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Cardiac valve cells and their microenvironment—insights from *in vitro* studies

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Abstract

During every heartbeat, cardiac valves open and close coordinately to control unidirectional flow of blood. In this dynamically challenging environment, resident valve cells actively maintain homeostasis, but the signalling between cells and their microenvironment is complex. When this homeostasis is disrupted and valve opening obstructed, haemodynamic profiles can be altered and lead to impaired cardiac function. Currently, late stages of cardiac valve diseases are treated surgically, as there are no known drug therapies to reverse or halt disease progression. Consequently investigators have sought to understand the molecular and cellular mechanisms of valvular diseases using *in vitro* cell culture systems and biomaterials scaffolds that can mimic extracellular microenvironment. In this Review we describe how signals in the extra cellular matrix regulate valve cell function. We propose that the cellular context is a critical factor when studying the molecular basis of valvular diseases *in vitro*, and one should consider how the surrounding matrix might influence cell signalling and functional outcomes in the valve. Investigators need to build a systems level understanding of the complex signalling network involved in valve regulation, to facilitate drug target identification and promote *in situ* or *ex vivo* heart valve regeneration.

Introduction

As the heart evolved from a single to multiple chamber structure, cardiac valves arose to control unidirectional flow of blood during cardiac cycles. For example, aortic valves open in response to higher blood pressure in the left ventricle compared with the aorta, and close when the pressure equilibrates. These valves function in a similar manner to valves in water dams or car engines. However, cardiac valves are living tissue with the ability to repair and remodel in response to damage. During an average human life span, heart valves open and

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close approximately 3 billion times,¹ withstanding various mechanical stresses, including fluid shear stresses and bending stretch.^{2,3}

The material composition and structure of cardiac valves confer their robustness and durability. In humans, cardiac valves are made of thin (~500 µm) pliable cusps, and only mitral and tricuspid valves have supporting chordae tendineae and papillary muscles.⁴ A closer examination of the tissue architecture of an aortic valve reveals three distinct layers of extracellular matrix (ECM), rich in collagens, proteoglycans, or elastin (Figure 1a).⁴ These ECM proteins impart unique macroscopic mechanical properties to valves, enabling them to withstand tension when closed and flexure when open. For example, the elastin fibers on the flow side of the valves (known as ventricularis) are radially aligned and elastic, which extend when the valves open and recoil when valves close.⁵ Proteoglycans in the middle layer, or spongiosa, function as a cushion for absorbing tension and friction between the top and the bottom layers.⁴ Finally, the fibrosa layer contains circumferentially oriented collagen fibers, which confer stiffness and strength to the valves.⁴

Cardiac valves are composed of valvular endothelial cells (VECs) that line the surfaces of the leaflets, and valvular interstitial cells (VICs) distributed throughout the leaflets (Figure 1b). Both VECs and VICs maintain tissue homeostasis for the day-today function of cardiac valves, as they secrete biochemical signals, matrix proteins, and matrix remodeling enzymes (Figure 1c). In response to injury or disease, these resident cells often activate in an attempt to repair the valve (Figure 1c). For example, VECs can undergo an endothelial to mesenchymal transition (EMT) to acquire a fibroblast or myofibroblast phenotype that leads to changes in the microenvironmental signals and facilitates tissue regeneration.⁶ However, under sustained injury (such as aging and valve calcification), persistently activated valvular cells can participate in disease progression through inappropriate remodeling of their surrounding ECM.⁷ For example, aortic VICs can deposit fibrotic collagen and calcified matrix.⁷ These matrix components alter the pliable structure of cardiac valves, leading to a decrease in effective valve opening (known as stenosis), increased blood flow speed, and increased differential pressure across the valves.⁷ In other cases (such as mitral valve diseases), VICs degrade the collagen content of the valves, which can lead to mitral valve prolapse⁸ and regurgitation.⁹

Semilunar valves and atrioventricular valves originate from different heart fields and lineages of cells during embryonic development.^{4,10,11} Their tissue organization and haemodynamic mechanics might also be related to their differential propensity to develop diseases later in the life. For example, aortic and mitral valves are more prone to diseases than the other types of valves.¹² Two main forms of calcific aortic valve diseases (CAVD) exist:⁷ aortic valve sclerosis, which involves tissue stiffening, fibrosis and early calcification;^{13,14} and calcific aortic stenosis (CAS), which involves extensive calcification and reduced valve opening. Aortic valve sclerosis is estimated to be present in ~ 29% of adults >65 years of age, whereas CAS is present in ~2% of the same age group.^{7,15} The hearts of patients with symptomatic CAS can undergo pathogenic remodelling towards heart failure unless the valves are replaced with either tissue or mechanical valves.¹⁶ To date, no drug-based therapy is available for treating CAS, other than managing risk factors, such as high blood pressure or hypercholesterolaemia.¹⁷ Another major valvular disease is mitral

valve prolapse, which is characterized by a bulging of the mitral valve cusp(s) into the left atrium and can lead to mitral regurgitation during ventricular systole.¹⁸ This disease is often treated by surgical repair.¹⁸ Each year, ~98,000 hospital procedures are performed for patients with cardiac valve diseases in the USA alone, which costs billions of US dollars.¹² These numbers highlight the prevalence of cardiac valve disease and the need for a clear understanding of the disease progression to enable early intervention or drug treatments for the primary pathogenesis.

Cell culture studies have advanced our understanding of valvular diseases. The field has certainly gained considerable insight into the characteristics and associated factors of CAVD using animal models^{4,19-21} and diseased human valves.^{22,23} However, how the detailed mechanisms regulate cellular phenotypes in these complex models has been hard to determine. *In vitro* cell culture models can complement animal studies when studying intracellular signaling and enable high throughput drug screening. However, a major caveat when interpreting these results is that the cells are generally cultured on traditional plastic plates, which are stiffer than an endogenous matrix, the cells are in a polarized environment (that is, the bottom of the cells is adhered to the plastic plates, but the top is free in solution, and biochemical signals can only be introduced as media-soluble factors. To address these shortcomings, new biomaterials have allowed the synthesis of matrix environments that more closely mimic native ECM and enable monitoring of cellular responses in a more physiologically relevant setting.²⁴

In this Review we describe how the cellular phenotypes and microenvironmental signalling in valvular diseases are regulated, and highlight recent developments and applications of biomaterial-based culture systems in facilitating this process. Other investigators have nicely summarized that as many as five different phenotypes of VICs might exist.²⁵ In this Review we focus on the two main pathogenic phenotypes, myofibroblast and osteoblast-like, which are strongly associated with progression of CAVD. Differentiation of valvular cells into these pathogenic cellular phenotypes is regulated by their microenvironment, which include diffusible biochemical signals, physical matrix cues, and cell–cell communication (Figure 2).

Cellular phenotypes in CAVD

CAVD is characterized by a progressive thickening, fibrosis, formation of calcified matrix, and new vascularization in the valve leaflets.^{7,14,19,26} Mineralized spherical particles (0.1–5 µm), with nanostructures similar to those found in other cardiovascular calcification lesions (such as plaque), but distinct from bone mineral, are seen in human valves with different levels of calcification (Figure 2).²⁷ The cause of valve calcification and stenosis is multifactorial; inflammation, oxidative stress, lipid overload, endothelial damage, apoptosis and transdifferentiation of resident cells can all contribute to disease initiation and progression.²⁸ Owing to the association of lipid overload and CAVD, several clinical trials based on statin-derived lipid-lowering drugs have been carried out. However, in these trials lowering lipid levels had limited benefit for preventing CAVD progression.^{17,29} These studies indicate that managing multiple initiating factors of the disease in the correct temporal manner and targeting downstream molecular and cellular pathology mechanisms,

might be necessary for treating CAVD. Valvular cells, VICs, and VECs adopt pathogenic phenotypes in response to the initiating factors of CAVD (Figure 2b) and lead to the structural and mechanical deterioration of the tissue. Understanding how these cells function is, therefore, critical for comprehending the molecular and cellular pathology of CAVD before calcification and stenosis develop.

The appearance of myofibroblasts are a primary pathogenic cellular phenotype and an indicator of early CAVD.⁸ Myofibroblasts mostly originate from VICs, VECs, resident or circulating progenitor cells, in response to microenvironmental cues (such as increased matrix stiffness or transforming growth factor- β 1) in the diseased valves (Figure 2b).³⁰⁻³⁴ Myofibroblasts proliferate, migrate to wound sites, express contractile stress fibers (Figure 2c) to reduce wound size, and produce ECM scaffold proteins and enzymes that remodel the matrix around them.³⁵⁻³⁷ Across human organs, such as skin,³⁸ liver,³⁹ lung,⁴⁰ kidney,⁴¹ and heart,⁴² activation of resident cells into myofibroblasts is a critical aspect of tissue repair and fibrosis. While myofibroblasts are beneficial during the initial phase of tissue healing, persistence of myofibroblasts can lead to fibrosis and matrix protein disorganization.^{43,44} Temporal control of the myofibroblast phenotype is, therefore, likely to be a key aspect of regulating CAVD progression (Figure 2b). In general, as a wound heals, activated myofibroblasts might undergo apoptosis when the mechanical stress of the wound is relieved (Figure 2b).^{45,46} Myofibroblasts can also become senescent and be killed by natural killer cells in mice with liver fibrosis (Figure 2b).⁴⁷ In addition, myofibroblasts can deactivate to a quiescent fibroblast state in cultured cardiac VICs or mice with liver fibrosis (Figure 2b).^{48,49}

Another pathogenic phenotype detected in calcified valves is the osteoblast-like phenotype (Figure 2b).²² Osteoblasts deposit bone tissue. Whether VICs can truly become osteoblasts is still debated;^{50,51} however, VICs have been observed to acquire many osteoblast-like properties when presented with biochemical and biophysical cues that are known to induce bone formation.⁵²⁻⁵⁵ For example,^{54,55} human VICs can be induced by BMP2 or cyclic stretch to express osteoblast gene markers (such as runt-related transcription factor 2 [Runx2] and transcription factor Sp7 [commonly known as Osterix]), and alkaline phosphatase activity.^{52,53} Functionally, in ~13% of severely calcified valves, heterotopic ossification has been observed using histological stains of the tissue.⁵⁶ While many investigators observe that VICs can undergo *in vitro* apoptosis-mediated dystrophic calcification, or activate osteogenesis when cultured in osteogenic media,⁵⁷⁻⁵⁹ the matrix that the cells produce on a petri dish might not be identical to bone matrix *in vivo*, indicating that calcification in CAVD might be distinct from that in bone formation.⁵¹ Possibly only a small population of cells can become osteoblasts and contribute to valve calcification *in vivo*. Like myofibroblasts, these osteoblast-like cells might derive from VICs, VECs, or other types of cells (such as circulating progenitor cells) in response to local matrix changes (such as increased matrix stiffness and TGF- β 1).^{6,33,34,58,60} Whether the same or distinct cells differentiate into myofibroblasts or osteoblast-like cells in valvular disease is still unclear (Figure 2b).⁶¹

Soluble molecular signals

Diffusible biochemical cues are acute and potent signals in directing biological outputs of valvular cells and are among the early initiating factors for cardiac valve diseases, including CAVD. Many biochemical signals have been identified by examining diseased human valves. In early sclerotic lesions of aortic valves, production of inflammatory cytokines, lipoproteins, and endothelial paracrine factors is induced.¹⁴ In advanced lesions and calcified valves, a number of growth factors or their receptors, including transforming growth factor β -1 (TGF- β -1),⁵⁹ bone morphogenetic protein 2 (BMP2),⁵⁶ vascular endothelial growth factor (VEGF),⁶² and low-density lipoprotein receptor-related protein 5,⁶³ are up-regulated. These signaling molecules secreted by valvular cells can elicit changes in valve tissue composition and mechanics by acting on the resident VICs and VECs. For example, VECs can secrete paracrine molecules and undergo EMT to become a mesenchymal type cell that mediates tissue repair.^{6,64,65} The EMT triggered in VECs by pathogenic biochemical factors has common molecular features with cardiac valve development (such as expression of mesenchymal markers and activation of NF- κ B);^{64,66} however, further characterization is needed to identify the precise mechanism by which this occurs. In addition, VICs secrete fibrous matrix proteins, matrix metalloproteinases (such as MMP1 and MMP2), biochemical factors, and can differentiate into myofibroblasts or osteoblast-like cells or undergo cell death-mediated calcification.^{7,25,67}

Proinflammatory cytokines

When the homeostasis of valve tissue is disturbed, immune cells (such as macrophages and T cells) infiltrate the damaged area, and secrete various pro-inflammatory cytokines, (for example TNF and IL-1 β) and MMPs.¹⁴ When human aortic VICs were treated with TNF, it promoted phenotypes associate with calcification;⁶⁸ however, IL-1 β increased the secretion of MMPs in these cells.⁶⁷ The inflammatory cytokines TNF and IL-6 can both enhance EMT of adult porcine aortic VECs (PAVECs), as measured by their invasion into a 3D collagen gel and gene expression.⁶⁴ Interestingly, all these cytokines promoted nuclear translocation and activation of NF- κ B.^{64,69} As a common signalling target of these proinflammatory factors, NF- κ B might be a useful drug target to ameliorate the detrimental effects of these cytokines towards promoting diseased phenotypes of VICs or VECs. Targeting the NF- κ B pathway has been exploited in treating other fibrosis-associated diseases in the liver and the lung^{70,71} and presents an attractive option for treating valve sclerosis. Furthermore, immune cells have been used to deliver cancer drugs,⁷² one could, therefore, also take advantage of the localization and secretion of biochemical factors by these immune cells to deliver signals to the site of cardiac valve diseases and prevent development of valvular sclerosis.

Factors associated with endothelium damage

The valve endothelium is a protective layer of cells intimately involved with the homeostasis of valve tissue.⁷³ VECs are the primary cells in valve endothelium and have some distinct properties from aortic endothelial cells, such as alignment and gene expression in response to shear stress.⁷³ In response to external stimuli (such as haemodynamic changes, oxidized LDL, proinflammatory cytokines, or bacterial toxins), VECs increase their expression of cell

adhesion molecules, such as ICAM and VCAM, to promote the adhesion and infiltration of monocytes and lymphocytes to participate in tissue repair.⁷³ VECs also secrete paracrine factors, including nitric oxide, endothelin-1, and C-type natriuretic peptide. Most biochemical factors secreted by VECs (except endothelin-1) function to reduce the pathogenic phenotypes of VICs. For example, nitric oxide reduces both myofibroblast and osteoblast differentiation of PAVICs,⁷⁴ likely through the canonical cGMP pathway to inhibit myofibroblast activation.^{74,75} Similarly, C-type natriuretic peptide treatment on PAVICs inhibits actin, aortic smooth muscle (ACTA2, or commonly known as α -smooth muscle actin, α -SMA) expression, collagen gel contraction, and blocks calcific nodule formation and expression of osteogenesis-related genes under osteogenic culture conditions.⁷⁶ By contrast, endothelin-1 is profibrotic and promotes contraction of valve cusps *in vitro*.⁷⁷ If the production of these protective molecules by cardiac valve endothelium can be manipulated *in vivo*, this modulation might lead to a therapy that controls or reduces VIC pathogenic phenotypes.

TGF- β superfamily

TGF- β -1 is a potent profibrotic chemokine and is implicated in fibrosis of organs in the body, including cardiac valves, lungs, kidneys, and liver.⁷⁸ Elevated TGF- β -1 levels have been detected in human calcified valves and is likely derived from infiltrated macrophages and resident mesenchymal cells.⁷⁹ Based on *in vitro* cell culture studies, TGF- β -1 induces myofibroblast activation of VICs, which is characterized by stress fibers expressing Acta2 and collagen deposition,^{30,43} and can promote apoptosis-associated calcification in VICs.⁵⁹ *In vitro* TGF- β -1 induces EMT-mediated invasion of VECs into collagen gels.⁶⁴ Furthermore, TGF- β -1 promotes secretion of profibrotic cytokines to change the local profile of biochemical signals.^{78,80} All these effects exacerbate fibrotic lesion development mediated by valvular cells, infiltrated immune, and progenitor cells. TGF- β -1 can act through either canonical (mothers against decapentaplegic homolog 2 and 3) or non-canonical (focal adhesion kinase 1, Phosphatidylinositol 4,5-bisphosphate 3-kinase, mitogen-activated protein kinase 11) signalling pathways to promote different cellular functions.⁸¹⁻⁸³ Besides TGF- β -1, BMP2 is also elevated in human stenotic valves, which can lead to osteogenic differentiation of resident cells by stimulating the expression of pro-osteogenic genes, such as Runx2 and Osteocalcin.⁵⁴

Many other biochemical signals that might be associated with CAVD are currently being investigated, including lipids,^{84,85} angiotensin II,^{86,87} reactive oxygen species,⁸⁸ Wnt,⁸⁹ VEGF,⁶² fibroblast growth factor 2,⁹⁰ and serotonin.^{91,92} These biochemical factors can activate different intracellular signaling pathways or gene expression to regulate VICs and VECs in a spatial, temporal and dose-dependent manner.⁴ However, many of these studies investigate the effect of the biochemical signals in isolation from the native matrix environment.^{87,90-92} While these studies have provided valuable insights into signalling regulation on valvular cells, the response of VICs and VECs to the many signals often depends on the context of the microenvironment in which they reside (such as, matrix composition and stiffness).

The extracellular matrix

The ECM is a complex environment, rich in biochemical and biophysical factors that can direct cellular function.^{24,93} ECM proteins interact with cells through specific integrin-binding epitopes, which subsequently activate intracellular signalling and regulate cellular phenotypes.⁹⁴ In addition, matrix molecules such as proteoglycans can locally sequester biochemical factors, and consequently, the ECM serves as a reservoir of biological signals that can be released on demand.⁹⁵ Simple delivery of biochemical factors as soluble media additives, therefore, might not fully simulate the effects of biochemical factors presented in cardiac valve tissue, where these signals are often bound to matrix proteins or might need to diffuse through a dense matrix. The ECM also maintains basal cellular tension through its mechanical properties, transmits external mechanical forces, often presents distinct topographical features, and renders positional information to cells.^{96,97}

Pioneering experiments with cells cultured in or on hydrogels comprised of natural ECM proteins (such as collagen and fibronectin) or synthetic biomaterials have revealed that the culture substrate can also regulate cellular function. For example, muscle satellite cells have an increased ability to self-renew and regenerate damaged muscle tissues when cultured on laminin-coated hydrogels with a matrix elasticity that closely matches their native niche.⁹⁸ Similarly, VICs can better maintain an unactivated fibroblast phenotype when cultured on hydrogels that mimic elasticity of the ECM, compared with plain plastic plates.⁹⁹ This finding suggests that the culture microenvironment should be controlled to preserve the native phenotype of VICs. Moreover, controlling the microenvironment might be especially important when studying VIC responses to exogenous microenvironmental signals, which might not be apparent if the cellular phenotype is altered by culture on plastic plates.

The cellular microenvironment can also influence cell fate determination when presented with biochemical signals. For example, PAVICs do not form calcified nodules on soft poly(ethylene glycol) (PEG) hydrogels even when coated with fibrin, which can increase VIC calcification on plastic plates.¹⁰⁰ Furthermore, TGF- β -1 could not activate the myofibroblast phenotype of VICs on compliant collagen or PEG gels,^{32,49} indicating that a stiff matrix environment might be a requirement of myofibroblast activation in valve cells. Culture on plastic plates provides only a 2D surface for growing cells, which can unnaturally polarize them (Figure 3); whereas most cell types residing in tissues, including VICs, are embedded in a 3D matrix environment. When cells are cultured in 3D bioscaffolds, they have different focal adhesion structures, as well as mechanotransduction, polarity and signaling responses to growth factors, than those cultured on 2D (Figure 3).¹⁰¹

ECM proteins in healthy valves

ECM proteins in a healthy cardiac valve promote valvular cell survival and function.¹⁰² PAVICs isolated from different layers of ECM have distinct levels of myofibroblast activation in response to environmental signals,¹⁰³ indicating that ECM composition and mechanical properties likely regulate the basal phenotype of these cells.

Collagens comprise ~90% of the ECM in the cardiac valve.¹⁰² In a healthy valve, collagen forms fibrils by bundling procollagen triple peptide helices in the extracellular space, and

this structure confers strength to the fibrosa in the direction of its alignment.¹⁰² However, in CAVD, excessive collagen deposition and disrupted alignment can lead to increased matrix stiffness but reduced flexibility as the valve opens and closes (Figure 1).⁷ Fibrillar collagen coated onto plastic plates can inhibit myofibroblast activation and calcific nodule formation of PAVICs compared with uncoated plates,^{104,105} indicating that collagen might help inhibit pathogenic differentiation of VICs. Conversely, collagen-based gels with different stiffness can promote calcific nodule formation in PAVICs, via osteogenic or myofibrogenic mechanisms, when cultured in calcifying media on 2D substrates.^{57,106} These results suggest that collagen can regulate the cellular phenotype through both biochemical and biophysical properties, which correlates with observations that the initiation of calcification occurs more often in the fibrosa of valves than the other two layers in the valve.¹⁰⁶ In addition, 3D *in vitro* collagen gels can promote VIC adhesion, spreading, proliferation and matrix synthesis.^{102,107,108} Collagen gels are a simple, but robust, platform for culturing valvular cells *in vitro*, which has led to growing interest in other matrix systems that might permit investigators to alter the cellular microenvironment (for example increase stiffness or change fiber alignment) to study disease progression.

Proteoglycans and glycosaminoglycans (GAGs) (such as versican and hyaluronic acid) are also abundant in the middle layer of the valve (the spongiosa).¹⁰⁹ These proteins do not provide the same stiffness of collagen; however, they are highly polar and attract water, which serves as a lubricant cushion to absorb compressive stresses in the tissue.¹¹⁰ In addition, proteoglycans and GAGs can help sequester, or function as ligands, for growth factors.⁹⁵ In CAVD, proteoglycans and GAGs might contribute to atherosclerotic lesion development by retaining lipids accumulation and attraction inflammatory cells in the valve ECM.¹⁰⁹ Managing the synthesis and structure of proteoglycans and GAGs has, therefore, been proposed as a promising therapeutic target to treat CAVD.¹⁰⁹ The GAG, heparin, is a potent activator of PAVICs when coated on plastic plates, promoting the formation of myofibroblasts and calcified nodules.^{111,112} However, heparin is also known to interact with growth factors such as FGF2 and TGF- β -1,¹¹³ so understanding GAG regulation of VIC phenotypes is complex. Another type of GAG is hyaluronic acid, which constitutes a matrix scaffold to maintain VIC survival, growth and ECM protein secretion, to enable the study of proteoglycan and GAG effects.^{114,115} Interestingly, hyaluronic acid degradation products from a PEG–hyaluronic acid-based hydrogel can promote elastin synthesis in PAVICs,¹¹⁶ indicating that this matrix cue can be fine-tuned to regulate VIC secretory properties and might be beneficial for valve tissue engineering strategies. As hyaluronic acid can bind to cell surface receptor CD44,¹¹⁷ and interact with other growth factor receptors,¹¹⁸ further investigation is needed to determine if the biological activity of hyaluronic acid-based biomaterials is mediated by CD44 or other interacting growth factor receptors.

Elastin is the major ECM component on the flow side of aortic valves and extends as a fibrous, interweaving structure deep into the valve (that is the spongiosa).¹¹⁹ While the effects of presenting the elastin protein to VICs have not been investigated in detail *in vitro*, mice with elastin haploinsufficiency develop aortic regurgitation with increasing age and VICs have reduced expression of genes that are associated with TGF- β -1-induced fibrogenesis.¹²⁰ This result indicates that elastin is an important component of the valve and

regulates gene expression of VICs. Elastin degradation and fragmentation have also been detected in diseased valves,¹²¹ and treatment with fragments of degraded elastin can induce calcification phenotypes in rat dermal fibroblasts *in vitro*.¹²² Other ECM proteins, such as fibronectin, vitronectin and periostin, also help maintain the mechanical integrity of the valves and regulating cellular functions.¹⁰² Interestingly, decellularized valvular matrices seeded with valve progenitor cells have been exploited as scaffolds for clinical valve replacement; for example, this approach has been used to successfully replace pulmonary valves in sheep¹²³ and humans.¹²⁴

While *in vitro* ECM-based culture systems are a promising advance for understanding VICs, these platforms are not without limitations. ECM proteins in the healthy valve are easily accessible materials and support the basic biological functions of VICs; however their biochemical and biophysical properties are usually intertwined and difficult to determine. Furthermore, in developing clinically relevant tissue constructs for valve replacement, naturally derived scaffolds can encounter problems with batch-to-batch manufacturing variations and interspecies immunogenic issues with the hosts.¹²⁵

Synthetic biomaterials and the valve ECM

Synthetic biomaterials such as those made from PEG or poly(vinyl alcohol) precursors, have been used as substrates to understand the effects of matrix reserved cues on VICs.^{126,127}

These hydrogels can be synthesized under ambient, mild conditions for cell culture and encapsulation. Synthetic ECM mimics, can be used as basic permissive environments that allow cells to develop in 2D and 3D culture in a passive manner, or as active promoting niches that present specific biological cues (for example, adhesive ligands, MMP degradable sequences, and locally sequestering growth factors).¹²⁸ Synthetic biomaterial scaffolds are often engineered to have elasticities similar to those measured in many soft tissues (Figure 3).¹²⁹ VICs not only survive in these synthetic biomaterials, but can actively remodel the gel environments and respond to chemical signals such as TGF- β -1 by producing more collagen matrix.^{130,131} However, a major challenge in developing synthetic ECM scaffolds for cell culture is to determine those signals that are necessary to create an environment that supports basic cell function, while permitting control of the material to dissect the complexity in matrix signalling.

Biochemical signals in the ECM scaffold—The ECM can regulate diffusible biochemical signals via covalent or noncovalent interactions.⁹³ Matrix proteins also have inherent peptide domains that can directly bind with integrins on cell surfaces, growth factors and other ECM proteins, or can be cleaved by ECM remodeling enzymes.⁹³ For example, collagen binds integrins, the discoidin domain receptors, or other ECM proteins through different peptide sequence domains.^{132,133} To recapitulate these different ECM functional domains in artificial bioscaffolds, peptides are now routinely used as surrogates for whole proteins, and can be helpful in ascertaining how matrix cues regulate valvular cells. For example, ECM-derived adhesive peptides (such as RGDS derived from fibronectin, YIGSR derived from laminin) partially recapitulate the effects of ECM proteins on VIC calcification phenotypes when cultured on plastic plates.¹⁰⁵ This result indicates that integrin binding is partially responsible for ECM-regulated VIC calcification. Plastic plates

non-specifically adsorb many serum proteins, some investigators have, therefore, exploited peptide-functionalized hydrogels modified with different ECM adhesive peptides (RGDS, VGVAPG derived from elastin, and P15 derived from collagen) to study how matrix composition regulates VIC activation and ECM deposition.¹²⁶ When culture substrates had a basal level of RGDS to promote PAVIC adhesion, adding the collagen-derived P15 peptide sequence led to an increase in Acta2 levels (Figure 3, blue bars).¹²⁶

Synthetic hydrogels also provide a greater degree of biochemical signal spatiotemporal control than traditional culture methods. Methods have been developed to tether biomolecules to hydrogels, maintain their activity, and examine their effects on stem cell functions.¹³⁴⁻¹³⁶ These approaches can be easily adapted to VICs to present active growth factors in locally and temporally controlled manner. However, in an *in vivo* setting, secreted biochemical factors might remain bound to the matrix until they are passively released, activated mechanically, or enzymatically.^{137,138} For example, rat lung myofibroblasts can activate TGF- β -1 mechanically by contracting their matrix.¹³⁷ As one example of controlling interactions between growth factors and matrices, investigators incorporated peptides with different sequences and structures with affinity for FGF-2 to control its local presentation in hydrogels.¹³⁹ In the future, the sequential release of multiple growth factors might enable concentration gradients that elicit differential cellular responses to better study how growth factors contribute to CAVD development and tissue regeneration.

Biophysical signals in the ECM scaffold—The ECM itself can regulate cells based on its biophysical material properties, such as elasticity.¹²⁹ Elasticity is physiologically meaningful material property relevant to disease progression, because native tissues become stiffer as fibrosis develops. A simplified measure of matrix elasticity is the ratio of an applied stress divided by the deformation of a material (strain), and is often reported as a Young's modulus (E) when a tensile stress is applied.¹⁴⁰ Traditional plastic plates for cell culture have an E of ~3 GPa, which is many orders of magnitude higher than the stiffness of valve leaflets.¹⁴⁰ The E of cardiac valves has been reported in the literature as ranging from 0.1 kPa to 2 MPa.^{3,141-144} This variability might be due to anisotropic properties of valves, differences in measurement methods, and strain levels used for calculation. Owing to this disparity in mechanical properties, synthetic hydrogels are designed to mimic the elasticity range of valve tissue, and assess the specific effects of matrix elasticity on VIC phenotypes (Figure 3). In one study investigators created a PEG-based hydrogel with an E gradient from 7 to 32 kPa.¹³⁸ In this environment >80% of PAVICs formed myofibroblasts near the stiffer region (32 kPa) and <10% differentiated into myofibroblasts near the softer end (7 kPa) after 3 days of culture in low serum media (Figure 3).¹⁴⁵ This study is an excellent example in which one might engineer different elasticities to direct VIC population phenotypes.

The elasticity of the culture environment can regulate myofibroblast activation of fibroblasts isolated from different tissues, including liver, skin and lung; very similar thresholds of activation have been observed to that of VICs.¹⁴⁵⁻¹⁴⁸ Matrix elasticity (in combination with inductive biochemical signals) can also regulate calcific nodule formation,⁵⁷ proliferation^{49,149} and apoptosis of fibroblasts.^{146,149,150} Many of these studies have been carried out by culturing cells on 2D substrates with different elastic moduli. However, one group has investigated the mechanobiology of VICs in 3D culture and found that cells

cultured on stiff environments and in the presence of TGF- β -1 were induced to myofibroblasts and exerted higher tension on their 3D matrix.¹⁵¹

Matrix elasticity can also modulate cellular responses to biochemical factors. For example, PAVICs have increased myofibroblast activation in response to TGF- β -1 on poly(acrylamide) gels coated with fibrillar collagen when E was >22 kPa, but did not respond to TGF- β -1 when E was <11 kPa.¹⁴⁷ Matrix elasticity might act via the transcriptional coactivators, Yorkie homolog (also known as Yes-associated protein; YAP) and WW domain-containing transcription regulator protein 1 (commonly known as TAZ); both proteins can function as sensors of matrix elasticity in human mesenchymal stem cells.¹⁵² YAP and TAZ are also present in VICs and become localized to the nucleus with increasing matrix elasticity.¹⁵³ Finally, matrix stiffness might regulate intracellular biochemical signalling of cells through mechano-signalling at focal adhesions.^{99,154}

While matrix stiffness has been recognized as an important biophysical cue that can regulate diverse cellular functions,^{98,129} other matrix biophysical signals, including nanotopography,⁹⁷ anchoring of matrix fibers,¹⁵⁵ and degradation-mediated cellular traction¹⁵⁶ should also be considered as contributing factors in directing cell fate. While beyond the scope of this Review, cells residing in valves are highly responsive to changes in haemodynamic profiles and external mechanical stress (such as cyclic stretch, pressure, shear stress), and have been excellently reviewed elsewhere.^{3,157-159} Mechanical stresses experienced by tissues are transmitted to cells and, therefore, should be carefully studied to facilitate the design of tissue-engineered heart valves that respond positively to *in vivo* haemodynamics.^{160,161}

Cell–cell interactions

Cell–cell interactions are important regulators of tissue homeostasis and valvular disease progression and include both homotypic and heterotypic interactions: VIC–VIC, VIC–VEC, and VEC–VEC. In VIC–VIC adhesion, cadherin-11 (Chd11) is one of the most abundant cadherins in PAVICs and mediates cell–cell junctions between myofibroblasts.¹⁶² Chd11 can also promote calcific nodule formation in cultured VICs.¹⁶³ However, while antibodies against Chd11 can ameliorate fibrosis in synovium¹⁶⁴ and lung,¹⁶⁵ these have not been tested for valvular sclerosis. VEC–VEC junctions are important to maintain the integrity of the valve endothelial lining, but can be disrupted when VECs undergo EMT in response to inflammatory cytokines⁶⁴ and other disease risk factors, such as altered shear stress.¹⁶⁶

In the healthy valve tissue, VECs are largely in contact with VICs near the boundary of the valves and signal between each other via paracrine signals.⁴ These cells must be in close proximity for the paracrine signals to function properly, therefore, the spatial position of the cells is important. To create a culture platform that closely mimics the relative geometry of VICs and VECs in a valve, investigators have encapsulated PAVICs in collagen gels, with PAVECs seeded on the surface.¹⁶⁷ PAVECs can stimulate PAVICs to adopt a quiescent phenotype, with reduced Acta2 expression or proliferation.¹⁶⁷ In addition, another group of investigators used magnetic levitation to build *in vitro* micro-tissue blocks of PAVICs or PAVECs, and cocultured them by stacking the PAVEC block on top of PAVICs.¹⁶⁸ This

coculture maintained aspects of the healthy phenotypes of both cell populations and permitted *in situ* matrix deposition.¹⁶⁸ Other investigators have designed a coculture system whereby PAVICs were seeded on PEG hydrogels fabricated in a transwell membrane and the PEG tensile *E* can be controlled to study its influence on cell–cell signaling.⁷⁵ Interestingly, using this culture system, PAVECs induced ~70% reduction in myofibroblast activation when PAVICs were cultured on softer gels (~3 kPa), but ~20-30% of reduction in myofibroblast activation on stiff gels (~27 kPa).⁷⁵ This differential response was partially dependent on nitric oxide-mediated signalling.⁷⁵ Future experiments might identify those biochemical and matrix biophysical signals that regulate these VIC–VEC interactions and how. Coculture systems that preserve the spatial proximity of VICs and VECs are versatile and should be more widely used by investigators examining complex interactions in cardiac valve biology. Such experiments might reveal novel mechanisms of regulation that can otherwise be obscured or modified if cells are simply cultured together on plastic substrates or without the ability to control the cell's spatial proximity.

Dynamic culture systems

The process cell remodelling is more dynamic during CAVD development than in the healthy valve. In the diseased valve, local cell populations change their phenotypes, secrete varying amounts and types of cytokines, and actively remodel the ECM (Figure 4).^{7,14} To recapitulate these dynamic signals and understand the temporal regulation of cellular phenotypes, we need to exploit advanced multifunctional culture substrates that allow manipulation of biochemical and biophysical matrix cues on demand. For example, biomaterials that permit control over their material properties in both space and time offer great potential for understanding the basic biology of matrix signalling and in facilitating tissue regeneration.¹²⁸

To study the effects of increasing concentration of biochemical signals on a cell's microenvironment, investigators have used light-initiated chemistry to pattern streptavidin or barnase (which bind biotin and barstar respectively) into a 3D agarose gel.¹⁶⁹ Growth factors linked to either biotin or barstar can then be introduced to the gel and bind to these prepatterned positions.¹⁶⁹ This approach enables the delivery of two different biochemical signals, both temporally and spatially, in a manner that preserves their activity. To modify biochemical ligands involved in cell adhesion, investigators have patterned cell adhesive peptides modified with photolabile groups (*o*-nitrobenzyl ether) into a 3D PEG hydrogel and then specifically cleaved them using light with a wavelength of 365 nm to remove the signals at defined loci and times.¹⁷⁰ To achieve recurrent patterning in the matrix microenvironment, Gandavarapu *et al.* developed an allyl sulfide functionalized PEG hydrogel system where one can pattern and exchange different biochemical cues modified with a sulfhydryl functional group multiple times.¹⁷¹ These systems might be used to study how locally patterned TGF- β -1 or other biochemical signals regulate VIC phenotypes in 3D, and importantly, how cells can revert to a homeostatic state with their surrounding matrix when levels of the activating signal are modulated.

Synthetic bioscaffolds can also be used to study how mechanotransduction can influence cell states. For example, a photo-responsive PEG hydrogel has been developed that can be

softened upon exposure to light, *in situ*, and in the presence of cells.¹⁷² Exploiting this dynamic substrate, investigators have shown that PI3K/AKT activity decreased in response to reduced matrix elasticity (from 32 kPa to 7 kPa), which consequently inhibits the differentiation of VICs to myofibroblast.⁹⁹ Using a similar photo-responsive PEG hydrogel, researchers have created topographical patterns at the micro-meter scale on soft PEG hydrogels (of ~3 kPa), which induce parallel PAVIC alignment according to the patterned features and myofibroblast activation.¹⁷³ This result indicates that both stiffness and topography of the matrix affect VIC differentiation. Biomaterials that stiffen over time have also been developed¹⁷⁴ and used for directing hepatic myofibroblast activation.¹⁷⁵ These materials mimic aspects of the matrix stiffening process during the development of valvular sclerosis, and together, should prove useful to dissect the signaling pathways involved in activating or deactivating cells in response to matrix elasticity changes. However, in our experience these substrates do not completely mimic *in vivo* tissue remodelling, but continual improvements are being made to more closely mimic the native ECM. Nevertheless, *in vitro* reconstituted biomaterials make it possible to understand the specific and the combined effects of different ECM remodeling on cellular phenotypes,

Future perspectives

If we can understand how valvular cells and microenvironmental signals mutually regulate each other, it might be possible to ameliorate valvular disease progression at an early stage by targeting these interactions. Furthermore, by directing cells to deposit a specific ECM composition in an engineered scaffold, one might be able to create viable replacement valves, with the potential for further *in situ* growth, for pediatric patients. To achieve such goals, understanding the mechanisms of regulation between cells and the ECM in both normal and diseased valves is required.

Given the role of VICs in mediating tissue repair, we need to better define their basal, myofibroblast, and osteoblast phenotypes. VICs might become activated when they are isolated from their native matrix,⁹⁹ therefore, determining how we can maintain their native phenotypes by designing appropriate culturing substrates would be beneficial. Importantly, measuring how differential gene expression contributes to ECM remodelling, stiffening, and other biological function in valvular tissue function is also needed.

Furthermore, how the temporal presentation of biochemical signals (such as TNF and TGF- β -1) regulates VEC and VIC function as CAVD progresses, and their interaction in regulating intracellular signalling, is unclear. High throughput screening methods might improve our understanding of the roles of growth factors in directing certain cellular functions (for example, via contraction or matrix protein production). Importantly, these factors must be studied and characterized in the context of a suitable microenvironment, to understand how growth factor signalling and availability can be coupled to the matrix.

Future studies should take advantage of synthetic biomaterials to determine how individual and coordinated signals of ECM elasticity, adhesion, and biochemical interactions influence valve cell function. In addition, biomaterials that can be dynamically altered to change their biophysical and biochemical properties will enable investigators to mimic the ECM changes

seen during the disease progression to study the underlying molecular and cellular mechanisms. Furthermore, using animal models of CAVD originating from different molecular causes to understand the cell-matrix biology during disease progression will become increasingly important. Ultimately, a systems biology-based approach will help identify critical factors and analyze the complex interactions of diffusible biochemical and ECM signals. However, new biomaterials and technologies will be required to deliver them in an appropriate manner, at the right dose, place, and time.

Conclusions

Cell culture models provide a high throughput and high speed platform on which to study how valvular cells maintain valve homeostasis, mediate disease progression, and respond to external biochemical signals. However, valvular cells need to be cultured in a physiologically relevant matrix environment to improve our understanding of the dynamic crosstalk between cells and the matrix microenvironment. Reconstructing models of normal or diseased valves *in vitro*, including properties such as appropriate matrix scaffolds and cell architecture has many advantages. For example, they can serve as models for drug screening, elucidating mechanisms of disease progression or reversal, and engineering tissue for valve replacement. We believe the field of valve biology is rapidly evolving to take advantage of these new technologies and enables a systems-level understanding of the complex interplay between biochemical signals, the physical matrix, and cell-cell communication. This knowledge will be a critical link for future efforts to direct valvular cell phenotypes and tissue regeneration.

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Key points

1. The interaction between valve cells and their microenvironment signals determine the functional output of cardiac valve tissues; cardiac valve diseases might follow a disruption in these interactions
2. *In vitro* cell culture studies can help understand cardiac valve disease pathobiology, which is enhanced by culturing cells on biomaterials that mimic the native valve cell matrix environment
3. Many biochemical signals regulate pathogenic phenotypes of cells in the valve; however, their effects are dependent on the matrix microenvironment
4. Synthetic biomaterials can be used to understand valve cell regulation by biochemical, biophysical, and other matrix-associated signals, especially related to the development of valvular diseases
5. Understanding the dynamic interplay between microenvironmental signals and valvular cell phenotypes will enable the identification of new therapies and the design of *ex vivo* tissue engineered valves

Figure 1. Valve cells and their matrix regulate tissue homeostasis and disease progression

a | Cardiac valves have three extracellular matrix layers: the ventricularis, spongiosa, and fibrosa. In this image, a cross-section of a porcine aortic valve was visualized by second-harmonic generation confocal imaging. Elastin in green, collagen in red. Scale bar: 100 μm .

b | Two main cell populations comprise the cardiac valve. VECs, which are CD31^+ (green), line the boundaries of the leaflets. VICs reside throughout the valve leaflet. Nuclear staining (DAPI; Blue), Scale bar: 100 μm . **c** | In healthy valves (left), very few VICs and VECs are activated to repair local tissue damage during normal valve function. Collagen fibers are circumferentially aligned and activated VICs undergo continual turn over into deactivated fibroblasts and other cell fates (such as apoptosis). In diseased valves (right), VICs and VECs are recruited to remodel the tissue, leading to excessive collagen accumulation, a degraded and disarrayed matrix, which in some cases becomes calcified. Valve cells lose the homeostatic equilibrium and remain in an activated state. Abbreviations: VEC, valvular endothelial cells; VIC, valvular interstitial cells.

Figure 2. Dynamic cellular phenotypes in cardiac valve diseases

a | Mineralized spherical particles (red) have been observed in human calcified valves, but not in normal valves. Scale bars 2 μm **b** | Cells in the valve adopt diseased phenotypes (myofibroblast and osteoblast-like cells) in response to environmental signals. Activated myofibroblasts can revert back to quiescence, senescence, or undergo apoptosis. Osteoblast-like cells might also undergo apoptosis. (1) Myofibroblasts are characterized by Acta2-positive stress fibers (green). Scale bar 50 μm . (2) VICs can be induced to mineralize, indicated by Alizarin Red S staining. Scale bar: 50 μm . Abbreviations: Acta2, Actin, aortic smooth muscle; VEC, valvular endothelial cells; VIC, valvular interstitial cells. Permission obtained from NPG © Bertazzo, S., *et al.* Nano-analytical electron microscopy reveals fundamental insights into human cardiovascular tissue calcification. *Nat. Mater.* 12, 576-583 (2013).

Figure 3. Matrix signals regulate valve cell phenotypes

a | Plastic culture plates have high stiffness and biochemical signals must be introduced as soluble factors in the media. The stiffness of biomaterial-based cultures can mimic native tissues, and enables cells to grow in 3D space, with biochemical signals presented throughout the matrix. **b** | Adhesive signals (yellow in the schematic) can be tethered to an inert matrix to study specific effects on cellular phenotypes. For example, an elastin-derived peptide (VGVAPG, green bars) or a collagen-derived peptide (P15, blue bars) was covalently linked to PEG hydrogels and shown to have distinct effects on α -SMA levels. **c** | Elastic stiffness can be modified to study intracellular cytoskeletal changes. In a modulus gradient of 7–32 kPa, >80% of PAVICs activated to myofibroblasts near the stiffer region and <10% became myofibroblasts near the softer end after 3 days of culture (red line). However, these differences were not observed on day 1 (blue line). Abbreviations: PAVIC, porcine aortic valve interstitial cell. Permission for panel b obtained from Gould, S. T. *et al.* Small peptide functionalized thiol-ene hydrogels as culture substrates for understanding valvular interstitial cell activation and de novo tissue deposition. *Acta Biomaterialia*, **8**, 3201–3209 (2012). Permission for panel c obtained from Kloxin, A.M. *et al.* In situ elasticity modulation with dynamic substrates to direct cell phenotype. *Biomaterials* **31**, 1–8 (2010).

Figure 4. Valve cells and the ECM microenvironment are dynamic and mutually regulated VICs interact with the ECM directly through integrins, with biochemical signals via receptors, and with neighboring cells via cadherins. In healthy tissue (left), the cells and ECM are in a homeostatic state. When microenvironmental signals of valvular disease are present (right), cells can be activated, and biological outputs are altered. Secretion of ECM proteins, matrix remodelling enzymes, and biochemical signals is increased; more contractile stress fibers and cell-matrix adhesions form; apoptosis increases where effective matrix interactions are lost. These cellular functions alter the microenvironment and valve tissue structure in diseased valves. Abbreviations: VEC, valvular endothelial cells; VIC, valvular interstitial cells.