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Review Article

Molecular and cellular mechanisms of valve calcification

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ABSTRACT

Aortic valve stenosis is the most common form of acquired valvular disease, with a prevalence of 1% to 2% in people over the age of 65 years. Untreated, the presence of severe symptoms is associated with a life expectancy of less than 5 years. Relatively little is known about the role of the cells within the valve or the regulatory pathways that are involved in the onset and progression of the disease. The aim of this article is to review the role played by valve interstitial and endothelial cells and highlight the role of pathways and individual mediators that have been implicated in playing a role in the disease process. This includes mediators that regulate pro- and anti-calcification mechanisms. The clinical significance of calcium within the valve is discussed, as are the therapeutic opportunities that may allow for development of a medical therapy for aortic stenosis. Understanding the molecular and cellular mechanism of valve calcification will allow development of alternative therapies to surgical replacement of the valve and improve prognosis of patients with aortic stenosis.

Keywords: calcification, aortic valve stenosis

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INTRODUCTION

Unlike atherosclerosis and vascular calcification, relatively little is known about the role of the regulatory pathways that are involved in aortic stenosis. These diseases share a number of characteristics and risk factors, suggesting they share a common mechanism [1]. While both are inflammatory diseases associated with similar risk factors, particularly high lipids, the architecture of the lesions in each disease is strikingly different. The differences in the mechanical environment of the valves and the vessel wall suggest that the mechanotransduction pathways involved in the genesis and progression of the inflammatory response in each disease may differ. Untreated, the presence of severe symptoms is associated with a life expectancy of less than five years, with surgical replacement of the valve currently being the only viable treatment [2,3]. Even in patients who may be asymptomatic, but have severe stenosis, there is still a high rate and risk of rapid deterioration in the function of the valve (Fig. 1) [4]. Calcification also affects the currently used tissue valve substitutes and in the future may have an impact on the performance of the tissue engineered heart valves which are currently being developed [5,6]. The burden of calcific aortic valve disease is increasing worldwide and is expected to triple to over 850,000 cases per year over the next 50 years and will therefore have an increasing impact on healthcare budgets in the years ahead [7]. Thus, efforts to understand the pathways involved in the pathogenesis of the disease and the mediators that affect them will be important for the development of medical and alternative surgical strategies to treat aortic stenosis.

The risk of aortic valve calcification increases with age of the patient and is associated with the incidence of diabetes, hypertension, smoking, male gender, elevated lipoprotein(a) low-density lipoprotein levels, metabolic syndrome and renal dysfunction [8–10]. Previously, calcification of the aortic valve was believed to be a passive degenerative process involving the deposition of calcium into the valve leaflets. However, valve calcification is now recognised as an active process that involves the activation of pro-inflammatory pathways and mediators.

CHARACTERISTICS OF THE DISEASE

The disease is characterised by an increase in leaflet thickness, stiffening and progressive calcification of the aortic aspect of the leaflets (Fig. 2) [8]. Interactions between valve interstitial cells,

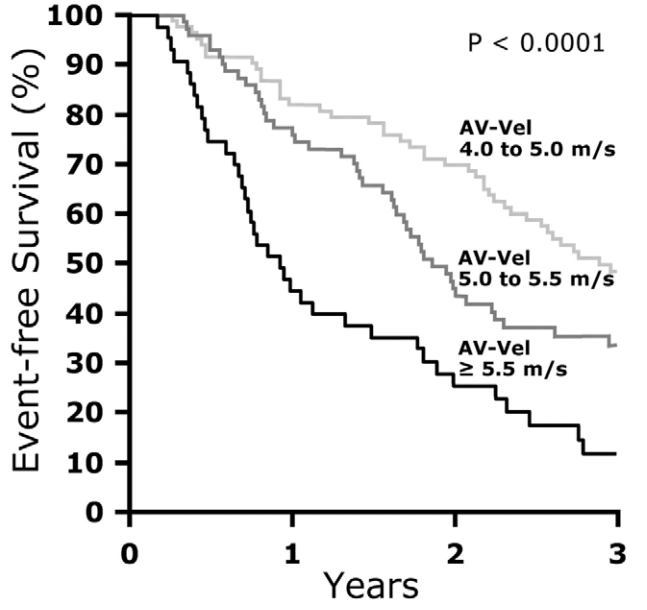

Figure. 1 Event free survival rate for patients with differing peak aortic jet velocity (AV-Vel) (from Rosenhek et al. reference [4].

endothelial cells, the extracellular matrix, mechanical stimuli and regulatory pathways involving inflammatory mediators, matrix remodelling enzymes, growth factors and angiogenic factors all play an important role in the calcification process. The effects of specific mediators in these pathways result in the valve interstitial cells adopting an osteoblast-like phenotype, the accumulation of calcium and the formation of calcified nodules [11–13]. Pathological changes other than those associated with the expression of bone cell markers are also evident in calcified valves. Down-regulation of the anti-angiogenic factor chondromodulin-I has been shown to occur in areas of diseased valves associated with increased expression of vascular endothelial growth factor-A, T-lymphocytes, mast cells, TNF- α, IL-6, neovascularisation and calcification (Table 1) [14].

The concept that valve calcification can be characterised as an inflammatory condition is supported by the expression of cell adhesion molecules by valve endothelium and the presence of inflammatory cells within the cusp [13,15]. Thermal heterogeneity studies in patients with aortic valve stenosis have demonstrated the presence of active inflammation in valves by correlation between the presence of cellular infiltrates and changes in temperature of the valve (Fig. 3) [16]. Early lesions are characterised by inflammatory infiltrates composed of non-foam cells and foam cell macrophages, and some T-cells [17]. While the presence of infiltrating inflammatory cells is evident in diseased valves, the events that trigger recruitment of inflammatory cells and the role played by cells that reside within the valve may also play an important role (Fig. 4). As the disease progresses, the spotty or finely distributed calcification spreads to form either radially orientated lesions or those that are associated with the regions of coaptation [18]. Eventually, the valve cells adopt an osteoblast

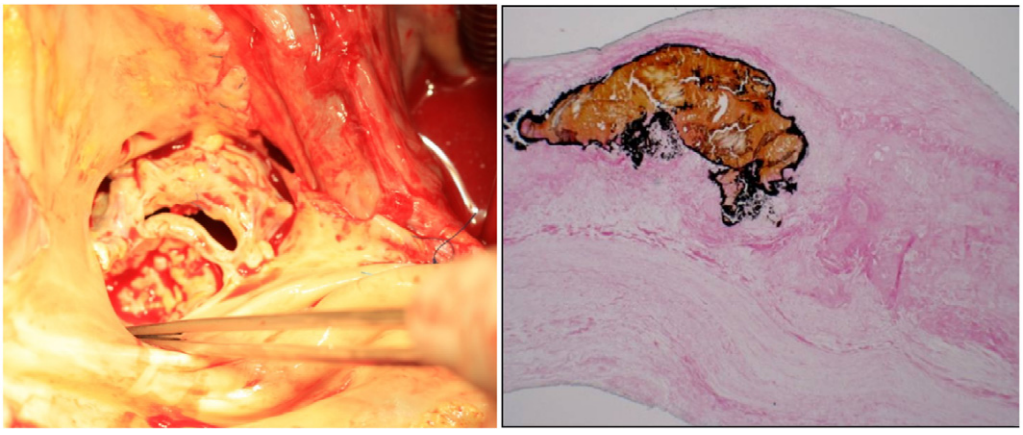

Figure. 2 A) Photograph showing the aortic surface of calcified bicuspid aortic valve aortic valves and (B) a photomicrograph showing the presence of a calcific lesion in the fibrosa of a valve cusp.

Table 1. Comparative expression of inflammatory cells and mediators in patients with aortic stenosis and aortic insufficiency (from Toutouzas et al. reference [16].

	AVS (n = 75)				AVI (n = 21)			
Grade	**0**	**1+**	**2+**	**3+**	**0**	**1+**	**2+**	**3+**
Histological features								
T lymphocytes	0%	2.6%	12%	85.4%	90.4% [a]	9.6%	0% [a]	0% [a]
Mast cells	0%	0%	14%	86%	81% [a]	9% [a]	0% [a]	0% [a]
Calcium deposits	0%	0%	3%	97%	76% [a]	23% [a]	0%	0% [a]
Immunohistological features								
CD3	0%	1.3%	10.6%	88%	80.9% [a]	19.0% [a]	0%	0% [a]
TNF-α	0%	2.7%	16%	81.3%	76.2% [a]	23.8% [a]	0% [a]	0% [a]
IL-6	0%	0%	17.3%	80%	80.9% [a]	19.0% [a]	0% [a]	0% [a]

CD3 = cluster of differentiation 3; IL = interleukin; TNF = tumor necrosis factor.
[a] $p < 0.05$ for comparisons between aortic valve stenosis (AVS) and aortic valve insufficiency (AVI).

phenotype, with increased expression of osteopontin, bone sialoprotein, osteocalcin and osteoblast-specific transcription factors [19,20].

Changes also occur in the extracellular matrix of the valve, characterised by a fibrotic thickening of the valve cusps [8]. Diseased valves express increased levels of angiotensin converting enzyme, angiotensin II, and the angiotensin type 1 receptor, which may mediate a profibrotic and proinflammatory state [21]. The repertoire of mediators that are potentially involved illustrates both the complexity of the disease process and the sophisticated function of the cells found in heart valves.

ROLE OF THE CELLULAR COMPONENTS OF THE AORTIC VALVE

As recently as 10–15 years ago heart valves were perceived to be inert flaps of tissue that opened and closed in response to the flow of blood through the heart. However, it is now accepted that interstitial cells within the heart and the endothelial cells that cover the surface of the leaflets make an important contribution to the function of the valve. By the same token, they are also targets for a range of mediators that are implicated to play a role in the genesis and progression of valve calcification.

Table 2. Heart valve interstitial cell phenotypes.

Cell Phenotype	Function
Embryonic Progenitor	Precursor for qVIC via activation steps. Derived from embryonic cardiac cushions
qVIC	Quiescent phenotype of VIC, maintains structure and function, prevent angiogenesis. SMC α-actin negative
pVIC	Precursor cell resident in the valve, can differentiate into obVIC phenotype
aVIC	Activated phenotype of qVIC, involved in proliferation, migration, remodelling. SMC α-actin positive
obVIC	Calcification, chondrogenesis and osteogenesis, secretion of bone markers and ECM. Derived from pVICs or aVICs

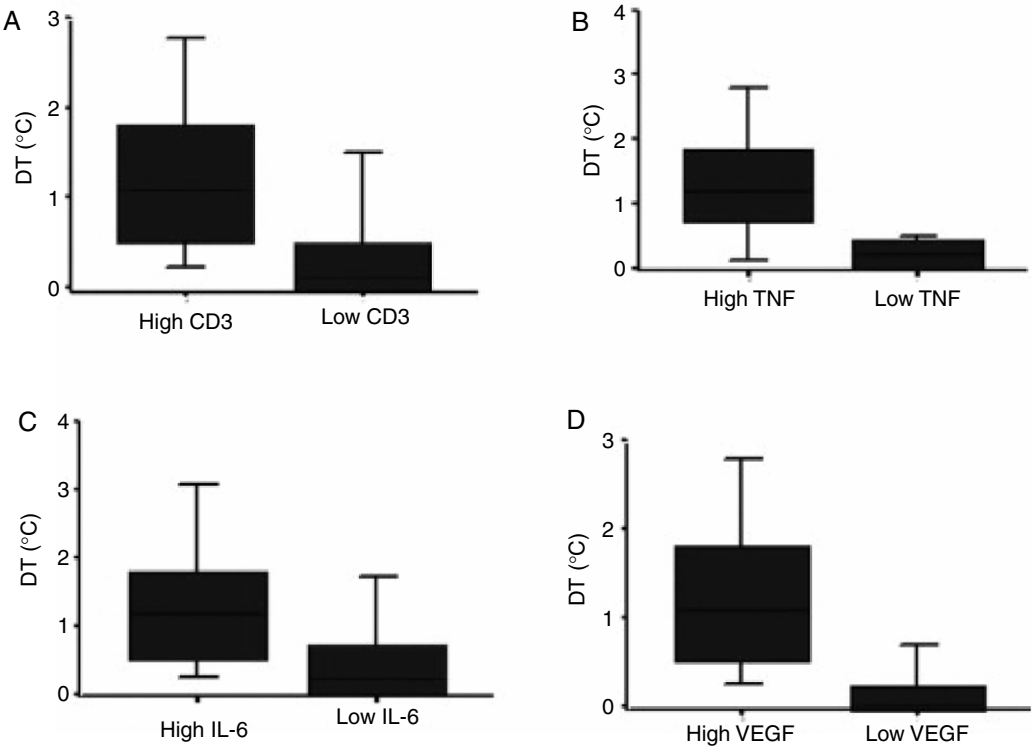

Figure. 3 Active inflammation in aortic valve leaflets measured by temperature difference in patients with aortic stenosis with intense compared to low expression of (A) CD3, (B) TNF- α, (C) IL-6, and (D) vascular endothelial growth factor (VEGF) (from Toutouzas et al. reference [16].

Valve interstitial cells

Valve interstitial cells are a heterogeneous and dynamic population of cells [22–28]. Synthetic, contractile, osteoblastic and stem cell phenotypes can be identified within the valve (Table 2). These cells synthesize extracellular matrix components, matrix remodelling enzymes, growth factors, cytokines and chemokines [29,30]. The presence of smooth muscle α-actin positive cells within the cusp has been demonstrated by immunohistochemical and molecular methods [27,31,32]. It is believed that the smooth muscle α-actin positive cells represent an 'activated' population of myofibrobalsts that differentiate into osteoblast-like cells. This has been challenged by recent observations using pure populations of different interstitial cell phenotypes (obtained by clonally expanding bovine valve cells). These studies suggest that the smooth muscle α-actin negative cells differentiate into cells responsible for bone formation in response to endotoxin and phosphate [33]. It has also been suggested that valves contain an osteoprogenitor subpopulation [34].

The role of valve interstitial cells in orchestrating inflammatory responses in valve cusps is not fully understood. It has been proposed that a 'response to injury' mechanism may orchestrate valve inflammation and calcification [35]. Durbin et al. suggested that valve endothelial cells and interstitial cells are potential participants in processes that attempt to repair the valve in response to injury or insult. However, phenotypic and functional characteristics of valve interstitial cells show them to be unlike vascular fibroblasts and smooth muscle cells, suggesting that the mechanisms of their 'response to injury' may not necessarily resemble that seen in the cells found elsewhere in the vascular system [26,28,31,36]. For some time it was believed the valve interstitial cells served only as structural cells that were responsible for maintaining the structural integrity of the valve cusp via the secretion of extra-cellular matrix proteins. It has been shown that these cells have unique characteristics, specific patterns of gene expression, an immunomodulatory role via the expression of specific cell surface molecules [26,28,31,32], and possess the capacity to secrete a variety of pro- and anti-inflammatory cytokines including IL-6, IL-10, IL-12 and TNF- α [37].

Valve endothelial cells

Endothelial cells function as a regulatory interface between the blood and the underlying tissue by being able to respond to their mechanical and humoral environment. The endothelial cells that cover the valve cusps are exposed to a unique haemodynamic and mechanical environment, with the ventricular surface experiencing high shear laminar flow while the aortic surface is exposed to a disrupted pattern of lower shear stress (Fig. 3). Valve endothelial cells have been shown to be

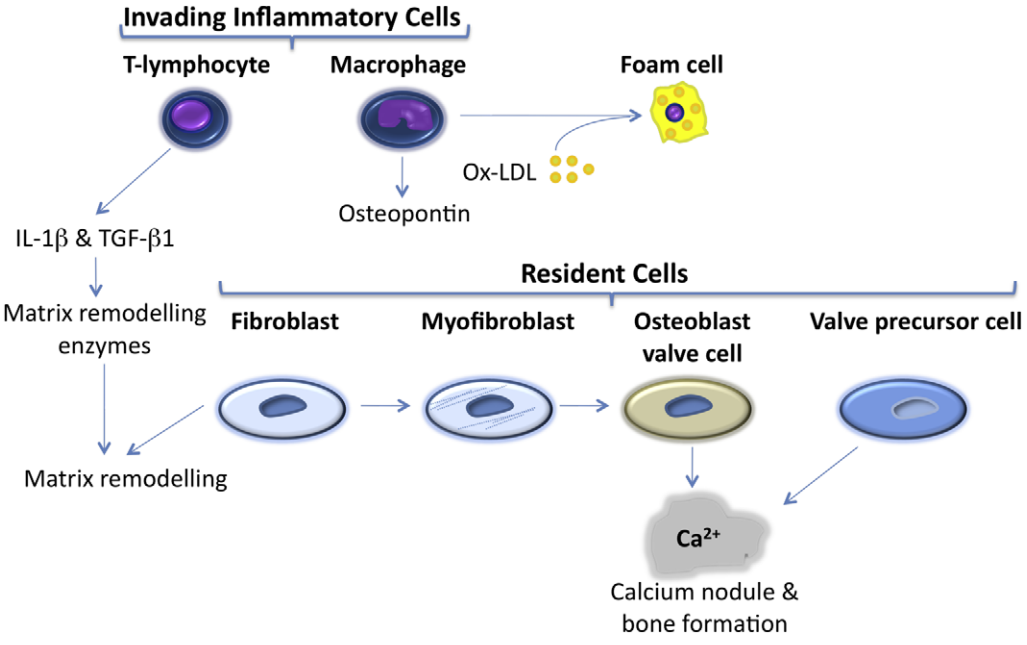

Figure. 4 Schematic representation of the cells that participate in the formation of calcified lesions in heart valves.

different from other endothelial cells in the vascular system [38,39]. Their function is regulated by an as yet undefined series of mechano-sensitive signal transduction pathways that regulate their response to various mechanical or inflammatory stimuli [40]. Indeed, gene expression of cells on either side of the valve has been shown to differ. Out of 584 genes, 285 genes had higher expression and 299 had lower expression on the aortic side of the valve relative to the ventricular side [39]. Analysis of these genes revealed that the aortic surface of the valve was associated with greater expression of genes implicated in valve calcification.

Studies on atherosclerosis, as well as a number of studies on valve calcification, have illustrated that the endothelium plays an important role in the disease process. While the significance of endothelial dysfunction in atherosclerosis is well accepted, its importance in aortic valve disease is beginning to emerge. A number of studies looking at different clinical cohorts suggest a strong relationship between systemic endothelial dysfunction and aortic valve disease [41].

The endothelium releases a variety of biologically active molecules, some of which have opposing effects on blood components or underlying cells [42]. In general, the valve endothelium is associated with a protective effect against valve calcification. The specific role of individual endothelium-derived mediators is discussed further on in this article.

INFLUENCE OF THE EXTRACELLULAR MATRIX

It has been shown that the stiffness of the extracellular matrix is able to influence the ability of valve cells to express osteoblast markers. As matrix stiffness increases, the ability of cells to form calcifying nodules increases. This effect is potentiated by TGF- $\beta 1$ via its ability to enhance smooth muscle α-actin expression [43]. The stiffness of valve tissue can be regulated by the valve endothelium, via contraction of interstitial cells [44]. Interstitial cell contraction can be mediated by TGF- $\beta 1$, a range of vasoactive agents and neuronal mechanisms, and can contribute to apoptosis-dependent calcification [43,45–47]. The calcifying response of valve interstitial cells can also be altered by changing the components of the extracellular matrix or the binding to synthetic peptides containing extracellular matrix derived sequences [48].

These studies illustrate the interplay between the valve interstitial cells, the endothelial cells and the extracellular matrix of the valve. Pathways that can influence any of these three components of the valve will in turn have the capacity to affect the function of the others. Understanding the modulators and mediators involved in the pathways that influence cell and matrix function is therefore a prerequisite to elucidation of the molecular and cellular events that lead to valve calcification.

REGULATORY PATHWAYS INVOLVED IN VALVE CALCIFICATION

A number of studies have focused on the response of valve interstitial cells to potential mediators that stimulate expression of osteoblast markers and/or a mineralization of the matrix (Fig. 5). Such molecules include BMPs, TGF- $\beta 1$, TGF- $\beta 3$, Wnts RANK/RANKL and ATP [12,49]. In contrast, attention has also been paid to the protective effects of the endothelium. The rationale for many of these studies comes from the changes in the expression of these mediators in clinical samples of calcified valve tissue or their involvement in bone synthesis. It should be noted that many of the experimental studies, particularly *in vivo* models of the disease in mice and rabbits, rely on the concomitant effects of hypercholesterolemia to induce pathological changes in the valves, illustrating the importance of this risk factor in the progression of the disease [13,50–54]. The focus for the mechanism of action for all these mediators appears to be on the regulation of RUNX2 expression. In bone, RUNX2 is considered the 'master regulator' of osteoblast formation by regulating the differentiation of pluripotent mesenchymal stem cells into immature osteoblasts, mature osteoblasts and, eventually, osteocytes. The formation of immature osteoblasts is also regulated by β-catenin and osterix [55,56]. Understanding the regulation of RUNX2 activation and degradation may therefore reveal targets that could be exploited by molecular and pharmacological interventions on render cells unresponsive to pro-calcific stimuli.

TGF- $\beta 1$

TGF $\beta 1$ is a member of the TGF β superfamily of proteins that also include other isoforms of TGF β (TGF $\beta 1$ and TGF $\beta 2$), BMPs, glial cell-derived neurotrophic factors, activins, inhibins, Anti-müllerian hormone, and Nodal. TGF $\beta 1$ is a secreted protein that binds to the TGF β-type II receptor and is required to associate with the type I receptor to initiate the Smad signalling cascade to mediate

effects on the control of cell growth, cell proliferation, cell differentiation and apoptosis [57]. It is believed that the accumulation of TGF β1 in the extracellular matrix of the valve tissue is due to ongoing endothelial injury leading to platelet and inflammatory cell infiltration. Previous studies have demonstrated the presence of TGF β1 in diseased cardiac valves [58]. TGF β1 may contribute to the calcification process by stimulating transdifferentiation of valve interstitial cells into osteoblast-like cells [12,59,60]. In addition, TGF β1 can also induce the expression of smooth muscle α-actin in valve interstitial cells and mediate the release of BMPs [61–63]. It is believed that these 'activated cells' or myofibroblasts play a key role in osteogenic differentiation [64]. TGF β1 may also contribute to aortic valve calcification through stimulation of apoptosis which leads to formation of calcium nodules [58,60]. These apoptotic bodies act as sources of calcium as well as trapping the calcium in the extracellular matrix. The effect of TGF β1 on apoptosis is enhanced when valve tissue is subjected to mechanical stretch [62]. In contrast, autophagic cell death, rather than apoptosis, has been suggested to cause the release of matrix vesicles in early degenerative aortic valves, thereby attracting inflammatory cells [65]. Alternatively, increased TGF β1 activity can result in calcification by stimulating the release of various extracellular matrix (ECM) proteins, including collagen I and III, essential elements in the formation of bone or cartilage and also found in sclerotic and calcified valves [66]. Finally, TGF β1 has been shown to directly affect endothelial function by disrupting adhered junctions and inducing endothelial barrier dysfunction [67]. This effect may represent an early event in endothelial to mesenchymal transformation and/or affect the permeability of the endothelial layer, thereby potentially facilitating transmigration of inflammatory cells from the blood.

Bonemorphogenic proteins

Bone formation in cardiac valves has been associated with the expression of bone morphogenetic (BMP) proteins 2 and 4 [11]. However, the precise role of BMPs in the calcification process is unknown. BMPs belong to a large family of structurally related proteins, known as the TGF β superfamily [68]. More than 20 members of this family have been identified and they can be sub-grouped according to their sequence homology. BMPs were originally named for their ability to induce ectopic bone formation but, like other members of the TGF β superfamily, they are proteins with effects not solely related to bone [69]. BMPs have been shown to be multifunctional cytokines with biological activity in a variety of cell types including monocytes, epithelial and neuronal cells. They are important in a variety of cellular processes including apoptosis, proliferation, differentiation, adhesion, chemotaxis and cellular survival. They play crucial roles in directing mesenchymal stem cell fate *in vitro*. They stimulate osteoblast differentiation and inhibit the differentiation of mesenchymal cells into the myoblast lineage [70].

BMPs also play an important role in cardiac development and are crucial for myocardium formation. During muscularisation of the atrioventricular cushions, the cardiomyocytes that protrude into the mesenchyme express BMP2, -4, -5 and -7 mRNA, whereas BMP6 mRNA is expressed in the cushion mesenchyme. During delamination of the valves, BMP4 and BMP6 mRNA are expressed at the ventricular side of the forming mitral valve, BMP4 mRNA at the ventricular side of the forming tricuspid valve and BMP2, BMP4 and BMP6 mRNA at the vascular side of the forming semilunar valves [71]. Consistent with its role in bone formation, marked BMP2 and BMP4 expression, as well as higher levels of TGF β1, have been demonstrated in stenotic valves [11,58,72]. It has been shown that BMP2 and BMP4 can induce a significant increase in the activity and expression of alkaline phosphatase and osteocalcin in isolated human valve interstitial cells [12].

Wnt proteins

Wnts are a family of secreted proteins that act through paracrine and autocrine mechanisms to regulate many aspects of cell fate and development. They form a family of highly conserved molecules that, when released, bind to receptors of the Frizzled and LRP families on the cell surface. Signal transduction leads to an increased level of β-catenin in the cytoplasm, which enters the nucleus and forms a complex with TCF to activate transcription of Wnt target genes [73]. Wnts are known to be involved in embryogenesis and have a specific role in the development of the endocardial cushions, from which the heart valves arise in the developing heart [74]. It is known that BMP2 activates the Wnt pathway, an alternative signalling cascade that is implicated in both bone formation and calcification [75]. BMP2 can stimulate the expression of MSX2 which in turn mediates the release of Wnt proteins by affecting the ratio of Wnts and Wnt antagonists Dickkopf-1 and -2 [76]. While in

some systems (the periodontal ligament cell line, PDL-L2) MSX2 has been shown to be a negative regulator of RUNX2 activity and mineralized nodule formation, it has recently been demonstrated that activation of MSX2 and subsequent Wnts signalling plays a role in vascular calcification [76,77]. Expression and/or localisation of Wnt 2, 2b, 3a 5b and 10, as well as Frizzled receptors 2, 3, 4, 5, 7, 8, 9, 10 and the co-receptor lipoprotein receptor-related protein (LRP) 5 has been reported in valve interstitial cells [78]. Studies have demonstrated that the Wnt signalling system is one of the most important local regulators of bone formation [79]. Binding of Wnt to frizzled receptors and LRP5/6 co-receptors activates several signalling pathways, including the canonical β-catenin pathway. This results in an inhibition of a phosphorylation cascade that stabilizes intracellular β-catenin levels and subsequently translocates β-catenin into the nucleus to form a transcriptionally active heterodimeric β-catenin / T-cell factor (TCF) / lymphoid enhancer factor DNA binding complex [80]. In addition, Wnt 3a can be localised in cells adjacent to areas of bone formation in human calcified valves. Recent evidence suggests a direct regulation of RUNX2 by canonical Wnt signalling, whereby RUNX2 acts as a target of β-catenin/TCF1 for the stimulation of bone formation in mesenchymal cells in mouse models (Fig. 6). It has been demonstrated that blockade of the Wnt inhibitor DDK-1 allows this mechanism to promote inhibition of osteoclasts [81]. In an animal model of aortic stenosis, rabbits fed a cholesterol diet induced complex bone formations in the calcified aortic valve with an increase in the LRP5 receptors, osteopontin, and p42/44 expression. *In vitro* analysis confirmed the LRP5/beta-catenin expression in myofibroblast cell proliferation [54].

RANK/RANKL

Calcified valves have differential expression of RANKL (ligand for receptor activator of NF- κB, RANK) and osteoprotegerin (OPG) that are both members of the cytokine system that regulate bone turnover. RANKL is expressed by a variety of cells including osteoblasts, fibroblasts, activated T-cells and bone marrow stromal cells. Via the stimulation of RANK, RANKL induces osteoclastogenesis and has been shown to increase the DNA binding of RUNX2 [52]. While it has been shown that the RANKL promoter can itself bind RUNX2, it has been demonstrated that this transcription factor does not regulate the expression of RANKL [21]. Importantly, in human valve cells grown in osteogenic media, RANK/RANKL was shown to induce expression of RUNX2. Interestingly, RANK and RANKL knockout mice and mice deficient in the p50 and p52 subunits of NF- κB all develop severe osteopetrosis [82–84]. The RANK/RANKL pathway is inhibited by expression of OPG that serves as a soluble decoy receptor of RANKL to limit activation of RANK. Deletion of the OPG gene results in severe calcification of the vasculature and expression of RANK/RANKL in calcified areas. It has been shown that TNF- α is capable of increasing the responsiveness of RANKL, via induction of RANK [85]. In addition, IL-1, IL-4, IL-6, TGF- β1, GM-CSF and IFN- γ have all been shown to affect the regulation of bone synthesis and reabsorption [86–89]. It is believed that RANK/RANKL plays a role in vascular calcification by increasing extracellular calcium concentration due to the stimulation of osteoclast differentiation and bone reabsorption. It has been reported that there is an inverse relationship between bone density and valve and vascular calcification in apoE -/- mice [50]. However, the signalling pathway by which RANKL can induce the expression of osteoblast cell marker is unclear. It has been shown in POS-1 cells, a model of osteolytic osteosarcoma, RANKL can induce the phosphorylation of ERK1/2; an event that mediates increased expression of BMP2 [90]. In addition, activation of RUNX2 can be blocked by the ERK inhibitor PD98059 in response to superoxide-mediated stimulation of osteoprogenitor cells. These data taken together suggest that ERK1/2 may mediate the RANKL mediated expression of osteoblast cell markers. This could occur via increased expression of BMP2 and/or direct activation of the osteoblast transcription factor RUNX2.

Nitric oxide

Nitric oxide (NO) is a simple gaseous molecule cleaved from endogenous L-arginine by the oxidation of the terminal guanidine group by an enzyme called nitric oxide synthase (NOS). There are principally 3 isoforms of NOS, a neuronal enzyme (NOS-1), an inducible isoform (NOS-2) and an endothelial isoform (NOS-3) [91]. NO is involved in the control of blood vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation and adhesion of inflammatory cells to the endothelium [42,92]. Patients with aortic valve stenosis were found to have increased circulating levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS [93]. Similar results were reproduced in animal models of aortic valve calcification [94]. Interestingly, endothelial

dysfunction in this patient population persists despite aortic valve replacement, suggesting that the dysfunction is part of a wider systemic process [95].

Several studies have shown that restoration of endothelial function mitigates aortic valve calcification. Using an *in vitro* model of porcine aortic valve interstitial cell calcification, Kennedy et al. showed that supplementation of cultures maintained in osteogenic medium (containing TGF β) with NO donors or agents that increase intracellular cGMP prevented an osteogenic response [59]. This suggests that NO mitigates calcification through a direct effect on aortic valve interstitial cells. In a study examining the role of statins on aortic valve calcification in a chronic high cholesterol diet rabbit model, treatment of the animals with atorvastatin significantly reduced the incidence of aortic valve calcification [54]. This was associated with an increase in NOS-3 protein concentration in aortic valves from statin-treated animals, as well as a significant increase in the levels of circulating nitrites, suggesting preserved or improved endothelial function in these animals. Exercise training was also shown to simultaneously preserve endothelial integrity and limit progression of aortic valve calcification in a low density lipoprotein receptor-deficient mouse model [96]. These recently published studies shed light on a once neglected but increasingly recognised role of the endothelium in preserving aortic valve structural and functional integrity. Importantly, NO has been shown to be able to regulate the stiffness of valve tissue and effect mediated by NO induced relaxation of valve interstitial cells [44]. Maintenance of the optimal stiffness of the valve therefore appears to involve the release of NO by the valve endothelium. This could play a key role in regulating the calcification process due to the association between matrix stiffness and calcification [43].

NO has recently been shown to directly interfere with the TGF β signalling pathway by inhibiting TGF β-induced phosphorylation of Smad2/3 in cultured vascular smooth muscle cells [97]. This direct effect of NO is cGMP-dependent and also results in inhibition of the differentiation of smooth muscle cells into osteoblasts. In a separate study comparing aortas from wild-type mice versus NOS-3 knockout mice, NO was shown to inhibit TGF β signalling by enhancing the proteosomal degradation of Smad2 in a cGMP/protein kinase 1 (PKG-1) dependent fashion [98]. In addition, aortas from NOS-3-deficient mice showed enhanced collagen type I expression (in addition to enhanced TGF β1 expression and nuclear translocation of Smads).

Oxidative stress

Oxidative stress is an important mediator of pathological processes both in the valve and the vasculature. The calcification of the aortic valve in hypercholesterolemic prone mice is associated with increased oxidative stress in the valve [51]. Several studies have identified the presence of reactive oxygen species, which include oxygen free radicals, oxygen ions and peroxides, in calcified aortic valves [77,94,99–101]. In addition, many of the clinical conditions that predispose individuals to the development of aortic valve calcification are associated with oxidative stress and endothelial dysfunction, such as hypertension, hypercholesterolemia, diabetes and smoking [102]. Oxidative stress is potentially mediated by several enzyme systems present in endothelial cells including the nicotinamide adenine dinucleotide phosphate oxidase-dependent (NADPH oxidase), uncoupling of endothelial nitric oxide synthase (NOS-3) (in which oxygen reduction is uncoupled from nitric oxide synthesis) and the xanthine oxidase system [103]. Although it remains unclear whether oxidative stress is both necessary and sufficient for aortic valve calcification, it is undoubtedly an aggravating factor in disease progression, causing reduced nitric oxide (NO) bioavailability, up-regulation of adhesion molecules, chemokines and apoptosis [104]. It has been shown in human stenotic valves that oxidative stress is increased in calcified regions. Uncoupling of NOS and a lack of anti-oxidant enzymes were both shown to contribute to the increase in oxidative stress [101]. Furthermore, *in vitro* studies show an increase in calcifying nodules in response to oxidative stress [59]. Studies examining the role of NO on oxidative stress have shown the ability of NO to reduce reactive oxygen species generation and related cell injury [105]. In a separate study examining porcine aortic valve interstitial cells grown in osteogenic medium supplemented with TGF β1, supplementation of NO resulted in decreased production of superoxide anions, possibly through superoxide scavenging, an effect which was independent of NADPH oxidase activity [59]. Thus, it appears that however small NO's contribution is to reducing oxidative injury, it is likely a contributing factor which requires further study, both experimentally and clinically.

Endothelin-1

Endothelin-1 (ET-1) is a 21 amino acid peptide that is released by endothelial cells. It can act on two distinct receptor sub-types (ET_A and ET_B receptors). It induces a strong and protracted contraction of vascular smooth muscle cells and is believed to be an endogenous functional antagonist of the dilator effects of NO. Increased numbers of ET-1-positive cells and ET_A receptor mRNA levels, as well as a downregulation of NOS-3 gene expression in human stenotic valves has been reported [106]. Aortic valves contract in response to ET-1, an effect mediated by both ET_A and ET_B receptors [107]. The contractile effect of ET-1 has been shown to increase the stiffness of valve cusps *in vitro* [44]. The opposing properties of ET-1 and NO suggest that a delicate balance exists between these two mediators that optimises the stress distribution in the cusps. Focal areas of endothelial dysfunction may be relevant to the initiation of calcification since it is known that lesions on the aortic surface of the valve are associated with regions of increased local stress [18].

Adenosine triphosphate (ATP)

Extracellular ATP is known to be involved in osteoblast formation. It is one of the most important extracellular regulatory molecules in the skeleton for bone remodelling [108]. Extracellular ATP is known to bind as a signalling molecule to the P_{2Y} -receptor on the cell membrane and cause activation of downstream signalling pathways that can mediate inflammatory responses [109]. However, adenosine, which is an ATP breakdown product, binds to a different type of purinergic receptor, the P_1 -receptor subtype and is known to have anti-inflammatory properties [110].

Treatment of valve interstitial cells with extracellular ATP and agonists of the P_{2Y} receptor has been shown to enhance the activity and expression of bone markers in human valve interstitial cell. In contrast, adenosine treatment reduced the activity and expression of alkaline phosphatise induced by osteogenic media [49]. These findings suggest a novel role for extracellular nucleotides in regulating the expression of osteoblast cell markers by valve cells which may represent a potential therapeutic target for valve calcification.

Genetic factors

Bicuspid aortic valve is the most common congenital cardiac malformation, affecting up to 1%–2% of live births. Interestingly, bicuspid aortic valves are associated with the early development of valve calcification [111]. Although some of that might be due to mechanical factors related to abnormal stress distribution and flow patterns on the cusps, genetic defects have also been implicated. Notch-1 mutations have been identified in two families with an increased incidence of bicuspid valves [112,113]. However, family members with normal valves were seen to develop early aortic valve calcification, suggesting a potential role of the Notch-1 signalling pathway in the regulation of osteogenesis. Indeed, the hairy-related family of transcriptional repressors, which are activated by Notch-1 signalling, repress RUNX2 transcriptional activity [113]. Thus, mutations in Notch-1, and therefore a loss in the repression of RUNX2, may contribute to the increased incidence of calcification in patients with bicuspid valves. In addition, decreased levels of NOS-3 might play an important pathophysiological role. Mice with reduced NOS-3 expression have been reported to be associated with an increased incidence of bicuspid aortic valves [114]. The decrease in NOS-3 in this patient population has been confirmed in humans by comparing the expression of NOS-3 in explanted bicuspid and tricuspid aortic valves [114].

Other mediators

There are a number of other potential mediators that could be involved in the onset and progression of valve calcification. Many of these have been studied in the setting of vascular calcification and include Matrix Gla Protein (an inhibitor of BMPs), TNF (which can induce MSX2-Wnt signalling and oxidant stress), fetuin-A (which prevents aggregation of calcium phosphate), vitamin D, glucose and insulin, leptin and adiponectin (adipose derived factors that promote and inhibit calcification) [115]. In addition, changes in the levels of natriuretic peptides have been discussed as biomarkers for aortic stenosis [116]. Whether any of the natriuretic peptides play a role in the pathogenesis of the disease is unknown. C-type natriuretic peptide (CNP), but not ANP or BNP expression, has been shown to be reduced in diseased human aortic valves. This was associated with reductions in its processing enzyme, furin, and its target receptors [117]. CNP is produced by vascular endothelial cells and further studies are warranted on its possible role in the pathogenesis of valve disease.

There are also a number of other endogenous mediators and pharmacological agents that have been implicated in structural and functional changes in the aortic valve; these include the use of the appetite suppressant Phentermine, drugs used to treat Parkinson's disease and 3,4-methylenedioxymethamphetamine (Ecstacy) [118–121]. The action of some of these drugs has been shown to be via stimulation of 5- HT_{2B} receptors, indicating a possible role for this receptor in the remodelling of the valve [122,123]. However, the changes seen following administration of these agents is not limited to the aortic valve and occurs without the development of calcific changes [124].

Taken together, there are a range of potential signalling pathways that may combine with risk factors and mechanical forces to induce the expression of RUNX2 (Fig. 7). Understanding the steps in this process will identify potential molecular and pharmacological targets for regulating the calcification process.

PROGNOSTIC SIGNIFICANCE OF VALVE CALCIUM

There are a number of methods used to quantify the calcium content of valves; these include Electron beam tomography, traditional x-radiography, dual-energy X-ray analysis, atomic absorption spectroscopy, which have all been shown to give a good correlation with the physical assessment of valve calcification [125]. The calcium content of valves, measured by 16-multislice computed

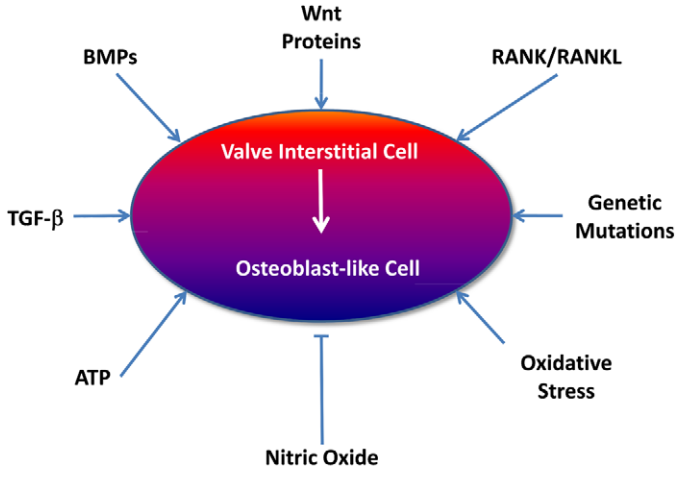

Figure. 5 Mediators implicated to play a role in valve calcification.

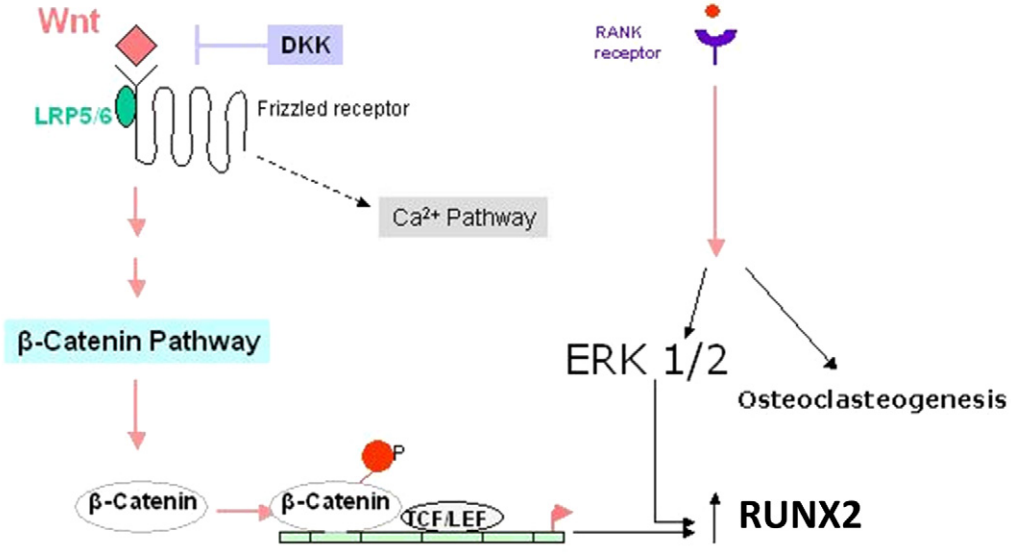

Figure. 6 Signalling pathways for Wnt proteins and RANK that lead to increased expression of RUNX2.

tomography, in patients with asymptomatic aortic stenosis is a predictor of an adverse short-term clinical outcome [126]. In addition, electron-beam computed tomography has been used to demonstrate a relationship between aortic valve calcium score and aortic valve area, suggesting its value in the diagnosis of severe aortic stenosis [127]. Correlation between valve calcium and aortic valve area has recently been shown to have 82% sensitivity, 80% specificity, 88% negative-predictive value and a 70% positive-predictive value [128]. In patients with a low ejection fraction (< / = 40%) aortic valve calcium was able to differentiate between patients with and without aortic stenosis in all but 3 of 49 patients. Since the only current option for treatment is surgical replacement of the valve, failure to treat the patient leads to a clear increase in mortality which is increased as the ejection fraction drops [129]. Thus, understanding the cellular and molecular mechanisms that mediate the calcification process will be fundamental to the development of strategies to prevent the reappearance of the disease in bioprosthetic, homograph or tissue engineered valve replacements, as well as being used to assess the efficacy of pharmacological tools that could be used for the medical treatment of patients with aortic stenosis.

CURRENT & FUTURE THERAPIES FOR AORTIC VALVE DISEASE

Surgical options

Currently, the only treatment for patients with symptomatic aortic valve disease remains replacement of the aortic valve. Traditionally, aortic valve replacement remains a surgical procedure, though in recent years, percutaneous methods for aortic valve implantation have also been developed. Irrespective of the method to substitute the native valve, it remains that native aortic valves require substitution, highlighting the fact that, to date, no treatments have been developed to alter the natural history of the disease or to slow the progression of aortic valve disease, which most often presents as aortic valve calcification. Thus, there is a desire to exploit the use of pharmacological agents to lessen or delay the need for surgical intervention.

Medical strategies

The limitations of the various options for heart valve replacement have focused interest in finding medical treatments for the disease. Considering recent advances into the understanding of aortic valve calcification as an active cell-mediated process involving inflammatory mechanisms and the fact that aortic valve stenosis shares many epidemiological risk factors with atherosclerosis such as hypertension, smoking and dyslipidemia, it is hoped identification of pharmacological targets will allow the development of medical strategies to prevent or inhibit the progression of the disease (Fig. 6). It has been shown in 'Reversa' mice that reducing plasma lipids with a genetic switch reduces pro-osteogenic signalling and valvular calcium deposition, however, this is not associated with an improvement in valve function [130]. The authors conclude that their findings demonstrate the

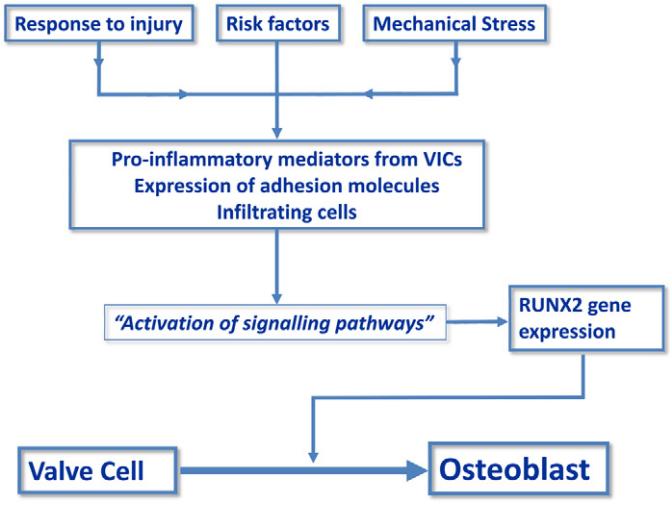

Figure. 7 Flow diagram showing the progression from initiation factors to differentiation of valve interstitial cells into osteobalst-like cells.

capacity to therapeutically modify the calcification process. Current clinical investigations are focused on the use of agents such as statins and ACE inhibitors, both of which have been shown to have efficacy in the treatment of cardiovascular disease.

Statins

Statins are known to exert a number of pleiotropic effects that occur independently of their ability to lower cholesterol. These include beneficial effects on endothelial function, stabilisation of atherosclerotic plaques, inhibition of smooth muscle cell proliferation and platelet aggregation, and a reduction in vascular inflammation. Evidence now suggests that these effects of statins are mediated by their ability to block the synthesis of isoprenoid intermediates. Inhibition of HMG-CoA reductase by statins prevents the synthesis of L-mavalonic acid and therefore other isoprenoid intermediates that are involved in the biosynthestic pathway for cholesterol. Isoprenoids act as important lipid attachments for the post-translational modification of a number of proteins that may affect their covalent attachments, subcellular localisation and intracellular trafficking. Inhibition of cholesterol synthesis specifically blocks the formation of geranylgeranyl pyrophosphate (GGPP), an isoprenoid intermediate that is associated with the isoprenylation of the small GTP-binding protein Rho. It has been demonstrated that inhibition of this pathway, as a consequence of blocking HMG-CoA reductase, is responsible for the up-regulation of NOS-3, the inhibition of smooth muscle cell proliferation and the enhancement of Ecto–5'-nucleotidase, an enzyme involved in ATP metabolism to adenosine.

There have been a number of observations relating to the potential use of statins in the treatment of patients with aortic valve calcification. Treatment with atorvastatin prevented increases in macrophages, proliferation cell nuclear antigen, osteopontin and gene markers of osteoblasts (alkaline phosphatase, osteopontin and RUNX2) in the aortic valves of cholesterol fed rabbits [131]. Clinical observations have shown that aortic valve calcium is reduced by over 60% in patients with valve disease who are receiving statins, of which just under 50% of the patients showed less progression of calcium accumulation in their valves [132]. In addition, the rate of degeneration of bioprosthetic valves has also been shown to be reduced in patients receiving statins, despite higher levels of cholesterol in the statin group. A similar ability of statins has also been demonstrated in the inhibition of calcification of vascular smooth muscle cells.

These studies support clinical observations where rosuvastatin has been shown to reduce the progression of aortic valve disease and improve inflammatory markers in patients with asymptomatic aortic stenosis. However, the SALITRE and SEAS trials reported a lack of effect of statin treatment on the rate of progression of aortic stenosis in patients with the disease [133,134]. The lack of effect of statins in the SALITRE and SEAS trials suggests that if any benefit is to be obtained with statins early initiation of treatment may be required to reveal a clinical benefit.

Renin-angiotensin system

Components of the renin-angiotensin system have been identified in diseased valves. ACE was seen to be co-localised with apolipoprotein B, while angiotensin II was present together with ACE within valve lesions [21]. These observations lead to the suggestion that the use of ACE inhibitors or angiotensin receptor blockers may be of benefit in the treatment of the disease; this is supported by the fact that angiotensin II is known to be involved in inflammation and oxidative stress. Retrospective studies have suggested that ACE inhibitors are able to slow the accumulation of calcium within the lesions, but not the progression of aortic stenosis [135]. Laboratory and animal studies are required to further elucidate the role of angiotensin II in the calcification process in order to obtain a clearer picture of the potential benefits and optimal time to pharmacologically target the renin-angiotensin system.

Other potential pharmacological strategies

The complex nature of aortic calcification and the array of potential mediators offer the possibility to identify new pharmacological targets. These may include agents that are able to augment protective mechanisms, anti-proliferative drugs as well as targeting the signalling pathways of mediators of bone formation [136]. Agents that block RANKL, alkaline phosphatase and the binding of phosphate have been advocated in the setting of medial calcification [115].

Nitrogen containing bisphophates share some properties with statins due to their ability to inhibit farnesyl pyrophosphate, an enzyme involved in cholesterol synthesis. In addition, nitrogen containing

bisphophates are also able to inhibit the deposition of calcium, potentially enhancing their statin-like effects [137].

Targeting receptor systems involved in bone metabolism may also be of some benefit. In bone, there is a balance between resorption and formation to maintain skeletal homeostasis. Recent evidence indicates that the sympathetic nervous system plays a crucial role in regulating bone deposition and resorption through β2-adrenoceptors (β2-ARs) [138]. Diseased aortic valve express both β1 and β2-ARs and it has been shown that stimulation of the β2-AR in cultured human valve interstitial cells can inhibit the expression of bone markers in response to stimulation with an osteogenic medium. Interestingly, effects seen with the β1-AR agonists appear to be indicative of a pro-osteoblastic response [139]. Since these receptors will be targets for sympathetic nerves found in valves, this offers the intriguing possibility that valve calcification may be determined, in part, by a balance between the effects of different subtypes of b-ARs and their activation by neuronal mechanisms [47,140].

CONCLUSIONS

Pathways that mediate calcification in the aortic valve are now being widely investigated. These studies have relevance to the disease process in native valves as well as any potential changes that may occur in bioprosthetic. Development of efficacious strategies that will regulate the calcification process will be of benefit to those patients who develop aortic stenosis. Controlling the calcific response of cells that will be used to make tissue engineered valves will have a direct impact on therapeutic potential for the next generation of heart valve substitutes.

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