

Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION



Cell Matrix Interactions in the Pathobiology of Calcific Aortic Valve Disease: Critical Roles for Matricellular, Matricrine, and Matrix Mechanics Cues

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Circ. Res. 2011;108;1510-1524

DOI: 10.1161/CIRCRESAHA.110.234237

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

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This article is part of a thematic series on **Pathobiology of Calcific Vasculopathy and Valvulopathy**, which includes the following articles:

Thematic series on the pathobiology of vascular calcification: An introduction [*Circ Res.* 2011;108:1378–1380]

Molecular imaging insights into early inflammatory stages of arterial and aortic valve calcification [*Circ Res.* 2011;108:1381–1391]

Calcific aortic valve stenosis: Methods, models, and mechanisms [*Circ Res.* 2011;108:1392–1412]

The roles of lipid oxidation products and receptor activator of nuclear factor-kappa B signaling in atherosclerotic calcification [*Circ Res.* 2011;108:1482–1493]

Fetuin-A regulation of calcified matrix metabolism [*Circ Res.* 2011;108:1494–1509]

Cell–matrix interactions in the pathobiology of calcific aortic valve disease: Critical roles for matricellular, matricrine, and matrix mechanics cues

Osteogenic BMP-Wnt signaling in valvular and vascular sclerosis

Calcium-phosphate homeostasis in the arterial calcification of CKD

Molecular genetics of calcific vasculopathy

Dwight A. Towler, Guest Editor

Cell–Matrix Interactions in the Pathobiology of Calcific Aortic Valve Disease

Critical Roles for Matricellular, Matricrine, and Matrix Mechanics Cues

Jan-Hung Chen, Craig A. Simmons

Abstract: The hallmarks of calcific aortic valve disease (CAVD) are the significant changes that occur in the organization, composition, and mechanical properties of the extracellular matrix (ECM), ultimately resulting in stiffened stenotic leaflets that obstruct flow and compromise cardiac function. Increasing evidence suggests that ECM maladaptations are not simply a result of valve cell dysfunction; they also contribute to CAVD progression by altering cellular and molecular signaling. In this review, we summarize the ECM changes that occur in CAVD. We also discuss examples of how the ECM influences cellular processes by signaling through adhesion receptors (matricellular signaling), by regulating the presentation and availability of growth factors and cytokines to cells (matricrine signaling), and by transducing externally applied forces and resisting cell-generated tractional forces (mechanical signaling) to regulate a wide range of pathological processes, including differentiation, fibrosis, calcification, and angiogenesis. Finally, we suggest areas for future research that should lead to new insights into bidirectional cell–ECM interactions in the aortic valve, their contributions to homeostasis and pathobiology, and possible targets to slow or prevent the progression of CAVD. (*Circ Res.* 2011;108:1510-1524.)

Key Words: biomechanics ■ calcific aortic valve disease ■ extracellular matrix ■ matricellular signaling ■ matricrine signaling

Valvular heart diseases result in >23 000 deaths annually in the United States, with calcific aortic valve disease (CAVD), encompassing aortic sclerosis and stenosis, being the most prevalent.¹ Stenotic valves with obstruction of left

ventricular outflow are present in approximately 2% of adults older than 65 years of age, based on 1997 data from the Cardiovascular Health Study.² Aortic sclerosis (defined by increased echogenicity and leaflet thickening without restric-

Original received February 27, 2011; revision received April 19, 2011; accepted April 26, 2011. In April 2011, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 15 days.

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DOI: 10.1161/CIRCRESAHA.110.234237

Non-standard Abbreviations and Acronyms	
TGF-βR	transforming growth factor- β receptor
ALP	alkaline phosphatase
CAVD	calcific aortic valve disease
Chm-I	chondromodulin-I
ECM	extracellular matrix
GAG	glycosaminoglycan
MMP	matrix metalloproteinase
PG	proteoglycan
SMA	α -smooth muscle actin
TGF-β	transforming growth factor- β
TN- C	tenascin-C
VEC	valvular endothelial cell
VIC	valvular interstitial cell

tion of leaflet motion) was prevalent in 26% of the same cohort. More recent data from the KORA/MONICA survey of German residents aged 25 to 74 years indicated an overall prevalence of aortic sclerosis of 28% and a prevalence of >35% in subjects older than age 65 years.³

The hallmarks of CAVD are the significant alterations that occur in the organization, composition, and mechanical properties of the valve extracellular matrix (ECM). Other features of CAVD include the presence of macrophages and T cells,^{4–9} oxidized low-density lipoprotein deposits,^{4–6,9} elevated oxidative stress,^{10,11} and inflammatory cytokines,^{7,12} neoangiogenesis,^{13,14} and the appearance of myofibroblasts,⁶ osteoblasts,^{9,15} and occasionally other ectopic mesenchymal cells.¹⁶ Ultimately, maladaptive ECM remodeling results in stiffened stenotic leaflets that obstruct flow and compromise cardiac function. Patients with severe aortic stenosis who do not receive surgical valve replacement have a mortality rate of 37% at 1 year after symptom onset.¹⁷ Even in the absence of left ventricular outflow obstruction, aortic sclerosis is associated with a 50% increased risk of cardiovascular death and myocardial infarction.¹⁸ Together, the growing prevalence of CAVD, its poor clinical consequences, and discouraging results from medical therapy trials¹⁹ emphasize the unmet scientific need for the identification of pathobiological mechanisms and new approaches to treat CAVD.

The structural and compositional alterations in valve ECM that define CAVD have traditionally been viewed as symptomatic of the disease, impacting biomechanical and hemodynamic function of the valve. However, the ECM not only provides structure but also influences cellular processes by signaling through adhesion receptors (matricellular signaling), by regulating the presentation of growth factors and cytokines to cells (matricrine signaling), and by transducing hemodynamic forces and resisting cell-generated tractional forces (mechanical signaling; Figure 1). Growing evidence suggests that the valve ECM contributes to disease progression through each of these mechanisms to profoundly influence many processes involved in CAVD, including pathological differentiation, fibrosis, calcification, and angiogenesis.

In this review, we summarize the changes that occur in the ECM in CAVD, highlight examples of the role of the ECM in regulating valve cell function and valve pathobiology through matricellular and matricrine signaling, consider the impact of ECM elasticity on valve cell fate and the implications for pathological development, and suggest some directions for future research in valve cell–ECM interactions.

Normal Aortic Valve Structure, Composition, and Mechanical Properties

The aortic valve is composed of three cusps or leaflets named the left coronary, right coronary, and noncoronary according to their location relative to the coronary artery ostia in the sinuses of Valsalva downstream of the valve. The leaflets attach at their base to the fibrous annulus region of the aortic root, with their free edges coapting to prohibit retrograde flow when the valve is closed. The leaflets, which are normally smaller than 1 mm in thickness in humans,⁶ are stratified into three layers: the fibrosa, spongiosa, and ventricularis (Figure 2A). The fibrosa on the aortic side of the valve leaflets is primarily composed of circumferentially oriented type I and III fibrillar collagens and functions as the main load-bearing layer. The middle layer is the spongiosa. It is composed primarily of proteoglycans with scattered collagen fibers and serves to link and lubricate the adjacent fibrosa and ventricularis layers as they shear and deform relative to each other during leaflet motion and pressurization.²⁰ On the left ventricular side of the leaflets is the ventricularis layer, composed of a network of collagen and elastin fibers. The elastin fibers are oriented radially and appear to reduce radial strains that occur when the valve is fully opened and aid in recoil.²¹ The adhesive proteins fibronectin and laminin are also present in the aortic valve.²²

Macroscopically, aortic valve leaflets function as a quasi-elastic tissue that is more mechanically compliant in the radial direction than in the circumferential direction.²⁰ This anisotropic behavior results from the unique structure and composition of the individual layers; therefore, it is not surprising that the layers exhibit distinct micromechanical properties. By tensile testing of dissected layers^{23,24} and flexural testing of intact valves,^{25,26} the fibrosa was found to be stiffer than the ventricularis, with both layers behaving anisotropically. A similar result was obtained using micropipette aspiration, which measures ECM elastic modulus (a measure of a material's intrinsic resistance to deform when mechanically stressed) focally at a length scale of 100 μ m.²⁷ Notably, there is significant heterogeneity in ECM modulus within the individual layers, with distinctly stiff and soft regions in the fibrosa and ventricularis, respectively.²⁷

The cellular components of the aortic valve include valvular endothelial cells (VEC) that form a monolayer that lines the leaflet surface and valvular interstitial cells (VIC) that populate all three layers of the leaflet. VEC likely indirectly contribute to valve homeostasis and ECM remodeling primarily through regulation of permeability,²⁸ adhesiveness to inflammatory cells,²⁹ and paracrine signaling to circulating cells and local VIC.³⁰ VIC are a heterogeneous population of fibroblasts, with a small population (<5%) of myofibroblasts and smooth muscle cells.^{31–35} In disease, the interstitial

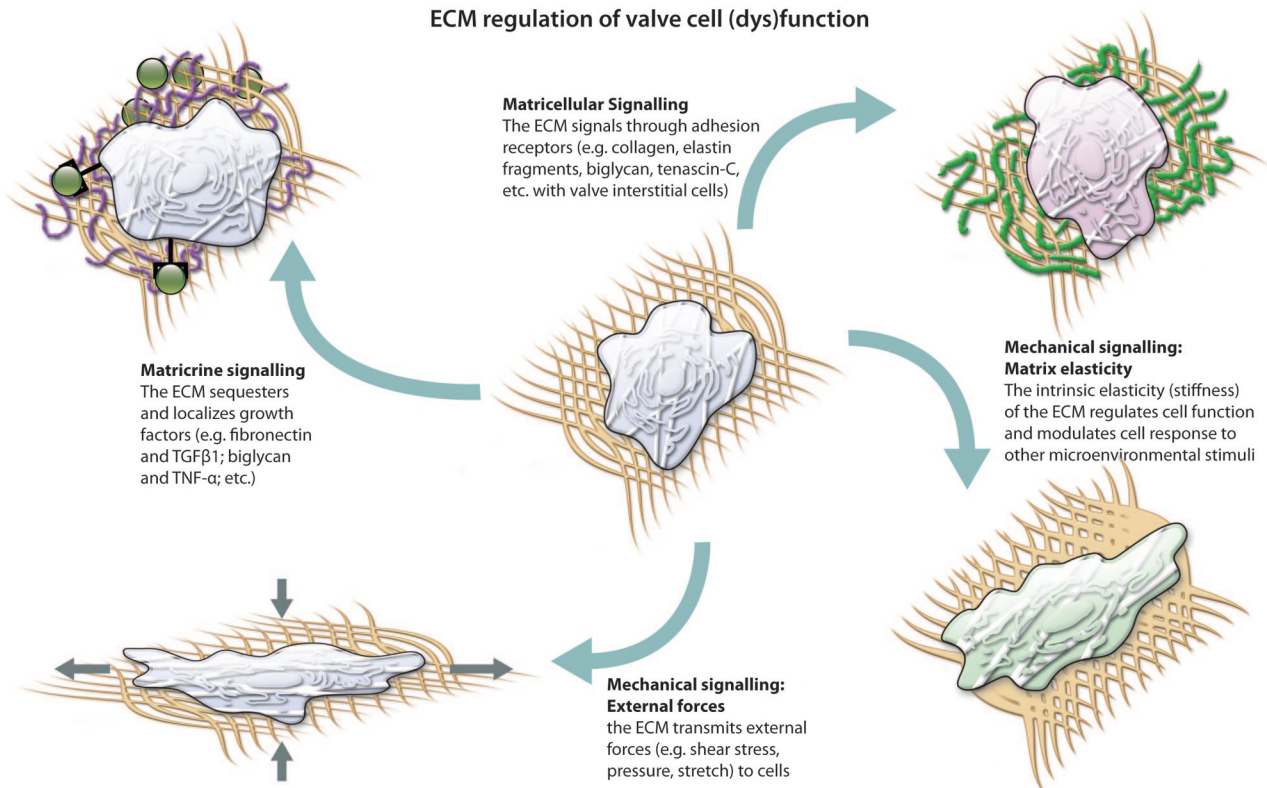


Figure 1. Mechanisms by which the valve ECM may regulate cell function to contribute to the pathobiology of calcific aortic valve disease. Illustration credit: Cosmocyte/Ben Smith.

population shifts to include a greater proportion of myofibroblasts (up to 30%^{32,33,36}) and osteoblast-like cells.¹⁵ In swine, these pathological VIC phenotypes likely originate from a sizeable subpopulation of mesenchymal progenitor cells with differentiation potential to the myofibroblastic, osteogenic, chondrogenic, and adipogenic lineages.³⁷ Although similar mesenchymal progenitors are thought to exist in human valves,^{21,38} and likely do, they have yet to be identified definitively. The primary function of VIC and tissue-resident progenitors is to maintain normal valve structure and function. The layer-specific expression of ECM proteins in the aortic valve suggests spatially heterogeneous subpopulations of VIC or local regulatory signals or both. Emerging evidence from our laboratory confirms that the phenotypic plasticity of porcine VIC is layer-dependent (M. Likhitpanichkul and C.A. Simmons, unpublished data), but it is not known whether these layer-specific subpopulations are responsible for the distinct ECM in each layer.

ECM Changes in CAVD and Their Effects on Matricellular and Matricrine Signaling

CAVD is characterized by fibrotic thickening of the valve leaflets, inflammation, neoangiogenesis, calcification, and the presence of other ectopic mesenchymal tissues (Figure 2B, C). Notably, lesions and calcification occur preferentially in the fibrosa layer.^{4,6} The reasons for the susceptibility of the fibrosa to disease are not fully understood, but side-specific susceptibility can provide important clues to the regulatory mechanisms involved in CAVD.³⁹ Histopathologically, the trilayer structure of the valve ECM is disrupted in CAVD, the

result of disorganized ECM protein synthesis and degradation, changes in the stoichiometry and localization of ECM components, and expression of ECM components usually only expressed in development or connective tissue formation. The consequence of these changes is not only structural but also biological, because the ECM provides matricellular and matricrine cues that influence cell responses that contribute to CAVD development. Here, we highlight key ECM proteins that are altered in CAVD and discuss their impact on cellular processes (Table). The reader is referred to a recent review by Hinton and Yutzey⁴⁰ for a discussion of ECM proteins involved in valve development, which in some cases recapitulate features of valve diseases other than CAVD in transgenic mice.

Collagen and Fibrosis

In normal human aortic valves, collagen is present in all three layers, but it is most abundant in the fibrosa layer (types I and III).⁴¹ Types I and IV collagen are also found in the subendothelial basement membrane.^{6,41} In diseased valves, dense fibrosis is observed,⁸ with collagen fibers in the fibrosa becoming disorganized.^{42–44} Collagen and ECM disarray is attributable to increased remodeling mediated by elevated activity of matrix metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-9), their tissue inhibitor-1 and tissue inhibitor-2,^{7,43,45–50} and cathepsins,^{51–53} increased synthesis but disorganized deposition of new fibers,⁵⁴ and physical disruption attributable to calcified nodule formation⁴³ and cellular infiltrates.⁴² Collagen fibers also appear in the ventricularis in disease.⁴³ In areas with underlying sclerotic lesions, the

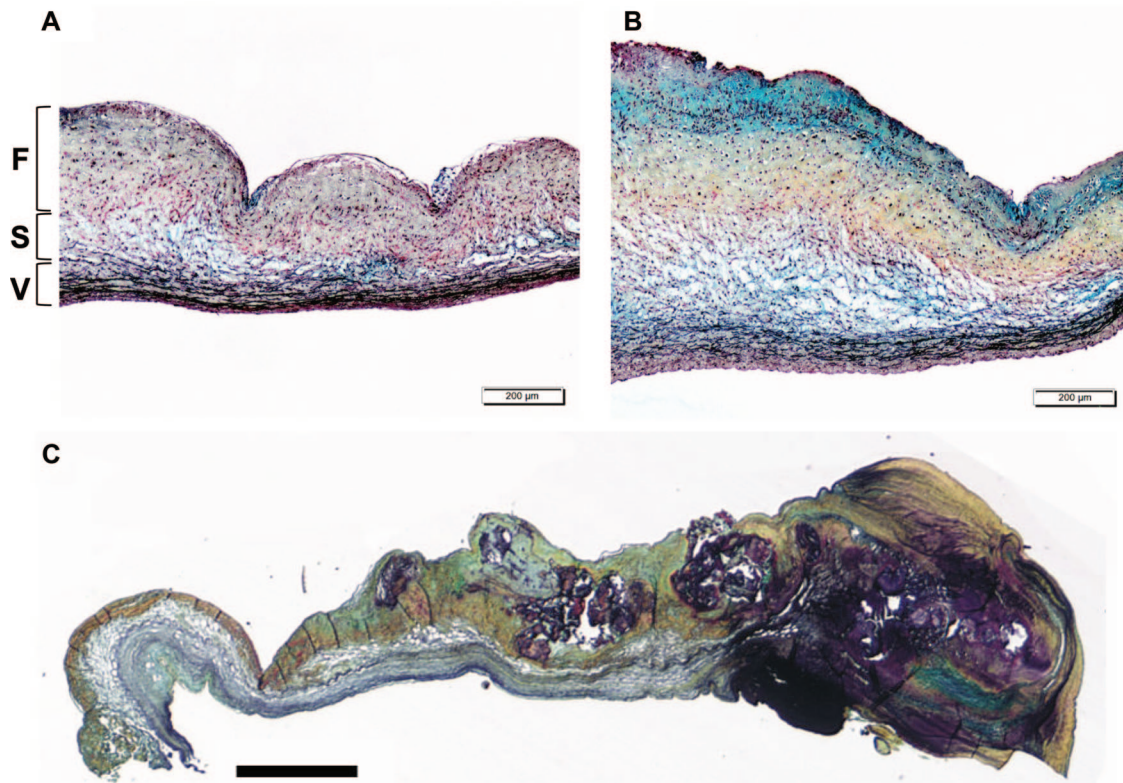


Figure 2. Aortic valve structure and composition. A, Normal porcine aortic valve leaflet stained with Movat pentachrome, demonstrating the trilaminar structure, the collagen-rich fibrosa (F), proteoglycan-rich spongiosa (S), and elastin-containing ventricularis (V). B, Aortic valve leaflet from a hypercholesterolemic pig showing early signs of valve disease, including thickening, increased matrix protein deposition (most notably a proteoglycan-rich region [blue] laid on the fibrosa), and elastin fragmentation (black). C, A calcified human aortic valve leaflet demonstrating obvious maladaptive extracellular matrix (ECM) remodeling, including significant calcification (purple in this preparation). Scale bars are 200 μm in (A) and (B), and 1 mm in (C). A and B, Images were kindly provided by Krista L. Sider, University of Toronto. C, Image is adapted from Stephens EH, Saltarelli JG, Baggett LS, et al. Differential proteoglycan and hyaluronan distribution in calcified aortic valves. *Cardiovasc Pathol*. 2010 Dec 23 [Epub ahead of print] and was kindly provided by Drs K. Jane Grande-Allen and Elizabeth H. Stephens.

basement membrane often appears thin, frayed, and reduplicated; in some cases, it appears to be absent.⁶ In rabbits, diet-induced CAVD results in significant collagen accumulation and thickening of the valve leaflets.^{42,55,56} It is unclear if collagen contributes to thickening of human valves, because collagen accounts for only 40% of the total protein in the noncalcified regions and 10% in the calcified regions, compared with 90% of total protein in healthy valves.⁵⁴ Accumulation of proteoglycans, lipids, and minerals, and possibly cellular proliferation, contribute to thickening of human leaflets.^{6,57}

Although it is clear that fibrosis and collagen remodeling are increased during CAVD, the pathobiological consequences are not well-defined. Alterations in the content and organization of collagen and other structural proteins likely impact the mechanical properties of the valve leaflet by changing the inherent elasticity of the leaflet tissue and contributing to leaflet thickening, which increases leaflet structural stiffness and resistance to deformation by hemodynamic forces. However, there is little information on the mechanical properties of diseased aortic valves because of the need for relatively large tissue samples for standard mechanical test methods and the lack of well-characterized large animal models of CAVD. Recent advances in micromechani-

cal testing have enabled measurement of mouse aortic valve leaflet mechanical properties,⁵⁸ but mouse leaflets lack the trilaminar structure of human and large animal (eg, swine) leaflets, and therefore may not be appropriate models to study valve matrix dynamics. The potential regulatory role of ECM mechanics on valve cell response is discussed further below.

Fibrotic changes may also influence cells directly by signaling through adhesion receptors. Structural proteins like collagen are typically not considered matricellular, but in vitro evidence suggests that collagen, collagen fragments, and associated proteins can play this role. Although the role of collagens and their fragments have been well-studied in atherosclerosis,⁵⁹ signaling by collagen and other structural proteins in the valve has been investigated primarily in the context of tissue engineering, with the goal of identifying biomaterials that predictably guide valve cell fate.^{60–62} In this context, VIC grown on stiff tissue culture-treated polystyrene coated with type I collagen or fibronectin generally remain in a quiescent fibroblastic state, whereas those on fibrin surfaces in particular have greater α -smooth muscle actin (SMA) expression and rapidly contract to form calcified aggregates containing apoptotic cells,^{60–62} indicative of myofibroblast differentiation.⁶³ Type I collagen and fibronectin inhibited SMA expression relative to untreated tissue culture-treated

Table. Summary of Extracellular Matrix Proteins in Normal and Diseased Aortic Valves and Their Potential Roles in Calcific Aortic Valve Disease (CAVD)

ECM Protein	In Normal Adult Valve	In CAVD	Possible Pathobiological Contribution
Collagen	Fibrosa: types I and III; fibrillar; oriented circumferentially	Disorganized, increased synthesis Increased presence in ventricularis	Fibrosis Leaflet thickening
	Basement membrane: type I and IV	Basement membrane appears thin or absent	Matricellular regulation of VIC myofibroblast differentiation
Proteoglycans and glycosaminoglycans	Primarily in the spongiosa	Increased in all three layers	Retention of lipoproteins and inflammatory cells
	Versican, biglycan, decorin, and hyaluronan are the most abundant	Often localized to tissue surrounding calcified nodules	Localizes pro-calcific and pro-inflammatory cytokines (e.g., TGF- β 1, TNF- α) Matricellular regulation of VIC
Elastin	Fibrillar; oriented radially; primarily in the ventricularis	Reduced and fragmented	Increases ECM stiffness Elastin fragments may induce VIC myofibroblast and osteogenic differentiation
Chondromodulin-I	Throughout the valve interstitium	Reduced, particularly in the spongiosa and ventricularis	Inhibits angiogenesis
Periostin	Localized to the subendothelial regions	Increased in the spongiosa and ventricularis; reduced in the fibrosa	Induces angiogenesis and MMP production Suppresses osteogenesis
Tenascin-C	Low expression, limited to subendothelial regions	Shifted to the interstitium Upregulated around calcified nodules	Increases ALP activity and MMP-2 expression in VIC
Bone-related proteins	Absent or unknown	Osteocalcin, osteopontin (bone sialoprotein) increased; others (eg, matrix Gla protein) unknown	Regulate mineralization

ALP indicates alkaline phosphatase; ECM, extracellular matrix; MMP, matrix metalloproteinase; TGF, transforming growth factor; TNF, tumor necrosis factor; VIC, valvular interstitial cell.

polystyrene, even in cultures challenged with transforming growth factor (TGF)- β ,^{60,61,64} a potent profibrotic growth factor. This contrasts studies in which osteogenic differentiation of calcifying vascular smooth muscle cells was shown to be promoted by type I collagen and fibronectin but inhibited by type IV collagen.⁶⁵ The effects of ECM proteins on VIC function appear to be mediated in part through their interactions with specific integrins.⁶⁶ The procalcific response on fibrin involves mitogen-activated protein kinase signaling, as pharmacological inhibition of ERK1/2 or its upstream kinase MEK in VIC on fibrin reduces aggregate formation, alkaline phosphatase (ALP) activity (an early marker of osteogenic differentiation), TGF- β 1 expression, and apoptosis.⁶⁷ VIC on fibrin-coated substrates were also more resistant to simvastatin inhibition of aggregation and apoptosis than VIC on laminin, suggesting ECM proteins can modulate VIC response to soluble cues.⁶⁸ In total, these studies clearly demonstrate matricellular regulation of VIC phenotype by proteins with biomaterials applications. Future studies focused on matricellular regulation by ECM proteins that interact with VIC in native valves are required to determine the relevance of this mechanism to the pathobiology of CAVD.

Proteoglycans and Glycosaminoglycans

In normal valves, proteoglycans (PG) and glycosaminoglycans (GAG) are present in all three layers, but they make up the bulk of the spongiosa. The most abundant PG in the aortic valve are versican, biglycan, and decorin, along with hyaluronan, a nonsulfated GAG that is not associated with a core protein.⁶⁹ With age⁷⁰ and during disease development,^{57,71} PG

and hyaluronan content increase in all three layers, often localized to the tissue surrounding calcified nodules.⁷¹

The role of PG and GAG in CAVD is almost completely unexplored, despite their presence in normal valves and abundance in disease. CAVD shares features of atherosclerosis that involve PG and GAG, including lipoprotein and lipid accumulation and inflammation; therefore, it is conceivable that similar mechanisms are at play in the valve. In atherosclerosis, inflammation is promoted by binding of macrophages to hyaluronan in lesions through the CD44 receptor.⁷² Increased PG content in lesions contributes to lipoprotein accumulation⁷³ through binding of negatively charged GAG with positively charged basic amino acids in the lipoproteins.⁷⁴ Modification of trapped lipoproteins, such as oxidation of low-density lipoprotein, can, in turn, alter PG synthesis and affect cellular function, including calcification. For example, oxidation of low-density lipoprotein induces vascular smooth muscle cells to increase synthesis of chondroitin or dermatan sulfate, the single GAG chain on decorin. In turn, chondroitin or dermatan sulfate elevates the bioavailability of TGF- β to activate osteogenic differentiation pathways and mineralization by vascular smooth muscle cells.⁷⁵ This could occur through a matricrine-related mechanism in which GAG chain addition inhibits sequestration of TGF- β by decorin. Sequestration of soluble proteins by PG can increase local growth factor and cytokine concentrations and availability. Of putative relevance to CAVD, TGF- β can bind to the protein cores of biglycan, betaglycan, and decorin, and tumor necrosis factor- α can bind to biglycan.⁷⁶ Biglycan and decorin are present in regions of early calcific nodule formation in human calcified aortic valves⁷¹ and may

aid to accelerate disease by localizing TGF- β , tumor necrosis factor- α , or other growth factors associated with CAVD. GAG side chains and hyaluronan can also facilitate biochemical signaling by mediating growth factor-receptor interactions. For example, heparan sulfate GAG interact with fibroblast growth factor and its receptor to form fibroblast growth factor–heparan sulfate–fibroblast growth factor receptor complexes⁷⁷ that are required for fibroblast growth factor signaling.⁷⁸ Fibroblast growth factor inhibits VIC myofibroblast differentiation *in vitro*,⁷⁹ but its role in the valve *in vivo* is not known.

PG and GAG can also regulate cell responses through matricellular signaling. For example, decorin has been shown to activate the TGF- β signaling pathway and promote TGF- β production in vascular smooth muscle cells.⁷⁵ Another example is the activation of toll-like receptor 2 in aortic VIC by biglycan to increase phospholipid transfer protein production through a nuclear factor κ B-dependent pathway, with implications for lipid retention and inflammation.⁸⁰

Elastin

Elastin is predominantly found in the ventricularis layer.^{57,81} In normal valves, elastin is organized in aligned fibers to provide structural support and elastic recoil.²⁰ In diseased valves, elastin fiber content is reduced in the ventricularis, and the fibers are disorganized and fragmented.⁵⁷ The loss and fragmentation of elastin fibers may be attributable to increased expression of elastinolytic proteases, including MMP-2 and MMP-9^{43, 45–48, 50} and cathepsins S, K, V, and G.^{51–53} Notably, cathepsin S-deficiency significantly reduced elastin fragmentation and calcification in the aortic valves and aortas of apolipoprotein E-deficient mice with surgically induced chronic renal disease.⁵¹ Although the cellular basis for this effect in the valve is not known, elastin fragments have been shown to induce ALP activity in fibroblasts and vascular smooth muscle cells.^{51,82} In dermal fibroblasts, degraded elastin peptides induced myofibroblast differentiation and upregulated ALP activity and expression of Runx2, an osteochondral transcription factor.⁸² In vascular smooth muscle cells, elastin fragments cleaved by cathepsin S were more potent than randomly cleaved elastin fragments in inducing ALP expression.⁵¹ Elastin damage in the aortic valve also leads to a slight increase in stiffness of the leaflets,⁸³ which, as discussed below, could influence VIC pathological differentiation.

Chondromodulin-I

Chondromodulin-I (Chm-I) is a glycoprotein found primarily in avascular tissues, including the eye and cartilage.^{84,85} It has been shown to inhibit endothelial cell proliferation and tubulogenesis.^{86,87} Chm-I is expressed in all four cardiac valves during development. Its expression persists postnatally in the valve interstitium, where it appears to maintain homeostasis by preventing angiogenesis, but it is significantly downregulated in regions of neoangiogenesis in human diseased valves.⁸⁸ The aortic valves of Chm-I knockout mice develop many features similar to CAVD, including thickened leaflets, calcium and lipid deposition, and neoangiogenesis, as evidenced by strong immunopositivity of von Willebrand

factor and vascular endothelial growth factor.⁸⁸ *In vitro*, VIC-derived Chm-I inhibits endothelial cell migration and tubulogenesis and induces apoptosis.⁸⁸ The role of angiogenesis in CAVD is not defined, but it may promote and accelerate inflammation;⁸⁹ regions with reduced Chm-I expression in diseased valves are associated with CD11b-positive and CD14-positive cells, suggesting macrophage infiltration.⁸⁸ Additionally, angiogenesis may promote calcification by delivering osteoprogenitors or inducing osteogenic differentiation.⁹⁰ Neovascular regions correlated spatially with regions with decreased Chm-I and increased expression of Runx2 in various human valvular diseases.⁸⁸

Periostin

Levels of periostin are high in the ECM during valvulogenesis after endothelial–mesenchymal transition, where it plays a critical role in type I collagen fibrillogenesis.⁹¹ In normal postnatal cardiac valves, periostin expression is decreased and localized to the subendothelium, with higher expression in the ventricularis layer than the fibrosa layer.¹³ In periostin-null mice, valve maturation and morphogenesis are severely impeded, resulting in leaflet abnormalities, including disorganized collagen bundles, lack of matrix stratification, ectopic aggrecan expression, and extensive calcification.^{91–93} This was accompanied by ectopic expression of the pro-osteogenic growth factor pleiotrophin, overexpression of Dlk1 (a negative regulator of Notch1 signaling), downregulation of downstream targets of Notch1, and increased expression of Runx2, osteocalcin, and osteopontin transcripts,⁹³ suggesting that periostin represses an osteogenic program during valve development via Dlk1/Notch signaling.

In adult human and adult mice fed a high-fat diet, periostin expression by myofibroblasts and infiltrated inflammatory cells was significantly increased, particularly in areas of neoangiogenesis, where Chm-I is downregulated; consistent with this, periostin induced endothelial cell tubulogenesis *in vitro*.¹³ Periostin also induced MMP expression by VIC, endothelial cells, and macrophages *in vitro*, and was colocalized with increased type I collagen expression in diseased valves. Moreover, in periostin-null mice fed a high-fat diet, leaflet thickening and MMP expression were both attenuated. This evidence suggests that periostin promotes angiogenesis and ECM remodeling in disease.

Together, these studies present a complex role of periostin in the aortic valve. Periostin prevents CAVD by repressing calcification associated with osteogenesis; however, it promotes features of CAVD, including angiogenesis and increased MMP. These apparently discrepant roles remain to be reconciled. Clues may come from its spatial expression patterns in disease: increased in the ventricularis and spongiosa, where angiogenesis occurs,¹³ and decreased in the fibrosa,⁹² where calcification occurs. Thus, periostin may play distinct roles in different layers, impacting different aspects of CAVD. Clearly, further study is required to resolve this issue, as well as the mechanisms by which periostin levels are regulated and by which it regulates valve cell phenotypes. Possibilities for the latter include indirect influence by promoting ECM integrity and organization by binding multiple ECM proteins, including tenascin-C (TN-C),

fibronectin, types I and V collagen, aggrecan, and heparin,^{94,95} or direct interaction with integrins to promote motility, invasiveness, and remodeling, as it does in atrioventricular cushion mesenchymal cells through Rho/PI 3-kinase signaling⁹⁶ and in endothelial and epithelial cells through focal adhesion kinase-dependent signaling.^{97,98}

TN-C

TN-C is a hexameric ECM glycoprotein that plays a critical role in connective tissue development and is transiently expressed during valvulogenesis. In normal valves, TN-C expression is low and limited to the basement membrane.⁴⁴ In CAVD, TN-C expression is significantly upregulated and shifted from the basement membrane to the interstitium, especially around, but not within, the calcified area.^{44,48} The cause of the upregulation of TN-C in CAVD is not known, although a number of growth factors that are upregulated in CAVD, including TGF- β , bone morphogenetic proteins, and tumor necrosis factor- α ,^{7,9,12} have been shown to induce TN-C expression.⁹⁹ Moreover, TN-C expression can be induced mechanically in fibroblasts,¹⁰⁰ and TN-C expression is higher in diseased valves from patients with increased loading on their valves because of hypertension.⁴⁴ In situ hybridization revealed that increased TN-C mRNA expression was detected in VIC that expressed SMA,⁴⁴ suggesting that myofibroblasts are responsible for secreting TN-C in diseased valves.

The pathological contribution of TN-C to CAVD is still not fully established. TN-C may promote ALP activity and collagen synthesis locally in calcifying regions of the valve, as it does in the osteosarcoma Saos-2 and ROS 17/2.8 cell lines.¹⁰¹ TN-C also may promote ECM remodeling, because it induces MMP-2 mRNA expression in VIC in vitro and colocalizes with MMP-2 protein expression in diseased valves.⁴⁸ TN-C may also regulate VIC function through its interaction with fibronectin to modulate VIC adhesion through syndecan-4 and its downstream signaling. For example, TN-C has been shown to block interactions between syndecan-4 and fibronectin, inhibiting recruitment of syndecan-4 to the focal adhesion complex.^{102,103} This reduces focal adhesion size and alters downstream focal adhesion kinase and RhoA signaling.^{102,104} RhoA signaling has been shown to be involved in VIC phenotypic expression, calcification, and response to statins in vitro.^{68,105}

Bone Matrix Proteins

Bone formation can contribute to the calcification of aortic valves, with 13% of calcified human aortic valves demonstrating laminar bone with mature marrow.⁹ Consistent with mineralization by bone formation, the expression of noncollagenous bone ECM proteins, including osteocalcin, osteopontin, and bone sialoprotein, is upregulated in diseased valves from humans and animals.^{15,42,55,106} Bone formation and increased levels of bone matrix proteins in CAVD are thought to result from differentiation of an osteoprogenitor subpopulation in VIC,³⁷ possibly through a transitional myofibroblastic state, although in vitro evidence suggests that this is not always the case.⁶³ In vivo lineage tracing studies, as have been performed in the vasculature,¹⁰⁷ are required to

resolve this outstanding question. Circulating hematopoietic stem cells^{108,109} and endothelial cells that undergo mesenchymal transformation¹¹⁰ may also contribute to the osteoprogenitor population.

Although it is clear that osteogenic processes occur in CAVD, the factors that regulate mineralization in CAVD are poorly understood. In particular, it is unclear why the fibrosa layer is more susceptible to mineralization. Mineralization is tightly regulated by noncollagenous proteins that bind calcium to promote mineralization (eg, osteocalcin and osteonectin) and those that inhibit mineralization (eg, matrix Gla protein).¹¹¹ It is conceivable that the susceptibility of the fibrosa to mineralization stems from distinct noncollagenous protein profiles in the individual layers. However, to date, noncollagenous proteins have been used only as markers of osteogenesis in the valve and their roles in orchestrating bone mineralization in CAVD remain unclear.

The Potential Role for ECM Mechanics in the Initiation and Progression of CAVD

Mechanical forces actively regulate VIC and VEC fate and function to drive valve development, homeostasis, and disease.¹¹² This is elegantly demonstrated in the progressive adaptation of the valve ECM and cell phenotypes to dynamic changes in functional requirements from fetal and postnatal development to adulthood and in diseased and engineered valve tissue.^{31,32,113,114} Therefore, in addition to its matricellular and matricrine roles, the ECM serves a mechanical role, because its composition and organization determine the inherent elasticity of aortic valve tissue. Tissue elasticity and the geometry of the valve leaflets together define the valve biomechanical function: thin pliable leaflets deform easily when subjected to hemodynamic forces, providing little resistance to blood flow, whereas thick, stiff, calcified leaflets resist deformation resulting in stenosis. Thus, changes in the valve ECM directly impact valve biomechanical function, with clinical consequences that define CAVD.

The ECM also defines the local micromechanical environment of cells in the valve by the resistance that it provides to cell-generated traction forces. Adherent cells sense the local elasticity of their ECM by pulling on the substrate via integrin-mediated adhesions and actinomyosin-based contraction.¹¹⁵ Traction forces are resisted in part by the cytoskeleton (for example, microtubules), but the majority are transmitted to the substrate at focal adhesions.¹¹⁶ Cells respond to the elasticity of the substrate by altering integrin expression, focal adhesions, and cytoskeletal organization to establish a force balance between the resistance provided by the substrate and the cell-generated traction force.¹¹⁷ Even in dynamically strained tissues like the cardiac valves, cytoskeletal prestress induced by tractional forces often exceeds external stresses applied to cells.¹¹⁸ And because integrin activation and cytoskeletal organization and tension regulate intracellular signaling pathways, many cellular processes are exquisitely sensitive to ECM elasticity.¹¹⁹

Transmission of External Hemodynamic Forces by the ECM

The external hemodynamic forces that shear and deform the valve leaflets throughout the cardiac cycle are a clear source

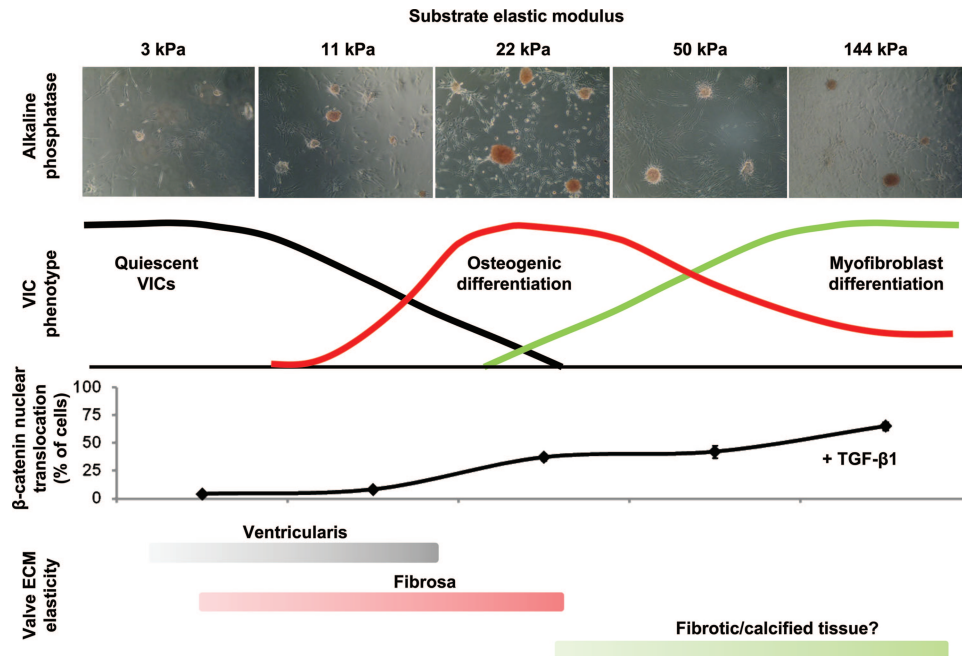


Figure 3. Role of extracellular matrix (ECM) elasticity in regulating valvular interstitial cell (VIC) phenotype. Based on porcine cell culture studies in osteogenic medium,⁶³ substrates with elastic moduli approximately 20 kPa favor osteogenic differentiation, demonstrated here by several aggregates that stain strongly for alkaline phosphatase activity (top). On softer substrates (<11 kPa), VIC appear to remain quiescent and do not form aggregates even in osteogenic culture medium, whereas on stiffer substrates (>25–50 kPa) VIC preferentially differentiate to myfibroblasts. VIC myfibroblast differentiation is induced by transforming growth factor (TGF)- β 1 but requires β -catenin;¹³⁵ β -catenin nuclear translocation increases with increasing ECM stiffness, consistent with there being more myfibroblast differentiation on stiffer substrates (third panel from the top). Only stiffer regions of the fibrosa of normal porcine aortic valves exceed the threshold elasticity for osteogenic differentiation and VIC responsiveness to TGF- β 1 (bottom), which suggests that there are focal regions in the fibrosa, but not the ventricularis, that are permissive to VIC pathological differentiation. As the valve ECM becomes fibrotic and calcified with disease, it presumably stiffens, but these measurements have not been performed to date.

of mechanical signals that actively regulate VIC and VEC fate and function.¹¹² Deformation of leaflet tissue is transferred to the cells via their adhesion to the ECM; therefore, external forces are defined at the cellular level in part by the properties of the ECM.²⁰

Supraphysiological stretch of aortic valve cusps *ex vivo* induces processes associated with CAVD, including increased expression of remodeling enzymes, proinflammatory proteins, bone morphogenetic proteins, and ALP by VIC.^{120–122} These changes occur predominantly in the fibrosa layer, perhaps because VIC in the fibrosa deform more than those in the ventricularis.¹²³ VEC also respond to supraphysiological stretch by increasing adhesion molecule expression *in vitro*¹²⁴ and by expressing osteocalcin in mitral valves *in vivo*.¹¹⁰ Thus, external force regulation of valve cell pathobiology may partially explain the increased prevalence of CAVD in patients with hypertension² and bicuspid valves,¹²⁵ and the localization of lesions in regions that are subjected to disturbed blood flow¹²⁶ and large bending stresses.¹²⁷

Regulation of Valve Cell Biology by ECM Elasticity

VIC are similar to many other cells in that their differentiation, function, and responsiveness to microenvironmental cues are dependent on ECM elasticity. Of putative relevance to CAVD, the pathological differentiation of VIC to myfibroblasts and osteoblasts is regulated by ECM elasticity in a manner similar to mesenchymal stem cells,^{128,129} consistent

with VIC containing a subpopulation of mesenchymal stem cells.³⁷ These and related studies are summarized here and in Figure 3.

The Effect of ECM Elasticity on VIC Myfibroblast Differentiation

The sensitivity of VIC myfibroblast differentiation to ECM elasticity was first demonstrated by Pho et al³⁶ using fibrillar collagen gels that were either thick, providing an elasticity that mimicked that of stiffer regions of the fibrosa (\approx 25 kPa), or thin, mimicking the stiffness of sclerotic tissue (\approx 110 kPa).⁶³ They found more myfibroblasts (identified by SMA-containing stress fibers¹¹⁸) on the stiffer substrates, an effect that required the actin-severing protein cofilin. Similarly, VIC differentiated to myfibroblasts on fibrin-modified stiff tissue culture-treated polystyrene but not on fibrin-modified soft polyethylene glycol hydrogels.⁶⁰ A photodegradable hydrogel was subsequently used to create stiffness gradients and show that the number of myfibroblasts increased with increasing substrate stiffness over the range of 7 to 32 kPa, with a reported threshold value for VIC myfibroblast differentiation of approximately 15 kPa.¹³⁰ These responses of aortic VIC to substrate elasticity are generally consistent with elasticity responses of other myfibroblast populations.¹³¹ Notably, VIC populations that were grown on 32-kPa matrices to induce myfibroblast differentiation had fewer myfibroblasts when the matrix stiffness was reduced to 7 kPa by local photodegradation.¹³⁰ Whereas this result is suggestive

of myofibroblast dedifferentiation, single cell tracing studies are required for confirmation. In contrast to aortic VIC, myofibroblast differentiation of VIC from mitral valves was insensitive to substrate elasticity (34 vs 323 kPa) or even reduced on stiff substrates in an age-dependent and location-dependent manner.¹³² Differences between ages and in aortic vs mitral VIC responses may reflect VIC adaptations to local ECM properties. Notably, VIC from different layers of the aortic valve have distinct substrate elasticity responses; for example, myofibroblast differentiation of aortic VIC from the softer ventricularis layer is more sensitive to substrate stiffness and mechanical stretch than that of VIC from the stiffer fibrosa layer (M. Likhitpanichkul, C. Moraes, and C.A. Simmons, unpublished observations). Although the implications of location-dependent and age-dependent VIC plasticity remain to be elucidated, one possibility is that adaptation to the local ECM micromechanical environment may aid in homeostasis but exacerbate certain pathological perturbations.³⁹

ECM Elasticity Modulation of TGF- β 1-Induced VIC Myofibroblast Differentiation

Mechanical tension is required for myofibroblast differentiation, but it alone is not sufficient; the formation of fully differentiated myofibroblasts requires active TGF- β .¹¹⁸ TGF- β 1 is present in human calcified aortic valve leaflets,¹² where it likely binds to the ECM as part of a latent complex until activated by protease-dependent¹³³ or integrin-dependent¹³⁴ mechanisms. Histologically, TGF- β 1 signaling components associate with myofibroblasts¹³⁵ but not with bone protein expression¹³⁶ in the valves of hypercholesterolemic swine and mice, respectively, which suggests that TGF- β signaling in CAVD is associated with myofibroblast differentiation, as it is in other fibrotic diseases, but not osteogenic processes. In fact, TGF- β 1 signaling may inhibit osteogenic calcification in the valve, because inhibition of TGF- β type I receptor *in vivo* promotes osteogenic differentiation of mouse bone marrow mesenchymal stem cells¹³⁷ and TGF- β 1 treatment *in vitro* suppresses osteogenic differentiation of osteoblasts¹³⁸ and VIC (J.H. Chen and C.A. Simmons, unpublished observations).

Importantly, TGF- β 1 induction of VIC myofibroblast differentiation requires mechanical tension, either by stretching the ECM¹³⁹ or by providing a stiff ECM.^{36,63,135} One elegant mechanism to explain this cross-talk is that a stiff or stretched ECM alters the conformation of the TGF- β 1 latent complex, which is bound to the ECM and the cell membrane, releasing TGF- β 1 and making it available to bind its cell membrane receptor and induce myofibroblast differentiation.¹⁴⁰ Increased cell contractility attributable to TGF- β 1-induced myofibroblast differentiation can further stiffen the ECM,¹⁴¹ setting up the possibility of a positive feedback loop of VIC activation. In contrast, a latent complex on a soft matrix (<5 kPa) is not opened by the cell pulling on it, because the ECM provides inadequate resistance to “pull back” and TGF- β 1 remains sequestered.

ECM elasticity also modulates the effects of TGF- β 1 on VIC myofibroblast differentiation through regulation of β -catenin signaling¹³⁵ (Figure 3). In sclerotic leaflets of hypercholesterolemic swine, SMA-positive myofibroblasts

colocalize with TGF- β and Wnt/ β -catenin signaling components. Consistent with this, TGF- β 1 directly induces rapid β -catenin nuclear translocation and SMA expression through a TGF- β type I receptor-dependent and Smad2/3 phosphorylation-dependent mechanism in primary cultures of swine VIC. This occurs in an ECM stiffness-dependent manner, with a minimum stiffness threshold of between 11 and 22 kPa for modest β -catenin nuclear translocation and myofibroblast differentiation and significantly more on stiffer substrates (>50 kPa). β -catenin is required for TGF- β 1-induced myofibroblast differentiation, because silencing or degrading it abolishes myofibroblast differentiation without altering Smad2/3 activity, even on the stiffest substrates. The implications of these findings for CAVD are considered further below.

The Effect of ECM Elasticity on VIC Calcification and Osteogenic Differentiation

In addition to its critical role in regulating VIC myofibroblast differentiation, ECM elasticity also influences calcification by VIC. Porcine VIC grown on fibrosa-like or sclerotic tissue-like collagen substrates in standard osteogenic medium (containing β -glycerophosphate, ascorbic acid, and dexamethasone) form calcified aggregates, but they can do so through distinct processes (Figure 4). On fibrosa-like matrices (\approx 25 kPa), aggregates contain viable cells, appear to be clonally derived, and calcify with strong expression of Runx2, ALP, osteonectin, and osteocalcin;⁶³ these aggregates resemble classical bone nodules formed by osteoprogenitors.^{142,143} On softer ventricularis-like matrices (<11 kPa), few ALP-positive aggregates form (Figure 3). Together, this evidence suggests that a fibrosa-like ECM elasticity promotes bone nodule formation through an osteogenic process. Strikingly, human marrow-derived mesenchymal stem cells preferentially undergo osteogenic differentiation on substrates with moduli in the same range,^{128,129} suggesting that this may be a common response for all mesenchymal progenitors. In contrast, VIC on stiff sclerotic tissue-like matrices (\approx 110 kPa) have weak ALP activity and lower expression of Runx2, osteonectin, and osteocalcin, and form calcified aggregates that contain a core of apoptotic and dead cells.⁶³ Aggregation on stiff matrices occurs through VIC myofibroblast differentiation, contraction of the cell sheet, and its detachment from the underlying culture substrate, a process that is inhibited by disrupting actin filaments and cytoskeletal tension,^{12,63,144} or inhibiting Rho kinase.¹⁰⁵ Calcification in this case requires apoptosis,¹² and apoptosis appears to be a result of anoikis and not the cellular contraction itself (R. Zhao and C.A. Simmons, unpublished observations). The relevance of anoikis as a mechanism to explain VIC apoptosis and calcification *in vivo* is questionable. Thus, data from *in vitro* VIC calcification experiments should be interpreted cautiously, because apoptosis-induced calcification on stiff substrates may be an *in vitro* artifact.

Implications of ECM Elasticity Regulation of VIC to CAVD

It is premature to conclude that valve ECM elasticity plays a regulatory role in CAVD, but the *in vitro* evidence is compelling. Growing evidence suggests that change in tissue

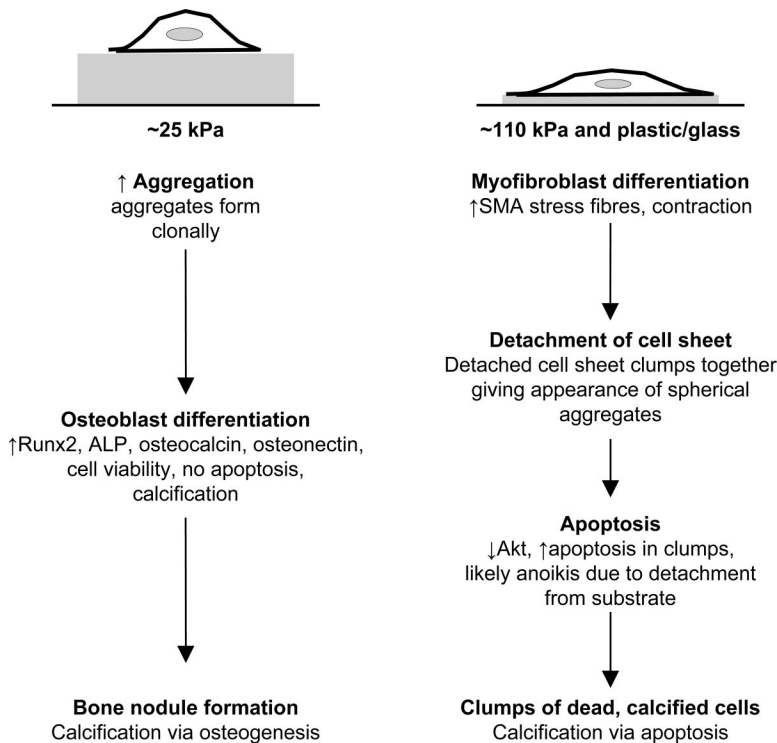


Figure 4. Mechanisms by which valvular interstitial cells (VIC) calcify in vitro.⁶³ VIC grown in osteogenic medium on thick collagen gels with elastic moduli approximately 25 kPa form calcified nodules through clonal growth, osteogenic differentiation, and bone formation. VIC grown on thin collagen gels (elastic modulus \approx 110 kPa) or other stiff culture substrates (ie, glass or tissue culture-treated polystyrene) in osteogenic medium differentiate to contractile myofibroblasts, which often pull away from the underlying substrate. The detached cell sheet contracts to form clumps of cells. Akt activity is reduced soon after contraction, followed by apoptosis, likely attributable to detachment from the substrate (anoikis). The apoptotic and dead cells in the clumps then calcify; calcification is inhibited if apoptosis is blocked.¹² We speculate that positive alkaline phosphatase staining sometimes observed in clumps formed on stiff substrates results from the cell clumps providing a soft substrate on which viable cells can grow and undergo osteogenic differentiation secondary to the initial apoptotic calcification event. Primary VIC osteogenic differentiation can occur on stiff substrates if cell sheet contraction is prevented by keeping cell densities low and avoiding other factors that promote myofibroblast differentiation, like transforming growth factor (TGF)- β 1. Calcified cell clumps typically can be distinguished from bone nodules morphologically by bare areas around the clumps where the cell sheet and ECM have pulled up from the underlying substrate.⁶³

elasticity is not only an outcome of scarring, aging, and other diseases (including atherosclerosis, cancer, and fibrosis of the heart, lung, liver, and kidney) but also contributes significantly to pathological development by influencing cell function directly and by modulating cellular sensitivity to microenvironmental and genetic perturbations.¹⁴⁵ This raises several interesting thoughts about the potential implications of ECM elasticity regulation of VIC biology.

First, the side-specific susceptibility of aortic valve leaflets to focal lesion development may reflect local regions in the fibrosa that are mechanically permissive to pathological development (Figure 3). Porcine VIC myofibroblast^{130,135} and osteoblast⁶³ differentiation occur only on substrates with elastic moduli exceeding approximately 20 kPa; this is also the threshold modulus for VIC responsiveness to TGF- β 1.¹³⁵ In normal swine leaflets without any evidence of disease, only focal regions within the fibrosa are intrinsically this stiff; most of the fibrosa, all of the ventricularis, and presumably all of the spongiosa are softer²⁷ and therefore are predicted not to permit VIC pathological differentiation. Furthermore, the in vitro data predict that osteogenesis in response to biochemical stimuli is favored on matrices with normal fibrosa-like elasticity, whereas myofibroblast differentiation is favored on stiffer ECM.⁶³ This suggests that the pathogenesis of CAVD is determined by a complex interplay between ECM elasticity and other microenvironmental cues, resulting in context-dependent pathological signaling and perhaps explaining why calcification is osteogenic in some diseased valves but not others.⁹

Second, VIC responsiveness to microenvironmental perturbations presumably evolves as the ECM composition, organization, and elasticity changes with age. This may partly explain why CAVD takes decades to develop. A similar

scenario has been proposed to explain higher rates of breast cancer malignancy with age and other factors that influence breast tissue stiffness.¹⁴⁶

Third, the putative changes in ECM elasticity that occur with disease development may influence VIC function and their sensitivity to pathological stimuli. This suggests the potential for a positive feedback loop in which, for example, stiffening of the ECM increases VIC sensitivity to profibrotic stimuli, leading to further stiffening of the valve. Notably, in several animal models with valve ECM abnormalities, disruption of the valve ECM (and presumably its elasticity) alters TGF- β signaling in VIC, leading to maladaptive ECM remodeling and valve disease.⁴⁰ One can imagine another scenario in which focal calcification stiffens the ECM locally, which in turn induces a fibrotic response in the surrounding region. Anecdotally, myofibroblasts are observed to surround calcified bone nodules in human valve allografts.¹⁴⁷ ECM stiffening will also impact local hemodynamics, which may in turn shift the endothelium to a more “disease-permissive” phenotype,¹²⁶ with increased expression of shear-sensitive paracrine factors that promote fibrosis and calcification (eg, TGF- β /bone morphogenetic protein¹⁴⁸) or decreased expression of those that prevent fibrosis and calcification (eg, C-type natriuretic peptide¹⁴⁹).

Based on current knowledge of valve ECM mechanics and pathogenesis, it is difficult to assess whether any of these mechanisms contribute to CAVD initiation and progression. An important step to that end will be the characterization of temporal and spatial changes in valve ECM elasticity over the course of CAVD and their correlation with VIC phenotypes and ECM components. A critical component of this will be the development, validation, and use of both small and large animal models of hemodynamically significant CAVD, which are currently limited.

Conclusions

Alterations in the structure and composition of the valve ECM are hallmarks of CAVD. However, ECM maladaptation and valve cell dysfunction are interdependent and intimately entwined with the ECM, orchestrating cellular and molecular events that contribute to CAVD initiation and progression through matricellular, matricrine, and mechanical signaling. Although the evidence for a regulatory role of the ECM in CAVD is compelling, several outstanding issues remain to be addressed.

First, relatively little is known about the earliest initiation events in CAVD. What are the molecular triggers for pathological ECM remodeling? Are changes in ECM elasticity exclusively the result of changes in matrix protein composition, or are more subtle mechanisms, such as matrix cross-linking, responsible in early disease?

Second, additional characterizations of the dynamic changes in valve ECM proteins and their impact on early disease development in particular are required. Proteomic profiling may provide substantial insight to this end.¹⁵⁰ Specific issues include the role of PG/GAG and noncollagenous proteins in the initiation of valvular fibrosis and calcification, and whether matrix proteins¹⁵¹ or adhesion molecules¹⁵² recently found to be involved in vascular calcification also play a role in CAVD.

Third, the molecular mechanisms by which the ECM directs cell function to contribute to valve pathobiology are poorly understood. What are the signaling pathways involved in matricellular and mechanical regulation of VIC phenotypes? Do these pathways converge with each other and with those activated by soluble proteins? If so, then what are the implications of cell integrative and context-dependent responses to CAVD etiology? And what can be learned from bioprosthetic valve calcification about the role of the ECM in CAVD?¹⁵³

Fourth, the interdependent relationship between the ECM and cells other than VIC has not been studied. Does the ECM influence inflammatory cells and vice versa? Are VEC responses to shear stress¹⁵⁴ or their capacity to transdifferentiate to VIC^{110,155} regulated by the ECM? Do VEC contribute to ECM remodeling, either directly or through paracrine signaling, and does this occur in a side-specific manner?^{126,156}

Fifth, given the putative importance of ECM mechanics, how does ECM elasticity evolve spatially and temporally over the course of aging, with and without CAVD? Do relationships between ECM elasticity and cell responses observed in vitro translate in vivo? Can advanced engineered biomaterial models that mimic valve tissue mechanics provide additional insights while avoiding confounding issues that make studying ECM elasticity effects challenging in animal models? These are just some of the open questions that, once answered, will provide new insights into reciprocal cell–ECM interactions in the aortic valve, their contributions to homeostasis and pathobiology, and possible targets to slow or prevent the progression of CAVD.

Acknowledgments

The authors thank Krista L. Sider for critically reviewing the manuscript and for providing the images in panels A and B of Figure

2. The authors thank Dr K. Jane Grande-Allen and Dr Elizabeth H. Stephens for providing the image in panel C of Figure 2.

Sources of Funding

This work is funded by operating grants from the Canadian Institutes of Health Research (MOP-102721), the Heart and Stroke Foundation of Ontario (NA6654), and the Natural Science and Engineering Research Council of Canada (NSERC; RGPIN 327627-06). J.H. Chen is supported by an NSERC Alexander Graham Bell Canada Graduate Scholarship. C.A. Simmons is supported by the Canada Research Chair in Mechanobiology.

Disclosure

None.

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