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In vitro Models of Aortic Valve Calcification: Solidifying a System

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Abstract

Calcific aortic valve disease (CAVD) affects 25% of people over 65, and the late-stage stenotic state can only be treated with total valve replacement, requiring 85,000 surgeries annually in the US alone [1]. As CAVD is an age-related disease, many of the affected patients are unable to undergo the open-chest surgery that is its only current cure. This challenge motivates the elucidation of the mechanisms involved in calcification, with the eventual goal of alternative preventative and therapeutic strategies. There is no sufficient animal model of CAVD, so we turn to potential *in vitro* models. In general, *in vitro* models have the advantages of shortened experiment time and better control over multiple variables compared to *in vivo* models. As with all models, the hypothesis being tested dictates the most important characteristics of the *in vivo* physiology to recapitulate. Here, we collate the relevant pieces of designing and evaluating aortic valve calcification so that investigators can more effectively draw significant conclusions from their results.

Keywords

Calcific aortic valve disease; Model; Quantification methods; *in vitro*

1. Introduction

Aortic stenosis, which has an estimated prevalence of 2% in patients between 70 and 80 years of age, is most often caused by calcific aortic stenosis, the late-stage presentation of calcific aortic valve disease (CAVD; note all acronyms and abbreviations used in this article can be found in Table 1) [2]. Prevalence of any aortic valve calcification was investigated in a randomized trial, and, for those aged 75–76, the prevalence was 48%; this further increased in the 80–81 and 85–86 year-old cohorts [3]. The incidence of this age-related disease is expected to grow dramatically as the US population over 65 nearly doubles over the next 25 years [4]. Calcific aortic valve stenosis is the main indication for valve

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replacement, which necessitates open chest surgery and is currently the only cure [5]. If the biological mechanism of valvular calcification was better understood, we could create more targeted, non-invasive therapeutics; a comprehensive review of CAVD therapeutic targets can be found in Hutcheson et al. [6]. To elucidate the important mechanisms that regulate the progression of CAVD, we first need to design models that recapitulate the *in vivo* human process.

In vivo models offer the complexity found in the human and can prevent overlooking an important variable. However, this complexity comes at the expense of confounding factors, especially because the experiments are performed in animals significantly different from humans. For example, leporine models must be fed very high cholesterol diets to induce the advanced disease observed in humans [7] or vitamin D2 to generate calcification [8]. Murine models require dietary and or genetic modification as well [9–11] to induce calcification; *Ldlr*−/− mice must be fed a high-cholesterol diet and while *Apoe*−/− mice develop hypercholesterolemia over time [12] it is unclear whether it progresses through the same mechanism as the human disease [13]. A full review of animal models of CAVD can be found in Sider et al. [13]. Since *in vitro* models allow better isolation and manipulation of variables and the *in vivo* models are far from perfect, we focus on *in vitro* models and their usefulness.

Once believed to be a passive process, aortic valve calcification is now thought to be an active process mediated largely by aortic valve interstitial cells (AVICs) [14]. AVICs are a heterogeneous population of fibroblast-like cells present in all three layers of the aortic valve and important in the structural maintenance of the valve, especially in maintenance of the extracellular matrix (ECM) [15, 16]. Progression of CAVD is marked by the formation of calcific nodules (CNs), which are cellular aggregates characterized in humans by a mixture of calcium phosphate phases [17]. Two well-established hypothetical mechanisms of CN formation exist: 1) transforming growth factor β1 (TGF-β1) mediates activation of myofibroblasts, causing calcification via apoptotic mechanisms [18], and 2) a population of myofibroblasts spontaneously transdifferentiate into osteoblast-like cells and these cells regulate mineralization (Figure 1) [19, 20]. In a study of human valves, 83% of the group demonstrated evidence of dystrophic calcification and 13% of those valves had mature lamellar bone and evidence of active bone remodeling [21]. It is unclear whether these processes occur simultaneously or sequentially [22]. Recent progress and the need for a robust *in vitro* system with which we can probe and clarify the mechanism of aortic valve calcification motivate this review.

2. Defining Aortic Valve Interstitial Cells

Human aortic valves consist of three layers: 1) the fibrosa faces the aorta and is composed mostly of type I fibrillar collagen arranged circumferentially in parallel bundles in a matrix of elastin, 2) the spongiosa is the middle layer composed of glycosaminoglycans that act as shock absorbers for the valve, and 3) the ventricularis faces the left ventricle and is primarily composed of elastin fibers oriented radially [23]. AVICs are present throughout the leaflets and are a heterogeneous population of myofibroblasts, fibroblasts, and smooth muscle like cells. Aortic valve endothelial cells (AVECs) sheath the surface of the leaflets and are

oriented circumferentially and form single cell monolayers, expressing Von Willebrand factor and nitric oxide (NO) [24–26]. Circulating cells have recently been implicated in the progression of calcification as well; elevated levels of endothelial progenitor cells with an osteoblastic phenotype and osteogenic precursor cells have been associated with severe and early heterotopic ossification, respectively [27, 28]. Early stages of CAVD develop lesions similar to atherosclerotic lesions, which suggests a role for inflammatory cells and biochemical signals [29, 30]. Elevated levels of macrophages and T-lymphocytes have been found in human calcified aortic valves [21, 31–33]. These cell populations all contribute to CAVD progression, but it is likely that it is through secretion of factors that influence AVIC behavior.

As the AVIC population is heterogeneous, we should consider the characteristics of various subpopulations. Recently, AVICs were categorized into five groups based on their phenotypic behavior: embryonic progenitor endothelial/mesenchymal cells, quiescent VICs (qVICs), activated VICs (aVICs), progenitor VICs (pVICs), and osteoblastic VICs (obVICs) [34]. We will refer to these subtypes for ease of discussion. Embryonic progenitors are usually present in the cardiac cushions and give rise to qVICs via endothelial to mesenchymal transformation (EMT). While these are very important in valve development, there is also evidence that these progenitors participate in adult valve repair. qVICs are responsible for maintaining physiological valve structure and function. The exact activity of these cells is undefined, but they are believed to regulate low-level matrix degradation and synthesis and inhibition of angiogenesis. pVICs are considered valve stem cells and they are likely responsible for VIC proliferation in response to tissue injury. pVICs may originate from AVECs that undergo an EMT-like process [34–36]. These EMT-related events are likely directly mediated by the mechanical forces present in the valve. In a recent study using chick explanted atrioventricular canals, EMT was found to occur preferentially in higher regions of strain [37]. This developmental process is likely recapitulated in an unregulated fashion during CAVD progression. This suggests that as the valve stiffens, more AVECs are transformed into pVICs and qVICs, allowing subsequent activation.

aVICs are qVICs that have become myofibroblasts characterized by alpha smooth muscle actin (αSMA) and increased contraction [34]. This activation occurs under pathological injury cues or abnormal mechanical stress via cytokines and growth factors produced by activated AVECs and macrophages. aVICs are associated with increased ECM secretion and degradation, matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) expression, proliferation and migration, and secretion of cytokines including TGF-β1. If apoptotic pathways become abnormal, aVICs can lead to calcification; this is referred to as the dystrophic pathway. obVICs are VICs that have undergone osteoblastic differentiation and promote calcification *in vitro*. This differentiation is induced by the addition of organic phosphate to culture media and subsequent calcification depends on the upregulation of alkaline phosphatase (ALP) activity. Adding bone morphogenic protein 2 (BMP2) and 25-hydroxycholesterol increased the rate of CN formation, as did TGF-β1, which induced calcification via an apoptotic mechanism [38]. BMP2 has been shown to be higher in stenotic human aortic valves [39] and upregulates osteogenic pathways involving Msx2 and Wnt signaling [40] and Runt-related transcription factor 2/core-binding factor

subunit alpha-1 (Runx2/Cbfα-1) [41]. It is likely that AVECs are regulating aVIC or obVIC function and that, given the presence *in vivo* of both BMP2 and TGF-β1, a combination of osteogenic and dystrophic pathways is occurring. Therefore, we are most concerned with the transitions to and behavior of aVICs and obVICs.

AVIC-AVEC co-culture systems have provided some insight into the complex regulation of AVICs. When porcine AVICs were cultured with the same number of AVECs in a 3D model, they demonstrated decreased αSMA, cell number, and increased total protein and sulfated glycosaminogly can content compared to AVICs cultured alone in the 3D collagen gel [42]. Given osteogenic differentiation media, AVICs in 3D collagen hydrogels showed much higher levels of calcification via Alizarin Red stain, but the addition of AVECs brought calcification, osteocalcin, Runx2, and αSMA back down to at least control levels [43]. A more recent co-culture model utilizes magnetic nanoparticles to layer AVICs and AVECs and allow them to freely float in media [44]. While it would necessitate a system capable of flow, including relevant circulating cells, precursor and/or inflammatory, in a co-culture system would help elucidate their role in calcification.

3. The Appropriate Model Organism

The ideal *in vitro* model would use primary human AVICs, but availability is the chief limiter of using human-derived samples. The next best cell would retain all characteristics of the human cells important to CAVD. Since it is believed that the important mediators of calcification are AVICs, we can narrow our search to finding a species with AVICs comparable to human AVICs.

Non-human primates are a logical choice because of their genetic similarity. However, maintenance of these organisms requires more space, time, money, and permissions than other organisms. Likely for these reasons, non-human primate AVICs have not been isolated, though *Macaca nemestrina* aortic smooth muscle cells have been isolated to investigate proteoglycan expression [45]. Porcine hearts are both anatomically and physiologically similar to human hearts. The growth of the heart in swine from birth to four months is analogous to that in humans from birth to mid-teens [46] and remodeling in atherosclerosis of micropigs closely resembles human pathology [47]. Interestingly, their valves contain the same αSMA-positive population of cells in the ventricularis [15]. Swine can also develop spontaneous valvular atherosclerotic lesions, a precursor to calcification [20, 48]. The first isolation of porcine AVICs noted that they appear more homogenous than murine or leporine VICs and had a high recovery rate after being frozen, leading to the extensive use of porcine AVICs in *in vitro* studies [49]. Though these cells are widely used and multiple research groups have reported calcification and mineralization, Cloyd et al. reported that porcine AVICs cultured in osteogenic media with TGF-β1 (which should activate both dystrophic and osteogenic pathways) did not form mineral deposits. They used Raman spectroscopy to show that even Alizarin Red-positive nodules did not exhibit mineralization [17]. While pig anatomy is highly similar to human anatomy, porcine AVICs *in vitro* is still a limited model. One important limitation specific to *in vitro* cell culture systems is the age of the cells. In 20% of long-term cell culture, AVICs become contact-inhibited monolayers and behave unstably [50]. Also, metabolic activity of porcine AVICs

was found to be passage number dependent [51]. Late-stage cultured AVICs demonstrated higher numbers of myofibroblasts [52–54]. Thus, porcine AVICs are generally used no later than passage 7. Though porcine AVICs have limitations, they are the best available model.

Ovine AVICs have been shown to form CNs when treated with TGF-β1 within 72 hours, and to calcify, assayed via Alizarin Red staining, within two weeks [18, 55]. Canine AVICs were also considered early in the development of CAVD research [38]. Specifically, beagles demonstrate age-related changes to aortic valves, including calcification; changes were especially apparent in the fibroblasts, suggesting a similar mechanism to human calcification [56]. *In vitro*, canine AVICs spontaneously formed CNs containing hydroxyapatite over two to three weeks, compared to human AVICs developing nodules in about six weeks under the same conditions [38]. Also, while an imperfect model, many similarities exist between canine and human myxomatous mitral valve disease, reinforcing the likeness between human and canine valves [57]. While canine AVICs were deemed very similar to humans', they are not often used, likely as a function of convenience – dogs have longer life spans than small animal models and are not maintained at a large scale for another purpose, as pigs are for food. Rabbits are used for *in vivo* studies, but not as often *in vitro*, likely because they require high cholesterol diets to develop calcification [5, 58–60].

Mice are another popular model organism, perhaps because of their low cost, easy management, short life spans, and availability of genetic mutants. Murine cell lines can be easily immortalized, allowing for near indefinite expansion and use without regard for passage limitations. AVICs could be harvested from a variety of genetically-altered models such as ApoE−/−, Notch1+/−, and LDLr−/− [10, 20, 61–65]. Though some of these models are the only ones to exhibit the hemodynamic effects of aortic valve stenosis, murine valvular structure is significantly different from human [20, 66]. Specifically, human valves have trilaminar structure, but murine valves only have a fibrosa and spongiosa [66]. While non-ideal, murine AVICs would provide a convenient model that facilitates genetic manipulation allowing for further exploration of CAVD mechanisms. A summary of the advantages and limitations of the AVICs derived from each model organism can be found in Table 2.

4. The Environment

4.1 Mechanical Characteristics

As a human ages, aortic valves remodel: AVIC density and proliferation decreases, elastin content increases, and collagen fibers become more aligned [67]. However, in CAVD, elastin content is fragmented and decreased, while collagen content increases and is disorganized, and the valve leaflet thickens. Remodeling of the ECM via MMP activity, and subsequent stiffening that is characteristic of CAVD has been shown to regulate cellular processes [31]. For example, AVICs cultured in the presence of TGF-β1 on type I fibrillar collagen gels or fibrin coated tissue culture plastic or hydrogels of ~25kPa formed osteogenic CNs, whereas nodules on ~120kPa formed through the dystrophic pathway via myofibroblastic differentiation [68, 69]. This suggests that after the initiation of disease, a positive feedback exacerbates the progression, at least in the dystrophic case.

Substrate composition has also been shown to affect calcification. AVICs cultured on fibrin or tissue culture polystyrene exhibited significantly more nodules than on collagen, fibronectin, or laminin [70]. In addition, the presentation of RGD to AVICs resulted in far more calcification than the presentation of YIGSR or DGEA. RGD, YIGSR, and DGEA are ECM-derived peptide sequences derived from fibronectin/fibrin/laminin/collagen, laminin, and collagen, respectively. Their receptors are $\alpha_v\beta_3/\alpha_5\beta_1/\alpha_1\beta_1$, 67-kDa laminin receptor, and $\alpha_2\beta_1$, respectively. Further investigation showed that disruption of the $\alpha_5\beta_1$ integrin or 67 kDa laminin receptor mediated binding between AVICs and ECM results in increased calcification [70]. Fibronectin coated tissue culture polystyrene suppressed calcification markers, while fibrin coated tissue culture plastic enhanced calcification as demonstrated by CN number, ALP activity, αSMA expression, Cbfα-1 expression, and calcium content via the o-cresolphthale in complex one method. However, both fibronectin and fibrin coating soft hydrogels suppressed calcification [71]. This suggests that substrate stiffness may be more important than specific ECM component interactions. However, the method in which stiffness is modulated (i.e. by increasing crosslinking) is often coupled to the presentation of ECM components, especially integrins.

Also important to consider are the effects of trying to recapitulate a 3-dimensional (3D) *in vivo* environment with a 2-dimensional (2D) environment *in vitro*. When porcine AVICs were encapsulated in peptide-modified polyethylene glycol hydrogels, results were consistent with 2D experiments [70]. However, in 3D spheroids, fibroblasts become much less sensitive to TGF-β1 than when arranged as a monolayer [72]. This suggests that the threshold for pathologic behavior induced by relevant biochemical cues may vary depending on the environment. Similarly, fewer isolated porcine AVICs express αSMA in 3D collagen type I gels when compared with those in 2D tissue culture flasks [73]. It appears that in a 3D environment, fewer aVICs are present and qVICs are less sensitive to TGF-β1 and thus more difficult to activate. This suggests that the concentration of TGF-β1 used to induce pathological behavior should vary dependent on the dimensionality of the system to best match *in vivo* levels. Similarly, porcine AVICs in a 3D collagen hydrogel were not susceptible to osteogenic media mediated calcification until mechanical stress was added [43]. It appears that a 3D environment may require more dramatic treatment to induce the same behaviors as a 2D environment.

4.2 Dynamics

Many traditional CAVD *in vitro* studies have been investigated in a static environment, but the valves exist in a dynamic environment; this likely affects calcification mechanisms. Interestingly, calcific lesions occur preferentially on the aortic side of the valve in the fibrosa, which is the stiffer side [74–76]. As the aorta stiffens with age, axial stiffening and circumferential compliance increase [77]; this results in higher mechanical loads placed on the circumferentially-aligned collagen fibers, along which AVICs reside [78]. Also, an increase in transvalvular flow greater than 0.3m/s per year is a clinical predictive marker for patients who might benefit from surgery, suggesting that increased flow contributes to pathological progression [79]. NO release by AVECs is regulated by flow; under laminar shear stress, NO is released and helps maintain valvular homeostasis via signaling to AVICs. However, low and oscillating shear stress, as would occur on the aortic side of a diseased

valve, inhibits this release [80]. Also, while the AVICs themselves are not be directly exposed to fluid flow, it has been shown that flow alone can differentiate fibroblasts (the majority cell type of the AVIC population) into myofibroblasts [81]. This positive feedback of a stiffening valve that can no longer properly regulate its AVICs to maintain homeostasis is evidence of the importance of the dynamic environment on disease progression.

Several groups have begun probing CAVD progression in dynamic *in vitro* models. Fisher et al. showed that CN formation is strain dependent and that strain drastically reduced the time to nodule formation – 48 hours versus three days to three weeks [82]. At the tissue level, in a bioreactor under cyclic strain, porcine aortic valve cusps showed greater evidence of calcification under 15% (pathologic) strain than 10% strain (physiologic) [83]. In a related study of vascular calcification, 7% cyclic, equibiaxial strain yielded greater mineralization than unstrained calcifying vascular cells [84]. Strain alone was able to induce higher levels of myofibroblastic phenotype as measured by αSMA and collagen synthesis than untreated, unstrained cells, suggesting that strain exacerbates calcification via the dystrophic pathway [85]. In 3D culture of porcine AVICs, osteogenic media was unable to induce calcification, but the addition of mechanical stress via anchoring the gel led to significant calcification, as well as increases in αSMA, Runx2, and osteocalcin mRNA levels [43]. These studies demonstrate the critical role of the stress and strain placed on AVICs.

4.3 Biochemical Cues

Many cytokines are known to modulate AVIC behavior, including inducing disease progression *in vitro*. TGF-β1 is upregulated in diseased human valves, and when applied *in vitro*, exacerbates nodule formation [18]. TGF-β1 has been shown to activate myofibroblasts in valves leading to increased αSMA expression via Smads and p38 [86, 87]. As these myofibroblasts become more contractile, they likely activate latent TGF-β1 from the ECM [88]. This positive feedback loop provides a strong potential mechanism for dystrophic disease progression. Some experiments have shown that fibroblast growth factor 2 (FGF-2) treatment can block nodule formation and matrix contraction of AVICs, effectively counteracting TGF-β1 treatment [54]. In addition, antagonism of $5HT_{2B}$, a TGF-β1-dependent cardiopulmonary serotonin receptor, has been shown to prevent myofibroblast differentiation and CN formation in porcine AVICs [87]. Another recent strategy is to target cadherin-11, a protein believed to mediate cell-cell tension in CAVD and that has higher expression in calcified human valves; siRNA knockdown of cadherin-11 *in vitro* prevented TGF-β1-mediated CN formation [89].

Early aortic valve morphogenesis is regulated by many signaling factors that are also important in bone formation. Transcriptional factor Sox9 activity in AVICs promotes a chondrocytic phenotype (obVIC), but prevents progression to osteogenic mineralization; Msx2 inhibits Sox9 function [90]. $Sox9^{fl/+}$; Col2a1-cre mice developed calcific lesions in their heart valves, supporting the important role of Sox9 *in vivo* [91]. Msx2 has also been shown to promote calcification via the Wnt signaling pathway, involving Wnt3a, Wnt7a, and nuclear translocation of β-catenin [92]. C-type natriuretic peptide (CNP) promotes endochondral bone formation and has been found in ventricular side AVICs, supporting its role as a protective factor. It also has been shown to prevent the differentiation of porcine

AVICs to myofibroblasts or osteoblasts *in vitro* [93]. The combination of bone morphogenic protein 4 (BMP4), TGF-β1, and cyclic stretch can induce CN formation *ex vivo* in human leaflets and can be inhibited by noggin [83]. Wnt receptor low density lipoprotein receptor-related protein 5 (LRP5) and β-catenin, factors in canonical Wnt signaling, show increased expression in diseased human aortic valves [94]. Reactive oxygen species (ROS) signaling has also been shown to upregulate Runx2 via AKT and Msx2 [95, 96], whereas Notch suppresses Runx2 signaling and sustains Sox9 level in AVICs, thus inhibiting mineralization [11, 97, 98]. ROS have also induced *in vitro* calcification in vascular smooth muscle cells via BMP2 activity and the osteogenic pathway [99]. Many experimental models involve treatment of AVICs with osteogenic media, usually supplemented with β-glycerophosphate, dexamethasone, and ascorbic acid. It is understood that these factors work in concert to promote osteogenesis over time; dexamethasone activates and regulates Runx2 expression via Wnt/β-catenin signaling, ascorbic acid is required for collagen1 formation and subsequent ECM-mediated upregulation of Runx2, and β-glycerophosphate is a source of phosphate required to produce hydroxylapatite mineral and inorganic phosphate regulates BMP2 via the ERK pathway [100]. One confounding effect of β-glycerophosphate is that it can induce dystrophic calcification detectable by Alizarin Red and von Kossa, which leads to false positive results for osteogenic calcification [100]; this may explain the Alizarin Red positive nodules lacking mineralization observed by Cloyd et al. [17]. Also, β-glycerophosphate and high levels of inorganic phosphate have been shown to induce different calcification from *in vivo* mineralization [101], suggesting physiological levels of inorganic phosphate should be used to more accurately model *in vivo* processes.

Consideration of inflammatory pathways also provides insight since chronic inflammation often precedes calcification. Tumor necrosis factor-α (TNFα) has been shown to accelerate calcification, assayed via ALP activity, Alizarin Red, and von Kossa, in human AVICs via BMP2-Runx2 pathway [102]. Signals from the TNFα pathway also regulate Msx2-Wnt mediated calcification in LDLr−/− mice [103]. TNFα, interleukin 1-β (IL1-β), and IL6, have been shown to regulate the Notch signaling [104] and toll-like receptor 4 (TLR4) stimulation is enhanced by Notch1 in human AVICs via NF-κB [105]. Silencing TLR4 attenuates BMP2 expression, and stimulating TLR2 or TLR4 induces CN formation in human AVICs [106]. oxLDL increases Wnt3a, which drives osteogenic differentiation through LRP5 [107]. This links the early stage chronic inflammation to the osteogenic pathway of calcification. Receptor activator of nuclear factor kappa-B (NF-κB) ligand (RANKL), a surface bound molecule of the TNF family, and peroxisome proliferator-activated receptor gamma (PParγ) have also led to increased calcification *in vitro* [108, 109].

It has been thought that statins may provide a protective effect against calcification, although this is controversial; *in vitro* experiments showed promise, but randomized human trials did not demonstrate the benefit of statin therapy [5]. NO donors have also been shown to reduce nodule formation, likely via soluble guanylyl cyclase activation [110]. Increasing the expression and activity of endothelial NO synthase (eNOS) in hypercholesterolaemic leporine aortic valves led to decreased calcification [111]. Porcine AVECs were able to inhibit AVIC calcification via NO secretion inhibiting the differentiation to obVICs. Additionally, blocking NO led to increased calcification even in 3D AVEC-AVIC co-culture

[43]. *Ex vivo* culture of porcine aortic valve cusps in osteogenic media demonstrated significantly more CN formation on the fibrosa side than the ventricularis, which was exacerbated with NO inhibition. In healthy human valves, eNOS levels were much higher on the ventricularis than the fibrosa, further supporting the important protective effect of NO [43].

5. Evaluation of Assays

5.1 Calcium Assays

Evaluation of valve calcification can be separated into two categories: direct, in which the level of calcium or mineralization is directly measured, and indirect, in which markers of the proposed dystrophic and/or osteogenic pathways toward calcification are measured. Direct evaluation has the advantage of determining whether the assay leads to a pathological outcome functionally, whereas the indirect measurements yield more mechanistic information (Table 3).

Direct evaluation techniques include von Kossa staining [19, 83, 89, 96, 98, 99, 102, 108, 112, 113], Alizarin Red staining [17, 43, 55, 60, 70, 71, 82, 83, 87, 89, 93, 95, 96, 98, 102, 103, 106, 110, 114–117], energy-dispersive X-ray spectroscopy (EDS) [19, 118], Raman spectroscopy [17, 119, 120], scanning electron microscopy (SEM) [17, 60, 118], transmission electron microscopy (TEM) [17, 118, 121], atomic absorption [117, 122], arsenazo III [60, 83, 112], and o-cresolphthalein complexone [71, 108, 117] measurements. While these are all used as measures of calcification, not all are perfectly specific and thus are often used in concert. The gold standard for calcium detection is atomic absorption spectroscopy. Atomic absorption spectroscopy is based on the principle that different elements absorb different wavelengths of light and it works by atomizing the sample, sending light usually from a hollow cathode lamp of a specific wavelength through the vaporized sample, and measuring the amount absorbed [123]. Samples with increased mineralization content exhibit higher absorbance levels compared to controls.

Probably the most common measure of calcification, Alizarin Red, or 1,2-dihydroxyanthraquinone, stains hydroxyapatite mineralized matrix red-orange. Calcium, but also magnesium, manganese, barium, strontium, and iron, forms complexes with the dye in a chelation process, and results in a birefringent stain. Calcium is usually in much higher concentration than the other elements, allowing the inference that the areas stained have calcium present. Alizarin Red is often used to stain CNs to verify their mineralization and to help quantify the nodule assay, either by making the nodules easier to count or by extracting the dye for more rigorous quantification. Typical methods for quantifying the amount of dye involve staining of the cells or tissue, washing extensively, extracting via acetic acid or cetylpyridinium chloride, neutralization with ammonium hydroxide, and colorimetric detection at 405nm or 550nm. The acetic acid-ammonium hydroxide method is three times more sensitive than the cetylpyridinium method and results in better signal to noise ratio, especially for weakly stained samples [70, 124]. This method is also advantageous over Arsenazo III quantification because it has a higher and wider linear range of detection [124].

Von Kossa is another common stain for mineralization, especially in tissue sections. The stain works by reducing the calcium ions with light and replacing them with silver deposits that appear dark grey or black in tissue [125]. This method is not specific for calcium phosphates [126], though it has been suggested that the yellow precipitates are specific [127]. Von Kossa can be further confused if performed on a C57BL/6 mouse, which has melanocytes that appear black in the aortic valve. Thus, von Kossa is performed frequently in combination with Alizarin Red staining.

Calcium content can be measured more directly by various methods, but it is important to note that these methods all require lysing of the sample, meaning that calcium from mineralized areas or calcific lesions is not differentiated from intracellular calcium. Arsenazo III is a metallochromogen that complexes with calcium at pH 6.75 without interference from any other cations commonly present in serum or plasma, and is measured at 650nm [128]. When compared with the o-cresolphthalein complexone method, accuracy and calibration stability increased [129]. The o-cresolphthalein complexone method involves a reaction of Ca^{2+} ions with o-cresolphthalein complexone in an alkaline solution (8-Hydroxyquinoline at pH 10.6) and reading the sample absorbance at 660nm [130]. While these methods do not have a range of detection as large as Alizarin Red quantified via the acetic acid-ammonium hydroxide method, they are still useful for samples with low levels of calcium.

Other elemental methods include SEM, TEM, and Raman spectroscopy. SEM yields topographical and compositional information about the sample's surface with a resolution on the order of nanometers. It can be performed on fixed, dehydrated, and gold-/platinum-/or carbon-sputter-coated samples or in wet conditions via environmental SEM (ESEM) [131]. TEM yields information about the sample's chemical identity based on how it absorbs electrons and has a resolution on the order of picometers [132]. It can be performed on fixed, dehydrated, and stained samples. EDS analysis allows one to determine particular elements and their proportions in the sample. It functions on the principle that different elements will absorb different energy x-rays and the amount absorbed corresponds to the amount of element present [133]. EDS can be performed during SEM and ESEM; the advantage to using ESEM is that the samples do not have to be coated and high accelerating voltages can be used. EDS performed during ESEM is better because of the lack of interference from the coating and because the lack of sample preparation yields more authentic data. EDS coupled with ESEM yields quantitative data as well as qualitative [131, 133]. Raman spectroscopy is unique in that it can be performed on live cells. This allows calcification to be measured over time. Raman has also been shown to be an effective diagnostic for human heart valve calcification. Given the appropriate training data, an algorithm based on spectral shifts could predict whether the tissue was calcified with 100% sensitivity and specificity [119, 120].

5.2 Indirect Assays

In addition to quantifying mineralization, there are assays commonly employed to assess the progression of calcification by investigating mechanistic markers in the context of CAVD. For example, characterizing the phenotypic changes of AVICs toward myofibroblasts is

commonly accomplished via immunofluorescence staining, western blotting, or ELISA for αSMA, collagen gel contraction assays, and wound assays. While ELISA is the most quantitative method for detecting changes in αSMA, immunofluorescence provides information about the protein's localization and both immunofluorescence staining and western blots provide a high enough resolution to see changes in expression level. Collagen gel contraction assays indirectly quantify the myofibroblastic differentiation of AVICs based on the principle that higher levels of αSMA will result in higher contractility, measured by the change in size of the collagen gel after being seeded with cells. The wound assay involves disruption of a cell monolayer with a pipette tip and it measures the tension between cells via the wound area. The larger the wound, the more neighboring cells there are pulling on those that were disrupted [89].

Alternatively, the osteogenic process of calcification is often evaluated via ALP activity, RT-PCR, immunofluorescence staining, ELISA, and/or western blotting for Runx2 and osteocalcin. ALP activity is measured by how much p-nitrophenyl phosphate is dephosphorylated by ALP, which turns the solution yellow and can be quantified by absorbance at 405nm [134]. Runx2/CBFα-1 is a transcription factor associated with osteoblast differentiation and osteocalcin/BGLAP is a protein secreted only by osteoblasts. Runx2 is often used as an early stage marker of osteoblast activity, and osteocalcin and ALP are later stage indicators of osteoblast activity. MMPs have also been investigated via zymography, collagenase activity, immunofluorescence staining, and western blots to determine which were most important for pathological matrix remodeling [135].

Atomic force microscopy (AFM) has also been used to characterize the composition of calcified valves *ex vivo* in an effort to better understand the mechanism of formation. The ultra-fine structure of calcified regions of a human aortic valve was examined on a nanometer scale and found to contain 30–70nm diameter closely connected crystals. They suggest the mechanism of formation is deposit from supersaturated interstitial fluid and the crystals then grow on the organic substrate regulated by volume diffusion of interstitial fluid [136]. Recently, an AFM technique for evaluating the mechanical stiffness of valves has also been developed. This technique allows researchers to characterize mechanical properties of small animal models of CAVD, which can be extended to larger animal models and other diseases as well, while leaving enough tissue for concurrent histological studies [75]. Also, AFM comparison of human aortic valves with current valve replacement materials can yield insight into development of better prosthetics and a possible mechanism of the calcification that is common in prosthetics [137].

6. Conclusions

Utilizing a combination of CAVD models to investigate the factors important to its progression will likely yield information critical to the development of new therapeutic strategies. *In vitro* models are useful because they allow the quick and controlled manipulation of a large set of variables. While this simplifies the task of clarifying each factor's contribution to the disease, it presents the challenge of determining which other variables it interacts with and how oversimplification of the model may lead to results that are not relevant *in vivo*. Similarly, the proper evaluation of the chosen assays is necessary to

yield significant insight into CAVD mechanism. Combining evaluation methods also increases the significance of results. For example, Alizarin Red staining may yield interesting and quantitative results, but when paired with von Kossa its specificity increases. Raman spectroscopy should be used more frequently as it allows evaluation of live cells. Indirect evaluation of calcification can yield important mechanistic and functional data via western blot, immunofluorescence, PCR, and ELISA or ALP activity, collagen gel contraction, wound assay, and zymography, respectively. Figure 2 presents a summary of the important variables to consider when designing and evaluating a relevant *in vitro* model of aortic valve calcification. From choosing an appropriate origin of cells to the combination of evaluation techniques, any *in vitro* assay should recapitulate the conditions of the normal and disease state in an efficient and informative manner. Thoughtful choices should lead to novel and more promising targets to prevent and reduce CAVD.

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Highlights

- Heart valve calcification is an idiopathic disease
- Suitable animal models for valve calcification are lacking
- In vitro models may provide mechanistic insights for calcification in vivo
- This review highlights in vitro valve calcification models

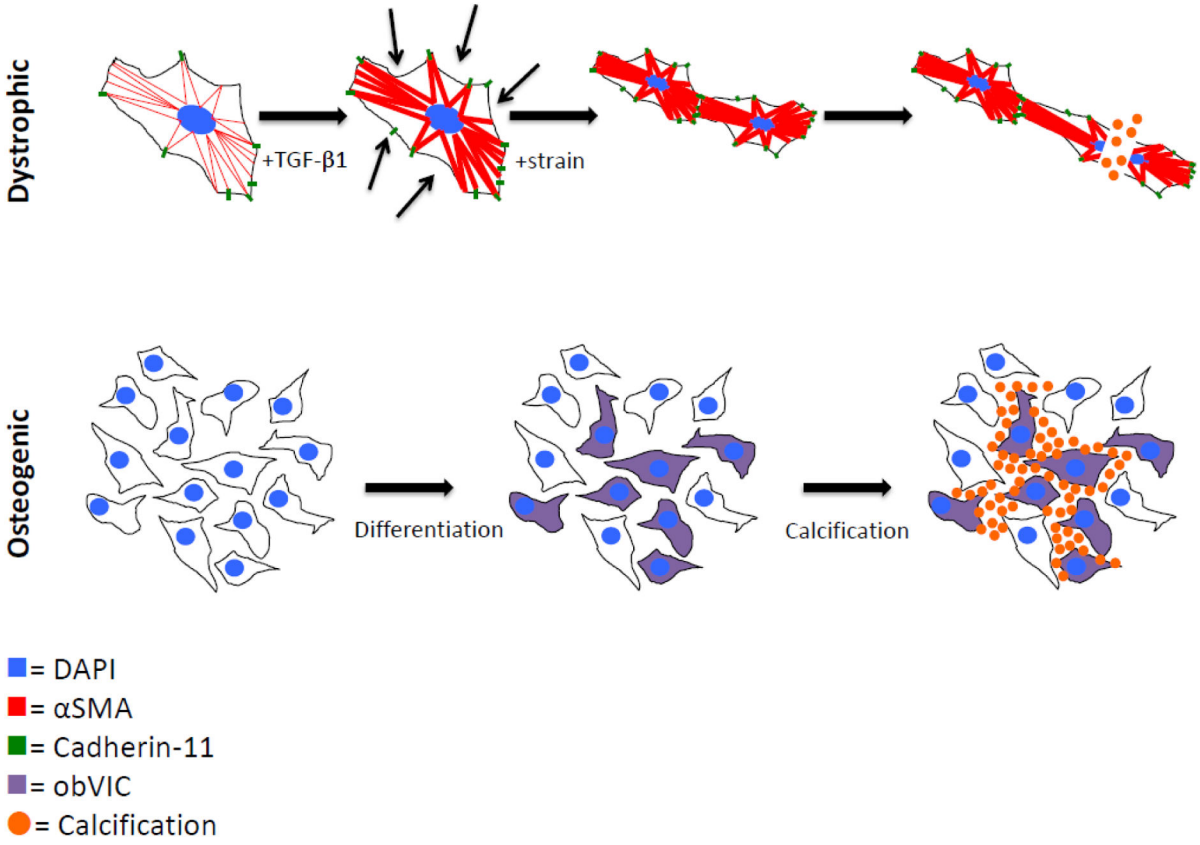


Figure 1.

Cartoons depicting proposed mechanisms of valve calcification. The dystrophic pathway is mediated by a TGF-β1 mediated increase in αSMA and cadherin-11, which increases the cells' contractility and strengthens their connections to each other. Under pathological strain, the increased and uneven tension tears cells apart, leading to calcification via apoptosis. The osteogenic pathway proceeds by osteogenic differentiation into obVICs, likely from qVICs. These obVICs actively form mineralized deposits.

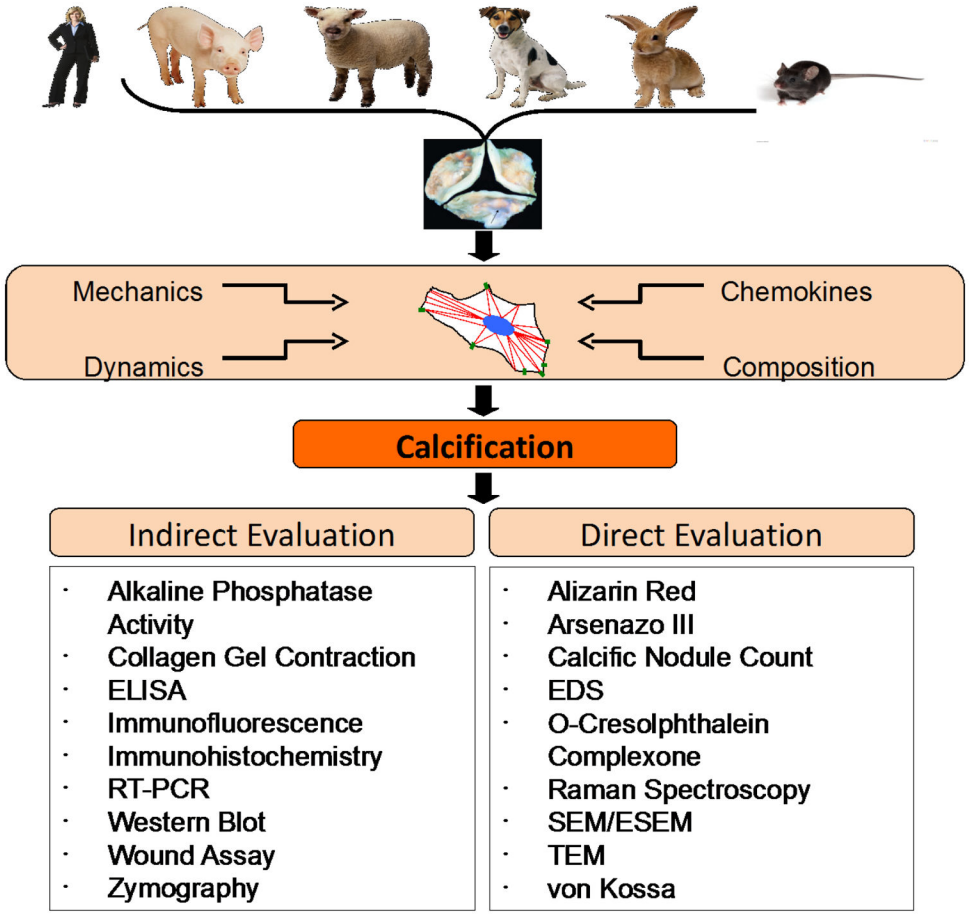


Figure 2.

Both the design and the evaluation of *in vitro* models of calcification must be considered. During design, it is important to consider the type and origin of the cell or cells and the composition, cues, and mechanical environment of these cells. During evaluation, a coupling of mechanistic and functional evaluation leads to powerful conclusions. Examples of calcification-related assays that fall under each category are listed.

Table 1

Acronyms and Abbreviations

αSMA	Alpha smooth muscle actin
AFM	Atomic force microscopy
ALP	Alkaline phosphatase
AVEC	Aortic valve endothelial cell
AVIC	Aortic VIC
aVIC	Activated VIC
BMP2	Bone morphogenic protein 2
BMP4	Bone morphogenic protein 4
β-catenin	Intracellular transducer of Wnt pathway
CAVD	Calcific aortic valve disease
CN	Calcific nodule
CNP	C-type natriuretic peptide
ECM	Extracellular matrix
EDS	Energy-dispersive X-ray spectroscopy
ELISA	Enzyme-linked immunosorbent assay
EMT	Endothelial to mesenchymal transformation
eNOS	Endothelial NO synthase
ESEM	Environmental SEM
FGF-2	Fibroblast growth factor 2
IHC	Immunohistochemistry
IL1-β	Interleukin 1-β
IL6	Interleukin 6
LRP5	Low density lipoprotein receptor-related protein 5
MMP	Matrix metalloproteinase
Msx2	Msh homeobox 2
NF-κB	Nuclear factor kappa-B
NO	Nitric oxide
Notch1	Notch homolog 1
obVIC	Osteoblastic VIC
Osteocalcin/BGLAP	Bone gamma-carboxyglutamic acid-containing protein
oxLDL	Oxidized low-density lipoprotein
PParγ	Peroxisome proliferator-activated receptor gamma
pVIC	Progenitor VIC
qVIC	Quiescent VIC
RANKL	Receptor activator of NF-κB ligand
RT-PCR	Reverse transcription polymerase chain reaction
ROS	Reactive oxygen species
Runx2/CBFα1	Runt-related transcription factor 2/core-binding factor subunit alpha-1

SEM	Scanning electron microscopy
Smad	Intracellular transducer of TGF-β pathway
Sox9	Transcription factor Sox9 of the SoxE family
TEM	Transmission electron microscopy
TGF-β1	Transforming growth factor β1
TIMP	Tissue inhibitor of metalloproteinase
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor alpha
VIC	Valve interstitial cell
Wnt3a	Signaling protein of the Wnt family
5HT2B	5-hydroxytryptamine receptor 2B

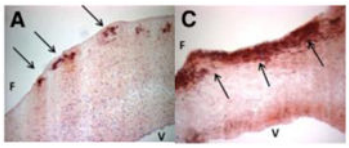
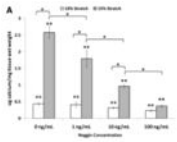
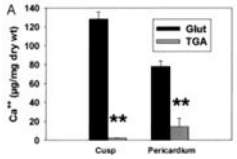
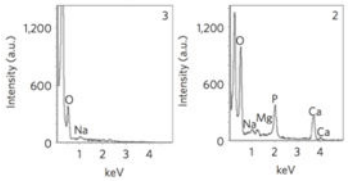
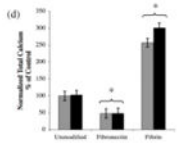
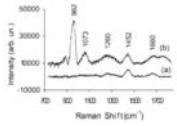
Table 2

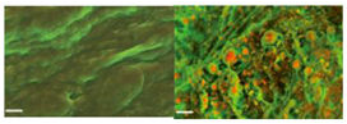
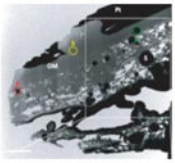
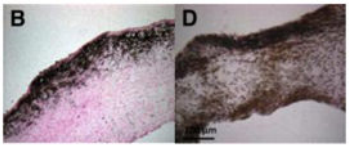
Examination of advantages and disadvantages associated with AVICs derived from common model organisms.

Organism	How are its AVICs useful?	Why are they imperfect?	Who has used these AVICs?
Human	Most appropriate	Difficult to obtain	[31, 38, 41, 95, 102, 105, 106, 108, 113, 121, 135, 138]
Porcine	Similar anatomy to human; easy to obtain; swine spontaneously develop calcification precursors	More homogenous than human	[16, 17, 22, 42–44, 48, 51–53, 69–71, 73, 82, 86, 87, 89, 93, 98, 110, 114, 115]
Ovine	CNs develop more quickly than human	More difficult to obtain than porcine	[18, 55, 117, 139, 140]
Canine	CNs develop more quickly than human; pathology naturally occurs	Difficult to obtain; require ageing	[38]
Leporine	Many osteogenic markers upregulated; easy to obtain	Require high cholesterol diets over time	

Table 3

Summary of direct techniques for evaluating calcification *in vitro* including advantages, limitations, and expected results in the normal and pathological states of human or porcine aortic valves (or rat, for atomic absorption spectroscopy).

Techniques for Evaluation of Calcification				
Technique	**Advantages**	**Limitations**	**Normal/Pathological Results**	**Notes on Images**
Alizarin Red	Easy to stain; relatively easy to quantify with large range; inexpensive	Other elements, like magnesium, iron, and manganese also stain red		Tissue sections from porcine aortic valves; F=fibrosa; V=ventricularis; Balachandran 2010.
Arsenazo III	No interference from cations commonly found in plasma; easy to quantify; more stable and accurate than o-cresolphthalein complexone	Cannot differentiate between intracellular and extracellular calcium		Porcine aortic valves; 10% strain is physiologic; 15% is pathologic; Balachandran 2010.
Atomic Absorption	Gold standard to determine sample composition	Requires vaporization of sample; expensive		Calcium in porcine cusp or bovine pericardium after glutaraldehyde or triglycidylamine crosslinking in transplant rat model; Connolly 2005.
Energy-Dispersive X-ray Spectroscopy	Easily quantifiable; can perform during SEM or ESEM; ESEM yields more authentic data (no coating interference)	Expensive		Human aortic valves; region with and without calcific lesions; Bertazzo 2013.
O-Cresolphthalein Complexone	Easily quantifiable	Not as stable and accurate as Arsenazo III		Porcine AVICs on various coated tissue culture polystyrene; withTGF-β1 is pathologic (black); Benton 2008.
Raman Spectroscopy	Can be performed on live cells; algorithms can use data to accurately diagnose valve calcification	Expensive		Human aortic valves; a is physiologic; b is pathologic; Otero 2004.

Techniques for Evaluation of Calcification				
Technique	**Advantages**	**Limitations**	**Normal/Pathological Results**	**Notes on Images**
Scanning Electron Microscopy	Topographical and compositional information; resolution ~nm; can be performed on hydrated samples (ESEM)	Difficult to quantify without EDS; expensive		Human aortic valves; scale bar is 3μm; green to orange represents increasing intensity; Bertazzo 2013.
Transmission Electron Microscopy	Chemical composition information; resolution ~pm	Expensive; difficult to perform on hydrated tissue		Human aortic valves; scale bar is 2μm; S=spherical particles; OM=organic matter; Pt=platinum; Bertazzo 2013.
von Kossa	Easy to stain; inexpensive	Melanocytes in valves of a black or brown mouse will appear as false positive stain; not specific for calcium phosphate		Tissue sections from porcine aortic valves; black is calcification; Balachandran 2010.