The Regulation of Valvular and Vascular Sclerosis by Osteogenic Morphogens

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Abstract: Vascular calcification increasingly afflicts our aging, dysmetabolic population. Once considered only a passive process of dead and dying cells, vascular calcification has now emerged as a highly regulated form of biomineralization organized by collagenous and elastin extracellular matrices. During skeletal bone formation, paracrine epithelial-mesenchymal and endothelial-mesenchymal interactions control osteochondrocytic differentiation of multipotent mesenchymal progenitor cells. These paracrine osteogenic signals, mediated by potent morphogens of the bone morphogenetic protein and wingless-type MMTV integration site family member (Wnt) superfamilies, are also active in the programming of arterial osteoprogenitor cells during vascular and valve calcification. Inflammatory cytokines, reactive oxygen species, and oxylipids—increased in the clinical settings of atherosclerosis, diabetes, and uremia that promote arteriosclerotic calcification—elicit the ectopic vascular activation of osteogenic morphogens. Specific extracellular and intracellular inhibitors of bone morphogenetic protein–Wnt signaling have been identified as contributing to the regulation of osteogenic mineralization during development and disease. These inhibitory pathways and their regulators afford the development of novel therapeutic strategies to prevent and treat valve and vascular sclerosis. (Circ Res. 2011;109:564-577.)

Key Words: BMP  ■  Wnt  ■  inflammation  ■  osteoblast differentiation  ■  vascular calcification
Atherosclerotic calcification increasingly afflicts our aging and dysmetabolic population.1 Vascular calcification was once thought to be a passive process of dead and dying cells. However, elegant studies by the bone and cartilage biologist H. Clarke Anderson in the 1970s identified alkaline phosphatase (ALP)-containing matrix vesicles as sites of atherosclerotic and medial vascular mineralization2,3; this provided intriguing evidence that actively regulated osteogenic processes were participating in vascular mineralization. Another bone biologist and physician-scientist, Marshall Urist, had identified a “morphogenetic matrix for differentiation of bone tissue” earlier in that decade,4 and in 1988 John Wozney, Vicky Rosen, and colleagues cloned and characterized the first genes encoding bone morphogenetic proteins, including bone morphogenetic protein (BMP)2.5 In 1993, Boström, Demer, and colleagues first demonstrated the expression of BMP2 in calcified human atherosclerotic plaques—and the capacity of BMPs to direct osteogenic differentiation of bone tissue” earlier in that decade,4 and in 1988 John Wozney, Vicky Rosen, and colleagues cloned and characterized the first genes encoding bone morphogenetic proteins, including bone morphogenetic protein (BMP)2.5 In 1993, Boström, Demer, and colleagues first demonstrated the expression of BMP2 in calcified human atherosclerotic plaques—and the capacity of BMPs to direct osteogenic differentiation of bone tissue.”

Since 1993, the notion that osteochondrogenic morphogens participate in the biology of arterial calcification has been robustly confirmed and extended to encompass newly discovered regulators of skeletal development.9 In this report, we review the regulation of valvular and vascular sclerosis by osteogenic morphogens. We highlight mechanistic insights afforded by these studies that provide the foundation for novel therapeutic approaches to treat arteriosclerotic valve and vascular disease.

BMP-Wnt Signaling: An Osseocentric Perspective

**Paracrine Signals Control Skeletal Osteogenesis**

Bone and cartilage are the two best-recognized tissues of the vertebrate skeleton, the former arising in intimate association with the vasculature.10 The skeleton contains two major anabolic cell types, the osteoblast and the chondrocyte.11
True bone formation or “ossification” occurs through two distinct mechanisms. Membranous ossification describes the mineralization that occurs in neural crest–derived craniofacial bones. Membranous ossification does not require a preceding cartilaginous template but occurs directly within a type I collagen–based extracellular matrix elaborated and organized by osteoblasts. By contrast, most bones of the body mineralize through endochondral ossification. This second process requires an initial, avascular type II collagen–based extracellular matrix template deposited by chondrocytes. Subsequent chondrocyte hypertrophy at the growth plate, type X collagen deposition, vascular endothelial growth factor (VEGF)-driven vascular invasion, chondrocyte apoptosis, and cartilaginous matrix mineralization ensues. This is followed by osteoclast-mediated matrix resorption and osteoblast-mediated bone deposition (again, type I collagen–based) that replaces the calcified cartilage template.

During skeletal development, paracrine epithelial-mesenchymal interactions control the osteogenic programming of skeletal anlage, best characterized during odontogenesis and endochondral ossification of the appendicular skeleton. As first elucidated in studies of limb morphogenesis, proximal-distal, anterior-posterior, and dorsal-ventral morphogen gradients interact to control skeletal patterning, growth, and ossification. For example, reciprocal interactions between epithelial FGF4/FGF8 in the apical ectodermal ridge (AER) and mesenchymal FGF10 maintain limb bud outgrowth. Ectodermal Wnt3a signaling is required for AER FGF8 production. Sonic hedgehog production by posterior mesenchyme called the zone of polarizing activity maintains AER function through gremlin, a negative regulator of BMP signaling. Limb BMP4 tone provides a powerful ventralizing signal that is modulated by gremlin (preserves the AER) and by Wnt7a elaborated from the dorsal ectoderm. Dynamic expression of Dkk1 in ventral limb bud, anterior and posterior mesenchyme, and the AER antagonizes Wnt3a and Wnt7a signals that maintain FGF8 and sonic hedgehog expression. Thus, a complex 4-dimensional interplay between Wnt, BMP, FGF, Hedgehog, Dkk1, and other paracrine signals controls the growth, lineage specification, and differentiation of osteochondroprogenitors in limb bud mesenchyme. Likewise, paracrine PTHrP signals at the mineralizing growth plate control the temporospatial timing of endochondral bone formation through negative regulation of Indian Hedgehog and the FGF- and VEGF-dependent vascularization required for endochondral ossification as mentioned above. Again, in the craniofacial skeleton, membranous bones and teeth form without the requirement of a preceding calcified cartilage template. Whether arising from endochondral or intramembranous processes, once true bone has formed paracrine BMP and endocrine PTH tone maintains osteogenic Wnt/β-catenin signals that support postnatal skeletal anabolism, mechanical integrity, and fracture repair.

**Transcriptional Mediators of Osteochondrogenic Differentiation**

Sox9-positive mesenchymal condensations demarcate the skeletal morphogenetic field. Robust osteogenic programming requires coordinated actions of L-Sox5, Sox6, and HIF. Robust osteogenic programming of mesenchymal progenitors requires gene regulatory programs directed by at least 6 DNA-binding transcription factors: Runx2 (a.k.a. Cbfa1), Msx1 and Msx2, LEF1, NFATc1, and Osx. β-Catenin, a transcriptional coadapter that promotes transcription directed by the TCF/LEF transcription factor family, is indispensable for osteoblast differentiation as well and maintains the elaboration of osteogenic transcriptional programs (Figure 1). Runx2 has all the attributes of a “master gene” differentiation factor for the osteoblast lineage and bone matrix gene expression in both membranous and endochondral bone. During embryonic development, Runx2 expression precedes osteoblast differentiation and is restricted to mesenchymal cells destined to become osteoblasts (Figure 1). In addition to its critical role in osteoblast commitment and differentiation, Runx2 appears to control osteoblast activity, the rate of bone formation by differentiated osteoblasts, in concert with ATF4. In the skull, additional signals provided by the homeodomain proteins Msx1 and Msx2 are critical for intramembranous bone formation—a process that...
that cannot proceed even in the presence of an intact Runx2 gene when Msx expression is lacking. Downstream of Msx, Runx2, and β-catenin genomic programs is osterix (Osx), a zinc finger transcription factor required for matrix mineralization in concert with NFATc1. Importantly, the expression of Msx1, Msx2, Runx2, and Osx during osteogenic differentiation is critically dependent on BMP signaling. Moreover, BMP2 signaling in mesenchyme maintains the specification of osteoprogenitors required for postnatal skeletal integrity.

**BMPs: Prototypic Osteogenic Morphogens**

BMPs are secreted polypeptides, a subgroup of the transforming growth factor (TGF)-β superfamily of growth factors. BMPs were first identified as a bioactivity in demineralized and pulverized bone powder capable of inducing ectopic endochondral bone formation in muscle. Over the ensuing years, more than 15 distinct BMP family members have been identified (the BMP system is extensively reviewed elsewhere). The BMPs are secreted in an active form, with function modulated by extracellular antagonists such as noggin, chordin, gremlin, cross-veinless 2 (CV2, also referred to as BMPER), cartilage oligomeric matrix 2 (COMP2), and matrix Gla protein (MGP; Figure 2). BMPs elicit their effects through activation of receptor complexes composed of type I and type II Ser/Thr kinase receptors, to which the BMPs bind independently. There are 7 type I receptors, termed activin receptors-like kinase (ALK1 to ALK7), which determine the specificity of BMP signal in concert with five type II receptors including the BMP type II receptor. When the ligand binds, the type II receptors phosphorylate and activate the type I receptors, which propagate the signaling by phosphorylating transcription factors referred to as Smads (Figure 2). Receptor complexes may also contain so-called type III coreceptors, which further modulate signaling. The BMP type I receptors (ALK1, ALK2, ALK3, and ALK6) activate the receptor-activated (R)-Smads, Smad1, Smad5, and Smad8, whereas the activin and the TGF/β type I receptors (ALK4, ALK5, and ALK7) phosphorylate Smad2 and Smad3. Activated Smads assemble into complexes together with the common mediator (co)-Smad4, which translocate to the nucleus and modulate gene expression in concert with Runx2, TCF/LEF, and other factors. This process is regulated in part through inhibition by the inhibitory I-Smads, Smad6, and Smad7 (Figure 2). BMPs can activate non-Smad signaling pathways as well. Studies by Rawadi, Baron, and colleagues first identified one of these non-Smad signals to be a paracrine Wnt/β-catenin relay that promotes alkaline phosphatase expression and matrix biomineralization in conjunction with and parallel to BMP/Smad signaling.
Wnts: Osteogenic Modulators and Mediators of BMP Action

The Wnts are a family of secreted, lipid-modified polypeptide ligands that classically signal through activation of heterodimeric receptor complexes containing LRP5, LRP6, or LRP4 and a GPCR coreceptor of the Frizzled (Fzd) gene family (Figure 3). LRP coreceptor complexes with ROR2 are clearly also important and reviewed in detail elsewhere. In bone formation and skeletal homeostasis, Wnt10b, Wnt7a, Wnt7b, Wnt3a, and Wnt1 have emerged as important, although other ligands are certain to contribute. With canonical or classic Wnt signaling mechanisms, nuclear accumulation of β-catenin ensues. β-Catenin is a transcriptional coadaptor indispensible for Runx2- and LEF1-mediated programming of osteogenic differentiation. Collaborative interactions with BMP- and TGFβ-regulated Smad complexes also occur. Even in the presence of skeletal osteochondrogenic transcription factor expression (Runx2, Msx1/2, LEF1, Oxs), robust elaboration of osteogenic programs is impossible in the absence of activated Wnt/β-catenin signaling. The canonical pathway sequentially utilized a casein kinase 1/dishevelled signaling cascade to phosphorylate GSK3 and inhibit the β-catenin destruction complex, thus increasing cellular β-catenin levels and nuclear accumulation. Additionally, a “noncanonical” pathway also requiring dishevelled but proceeding through protein kinase Cδ activation has been identified to support Oxs expression and bone formation. In a manner analogous to the negative regulation of BMP signaling, secreted Wnt binding proteins known as secreted frizzled-related proteins inhibit Wnt ligand binding and activation of LRP5/6-Fzd cell surface receptors. Two classes of nonsignaling “ligands”—members of the Dkk family and SOST/sclerostin—bind to the osteogenic LRP5s and inhibit signaling (Figure 3). In addition, intracellular inhibitors of Wnt/β-catenin signaling such as ICAT (inhibitor of β-catenin and TCF/LEF) antagonize protein-protein interactions between β-catenin and nuclear transcription factor complexes bound to DNA (Figure 3). Thus, the sequential and parallel interactions between the BMP and Wnt signaling cascades control osteochondral mineralization, with intracellular and extracellular fine-tuning of signal duration and strength.

Role of BMP Signaling in Vascular Biology and Calcific Vasculopathy

BMP Signaling in Vascular Development and Disease

When BMP2, BMP4, and BMP6, were detected in calcified areas of atherosclerotic lesions, it was therefore pre-
sumed that they enhanced vascular calcification—even more so when it became evident that vascular calcification is largely driven by osteogenesis in the vascular media. Indeed, a causal relationship between BMP activity and vascular calcification has now been established. However, BMP signaling is not only driving ectopic calcification but is essential for cardiovascular development, with critical roles in the establishment of endothelial tubes during vasculogenesis, the recruitment and differentiation of vascular smooth muscle cell (VSMC) precursor cells, and vascular patterning. Mutations in the genes coding for the ALK1 receptor and endoglin, an ALK1 coreceptor, cause hermaphrodite hereditary telangiectasia, which is characterized by abnormal angiogenesis and arteriovenous malformations. Furthermore, mutations in the BMP type II receptor and ALK1 receptors have been linked to pulmonary arterial hypertension, characterized by a dysregulation of vascular plasticity, proliferation, and differentiation in VSMC. ALK1 activation in turn induces MGP, an angiogenic factor and induces expression of ALK1, 67 suggesting that regulation of BMP activity is essential for maintaining a normal media. The potential anticalcific roles of other BMP antagonists, such as noggin, CV2, gremlin, chordin-like 1, and cartilage oligomeric proteins are not yet known although gremlin is overexpressed and may have antiosteogenic effects in the calcified media in uremia.

In addition to the aorta, MGP is also expressed in organs such as the lungs, where it plays a role in the formation of the pulmonary arterial tree by inhibiting BMP4. BMP2 in particular has an inhibitory effect on VSMC proliferation and differentiation, whereas BMP7 promotes the VSMC phenotype in a manner reminiscent of TGF-β. Furthermore, BMP inhibition—potentially in later steps—appears to be a key factor in maintaining VSMC differentiation. Loss of MGP, an inhibitor of BMP2 and BMP4, causes extensive calcification of elastic and muscular arteries. The loss of MGP results in a significant increase in aortic BMP activity and leads to osteochondrogenic transdifferentiation of VSMC with subsequent mineralization, supporting the notion that regulation of BMP activity is essential for maintaining a normal media. The potential anticalcific roles of other BMP antagonists, such as noggin, CV2, gremlin, chordin-like 1, and cartilage oligomeric proteins are not yet known although gremlin is overexpressed and may have antiosteogenic effects in the calcified media in uremia.

BMP activity is important for the regulation of phenotypic plasticity, proliferation, and differentiation in VSMC. BMP2 in particular has an inhibitory effect on VSMC proliferation and differentiation, whereas BMP7 promotes the VSMC phenotype in a manner reminiscent of TGF-β. Furthermore, BMP inhibition—potentially in later steps—appears to be a key factor in maintaining VSMC differentiation. Loss of MGP, an inhibitor of BMP2 and BMP4, causes extensive calcification of elastic and muscular arteries. The loss of MGP results in a significant increase in aortic BMP activity and leads to osteochondrogenic transdifferentiation of VSMC with subsequent mineralization, supporting the notion that regulation of BMP activity is essential for maintaining a normal media. The potential anticalcific roles of other BMP antagonists, such as noggin, CV2, gremlin, chordin-like 1, and cartilage oligomeric proteins are not yet known although gremlin is overexpressed and may have antiosteogenic effects in the calcified media in uremia.

Regulation of Vascular BMPs and BMP Antagonists: The Activated Endothelium

An activated endothelium may be a significant source of BMP and a potential calcific stimulus. BMP expression is easily activated in the endothelium in response to a number of different pathogenic stimuli, many of which are well known to stimulate atherosclerosis and vascular calcification. Sorescu, Jo, and colleagues were the first to demonstrate that BMP4 is induced in aortic endothelial cells (ECs) by oscillatory shear stress, reactive oxygen species (ROS), and inflammatory cytokines. Subsequently, Csiszar, Ungvari, and colleagues showed that BMP2 is similarly induced by inflammatory cytokines and ROS and functions as an inflammatory mediator.

Interestingly, BMP inhibitors are induced in ECs in response to similar stimuli as BMP2 and BMP4, or by an increase in vascular BMP activity. In vitro, follistatin, noggin, and MGP and CV2 are induced by oscillatory shear stress or by high glucose levels. Most of these inhibitors are also induced in the aortic wall of fat-fed apoE null mice or diabetic mice and rats. Enhanced BMP activity causes induction of some inhibitors such as MGP and CV2 but not others such as noggin or chordin, pointing to a finely tuned mechanisms for regulation of BMP activity. Interestingly, HDL, a vasculoprotective factor, promotes higher expression of MGP in ECs and aortas of apoAI transgenic mice, thereby pushing the balance toward BMP inhibition. In addition, CV2 is induced by statins in ECs and also promotes BMP inhibition. Thus, the balance between endothelial BMPs and their inhibitors may determine the health of the endothelium, and an efficient limitation of endothelial BMP activity may confer protection against atherosclerosis. This also emphasizes the consideration of “BMP activity” or “BMP tone” as a context-specific working concept arising from the expression of specific BMP ligands, receptors or antagonists.

It should also be kept in mind that the adventitial microvasculature is also exposed to oxidative stress and inflamma-
tory mediators similar to the aortic endothelium and probably responds with higher BMP2/4 secretion. In addition, microvascular pericytes may contribute multipotent cells to the calcific process. Thus, a calcific process could also be triggered from capillaries in the periphery of the vessel.

Vascular BMP activity is stimulated in cells other than ECs. BMP2 production is upregulated in SMC treated by high phosphate in the form of nanocrystals and in pericytes and mesangial fibroblasts by high glucose. In addition, medial expression of BMPs, BMP receptors, and inhibitors is increased in diabetic mice. A subset of myofibroblasts also expresses BMP2 in response to diabetes and hyperlipidemia, even though myofibroblasts have been reported to downregulate BMP6 as a potential step in vascular remodeling.

**Role of BMPs in Triggering the Calcification of Atherosclerotic Lesions**

Endothelial BMP2 and BMP4 cause an induction of endothelial adhesion molecules in the ECs, in several instances using noncanonical signaling pathways. This provides a direct link between BMP activity and the initial stages of atherosclerosis, which sets the stage for atherosclerotic lesion development and ultimately lesion calcification. Once lesion formation gets under way, there may be local reactivation of developmental conditions where enhanced BMP activity could trigger osteogenesis in susceptible cells. Indeed, MGP, noggin and chordin are downregulated in dedifferentiated developmental conditions where enhanced BMP activity gets under way, there may be local reactivation of developmental conditions where enhanced BMP activity could trigger osteogenesis in susceptible cells. Indeed, MGP, noggin and chordin are downregulated in dedifferentiated VSMCs, which would make them more susceptible to BMP action. A number of BMP antagonists are later detected at increased levels in calcified vessels and may in that context help limit the progression of the ectopic calcification.

As already discussed, the endothelium may have a role in triggering vascular calcification, and several populations of multipotent cells that respond to BMPs have been identified in the vasculature. These include the calcifying vascular cells (CVC) isolated from the aortic media, myofibroblasts present in the adventitia of diabetic and hyperlipidemic LDLR−/− mice, and microvascular pericytes well known to have osteogenic potential (Figure 1). Circulating stem cells may also contribute osteogenic changes in the artery wall, and VSMC “transdifferentiation” certainly contributes to the vascular endochondral ossification mechanisms in MGP−/− mice, as elegantly demonstrated by Giachelli and colleagues. However, the relative contribution of the respective cell population to local osteogenesis and vascular calcification in different arteriosclerotic disease states remains to be established.

The anticalcific role of MGP may depend on normal EC differentiation. Recently, MGP transgenic mice were crossed with ApoE−/− mice, a model of atherosclerosis and lesion calcification, to study the effect of BMP inhibition on lesions. The increase in MGP resulted in diminished SMAD1/5/8 signaling, inflammation, lesion formation, and calcification after fat feeding. The majority of the MGP was found in adventitial myofibroblasts in the aorta of fat-fed diabetic LDLR−/− mice. BMP2 was instrumental in augmenting the BMP-2/Msx2-Wnt pathway leading to an osteogenic phenotype in a subset of the myofibroblasts. It was later discovered that diabetes in mice and rats activated BMP signaling throughout the vascular wall. Interestingly, the location of BMP2 and BMP4 differed in diabetic aortas in that BMP-4 was found in the endothelium and BMP2 all the way through the vascular wall. The high BMP4 was associated with high MGP, ALK1, ALK2, and MGP, which were all regulated as a group in ECs. When a MGP transgene was introduced to limit the BMP activity, MGP expression increased predominantly in the endothelium, even though BMP activity and calcification diminished in the entire media, suggesting cross-talk between the endothelium and the media.

Diabetes is a common cause of chronic kidney disease (CKD), another powerful stimulator of vascular calcification. The calcification in CKD has been primarily attributed to increased phosphate levels, which strongly promotes SMC mineralization in vitro and in vivo. High phosphate levels stimulate secretion of BMP2 in SMCs and BMP2 in turn upregulates the type III sodium-dependent phosphate cotransporter PiT1/SLC20A1, which mediates high phosphate-induced mineralization. Treatment with BMP7 counteracts vascular calcification in CKD, in part due to reduced phosphate levels and direct effects on SMC differentiation.
Therefore, it is important to understand all aspects of vascular BMP signaling in order to correctly target BMPs for anticalcific effects.

**BMPs, MGP, and Calcific Uremic Arteriopathy**

A particularly severe and rapidly deadly form of vascular calcification—calcific uremic arteriopathy (CUA, a.k.a. calciphylaxis)—arises in a subset of patients with CKD treated with warfarin for anticoagulation99–101 and rarely in patients with severe chronic liver disease. Widespread calcium phosphate deposition in pulmonary, intestinal, and dermal soft tissues—with predilection for the lower extremity, thighs and gluteal regions, and the abdominal pannus—is characteristic for CUA. Histopathologically, arteriolar (<0.6 mm diameter) medial calcification occurs—with concomitant fibroproliferative intimal vessel occlusion, dermal fat infarction, and skin necrosis. Immunohistochemistry demonstrates the expression of BMP4 in the periarteriolar adventitia of dermal CUA.102 Warfarin inhibits the MGP γ-carboxylation that is necessary for inhibition of BMP2/4 signaling61 as well as fetuin-dependent matrix vesicle clearance.103,104 Thus, it is highly probable that the warfarin-induced compromise in dermal arteriolar MGP function contributes to the pathobiology of CUA. However, the precise reasons for the histoanatomic disease predilection remain to be explained—and other γ-carboxylated proteins such as Gas6105 may also contribute to disease through Axl tyrosine kinase regulation of VSMC osteochondrocytic differentiation.

**Wnt/β-Catenin Signaling in Cardiovascular Calcification**

**Calcific Aortic Valve Disease: Consequences and Clinical Considerations**

Calcific aortic valve disease (CAVD) afflicts approximately 2% of our population over the age of 60 years.106 Progressive fibrosis and valve calcium accumulation impairs leaflet compliance and coaptation, impeding outflow, increasing myocardial work load, and permitting variable regurgitation. Once symptoms occur—for example, chest pain, syncope, or congestive heart failure—progressive aortic valve stenosis conveys a 2-year mortality rate that approaches 50% in the absence of surgical intervention.107 Using echocardiographic criteria, Rosenhek and colleagues demonstrated that the presence of aortic valve calcium in patients with asymptomatic mild or moderate aortic stenosis was the single most significant predictor of clinical progression.108,109

Numerous epidemiological studies identified risk factors for CAVD development, which are similar to those of vascular atherosclerosis, including smoking, male sex, body mass index, hypertension, elevated lipid and inflammatory markers, bicuspid aortic valve, type II diabetes mellitus, and/or metabolic syndrome and renal failure.110 Although aortic stenosis may occur in individuals with otherwise anatomically normal tricuspid aortic valves, congenital valve abnormalities markedly increase the risk. Importantly, nearly half of older individuals with aortic stenosis also have a bicuspid aortic valve (BAV), an aortic valve that develops with 2 functional leaflets instead of the normal 3.106,111,112 BAV occurs in about 2% to 3% of the population and is the most common congenital cardiac malformation. Although the causes for the development of BAV are unclear, genetic factors have been identified in some cases. BAV disease tends to progress more rapidly for reasons that are poorly understood. Genetic mutations associated with BAV that cause cellular dysfunction may also predispose an individual to other congenital heart defects or to dilation and dissection of the ascending aorta.111

Detailed clinical histopathology reveals valve inflammation as a common theme whether CAVD arises with BAV, hypertension, dyslipidemia, or any of the other risk settings highlighted above. Furthermore, the pathological lesions of calcified aortic valves demonstrate the complexities and heterogeneity of soft tissue mineralization. Calcium accrual in areas of fibrosis, necrosis, atherosclerosis/lipid accumulation, hypertrophic cartilage, and ectopic bone can all be observed.113,114 Otto and colleagues described the fibro-fatty expansion and inflammation of the lamina fibrosa, displaced and/or split elastic lamina, and intracellular and extracellular lipid accumulation with stippled calcification in the earliest of valve lesions.115 With progression, woven bone formation—complete with marrow elements—and nodular amorphous calcium phosphate accumulates, the latter presumably through epitaxial mineral deposition on virtually acellular concretions. Nevertheless, the molecular “fingerprints” of active osteochondrogenic mineralization are present in all calcifying human aortic valve specimens—even when overt ossification (ectopic bone replete with marrow) is not observed.113,116–118 Features of both membranous and endochondral osteogenic programs are elaborated by calcifying valve interstitial cells (VICs),118 with osteogenic potential highly dependent on the stiffness of the extracellular matrix environment.119 Additional osteoprogenitors may derive from chondrocytes originating at the base of the semilunar valve leaflet insertions120 and from valve ECs through EMT (see below). The frequent (~15%) ectopic bone formation and virtually omnipresent elaboration of osteochondrocytic programs in CAVD prompted evaluation for the potential contributions of Wnt/β-catenin signaling.9

**Wnt/β-Catenin Signaling and the Biology of Calcific Aortic Stenosis**

The first evidence that activation of Wnt/β-catenin signaling participated in the calcification of human aortic valves came from quantitative western blot analysis comparing calcifying human tricuspid aortic valves versus normal aortic valves and mitral valves undergoing myxomatous degeneration. Accumulation of β-catenin, the prototypic mediator and marker of canonical Wnt signaling necessary for osteoblast formation,23 was upregulated 3.5- to 4-fold in calcifying tricuspid aortic valves as compared with normal aortic valves or myxomatous mitral valves.116 Expression of the osteochondrogenic transcription factor Runx2/Cbfal was also increased along with the chondrocytic transcription factor Sox9 and the bone-specific matrix proteins bone sialoprotein and osteocalcin. Additionally, expression of the Wnt coreceptor LRP5 and the ligand Wnt3 were identified as being increased in calcified aortic valves as assessed by immunohistochemistry.116 Sub-
sequent studies by Miller, Heistad, and colleagues confirmed the upregulation of Runx2 and Msx2 in calcifying valves, with overlapping yet distinct patterns of expression. Analyses of preclinical models have provided mechanistic insights into the role of Wnt/β-catenin signaling in diabetes- and dyslipidemia-induced arteriosclerotic calcification.118 Transgenic augmentation of aortic valve and vascular Msx2 expression upregulates multiple arterial Wnt ligand expression and increases in arterial calcium deposition. Implementing the TOPGAL mouse that possesses a LacZ-based reporter for endogenous activation of β-catenin dependent-transcription, concomitant upregulation of Wnt/β-catenin signaling was observed in calcifying aortic valves as well as arterial tunica media.121 Consistent with this, the capacity of VICs to undergo myofibroblastic conversion ex vivo is also dependent on β-catenin signaling that synergizes with TGFβ.40 The specific Wnt ligands and LRPs receptors participating in valve calcification have yet to be characterized, although LRP5 and Wnt3 are potential candidates based on evaluation of calcifying human valve specimens.

At this point, the important role of the endothelium—including both the aortic and ventricular surfaces of valve endothelium—should be reemphasized. The biology of the aortic valve is regulated by valve ECs adjacent to valve interstitial myofibroblast cells.122 These ECs maintain the health of the valve and mediate valve disease in the presence of cardiovascular risk factors and/or genetic signals. In the skeleton, angiogenesis and osteogenesis are tightly coupled processes.10 In keeping with this relationship, Rajamannan et al171 have provided evidence of osteogenic-angiogenic “coupling” in ossifying human rheumatic valves analyzed following removal for valve replacement surgery. The full spectrum of paracrine signals participating in valve cell-cell interaction—and the signals that trigger activation and differentiation of VICs along the osteogenic lineage—have not been fully established. However, BMP-Wnt signaling in endothelial integration of valve matrix metabolism and remodeling will continue to emerge as being important. As mentioned above, the inflamed endothelium elaborates BMP2 and BMP4. Triggers include hemodynamic shear stress, abnormal endothelial nitric oxide synthase function, inflammatory cytokines and growth factors, and the intracellular metabolic environment arising from dyslipidemia, hyperglycemia, and uremia. Moreover, as D’Amore and colleagues first noted, endothelial cells express LRPs and LRP5, in addition to multiple Wnt ligands,123,124 and platelet-derived Dkk1 induces an inflammatory EC phenotype.125 Intriguingly, valve endothelial cells have the capacity to undergo EMT,126 a process whereby cadherin-mediated cell-cell interactions are reorganized, endothelial differentiation programs are downregulated, and myofibroblastic gene regulatory programs are activated. On acquiring the myofibroblast phenotype, valve ECs post-EMT have the capacity to undergo osteogenic differentiation,69 resembling in this fashion the emerging role of the circulating endothelial progenitor cell (ePC) as a vascular osteoprogenitor.90,127 During development, members of the TGFβ superfamily drive and coordinate valve EMT in concert with Msx gene family members and β-catenin dependent signals.91,128 Finally, ECs of the valve ventricular surface elaborate gene regulatory programs distinct from those of the aortic surface.129 The molecular regulators control postnatal valve EC fates and phenotypes have yet to be fully characterized. Of note, both Smad2 and β-catenin signaling have been implicated in the epithelial-mesenchymal transitioning that contributes to myofibroblast load in idiopathic pulmonary fibrosis.130 Lessons learned from these studies may help guide productive experimental approaches to valve sclerosis that occurs either in the presence or absence of calcium accrual.

**Wnt/β-Catenin Signaling in Diabetic Arteriosclerosis and Vascular Fibrosis**

When fed high-fat diets characteristic of Westernized societies, male LDLR-deficient mice develop diet-induced obesity, dyslipidemia, and hyperinsulinemic diabetes characteristic of type II diabetes mellitus and the metabolic syndrome.131,132 Recent studies from the Multiethnic Study of Atherosclerosis identify that these metabolic syndrome parameters convey risk for aortic and arterial calcification.133 Consistent with this, male LDLR−/− mice on high-fat diets develop progressively severe arteriosclerosis and fibrosis, with medial and atherosclerotic vascular mineralization.131,132,133,135 Early on, aortic upregulation of BMP2 and Msx2 is observed with subsequent Runx2 expression. Msx2-expressing adventitial myofibroblasts elaborate osteogenic Wnt3a, Wnt7a, and Wnt7b expression with concomitant paracrine activation of type I collagen and osteinx gene expression, alkaline phosphatase activity, and osteogenic mineralization.121,134,136,137 Treatment with Dkk1, an inhibitor of LRP4/5/6 specifically downregulated by Msx2, inhibits osteogenic mineralization by aortic myofibroblasts and other mesenchymal cell types.121,138

As first predicted by Demer, Tintut, and colleagues,139 inflammatory cytokine and oxidative stress cues appear to play an important role in the initiation of diabetic arteriosclerosis.55,134,140 The prototypic inflammatory cytokine TNF, largely derived from adipose tissue macrophages, enhances oxylipid-dependent mineralization of calcifying vascular cells and adventitial myofibroblasts on rigid matrices such as tissue culture plastic. In addition to upregulating BMP2 production in endothelial cells, TNF directly activates atherosclerotic Msx2 and Wnt/β-catenin signaling, the latter localized again to the tunica media with the TOPGAL reporter mouse.134 Pharmacological inhibition of endogenous oxidative stress signals generated by mitochondrial and NAD family flavoenzymes inhibits TNF activation of myofibroblast Msx2 expression.135 Recent data by others have confirmed that oxylipids such as oxLDL promote aortic VSMC mineralization in part through upregulation of osteogenic Msx2 signaling,141–143 although the Wnt/Dkk and β-catenin responses were not specifically evaluated. Moreover, oxLDL upregulates BMP2 expression in vascular myofibroblasts through tolloid receptors TLR2 and TLR4.144 Thus, in the vasculature, osteogenic BMP-Wnt signaling is entrained to innate immunity and inflammatory redox cues that initiate and propagate tissue fibrosis with osteogenic differentiation of local progenitors. Moreover, ROS signaling enhances Runx2-directed transactivation that drives and reinforces
the osteochondrogenic phenotype of transdifferentiating VSMCs\(^{145}\) (Figure 1). It is tempting to speculate that the “diabetesy” associated inflammatory redox signals that drive BMP-Wnt signaling may promote macrovascular EMT and thus further contribute to fibrosis, calcification, and mural thickening in diabetic arteriosclerosis. Novel strategies that combine inhibition of inflammatory cytokine signaling with enhanced egress of inflammatory oxylipids\(^{146}\) may serve to inhibit arteriosclerotic disease initiation and progression through vascular downregulation of these osteogenic morphogens.

Modulation of Vascular BMP-Wnt Signaling to Prevent or Ameliorate Vascular Calcification: Promises and Pitfalls

Selective modulation of vascular BMP activity is a promising target for treatment or prevention of arterial calcification. Decreased BMP activity resulting from excess MGP limits calcification\(^{35,55}\) and BMP type I receptor inhibition by small molecules has been successfully used to limit heterotopic calcification in mice with fibrodysplasia ossificans progressiva.\(^{147}\) However, there are still many questions that need clarification in the targeting and design of BMP inhibitors. For example, what cells would be the best targets—endothelial, smooth muscle, or adventitial cells? What would be the best molecular targets—ligands, receptors, or inhibitors? Similar strategies can be envisioned that would target the Wnt signaling cascade. However, because postnatal bone homeostasis and skeletal integrity is dependent on BMP-Wnt signaling, can strategies be identified that inhibit osteogenic vascular BMP-Wnt actions without mechanism-based toxicity in the skeleton? Similar issues arise when one considers many other mechanisms for modulating osteogenic morphogens in vascular disease. For example, Notch1 sensitizes VSMC to pro-osteogenic BMP2 signaling,\(^{148}\) including the upregulation of alkaline phosphatase through Msx2-dependent actions\(^{149}\) while inhibiting bone marrow osteoprogenitor differentiation.\(^{150}\) This suggests that antagonism of Notch1 signaling might safely help ameliorate pathogenic BMP signaling in VSMC-mediated arterial calcification. Yet, Notch1 also inhibits osteochondrogenic signaling in calcifying aortic valve cells.\(^{151,152}\) Moreover, decreases in Notch1 tone predispose to calcinotic bicuspid aortic valve formation\(^{152–154}\) and potential vascular tumor formation with hemorrhage.\(^{155}\) Given the emerging difference in valvarus versus vascular Notch1 actions, it remains to be determined whether modulation of the Notch1 signaling pathways offer a significantly wide therapeutic window for pharmacological intervention.

Alternatively, strategies can be envisioned that aggressively target the pivotal signals upstream of vascular BMP-Wnt activation to prevent disease initiation and slow arteriosclerotic progression. As discussed, inflammation, an abnormal oxidative stress environment, and oxylipids stimulate the vascular endothelium to upregulate the secretion of BMPs important in osteogenic cell signaling.\(^{35,74,75,135}\) These same signals downregulate important mineralization defense mechanisms, including the degradation of inorganic pyrophosphate by alkaline phosphatase\(^{132,156}\) and fetuin.\(^{157}\) Furthermore, mechanical stretch plays a permissive role, allowing vascular osteoprogenitors to transition to a calcifying phenotype.\(^{119}\) Thus, as contributors to pathobiology each of these processes are potential targets for pharmacological intervention. HMG CoA reductase agents, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers, provide one approach. Indeed, in the Japanese Aortic Stenosis Study, the use of angiotensin receptor blockers was associated with reduced risk of disease progression in CAVD.\(^{158}\) Interestingly, in preclinical models pulsatile hPTH (1–34) administration simultaneously reduces vascular\(^{156}\) and skeletal\(^{159,160}\) oxidative stress signals, while simultaneously reducing vascular calcification and enhancing bone mass accrual.\(^{137}\) Thus, novel anti-inflammatory ApoAI mimetics such as D-4F\(^{161,162}\) may help promote the egress of inflammatory oxylipids generated in the setting of diabetes and dyslipidemia in ways that simultaneously improve vascular and bone health.\(^{146}\) However, in any one individual, certain pathophysiological stimuli (hyperphosphatemia, hyperlipidemia, hypertension, hyperglycemia) may be more critically important for intervention than others\(^{1}\) and may differ with stage of disease. This may account for the disparate responses observed with respect to the impact of statin therapy on CAVD.\(^{163}\) With declining renal function, hyperphosphatemia plays an increasingly important role in vascular calcification.\(^{164}\) Finally, once a significant amount of vascular calcium has accrued—or true ectopic ossification has occurred—a “point of no return” may be reached at which medical intervention by any means is no longer possible. Thus, careful patient characterization and selection will be required when testing known or novel therapeutic interventions to prevent or retard vascular calcification.

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Disclosures

None.

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