American Heart Association | American Stroke Association

Atherosclerotic Calcification: Wnt Is the Hint

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Cardiovascular diseases remain the primary cause of death.[1] Myocardial infarction (MI), angina, and stroke take >16 million lives every year, but the underlying mechanisms by which these often-fatal cardiovascular events occur tend to escape attention.[2] Atherosclerosis is the underlying pathological inflammatory vascular disease not only responsible for most MIs and strokes, but it accounts for 29% of deaths worldwide.[1,3]

Once believed to be a passive process, it is now understood that atherosclerosis takes an active route involving several cell types, with contributions from a multitude of organ systems, molecular mechanisms, and other pathological conditions, including, but not limited to, hypertension, hyperlipidemia, and type 2 diabetes mellitus.[1,3–5] Atherosclerosis is characterized by the accumulation of lipids, fibrous elements, and inflammatory cells within the vascular wall of medium and large muscular and elastic arteries.[1,3,6] Atherosclerotic plaque formation leads to intimal thickening and luminal stenosis.[1] Reduction of blood flow ensues, leading to ischemia of the brain, heart, and extremities and clinically manifesting as MI, angina, or stroke.

The main features of atherosclerosis are endothelial dysfunction, intimal thickening, inflammation, and vascular calcification (VC).[4,7,8] These processes are considered a consequence to vascular injury.[1] VC is characterized by calcium deposition in the walls of the vasculature.[5] Expansion of the calcified lesion leads to thrombus formation and, if significant enough, vascular occlusion. Although the exact mechanisms for VC are unknown, there has been an increase in interest about the role Wingless (Wnt) signaling plays in disease pathogenesis. Briefly, the Wnt signaling pathway is an evolutionarily conserved pathway across kingdom Animalia and plays a crucial role in pattern formation during embryogenesis.[9] The Wnt signal transduction pathway plays a crucial role in organ formation in embryonic development, cell proliferation, polarity, migration, and differentiation.[10] Thus, deregulated Wnt signaling is associated with many human diseases.[11] Therefore, it is justifiable to suggest that research delineating atherogenesis, with focus on the contribution of Wnt signaling in the pathogenesis of atherosclerotic calcification, is warranted.

Components of the Vasculature System

Endothelium

The vascular endothelium has a multifaceted role, acting simultaneously as a permeability barrier, a nonthrombogenic surface, a regulator of vascular tone and transendothelial flow, and an inhibitor of vascular smooth muscle cell (VSMC) growth and migration.[12] Under normal circumstances, endothelial cells (ECs) release an array of vasodilators and anticoagulants, such as NO and prostacyclin, which reduce platelet aggregation, monocyte and leukocyte adhesion, and VSMC proliferation.[13] Under pathological conditions, ECs release factors, such as thromboxane and endothelin-1, that increase platelet aggregation, monocyte adhesion, vasoconstriction, and VSMC proliferation. Wnt signaling has been shown to be involved in EC permeability, proliferation, and survival.[14–16] Naturally, β-catenin binds to cadherins to stabilize adhesion of neighboring ECs.[17] During cellular migration, however, β-catenin localizes in the nucleus, resulting in a loss of tissue integrity.[18]

Vascular Smooth Muscle Cells

Smooth muscle cells of the vasculature are found as 1 of 2 possible phenotypes: the first is the contractile phenotype, the predominant form of VSMC, in the tunica media; and the second is the activated synthetic phenotype, in the tunica intima.[12] The differentiated contractile VSMCs can be

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stimulated by adrenergic receptors or angiotensin II. They have a low proliferation rate that is maintained in check by the endothelium-derived NO.[12] Vascular injury triggers the phenotypic change to an active synthetic phenotype with an increased proliferation rate and secretory ability.[19] Wnt signaling promotes VSMC survival,[20] whereas silencing Wnt signaling reduces cell proliferation.[21] Along with cellular proliferation, some data suggested that Wnt signaling may play a role in the VSMC migration during intimal thickening,[2,22] motility,[23] and osteogenesis.[24] Moreover, a recent study has demonstrated a role for Wnt5a in cholesterol efflux by VSMCs.[25]

Adventitial Fibroblasts

Fibroblasts predominate the vascular adventitia. They have low proliferation rates and can synthesize extracellular matrix proteins and ground substance. In response to injury, fibroblasts rapidly proliferate, migrate, and differentiate to myofibroblasts.[12] Canonical Wnt signaling has played a prominent role in fibroblast proliferation and differentiation.[26,27]

Endothelial Dysfunction, Intimal Thickening, and Luminal Stenosis

Some naturally occurring stimuli within the body have the ability to injure the vessel wall and lead to endothelial dysfunction. For example, the turbulent flow that occurs in bifurcated regions of the vasculature is enough to activate endothelium, which is perhaps why atherosclerotic plaque formation often occurs in sites like carotid and coronary arteries.[7] Long-term exposure to risk factors, such as hyperlipidemia, hypertension, and diabetes mellitus, also contributes to endothelial injury.[1,4] There are 2 ways by which the endothelium can be activated. During minimal vascular injury, whereby the short-lived inflammatory response can effectively remove or solve the problem, vessel remodeling is less likely to occur.[1] In contrast, sustained exposure to the above mentioned risk factors increases vascular adhesiveness, leakage, and accumulation of extracellular lipid within the vessel wall, thereby inducing foam cell formation and inflammatory response propagated by sustained release of growth factors and cytokines.[3,28,29]

Vascular endothelium is considered to be dysfunctional when inflammation is sustained, altering the endothelial homeostatic properties.[1] When dysfunctional, ECs exhibit increased expression of adhesion molecules and release cytokines, resulting in monocyte, lymphocyte, and platelet adhesion, enhancing the inflammatory response.[7,12] Adhesion of monocytes is considered the hallmark of atherosclerosis and considered atheromatous from this point forward.[4] Dysfunction also alters endothelial permeability, allowing intraplaque invasion of monocytes and macromolecules, like low-density lipoprotein.[19] Under normal conditions, NO inhibits VSMC migration. In endothelial dysfunction, NO levels are reduced, allowing VSMC proliferation and migration into the intima, contributing to the next key process in atherosclerotic progression: pathological intimal thickening.[7,12]

The sustained inflammatory response not only drives proliferation and migration of VSMCs, it also increases the inflammatory cell population by recruitment and intraplaque proliferation of macrophages and lymphocytes. Synthesis of extracellular matrix by VSMCs, along with inflammatory cell invasion, thickens the arterial wall.[1,30] When lipids begin to accumulate in the vessel wall, modification via aggregation induces lipid uptake in macrophages, creating foam cells.[6,7] Furthermore, macrophages can secrete cytokines to recruit more monocytes or induced proliferation of resident monocytes, creating a feed-forward cycle for an ad infinitum inflammatory state.[1,31]

Apoptosis of VSMCs and macrophages, along with loss of extracellular matrix, contributes to plaque instability and may lead to eventual rupture, which accounts for ≈50% of MIs and acute coronary syndromes attributable to occlusion of the artery.[1,28] A plaque burden of 70% or greater has significantly increased the risk of rupture.[32] However, approximately half of those who died from luminal thrombosis, leading to complete artery occlusion (resulting in sudden coronary death), had <75% stenosis, and ≈10% of sudden coronary deaths occur without atheromatous lesion formation.[33,34] Therefore, lesion composition may be just as relevant to risk of rupture as lesion size.[35]

Microvessel formation in the lesion, otherwise known as angiogenesis, contributes to plaque development and increases the risk of rupture.[8] In addition, there are suggestions that an obstructive calcified lesion is more stable than a nonobstructive thin-cap fibroatheroma, because the calcified plaque leads to stable angina pectoris, whereas the fibroatheroma has a tendency to rupture.[36] Thus, calcification may be paradoxically beneficial, despite its associations with cardiovascular morbidity and mortality.[30] Conversely, the increased incidence of disease and death with VC may not be a direct effect of calcification itself, but rather the fact that calcification is found in advanced atherosclerotic lesions.[37]

Vascular Calcification

VC is the pathological deposition of calcium in vascular structures and is a significant area of study because it commonly affects our aging population and those experiencing diabetes mellitus, dyslipidemia, heart valve disease, and end-stage renal disease.[5] VC is a marker for atherosclerosis

and is associated with several cardiovascular pathological features, including hypertension, congestive heart failure, cardiac hypertrophy, and ischemia, and increased risk of MI and stroke.[38,39] VC is a significant risk factor for morbidity and mortality in cardiovascular disease.[7,19,37,40–44] Once thought to be a passive degenerative process, it is now evident that biomineralization of the arterial wall is a complex regulated process.[5,45,46] There are 4 histoanatomic variants of VC, which include medial artery calcification, atherosclerotic intimal calcification, cardiac valve calcification, and soft tissue calciphylaxis or calcific uremic arteriolopathy.[38,47] A major breakthrough in the study of VC was the realization that the ectopic deposition of calcium in vascular structures is similar to intramembranous and endochondral ossification in bone development.[48] As early as the 19th century, bonelike tissue was identified within atherosclerotic arteries.[49]

In general, arterial calcification occurs in the intimal layer of the wall and in the aortic valve, and it is commonly found in the aorta and coronary, carotid, and renal arteries.[50,51] However, the prevalence of arterial calcification varies widely between populations.[51] Both vascular and cardiac valve calcification are considered complications of atherosclerosis because risk factors that predispose and/or potentiate plaque formation may also predict progression of calcification.[52–54] Intimal calcification itself is a key contributive process of advanced atherosclerotic lesion formation, and vascular calcium detection by computed tomography can be used as a subclinical marker of atherosclerosis. The fact that coronary artery and aortic valve calcification often occur concurrently reinforces the suggestion that atherosclerosis is a precursor to these variants of calcification.[4,7,33,42,55,56]

The progression of atherosclerosis results in a structural and cellular remodeling of the vasculature.[4,7,41,57] Changes that alter mineral metabolism, modify calcification regulators, or modulate vascular cell phenotype can have catastrophic consequences in vascular structures.[40,55,58–60] Advanced calcific lesions can develop woven bone and even commence hematopoiesis. This remodeling of the vasculature can extend to the point of acquiring some functional characteristics of bone, offering the perspective that VC occurs in a manner similar to bone development.[48,60–62] Briefly, osteogenesis involves formation of mesenchymal cell condensations, which differentiate into bone-forming cells for intramembranous or endochondral ossification.[63,64] Direct differentiation of mesenchymal cells to osteoblasts in intramembranous (IM) bone development allows deposition of mineralization-ready bone matrix, whereas EC ossification first requires the deposition of a cartilage template by chondrocytes, on which osteoblasts will elaborate to form bone.[65] Mineralization of bone is initiated by shedding of matrix vesicles by osteoblasts and chondrocytes.[66,67] These matrix vesicles contain hydroxyapatite crystals that act as nucleation sites for the mineralization cascade.[66,67] Several genetic studies support that both IM and EC mechanisms are regulated by multiple signaling factors and pathways, including bone morphogenic proteins (BMPs), muscle segment homeobox protein homolog 2 (Msx2), Runt-related transcription factor 2 (Runx2), sex determining region Y-box 9 (Sox-9), and Wnt.[5,68,69] BMPs regulate bone formation and osteogenic differentiation.[70] Msx2 expression is crucial for cranial bone development and IM ossification.[55] Runx2 is a master transcription factor also required for osteoblast differentiation and chondrocyte maturation.[65] Sox-9 is a master regulator of chondrogenic mesenchymal condensations.[55,65] Wnt signaling mechanisms regulate stem cell proliferation and organogenesis; they also regulate bone mass.[64] Altered expression of these factors impairs proper bone development by disturbing osteogenic cell differentiation or tissue mineralization.

There are 2 pathways for bone formation: intramembranous ossification or endochondral ossification.[64] Intramembranous ossification requires the formation of mesenchymal condensations that eventually directly differentiate into osteoblasts and mineralize into bone.[64] Most bones of the body, however, undergo endochondral ossification, whereby a cartilage template is formed first, followed by mineralization. Low levels of β-catenin signaling allow increased expression of SOX-9, a regulatory factor of cartilage, resulting in endochondral formation, whereas increased β-catenin levels drive Runx2 expression and intramembranous ossification. This suggests a regulatory factor controlling VC.

The mechanism of VC seems to involve several pathways as well as exogenous influence. It has been established that Msx2 drives osteogenic differentiation by activating Wnt signals and upregulating alkaline phosphatase.[5,61] Moreover, Msx2 upregulates Wnt/β-catenin–dependent T-cell factor/lymphoid enhancer-binding factor transcription, as well as upregulating Wnt3a and Wnt7a while downregulating the Wnt inhibitor Dickkhopf homologue-1.[5] The study of bone metabolism has progressed much further than that of calcified vascular tissue,[48] and the mechanisms underlying the active process and pathogenesis of VC remain unclear. The crucial role of Wnt signaling in bone metabolism makes the Wnt signal transduction pathways a promising field of study in determining the molecular mechanisms underlying VC. Developing a better understanding of the pathogenesis of VC will help contribute to the advancement of treatments and therapies for this vascular pathological feature.

Wnt Signaling Pathways

The name Wnt originates from the fusion of the words *wingless*, the Drosophila segment polarity gene, and *integrated* or *int-1*, the mammalian homolog of *wingless*.[71] The Wnt family of proteins consists of 19 secreted lipid-modified

glycoproteins that bind to the Frizzled receptor family.[11] There are 10 Frizzled isoforms in humans, and these Frizzled proteins have 7 transmembrane domains and are classified as a distinct family of G-protein–coupled receptors.[72] Once Wnt binds to Frizzled and the associated coreceptor complex, the signal is transduced to the cytoplasmic protein Disheveled. At this level, the cascade branches off into 3 major signal transduction pathways, the canonical or Wnt/β-catenin pathway and the noncanonical planar cell polarity and Ca^{2+}-dependent pathways (Wnt/Ca^{2+} pathway).[2]

The canonical Wnt/β-catenin signaling pathway is the most studied Wnt pathway and has played an essential role in many biological processes, such as development, cell proliferation, and bone metabolism.[73] At rest, under unstimulated conditions, a protein scaffold of Axin, adenomatous polyposis coli,[74] and glycogen synthase kinase-3β (GSK3) mediate the phosphorylation of β-catenin. This results in the polyubiquitination and proteasomal degradation of β-catenin.[75,76] In active Wnt signaling, Wnt binds Frizzled receptors and low-density lipoprotein receptor–related protein (LRP) 5/6 coreceptors, resulting in the activation of Disheveled, which inhibits GSK3 activity and disrupts the adenomatous polyposis coli/Axin/GSK3 complex that targets β-catenin for destruction.[76–78] As a result, β-catenin is stabilized, accumulates in the cytoplasm, and subsequently translocates to the nucleus.[73] In the nucleus, β-catenin associates with members of the lymphoid enhancer-binding factor/T-cell factor family of transcription factors and several other factors, enabling this complex to act as a transcriptional activator or repressor of Wnt target genes.[76,79,80] Canonical Wnt signaling is illustrated in Figure 1. As mentioned previously, Wnt molecules interact with several different surface receptors and can produce a variety of cellular outcomes, depending on the presence of coreceptors and which receptor complexes can be formed.[81]

The 2 main noncanonical/β-catenin–independent Wnt signaling cascades are the Wnt planar cell polarity and the

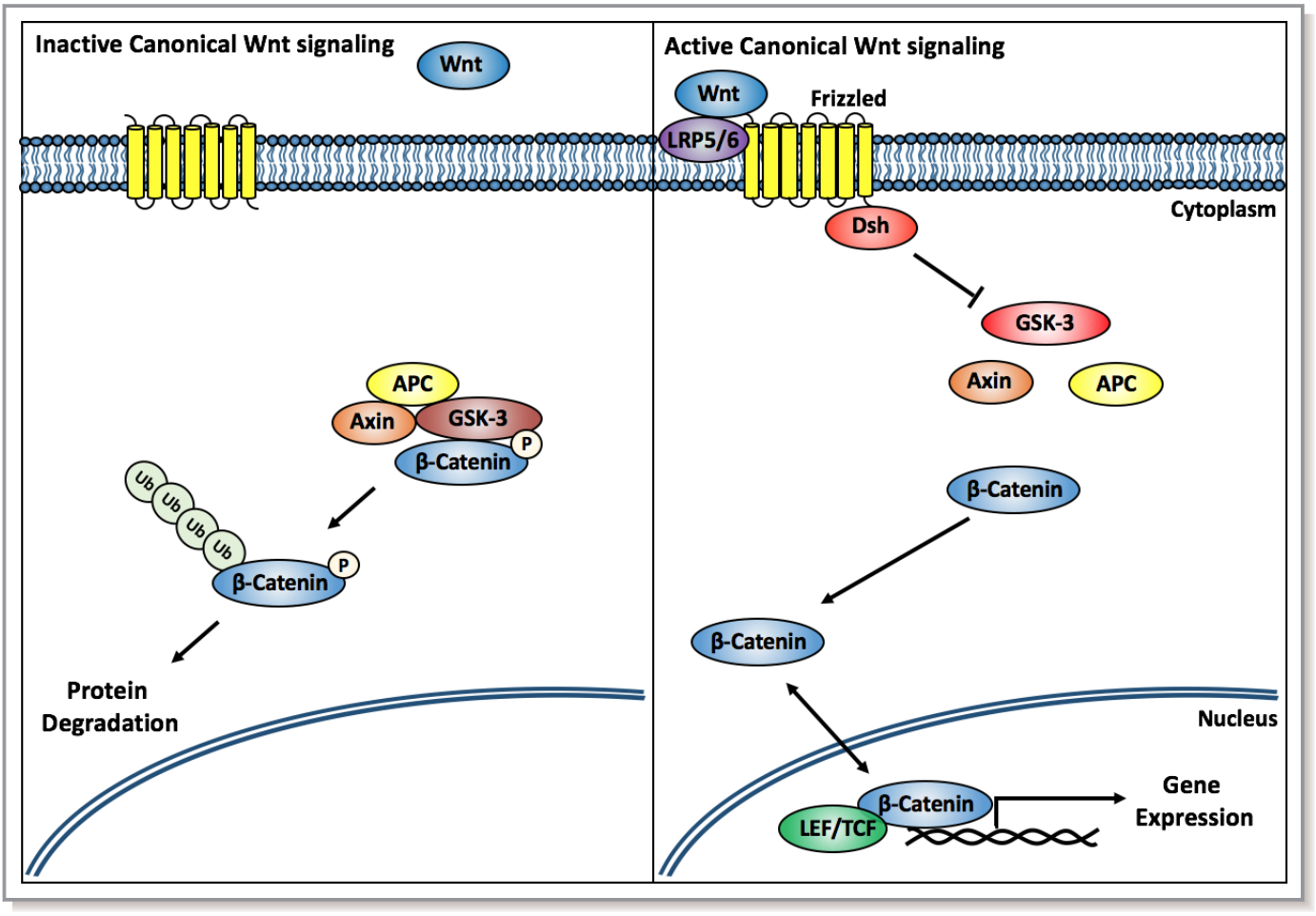

Figure 1. Schematic representation of inactive and activated canonical Wnt signaling. Without activation of the Frizzled receptor and the low-density lipoprotein receptor–related protein (LRP) 5/6 coreceptor, the Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase-3 (GSK3) complex forms and phosphorylates β-catenin, leading to its ubiquitination and subsequent degradation. Activation of canonical Wnt signaling leads to β-catenin nuclear localization and change in expression of Wnt target genes. LEF/TCF indicates lymphoid enhancer-binding factor/T-cell factor.

Wnt/Ca^{2+} signaling pathways. However, there are other noncanonical Wnt signaling pathways, such as the Wnt-Ras-related protein 1 (RAP1) pathway, the Wnt/receptor tyrosine kinaselike orphan receptor 2 receptor pathway, the Wnt–protein kinase A pathway, the Wnt-GSK3-microtubule pathway, the Wnt–c-Jun N-terminal kinase signaling pathway, the Wnt–related to tyrosine kinases pathway, and the Wnt–mammalian target of rapamycin pathway.[47] There is a great deal of overlap between these noncanonical pathways and between noncanonical Wnt signaling and the canonical Wnt–β-catenin pathway.[82] The traditional view of Wnt signaling suggests that Wnt1, Wnt3a, Wnt8, and Wnt8b are involved in canonical Wnt signaling, whereas Wnt4 and Wnt5a act in the noncanonical Wnt signaling pathway. However, classification of these molecules is difficult because of the significant cross talk between the pathways.[83] In the noncanonical planar cell polarity pathway, Wnt signaling leads to the activation of the small GTPases Rho and Rac, which mediate cytoskeletal rearrangements, resulting in lateral asymmetry.[77] In the Wnt/Ca^{2+} signaling cascade (Figure 2), Wnt binds to the extracellular cysteine-rich domain on the N terminus of its cognate Frizzled receptor.[72] This promotes the interaction of Frizzled with Disheveled-Axin-GSK3, and GSK3 mediates the phosphorylation of receptor tyrosine kinaselike orphan receptor 1/2, a Wnt-Frizzled coreceptor.[83] The phosphorylation of the receptor tyrosine kinaselike orphan receptor coreceptor leads to the activation of phospholipase C, which mediates a transient increase in inositol 1,4,5-triphosphate and 1,2-diacylglycerol from membrane-bound phospholipid phosphatidylinositol 4,5-bisphosphate.[83] Inositol 1,4,5-triphosphate subsequently interacts with the calcium channels on the endoplasmic reticulum membrane. This results in a release of Ca^{2+}, which interact with calmodulin to activate calmodulin-dependent protein kinase II.[83,84] The mobilized calcium ions from the endoplasmic reticulum also interact with 1,2-diacylglycerol to activate protein kinase C.[85] Calmodulin-dependent protein kinase II and protein kinase C then activate various nuclear transcription factors (eg, nuclear

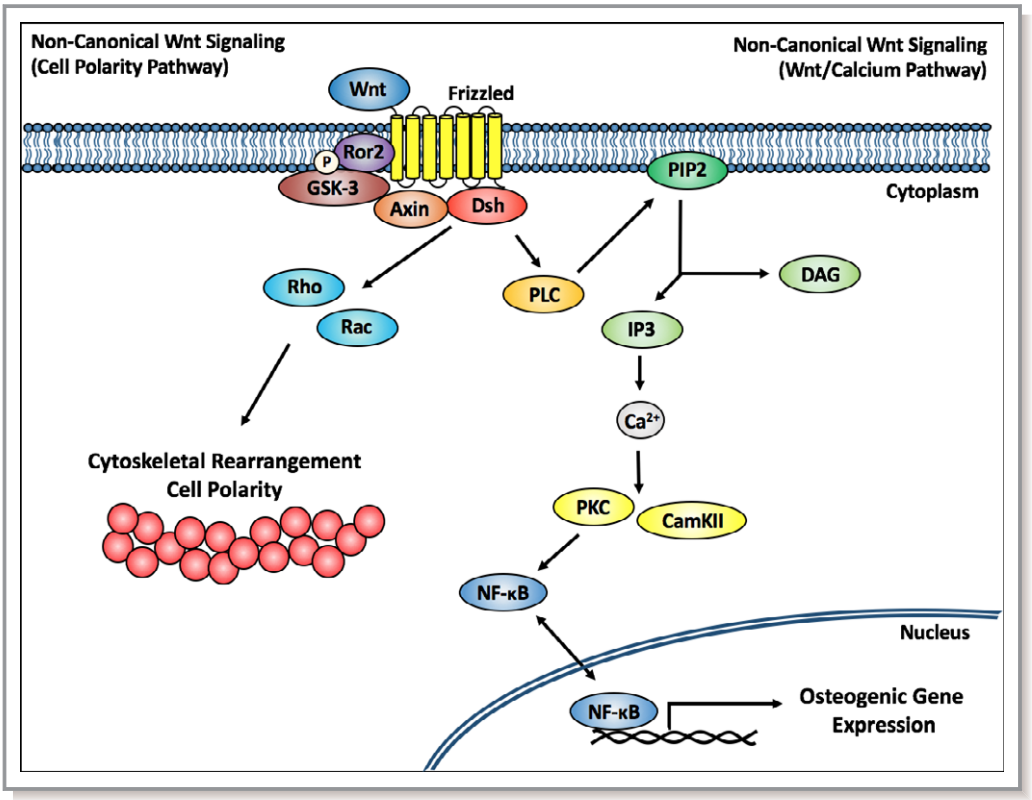

Figure 2. Schematic representation of the cell polarity and Ca^{2+} noncanonical Wnt signaling pathways. In the cell polarity pathway, activation of particular Frizzled receptors leads to cytoskeletal rearrangement via activation of Rho and Rac GTPases. In the Ca^{2+} pathway, Wnt-Frizzled receptor binding leads to increased intracellular Ca^{2+} concentrations from the endoplasmic reticulum. Under these conditions, nuclear factor-κB (NF-κB), cAMP responsive element binding, and nuclear factor associated with T cells transcription factors can translocate to the nucleus and promote an osteogenic gene profile. CamKII indicates Ca^{2+}/calmodulin-dependent protein kinase II; GSK3, glycogen synthase kinase-3; IP3, inositol 1,4,5-triphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate 2; PKC, protein kinase C; PLC, phospholipase C; and Ror2, receptor tyrosine kinaselike orphan receptor 2.

factor-κB and cAMP responsive element binding), and the released calcium ions can activate phosphatase calcineurin, a widely expressed protein that can then dephosphorylate and thus activate cytoplasmic nuclear factor associated with T cells.[83,86] Nuclear factor-κB, cAMP responsive element binding, and nuclear factor associated with T cells can translocate to the nucleus and regulate the transcription of Wnt target genes.[83] The noncanonical cell polarity and Ca^{2+} Wnt signaling pathways are illustrated in Figure 2.

Implication of Wnt Signaling in Atherosclerosis Pathological Features

Wnt signaling plays an important role in several key biological processes, including cardiac development and angiogenesis. More recently, evidence is emerging for the role of Wnt signaling in atherosclerosis and, in particular, VC (summarized in Figure 3).[4] Behind the skeleton, the arterial vasculature is the second most extensively calcified structure in the human body.[39] Recent evidence demonstrates that Wnt ligands and their receptors play a role in calcification itself as well as the atherosclerotic events that predispose vessels to this pathological calcification. As mentioned previously, monocyte adhesion to ECs is a hallmark of atherosclerosis. Canonical Wnt signaling has been demonstrated to directly increase this monocyte adhesion.[87] Interestingly, Wnt signaling in ECs has been shown to activate proinflammatory gene expression via inhibition of canonical Wnt signaling and activation of the noncanonical Ca^{2+}-dependent pathway via ligand Wnt5a.[88,89] Several studies have demonstrated a direct link between canonical Wnt signaling via ligands Wnt1, Wnt2, and Wnt3a and arterial smooth muscle cell proliferation, which underlies the intimal thickening stage of atherosclerosis.[20,21,90,91] Canonical Wnt signaling and LRP5 have also been directly implicated in the promotion of foam cell formation.[6] Sclerostin, an inhibitor of canonical Wnt signaling, has also been of interest to prevent atherosclerosis. A recent study used human aortic aneurysm samples and transgenic mice overexpressing sclerostin in apolipoprotein E–deficient mice.[92] Sclerostin was downregulated in aortic aneurysms, and overexpressing sclerostin in atherogenic mice resulted in a decreased inflammatory response that inhibits development of atherosclerosis and aortic aneurysms.

Implication of Wnt Signaling in Atherosclerotic Calcification

VC in diabetic *LDLR*$^{-/-}$ mice is associated with upregulation of Wnt signaling via a mechanism involving BMP2.[5] The latter has been demonstrated in atherosclerotic lesions, particularly in vascular myofibroblasts and endothelium.[93] BMP2 expression is activated by pathogenic stimuli, such as tumor necrosis factor-α,[94] oxidized lipids,[94] and hyperglycemia[95,96] More important, BMP2 has been demonstrated to regulate

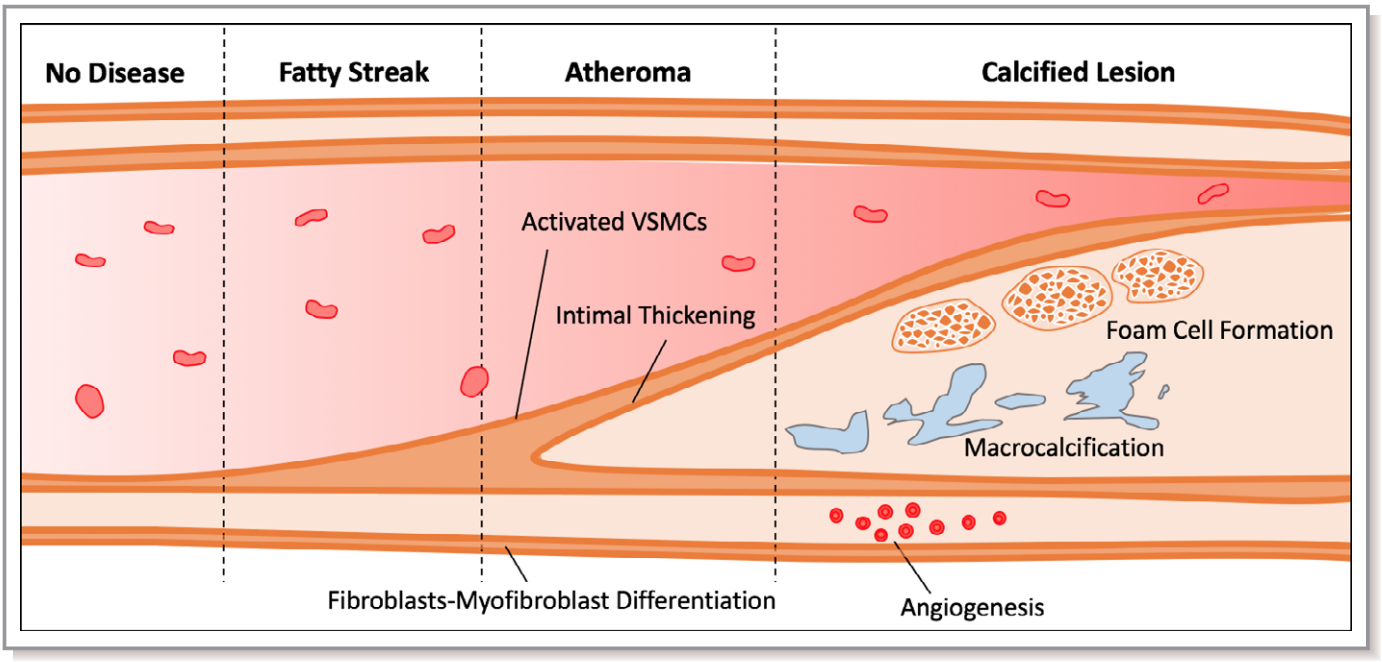

Figure 3. Involvement of Wnt signaling in the pathogenesis of atherosclerosis. Wnt signaling is associated with monocyte attachment, inflammation, cell proliferation, foam cell formation, and atherosclerotic calcification, ultimately leading to arterial stenosis. VSMC indicates vascular smooth muscle cell.

osteogenic programs contributing to VC.[55,62] One of the main ways in which BMP2 regulates osteogenic gene expression programs is through the induction of transcription factor Msx2, which controls craniofacial mineralization.[97] Msx2 has also promoted osteogenic differentiation, and it suppresses adipogenesis of vascular myofibroblasts.[96] Msx2 enhances Wnt signaling through the upregulation of several Wnt ligands[98] and the downregulation of dickkopf homologue 1,[99] a canonical Wnt pathway antagonist. Indeed, Msx2 and Wnt seem to regulate each other's expression in a bidirectional way.[100,101] For instance, Msx2 stimulates Wnt-dependent T-cell factor/lymphoid enhancer-binding factor transcription, increases β-catenin nuclear localization, and increases the expression of canonical Wnt ligands, Wnt3a and Wnt7, and noncanonical ligand, Wnt5.

Wnt Signaling in Pericytes

Wnt/β-catenin signaling has also contributed to VC and atherosclerosis by controlling the aberrant differentiation of pericytes in the vasculature.[102] Although pericytes were initially thought to be a feature of the microvasculature, they have been found to be present across the entire vasculature.[103] Their recruitment to the intima, media, and sites of arterial calcification in several pathological conditions, such as atherosclerosis, suggests their involvement in these diseases.[102,104,105] It has been demonstrated that the activation of Wnt/β-catenin signaling by transforming growth factor-β3 induces the chondrogenic and inhibits the adipogenic differentiation of pericytes,[102] which are thought to be uncommitted mesenchymal progenitor cells.[106] Studies have shown calcifying vascular cells to express pericyte markers, such as α-smooth muscle actin and 3G5.[93] These studies, in combination with the knowledge that pericytes can undergo chondrogenic, osteogenic, and adipogenic differentiation,[107,108] contrasts the function of vascular cells, which can only undergo chondrogenic and osteogenic differentiation.[109] This prompted the hypothesis that calcifying vascular cells originate from pericytes that have been exposed to Wnt signaling.[102]

Implication of Canonical Wnt Signaling in VC

In support of its role in VC, Wnt/β-catenin signaling plays a crucial role in bone development and homeostasis. During development, canonical ligand Wnt3a maintains apical ectodermal cells of limb buds in a highly proliferative state and is, thus, essential to embryonic skeletal development.[110,111] Loss-of-function mutations of Wnt coreceptor LRP5 cause reduced bone mass, skeletal fragility, and osteoporosis.[112] LRP5$^{-/-}$ mice have increased tibial fractures because of an overall decrease in bone mass and reduced osteoblast proliferation and function with no effect on bone resorption, whereas single-allele knockouts of LRP5 lead to an intermediate phenotype of bone mass. This suggests a dose-dependent response for *LRP5* alleles on bone mass density and that LRP5 regulates osteogenesis through its effects on osteoblast proliferation, differentiation, and function.[113,114] Wnt coreceptor LRP6 is thought to control postnatal bone mass,[113] and LRP6$^{+/-}$ mice lacking LRP5 had an even further decrease in bone mass density.[115] LRP5/6 knockout models in hypercholesterolemic (apolipoprotein E–deficient) mice developed less calcification in the aortic valve compared with control models.[116] On the other hand, gain-of-function mutations of LRP5 are associated with a high bone mass phenotype.[117,118] Interestingly, no change in osteoblast activity was observed in mice with increased expression of LRP5.[119] These mice did, however, have decreased osteoblast apoptosis, suggesting that the high bone mass phenotype is because of increased osteoblast cell count rather than function. Genomic sequencing of unrelated families with high bone density identified 6 different gain-of-function mutations in the *LRP5* gene.[120] Loss-of-function mutations in LRP5 were identified to be associated with osteoporosis.[112] In the context of atherosclerosis, a study of a family with early coronary artery disease, metabolic syndrome, and osteoporosis showed an LRP6 mutation that impairs Wnt signaling, suggesting a genetic link between LRP6 and Wnt signaling with coronary artery disease.[121]

Implication of Noncanonical Wnt Signaling in VC

Despite the fact that there is evidence implicating Wnt signaling in osteogenic differentiation and development, until recently, little was known about the role of noncanonical Wnt ligands in vascular and valvular calcification and atherosclerosis. The prototypical noncanonical ligand Wnt5a has been implicated in mouse bone homeostasis.[122] Additional studies have shown that Wnt5a$^{+/-}$ mice have reduced osteogenesis and increased adipogenesis in bone marrow mesenchymal progenitors and low bone mass.[123] Through the calcium-dependent Wnt signaling pathway, Wnt5a is thought to activate histone methyltransferase SET domain bifurcated 1, leading to the formation of a corepressor complex that suppresses peroxisome proliferator-activated receptor γ (PPARγ) function and thus mediates the osteoblastic differentiation of mesenchymal stem cells (MSCs).[123] A recent study has demonstrated a link between Wnt5a, toll-like receptor 4, and pathological inflammation in human macrophages.[124] Furthermore, the expression of Wnt5a has been demonstrated in murine and human atherosclerotic tissue.[19] In addition to finding strong Wnt5a expression in macrophage-

rich regions of atherosclerotic lesions of apolipoprotein E–deficient mice on atherogenic diets, Wnt5a was also expressed in other regions of developed human plaques, such as in smooth muscle cells.[19]

The potential involvement of Wnt5a in VC supports the investigation of other noncanonical Wnt ligands, particularly those working through the Wnt/Ca^{2+} pathway, and their roles in the molecular mechanisms underlying atherosclerosis. In particular, Wnt5b is an interesting candidate to study, because it is a paralog of Wnt5a, with 80% amino acid identity to the human Wnt5a protein.[125] By a recent bone mineral density genome-wide association study, Wnt5b (along with other Wnt signaling molecules, such as AXIN1, LRP5, Catenin beta-1 (CTNNB1), WNT3, and several others) has been shown to be involved in key biological pathways involving the skeleton.[126] Both Wnt5a and Wnt5b have been involved in coordinating chondrocyte proliferation and differentiation during endochondral ossification of long bones in mice.[127] More specifically, both Wnt5a and Wnt5b are expressed in the chondrocytes of developing long bones, and Wnt5a signaling regulated proliferation and differentiation of zone I epiphysis chondrocytes and the highly proliferative zone II chondrocytes. Although Wnt5a inhibits the transition of cells from zone I to zone II, Wnt5b promotes the transition from zone I to zone II and thus has an antagonistic effect in regulating chondrocyte proliferation.[127] A functional role for Wnt5b in adipogenesis was elucidated in 3T3-L1 preadipocytes, where Wnt5b overexpression promoted adipogenesis by antagonizing the Wnt3a-mediated Wnt/β-catenin pathway.[128] In subsequent studies in the same preadipocyte cell line, both Wnt5a and Wnt5b inhibited β-catenin–dependent Wnt signaling; however, Wnt5b overexpression, in particular, was determined to be a potent stimulator of adipogenesis via inhibition of canonical Wnt signaling and increased PPARγ expression.[129] This increased PPARγ expression is of particular interest because PPARγ involvement in lipid metabolism is particularly relevant in the study of atherosclerosis.

PPARγ is expressed primarily in adipose tissue and is also found in the heart.[130] PPARγ is expressed in ECs, VSMCs, and macrophages.[131] In addition to the well-established role of PPARγ in lipid metabolism, there is increasing support for its role in modulating inflammation, macrophage differentiation, and atherosclerosis.[132] PPARγ has had inhibitory effects on VSMC proliferation and migration[133,134] as well as EC growth and migration.[135] PPARγ activation reduces monocyte recruitment to atherosclerotic plaques.[136] PPARγ also has a modulatory effect on inflammation and is known to decrease monocyte/macrophage production of several inflammatory cytokines.[137] There is a large amount of evidence supporting the antiatherosclerotic properties of PPARγ, whether indirectly by improving control of lipid and blood glucose levels or directly by increasing plaque stability and direct actions on ECs, VSMCs, and macrophages.[135] This evidence suggests the potential of PPARγ in the treatment of atherosclerotic disease[133] and makes its relationship with Wnt5b and Wnt signaling a crucial area of study.

Another interesting Wnt ligand in the study of atherosclerosis is Wnt11. The embryonic expression pattern of Wnt11, particularly its restricted expression to the perichondrium of the developing skeleton, has long suggested its role in skeletal development.[138] Interestingly, the chromosomal location of Wnt11 to 11q13.5 is near previously linked markers of high bone mass.[138,139] In addition, Wnt11 expression is upregulated during mesenchymal osteogenesis.[140] Wnt11 activation has been associated with bone canonical Wnt/β-catenin signaling and noncanonical signaling.[141] Activation of Wnt11 through β-catenin–independent Wnt signaling is essential for embryonic cardiac development.[142] In terms of its role in osteogenic development, Wnt11 activation in MC3T3E1 preosteoblasts increases β-catenin accumulation, promoting BMP-induced osteoblast maturation and mineralization through canonical Wnt signaling.[143] Wnt11 induces the expression of R-spondin 2, a secreted enhancer of Wnt signaling, which stimulates osteoblastogenesis through increasing the expression of osteogenic factors, such as *Dmp1* (dentin matrix protein 1), *Phex* (phosphate-regulating endopeptidase homolog), and *Bsp* (bone sialoprotein).[143]

When studying the effects of Wnt signaling on the osteogenic differentiation of adult human MSCs, an interesting model of cross regulation between canonical and noncanonical Wnt signaling in the regulation of osteogenesis was proposed.[140] In this model, the MSCs exist in a proliferating progenitor cell stage or in a lineage-committed differentiated stage. Canonical Wnt ligands Wnt3a and Wnt9a promote MSC proliferation and maintenance of the progenitor cell pool. Meanwhile, noncanonical signaling, mediated by Wnt5a and Wnt11, is upregulated during MSC osteogenic and chondrogenic differentiation[144] and is involved in regulating and promoting MSC differentiation.[140] In this model, MSCs are maintained in the progenitor state by canonical signaling and will undergo differentiation on an exogenous signal involving noncanonical Wnt signaling that will release the cells from suppression of differentiation. These cells are then able to undergo osteogenic, chondrogenic, or adipogenic lineage commitment.[140] This model suggests that Wnt/β-catenin signaling can promote osteogenesis by expansion of the progenitor cell compartment.[140]

Involvement of Wnt Signaling in Aortic Valve Calcification

There is strong evidence suggesting the involvement of Wnt signaling cascades in cardiac valve calcification associated with human degenerative valve disease.[145] For example, LRP5 Wnt

coreceptor regulates bone matrix protein expression in the aortic valves and vasculature of mice with hypercholesterolemia.[46] Through its interaction with Wnt proteins, LRP5 activates β-catenin and is, thus, an important factor in mediating osteogenesis. Calcified aortic valves had a bonelike phenotype, whereas mitral valves had a cartilage phenotype; LRP5/Wnt3 signaling markers were present in both types of disease valves, but expression was more prominent in aortic valves.[145] These findings suggest that the LRP5/Wnt3 signaling pathway could be mediating an endochondral ossification process in the progression of valvular heart disease.[145]

Recently, we studied the role of noncanonical ligands Wnt5a, Wnt5b, and Wnt11 in aortic valve calcification.[146] Calcified valves had significantly elevated mRNA expression of all 3 Wnt ligands compared with noncalcified valves. Immunohistochemical analyses in atherosclerotic aortic valves demonstrate elevated Wnt5a expression within and surrounding areas of calcification, whereas Wnt5b and Wnt11 are found in fibrosis, inflammation, lipid, and calcification. Both Wnt5b and Wnt11 immunostaining had significant positive correlations with aortic jet velocity, mean and maximum pressures across the aortic valve. In addition, in vitro studies of the effects of Wnt5a, Wnt5b, and Wnt11 on human aortic valve interstitial cells demonstrated that all 3 Wnts increased apoptosis, osteogenic gene expression, and calcification (via alkaline phosphatase activity) in these cells.

More recently, we have shown that lipoprotein (a), known to be genetically associated with aortic valve calcification, caused increased deposition of minerals and microvessel formation in human aortic valve interstitial cells through induction of Wnt signaling pathways.[147]

Conclusion

Investigating the role of Wnt signaling in vascular and valvular calcification is fascinating given the crucial role that both canonical and noncanonical Wnt ligands play in skeletal development and the accumulating evidence suggesting that atherosclerotic calcification recapitulates bone development. The current body of evidence is suggestive of both canonical and noncanonical Wnt ligands contributing to pathological calcification of the aortic valves and vasculature. Further research into the exact signaling pathways underlying these processes is warranted to better understand this phenomenon. However, it is more likely that multiple pathways contribute to calcification, making it difficult to identify a single molecular target to treat VC. Current pharmacological therapies targeting Wnt signaling are still in their infancy.[148] Indeed, several pharmaceutical molecules that are known to modulate the Wnt signaling pathway have been developed and tested in cancer and neurologic disorder therapy.[149,150] A couple of others are used in the treatment of osteoporosis.[151] Indeed, on the basis of the evidence we provided above, there is still tremendous potential to use Wnts and their effectors in treating VC. Thus, we suggest that future experiments attempt to identify novel targets that either regulate or contribute to Wnt signaling. In doing so, these new targets, as well as those that are in current use clinically, may ultimately contribute to reducing cardiovascular morbidity and mortality.

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Disclosures

None.

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