesenchymal transition</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>MV</td>
<td>matrix vesicle</td>
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<td>oxLDL</td>
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<td>ROS</td>
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<td>TLR</td>
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<td>tumor necrosis factor</td>
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<td>VEC</td>
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<td>VIC</td>
<td>valve interstitial cell</td>
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It ends with important biological and clinical questions that must be answered to enable sophisticated disease staging and the development of new strategies to medically treat CAVD.

**Histopathologic Heterogeneity of CAVD**

The surgical view of CAVD is shaped by the gross anatomic appearance of rock-hard calcific nodules distorting and stiffening the normally pliant aortic leaflets. Detailed histopathologic studies first established that much of the material in these calcified nodules is amorphous calcium phosphate, that is, acellular aggregates not organized into any recognizable histological nodule organization. Srivatsa et al subsequently identified bone protein expression and true ectopic bone formation by histology in calcifying native human aortic valves. Open arrows, osteocytes; close arrows, osteoblasts. Of note, Mohler et al subsequently identified that ≥13% of calcifying valve specimens have histological ectopic bone replete with hematopoietic elements. See text for details. Reproduced from Srivatsa et al with permission from the publisher. Copyright © 1997, American Society for Clinical Investigation.

**Figure 1. True ectopic bone formation occurs in a substantial portion of calcified human aortic valves.** Srivatsa et al identified bone protein expression and true ectopic bone formation by histology in calcifying native human aortic valves. Open arrows, osteocytes; close arrows, osteoblasts. Of note, Mohler et al subsequently identified that ≥13% of calcifying valve specimens have histological ectopic bone replete with hematopoietic elements. See text for details. Reproduced from Srivatsa et al with permission from the publisher. Copyright © 1997, American Society for Clinical Investigation.

Endothelial dysfunction is likely to precede even these changes, reflecting the important role of the VECs as the initial barrier to metabolic, mechanical, and inflammatory insults. Moreover, patients with aortic valve sclerosis exhibit evidence of global endothelial dysfunction. VECs can also contribute to calcifying vascular cell types via the endothelial-mesenchymal transition (EnMT) elicited response to cues that promote arteriosclerotic calcification. As disease progresses, an exuberant inflammatory infiltrate consisting of T cells and macrophages is elaborated. This inflammatory infiltrate is common both to CAVD of aging and the precocious biomineralization that arises with bicuspid aortic valves. Interestingly, very recent data suggest that an adaptive immune response may be activated in CAVD because clonally expanded effector-memory T cell populations are present both in the valve and in the circulation of patients with severe CAS. The contribution of acquired immunity to CAVD remains to be determined. However, innate immune responses are clearly involved; nucleotide-binding domain and leucine-rich repeat containing family, pyrin domain containing 3 (NLRP3) and toll-like receptor (TLR) signals recognizing pathogen-associated molecular patterns, oxidized LDL, and amorphous calcium phosphate are activated and pathogenic in CAVD (see below).

**Vesicle-Mediated Mineralization: An Ultrastructural Component Common to Both Calcifying Valves and the Skeleton**

The earliest calcium deposition is observed in a stippled pattern associated with the base of lesions, viz., at the fibrofatty interface with the fibrofatty expansion of the valve spongiosa.
Amorphous calcium phosphate deposits can form readily via epitaxial mineral deposition on a number of nidi, including cholesterol crystals, fragmented elastin fibers, and collagen. Murshed et al were among the first to demonstrate that coexpression of collagen and alkaline phosphatase (ALP) in the elastin-rich environment of skin was adequate to drive mineralization. By hydrolyzing inorganic pyrophosphate and dephosphorylating osteopontin, natural inhibitors of calcium phosphate deposition, ALP enables robust tissue mineralization that would rapidly occur at the prevailing and physiological calcium phosphate concentrations that are fully permissive for mineral deposition. Rajamannan et al deployed immunogold electron microscopy to demonstrate the increased presence of ALP protein in CAVD.

The ALP ectoenzyme is not randomly secreted into the extracellular milieu but is shed from cells bound to matrix vesicles (MVs), spherical lipid bilayers of ≈30 to 100 nm diameter, which organize mineralization in physical association with elastin and collagen fibrils. The prescient bone biologist, H. Clarke Anderson, first demonstrated the presence of ALP-positive MVs in atherosclerotic plaques. Kim, Schoen, and colleagues went on to show the contributions of ALP-laden MVs to the valve sclerotic mineralization process in native and bioprosthetic valves. A novel MV phosphatase discovered by Kiffer-Moreira et al, Phosphil, may also play a particularly important role in MV-mediated biomineralization. However, although osteogenic gene expression, including ALP, can drive arterial calcification with or without overt ectopic bone formation, it has been recently appreciated that regulated biomineralization also can initiate even in conditions of relatively low ALP activity, albeit more slowly. MVs also contain annexin A5, annexin A6, and phosphatidyl serine, molecules that readily bind calcium and nucleate mineral deposition in the absence of ALP activity. Of note, ubiquitin staining of CAVD specimens revealed evidence of enhanced autophagy and cell death with a relative paucity of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive cells, suggesting that MVs released during autophagic death may be distinct from those of apoptosis. It should be noted that, once initiated, deposition of amorphous calcium phosphate may progress in the absence of MVs, particularly if inhibitors of mineralization, such as pyrophosphate, phosphi-osteopontin, and fetuin, are deficient. Indeed, the proinflammatory, thrombin-degraded form of osteopontin is observed at sites of severe CAVD. The biochemical nature of the earliest mineral deposits in CAVD, and the relative roles for ALP, phospholipids, annexins, and other nucleators versus mineralization inhibitors in disease progression, remains to be fully characterized. Once mineral deposition has been initiated, the arrival of circulating osteoprogenitors derived from myeloid cell lineage may play a particularly important role in programming stage-specific disease responses.

Circulating Osteoprogenitors and True Ectopic Bone Formation in Valve Ossification

In the advanced CAVD lesions described by Fitzpatrick, Mohler, and Rajamannan, both amorphous calcium phosphate concretions and ectopic woven bone formation were observed. This points to the molecular and cellular heterogeneity of the biomineralization processes that control valvular calcium accrual (Figure 3). Only recently have the potential reasons for this heterogeneity been mechanistically considered, likely relating in part to the recruitment of circulating osteoprogenitors capable of recapitulating the complete bone microenvironment. Khosla, Pignolo, and colleagues have identified circulating osteoprogenitor cell populations that participate in ectopic osteogenic injury responses during fracture repair and valve ossification. The origin of these circulating cells is almost certainly the bone marrow because circulating osteogenic precursor (COP) cells appear in higher numbers during growth phase and young adulthood. Indeed, the well-known relationship between younger age and bioprosthetic valve failure may relate to this observation. In the ectopic bone seen in 13% of patients with CAS, Mohler and Pignolo described sequential phases of inflammatory infiltration, fibroproliferation, neovascularization, cartilage formation, and endochondral ossification. Type I collagen(+) CD45(+) COP cells appear in human valves at the fibroproliferative (70%) and neovascularization (30%) phases of disease, whereas CD45(+) cells were...
identified in both ossifying and nonossifying valve segments.\(^5^2\) Because COP cells were only observed in segments adjacent to valves with true heterotopic ossification, the authors suggest that this cell population participates in the late-stage histopathologic evolution of CAVD.\(^5^2\)

More recently, the myeloid calcifying cell was described by Fadini et al.\(^5^5\) These cells are positive for both ALP and osteocalcin. Studies of sex-mismatched bone marrow transplant recipients and studies of individuals afflicted with chronic myelogenous leukemia confirmed bone marrow derivation of myeloid calcifying cell osteoprogenitors.\(^5^9\) Importantly, circulating myeloid calcifying cells are increased in the setting of type II diabetes mellitus, and may help explain the increases in CAVS arising in both native and bioprosthetic valves in this setting.\(^5^5\)

Very recently, Hajdu et al.\(^5^6\) provided evidence that in murine models CD45(+)CD133(+)CD34(−) bone marrow–derived cells contribute to the valve interstitial cell (VIC) population on the ventricular side of semilunar valves in the absence of valve injury during normal valve homeostasis. A subset of these cells located near sites of valve coaptation expressed myeloid markers.\(^5^6\) The precise relationships between this latter cell type, COP cells, myeloid calcifying cells, and the fibrocyte, first described by Bucala,\(^5^7\) have yet to be firmly established. However, Pignolo speculates that the COP cells his group identified likely represent members of the myeloid-derived fibrocyte lineage.\(^5^8\) Because rigorous functional assays are coregistered with more sophisticated cell-surface lineage markers, these relationships will no doubt emerge. Furthermore, as described below, the mechanical and paracrine milieu that regulates the osteofibrogenic potential of valve tissue-autonomous cell types may be significantly altered by valve accumulation of myeloid-derived lineages, including both COP cells and macrophages.

**Morphogens and the Pathobiology of CAVD**

The aortic valve forms from both neural crest derived and endodermal, viz, endocardial, cushion progenitors,\(^5^9\) with migratory neural crest cells programmed in part via interactions with cells of the secondary heart field.\(^5^9\) Many of the key paracrine signaling molecules of the Wnt,\(^6^0\) Notch\(^6^0\) signaling family as well as of the transforming growth factor (TGF)-β superfamily (including BMP),\(^6^1\) which are critical for control of bone formation,\(^6^2\) also play vital roles in the earliest stages of aortic valve morphogenesis. Moreover, as Lincoln and Yutzey first highlighted, a number of transcriptional regulators necessary for cartilage, bone, and tendon formation, including Sox9, NFATc1, Msx1, Msx2, and scleraxis, are also highly expressed in developing aortic valves.\(^6^3\)

Although this may not seem immediately germane to CAVD, the roles played by these molecules in skeletal morphogenesis are enlightening when viewed in the context of diseased valve biology. Outside of the skull, bone formation largely occurs via endochondral ossification, a process whereby an initially avascular cartilaginous template provisionally mineralizes via MV and chondrocyte apoptotic mechanisms.\(^6^4\) Osteoclast-mediated calcified matrix remodeling and vascular invasion with vascular-associated osteoprogenitors result in true bone formation.\(^6^5\) The transcription factor Sox9 plays multiple vital and sequential roles in endochondral bone formation, starting with the specification of mesenchymal progenitors that form the first cartilaginous anlage.\(^7^0\) The VIC population contains abundant Sox9-positive mesenchymal cells that are osteogenic progenitors.\(^7^0\)\(^7^1\) As Lincoln et al.\(^7^1\) demonstrated, Cre-mediated conditional deletion of Sox9 from EC and mesenchymal cells demonstrated its critical role in valve morphogenesis, VIC proliferative expansion, proteoglycan expression, and CAVD. As in the skeleton, although specifying a cell type with the capacity for biomineralization, valve Sox9 demarcates and maintains the VIC as a structurally synthetic but nonmineralizing mesenchymal cell with self-renewing potential.\(^7^1\) As a transcription factor important for elaboration of specific extracellular matrix programs, Sox9 binds WWCAAWX(N)CWTTGWW (W=A or T) DNA cognates, then directs the expression and

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**Figure 3. Sources of aortic valve osteoprogenitors: a heterogenous mix.** Mineralizing cell types relevant to the pathobiology of calcific aortic valve disease have been shown to arise from the osteogenic transdifferentiation of valve interstitial cells (VICs), circulating osteoprogenitors, and the endothelial-mesenchymal transition. Annular chondrocytes that produce the cardiac valve skeletal scaffold may contribute as well. Osteogenic nodules initially form in the interstitium oriented toward the aortic surface. However, once initiated, epitaxial deposition of amorphous calcium phosphate can occur without cell-mediated regulation. True ectopic bone formation occurs in >13% of specimens and likely requires the recruitment of a circulating osteoprogenitor that is capable of organizing a marrow microenvironment replete with hematopoietic elements.\(^7^0\) COP indicates circulating osteogenic precursor cell; EC, endothelial cell; ePC, endothelial progenitor cell; and oxLDL, oxidized low-density lipoprotein. See text for details (Illustration Credit: Ben Smith).
ultimate secretion of chondrocytic type II, IX, and XI collagens, and multiple proteoglycans, including aggrecan, biglycan, and fibromodulin.72 The latter 2 are particularly important because biglycan and fibromodulin organize the extracellular niche environment for progenitors capable of creating dense regular connective tissue.73 Loss of Sox9 expression in VICs is accompanied by progression to biomineralization,73 similar to the programming one expects from studies of the endochondral growth plate in the developing skeleton.70 Sox9 binding to cognates in Runx2 and Oxs genes, 2 transcriptional regulators necessary for osteogenic mineralization, has been proposed to inhibit elaboration of the osteoblast phenotype during chondrocyte specification.72 Thus, a model emerges in which sustained Sox9 expression and activity in VICs preserves the proliferative and synthetic chondrocytic phenotype necessary for valve function and durability, and prevents the elaboration of osteogenic mineralization programs.71,74 Of note, because Msx2 binds to Sox9 and inhibits its function,75 some of the pro-osteogenic actions of this homeodomain transcription factor may in fact relate to antagonism of Sox9 actions in VICs and other osteochondroprogenitors above and beyond its activation of canonical Wnt signaling.76

Another molecule familiar to skeletal biologists, C-type natriuretic peptide (CNP) or C-type natriuretic peptide, is also important in the biology of valve calcinosis. CNP functions to promote endochondral bone formation via activation of B-type CNP receptors, a transmembrane guanylate cyclase that signals in part via downstream cyclic GMP–dependent protein kinases protein kinase G (PKG)-I and PKG-II.77,78 Simmons et al79 first identified that VECs on the ventricular surface express very high levels of CNP, as compared with the aortic VECs on the aortic surface of the aortic valve. They went on to show that VECs located in the ventricular portion of the valve interstitium also expressed high levels of CNP.80 Because the ventricular face of the aortic valve is resistant to the procalcific mechanical and metabolic milieu that drives CAVD,7 they examined the effects of CNP on VIC-dependent mineralization. CNP and PKG signaling were shown to prevent VIC osteogenic nodule formation and mineralization.80 Thus, paracrine interactions between ventricular surface VECs and subjacent VIC populations may serve to restrict osteogenic potential of valve cells, and may explain in part the temporospatial evolution of CAVD.

Other pathways important in skeletal morphogenesis are also involved in the regulation of cardiac valve morphogenesis and CAVD. TGF-β, BMPs, and Wnt polypeptides act via corresponding activin like kinase (ALK)- and LDL receptor-related protein (LRP)-receptor signaling complexes to promote bone formation, mineralization, and skeletal homeostasis throughout vertebrate life.66 Cairns et al12 first identified the contributions of Wnt/β-catenin signaling to CAVD. Insightful studies demonstrated expression of the osteogenic Wnt receptor LRP5 and upregulation of signaling mediator β-catenin in calcifying human aortic valve specimens.13 Subsequent analysis of apolipoprotein E–deficient; LRP5−/− mice demonstrated that global deficiency of this canonical Wnt signaling system reduced aortic valve calcification.61 Of note, circulating inhibitors of Wnt signaling are elevated in patients with CAVD62; because many of these inhibitors are upregulated by canonical Wnt pathway activation as mechanisms for feedback inhibition, these molecules may serve as useful biomarkers for disease severity.62

TGF-β superfamily/ALK receptor/Smad signaling cascades, initiated by ALK1 and ALK5 activation in ECs and ALK5 in pericytes, are indispensable for normal vasculogenesis from the very earliest stages of vertebrate development.83,84 Likewise, active Wnt/LRP receptor/β-catenin signaling is necessary for the function of EC progenitors,85 and, with BMP/ALK receptor/Smad pathways, it promotes the EnMT necessary to create cardiac valves66 and epicardial fibroblasts.87 These same morphogenetic pathways seem to participate in the pathobiology of cardiovascular calcification and postnatal valve homeostasis. Miller et al88 were among the first to demonstrate that hypercholesterolemia-driven CAVD activated osteogenic BMP/Smad1/5 and fibrogenic TGF-β/Smad2 signaling in a hemodynamically significant murine disease model. Intriguingly, once initiated, cholesterol lowering was capable of reducing calcification but not fibrosis or Smad2 signaling in diseased valves. Ankeny et al89 related activation of this fibro-osteogenic signaling cascade in human aortic valves to selective downregulation of Smad6, an intracellular inhibitor of BMP and TGF-β signaling.

Yao et al90 elegantly demonstrated that inhibition of this BMP-activated pathway may be safely achieved to limit cardiovascular calcification by autocrine/paracrine elaboration of matrix Gla–protein. Matrix Gla–protein is a vitamin K-dependent, noggin-like, faux receptor inhibitor of BMP2 and BMP4.91 Although a detailed analysis of aortic valve function was not undertaken, it is intriguing to note that exposure to warfarin, an inhibitor of matrix Gla–protein γ-carboxylation and function, is significantly associated with human CAVD risk.92 Using an ex vivo model, Poggio et al93 demonstrated that cyclic stretch and BMP4 could activate VIC-mediated calcification of human aortic valve leaflets, and that noggin inhibited this process. Similarly, Derwall et al94 demonstrated that Fc-ALK3, an engineered BMP faux receptor, could also safely reduce cardiovascular calcification in low density lipoprotein receptor−/− (LDLR−/−) mice. The ALK2/ALK3 inhibitor LDN-193189, a compound with lesser inhibitory potency for ALK5, was able to recapitulate the effects of Fc-ALK3 administration in this model.94 A full toxicology study was not performed and, unfortunately, valvular structure and function were not addressed.

Other studies have recently pointed to important roles for ALK signaling in valve homeostasis and highlight the therapeutic challenges. The TGF-β receptor ALK5 has been targeted by postnatal pharmacological inhibition in hopes of mitigating cardiopulmonary, renal, and hepatic fibrosis.95 Moreover, as discussed below, mechanically challenged VICs become hyper-responsive to TGF-β, elaborating myofibroblast phenotype and osteogenic potential.96 However, Anderson et al97 recently demonstrated that ALK5 inhibition with either AZ12601011 or AZ12799734, small molecule inhibitors of ALK5, induced cardiac valve inflammation with neutrophil infiltration, VIC proliferation, and hemorrhagic degeneration in adult rats. These data point to the critical role for
ALK5 activity in maintenance of valve integrity throughout life, but highlight the difficulties of direct targeting of ALK receptors for treatment of cardiovascular disease.\(^\text{95,97}\) Strategies that electively modify ALK signaling tone via extracellular inhibitors of specific ligands seem to hold therapeutic promise.

**Inflammation, Oxidation, and DNA Damage; Intracrine Activation of Osteogenic Programs in CAVD**

A cellular feature common to virtually all clinically significant forms of macrovascular calcification, including CAVD, is inflammation.\(^\text{7,17}\) Tumor necrosis factor (TNF),\(^\text{98,99}\) interleukin (IL) 1-\(\beta\),\(^\text{100}\) advanced glycosylation end products,\(^\text{101,102}\) IL6,\(^\text{103}\) and oxidized LDL (oxLDL) cholesterol\(^\text{104,105}\) have all been shown to activate vascular biomineralization and vascular osteogenic signaling processes. Valve calcification is increased in mice lacking interleukin-1 receptor antagonist (IL1RA), an inhibitor of IL1 signaling.\(^\text{106}\) Because concomitant TNF deficiency reduces calcification arising with IL1RA deficiency,\(^\text{106}\) TNF bioactivity seems to be an important component, potentially related to pro-osteogenic oxidative stress signaling.\(^\text{107}\) Furthermore, osteogenic BMP and Wnt signaling cascades are entrained to TNF activity in the vessel wall.\(^\text{108}\) However, with respect to CAVD, the actions of oxLDL deserve special attention. oxLDL is a proinflammatory pathogenic component of dyslipidemia, arising from spontaneous extracellular chemical oxidation of LDL cholesterol.\(^\text{109}\) oxLDL signals in part via heteromeric TLR complexes, the receptors for viral and bacterial pathogens that activate innate immune responses to nonspecifically fight infection.\(^\text{109}\) Elevated levels of oxLDL are associated with worsened fibrocalcific responses in CAVD.\(^\text{21}\) Zeng et al\(^\text{110}\) recently identified that vascular BMP2 expression was inducible by TLR4 agonists and by a biglycan-TLR2 relay in VICS.\(^\text{22}\) In this model, BMP2 and ALP induction by oxLDL were reduced by RNAi-mediated TLR2 knockdown. Mathieu confirmed biglycan signaling via TLR2 in human CAVD.\(^\text{111}\) However, López et al\(^\text{112}\) also showed that TLR4 agonists can activate human VICS. The relative in vivo contributions of TLR2, TLR4, and other coreceptors in this pathway to CAVD remain to be delineated.

A key component of inflammation is intracellular oxidative stress, which produces signaling cascades that generate reactive oxygen species (ROS), such as hydrogen peroxide and superoxide. Miller et al\(^\text{113}\) were the first to demonstrate the pro-osteogenic, pathogenic role of ROS generation in CAVD. Using a combination of human histochemistry, histopathology, and murine disease models, they showed that hydrogen peroxide generation downstream of nitric oxide synthase uncoupling played a vital role in disease biology. Moreover, they demonstrated that enzymatic defenses that dissipated oxidative stress were downregulated in diseased valves.\(^\text{113}\) In vascular smooth muscle cells and vascular myofibroblasts, hydrogen peroxide activates both osteogenic Cbfal/Runx2 and Mxs2/ Wnt\(^\text{107}\) signaling cascades to promote mineralization. Both of these regulatory cascades were shown by Miller et al\(^\text{113}\) to be activated in calcifying human aortic valves. Liberman et al\(^\text{113}\) noted elevated hydrogen peroxide levels adjacent to ectopic calcification in a rabbit model of CAVD, independently confirming the relationship with oxidative stress. Moreover, oxLDL increases VIC production of Wnt3a, a morphogen that drives osteogenic differentiation via LRPP,\(^\text{116}\) The VIC population arises in part from circulating hematopoietic stem cells\(^\text{98,117}\); thus, as proposed by Rajamannan,\(^\text{116}\) an osteogenic stem cell niche, replete with marrow elements,\(^\text{17}\) may be ectopically created in aortic valves by the sustained paracrine Wnt signals elaborated in response to oxLDL.

Recently, Branchetti et al\(^\text{118}\) reported an exciting new component of osteogenic ROS signaling in VICS. They showed that hydrogen peroxide activated the DNA damage response, with Akt activation mediating the upregulation of the osteogenic transcription factors Runx2 and Mxs2.\(^\text{118}\) Transduction of VICs with adenovirus expressing catalase prevented osteogenic transcription factor induction.\(^\text{118}\) Because hydrogen peroxide also mediates TNF induction of Mxs2 and Wnt7b in calcifying vascular myofibroblasts,\(^\text{107}\) modulation of ROS signaling and the osteogenic phase of the DNA damage response may provide new therapeutic options for both valve and vascular sclerosis. Moreover, because type II diabetes mellitus and the metabolic syndrome result in significant mitochondrial dysfunction that propagates cellular ROS accumulation,\(^\text{119}\) targeting this osteogenic intracrine signaling cascade may prove to be most therapeutically effective in that clinical setting. These data converge with intriguing insights from Chau et al,\(^\text{20}\) demonstrating a role for BMP-Smad1 signaling in the DNA damage response. Smad1 not only promotes osteogenic Mxs2 and Runx2 gene expression,\(^\text{21}\) but also functions as a potent activator of Runx2-directed transcription.\(^\text{21}\) Thus, a potential intracrine signaling cascade emerges whereby the DNA damage response may promote osteogenic calcium accrual in VICS as relevant to CAVD (Figure 4).

Another morphogenetic signaling pathway that is central to bone formation, valve morphogenesis, and CAVD is the Jagged/Notch pathway. In the skeleton, Notch1 signaling maintains proliferative expansion of bone marrow mesenchymal cells, whereas inhibiting precocious osteoblast differentiation and depletion of this osteoprogenitor pool.\(^\text{121}\) In the developing heart, Jagged1 signals provided by ECs support Notch1-mediated EnMT necessary for cardiac valve morphogenesis.\(^\text{124}\) Notch1 deficiency in mice and humans causes a spectrum of aortic valve diseases, including bicuspid aortic valve and CAVD.\(^\text{125,126}\) Ex vivo, Notch suppresses osteogenic Runx2 signaling and mineralization in VICS.\(^\text{74,127,128}\) Moreover, Acharya et al\(^\text{14}\) demonstrated that Notch1 sustains expression of Sox9 in VICS, inhibiting osteogenic mineralization as predicted from the model of Yutzey (Figure 5). Of note, in this respect, VIC genomic responses to Notch1 activation diverge from those elicited in skeletal chondrocytes, where Sox9 is a direct target of Notch1 and RBPJK-mediated suppression.\(^\text{129}\) Whether Notch1 signaling is altered by metabolic and mechanical stimuli that promote cardiovascular calcification remains to be determined. However, haploinsufficiency for a RBPJK, a key transcriptional mediator of Notch1 signaling, predisposes to diet-induced CAVD in mice.\(^\text{127}\)

**The Impact of Mechanical Environment on CAVD**

As mentioned above, TGF-\(\beta\) receptor I and ALK3 signaling, activated by TGF-\(\beta\) superfamily members, is vital to the
earliest stages of embryonic vasculogenesis, vascular remodeling, and cardiovascular morphogenesis. Even at this very earliest stage of vascular physiology, mechanical forces alter vascular remodeling and cellular potential directed by paracrine morphogen signaling. Of note, VICs exhibit profound sensitivity to mechanical cues, selectively elaborating osteogenic potential under the correct mechanical environment. To maintain a quiescent, nonosteogenic, nonmyofibrogenic phenotype, VICs must experience a matrix stiffness <10 kPa. The mineralizing osteogenic phenotype is most apparent when VICs are exposed to a matrix stiffness of 25 kPa, a value that approximates that experienced by bone-forming cells during osteoid.133 At a stiffness >100 kPa,131,132 the osteogenic potential diverges from that of Notch1 signaling in skeletal chondrocytes.129 The osseous tissue response to TGF-β downstream of TGF-β signaling,134,135 and must experience a matrix stiffness <10 kPa to resume the original quiescent state. Of note, independent studies implementing BMP2/BMP4 functionally assessed by development of a contractile phenotype, VICs respond to TGF-β downstream of TGF-β signaling,134,135 and must experience a matrix stiffness <10 kPa to resume the original quiescent state. Of note, independent studies implementing BMP2/BMP4 functions to sustain Sox9 expression in VICs, an action that diverges from that of Notch1 signaling in skeletal chondrocytes.129

Figure 4. DNA damage in the osteogenic programming of valve interstitial cells (VICs): an emerging osteogenic regulatory cascade. Recent studies from Branchetti et al118 converge with that of Chau et al120 to suggest the indicated osteogenic signaling cascades downstream of cellular DNA damage. The ataxia telangiectasia mutated (ATM) kinase mediates activation of (1) Smad1 signals; (2) p53-dependent cell cycle arrest vs apoptosis; (3) H2AX and Ku antigen–dependent DNA damage repair; and (4) KH-type splicing regulatory protein (KSRP)–dependent microRNA processing. See text for details.

CAVD and the EnMT

As mentioned above, the EnMT plays a central role in cardiac valve development and the generation of fibroblasts from epicardium during ischemic repair.77 Studies by Paruchuri et al16 first demonstrated coexpression of CD31 and smooth muscle cell α-actin in a subset of ovine VICs. They went on to show that human pulmonary VECs were capable of responding to TGF-β2 to elaborate a VIC phenotype. Human pulmonary VEC and ovine aortic VEC clones behaved similarly, with EnMT downstream of TGF-β2 dependent on Notch signaling.65 The osteogenic potential of clonal ovine VEC populations was also clearly demonstrated.15 Ex vivo, mechanical strains on the order of 10% to 20% were shown to enhance EnMT via TGF-β2/Smad and Wnt/β-catenin signaling cascades, respectively.14 Strains applied orthogonal to anisotropic VEC alignment (mediated by extracellular fibronectin) exerted the greatest stimulus for EnMT, as functionally assessed by development of a contractile response to endothelin.14 The extent to which EnMT contributes to CAVD in vivo has yet to be determined. Intriguingly, activating mutations in human ALK2 have been demonstrated to promote heterotopic bone formation in fibrodysplasia ossificans progressiva, occurring in great part via osteoprogenitor development from the endothelial-mesenchymal transition.138 However, although these patients develop massive ectopic bone deposits in muscle in association with minimal trauma, cardiac valve calcification is not characteristic of this disorder.139

Future Directions: Promises and Pitfalls

Clearly, CAVD is both common and complicated. Although we know so much more about the pathobiology than we did a decade or so ago, we still lack key clinical tools and nonsurgical therapeutic options. Novel diagnostics, biomarkers, and therapeutic strategies are needed. Not everyone with echocardiographically defined CAVS progresses to end-stage CAS that requires aortic valve replacement surgery. Although we
know some of the risk factors, including echocardiographic estimates of valve calcium load, in early stage disease, we cannot predict with any certainty who will and will not progress. Development of robust biomarkers for disease progression will not only provide the clinical tools necessary for patient risk stratification and assessment of response to therapeutic intervention, but also may illuminate novel pathways that can be targeted medically to prevent disease progression. The need to capture this risk of disease progression is particularly acute in patients with bioprosthetic valves but may be fundamentally different from patients with native valve sclerosis. The seminal realization by Demer that oxylipids and cellular ROS signals are common to both bone loss and arterial calcium accrual suggests that cellular and molecular signatures indicative of vascular and skeletal oxidative stress will prove useful. Moreover, a point of no return will exist that can be targeted medically to prevent disease progression. Development of robust biomarkers for disease progression will not only provide the clinical tools necessary for disease portend overlapping yet distinct disease biology. Because elastin fragments liberated by cathepsin S protease as these may afford effective therapeutic mechanisms with the ALK5 inhibitors, indicating that more pharma- tication and progression. However, the potential risks associat- ed with targeting the ALK receptor kinases became apparent with the ALK5 inhibitors, indicating that more pharmaco- logical tools and studies are required. Moreover, Kalluri very recently demonstrated that small peptide agonists for ALK3 actually promoted the reversal of renal fibrosis and renal regeneration. Thus, technologies are needed to ensure tissue-specific targeting of agents modulating morphogenetic signaling pathways. Of note, in a model of uremic cardio- vascular calcification, Akaiwa et al demonstrated that inhibition of cathepsin S could mitigate progressive CAVD. Because elastin fragments liberated by cathepsin S protease activity are bioactive peptides that promote osteogenic differentia- tion in concert with TGF-β, novel strategies such as these may afford effective therapeutic mechanisms with acceptable safety profiles.

A few important clues lie within the clinical literature with respect to potential medical strategies for CAVD treat- ment. Older patients with osteoporosis treated with amino- bisphosphonates experience less aortic valve and aortic calcium accrual. Moreover, bisphosphonates reprogram the circulating osteoprogenitors, suggesting that certain osteotropic drugs can favorably impact the bone-vascular axis. However, younger women treated with bisphosphonates experience increases in aortic valve calcium load, indicating that the rate-limiting mechanisms regulating vascular calcium metabolism change with age. Once again, a better understanding of the mechanisms controlling the initiation and progression of CAVS is needed. As a result of the morphogenetic, metabolic, mechanical, inflammatory, and neuroendocrine regulation of CAVD taking shape from ongoing research, new diagnostic and therapeutic strategies will emerge that will better help us address the needs of our patients with valvular heart disease.

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Human aortic valve calcification is associated with an osteoblast phenotype.


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206 Circulation Research July 5, 2013


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