Regulation of Valve Interstitial Cell Phenotype and Function Using Biomimetic Hydrogels

by

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K. Jane Grande-Allen

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Engineering in the Graduate School of Duke University

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ABSTRACT

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Abstract

The aortic valve regulates the unidirectional flow of oxygenated blood from the left ventricle to the systemic circulation. When severe congenital defects occur in aortic valves, valve replacement is inevitable in children. However, current options including mechanical valves and bioprosthetic valves, lack the ability to grow and remodel, which necessitates multiple valve replacements as children grow. Tissue engineering provides a possible avenue to generate a living valve substitute that can grow and remodel via combining cells, scaffolds and environmental cues. The cells used in this work were valvular interstitial cells (VICs), the predominant cell population in the valves, and responsible for extracellular matrix (ECM) synthesis in the valve tissue. VICs are highly heterogeneous and dynamic in phenotype, with the majority assuming a quiescent, fibroblast phenotype in healthy adult valves¹,². During valve injury or disease conditions, VICs may undergo myofibroblast activation or osteogenic differentiation³,⁴. Myofibroblast activation is characterized by the expression of smooth muscle α-actin (αSMA), and may cause valve fibrosis³,⁴; osteogenic differentiation is characterized by the upregulation of alkaline phosphatase (ALP), followed by tissue calcification, which is the leading cause of valve disease in the elderly (>60 years of age) and the failure of bioprosthetic valves⁵. However, the most common method of in vitro VIC culture on two-dimensional (2D) stiff substrates leads to myofibroblast activation of VICs. For
better physiological relevance and future application in valve substitutes, there is a need to understand and regulate VIC behaviors within three-dimensional (3D) scaffolds that are more reminiscent to their native environments. This dissertation describes the development of a poly(ethylene glycol) (PEG)-based hydrogel platform to support VIC growth in 3D, and the exploration of free and immobilized bioactive cues to dictate VIC phenotype and behaviors toward the development of a living valve substitute.

Otherwise bioinert, PEG hydrogels were functionalized with cell-adhesive ligands RGDS and proteolytically degradable sequences (GGGPQGIWGQGK). The functionalized hydrogels supported VIC growth, proliferation and ECM remodeling (secretion of matrix metalloproteinase-2 and deposition of collagens) in 3D during the culture period of 4 weeks. The soft hydrogels with compressive moduli of ~4.3 kPa quickly reverted VICs from myofibroblast activation to a quiescent phenotype upon encapsulation, evidenced by the loss of αSMA expression. The functionalized PEG hydrogels are preferable to 2D stiff substrates for preservation of the native phenotype of VICs and resistance to calcification.

In an effort to potentially promote deposition of ECM components by encapsulated VICs, ascorbic acid (AA), which is a cofactor in the post-translational modification of collagen molecules and has been reported to increase collagen section by several other cell types, was added to the culture media of cell-laden hydrogels. AA treatment promoted VIC-mediated ECM remodeling without negatively influencing
their quiescent phenotype in hydrogels. AA also enhanced VIC spreading and proliferation while inhibiting apoptosis.

ECM-mimicking adhesive peptides with specific affinity to different receptors were immobilized on PEG hydrogels in order to regulate VIC adhesion, phenotype and ECM production. Expression of adhesion receptors by VICs was assessed via flow cytometry and used to guide the choice of peptides studied. The peptide RGDS with affinity to multiple integrin receptors, and specific receptor-targeting peptides DGEA (integrin α2β1), YIGSR (67 kDa laminin/elastin receptor; 67LR), and VAPG (67LR) were chosen based on the receptor expression profiles as well as the potential outcomes of each receptor binding. DGEA, YIGSR, and VAPG alone were insufficient to induce stable VIC adhesion. As a result, these peptides were studied in combination with 1 mM RGDS. For VICs cultured on 2D hydrogel surfaces, YIGSR and VAPG down-regulated the expression of αSMA (myofibroblast activation marker) whereas DGEA promoted VIC adhesion and VIC-mediated ECM deposition while inhibiting the activity of ALP (osteogenic differentiation marker). Further, YIGSR and DGEA in combination promoted ECM deposition while inhibiting both myofibroblastic and osteogenic differentiation. However, VICs behaved differently when cultured within 3D hydrogels, with VICs assuming a quiescent, fibroblastic phenotype without any calcification under all peptide conditions tested. DGEA promoted ECM deposition by VICs within hydrogels without causing VIC activation.
The results of this research provide a clearer understanding of VIC biology and pathology under biomimetic conditions and lay the groundwork for constructing living valve substitutes using the tissue engineering approach. The hydrogel platform developed in this work may also be applied to study the initiation and progression of valvular diseases.
Dedication

To my family; I could not have done this without your unconditional love and support.
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List of Abbreviations

AA: ascorbic acid
ALP: alkaline phosphatase
AV: aortic valve
αSMA: smooth muscle α-actin
BGS: bovine growth serum
BMP4: bone morphogenetic protein 4
BS: blebbistatin
CAVD: calcific aortic valve disease
CBF-α1: core binding factor-α1
Col: collagens
DAPI: 4',6-diamidino-2-phenylindole
DCM: dichloromethane
DGEA: GGGDGEAGG
ECM: extracellular matrix
EMT: mesenchymal transformation
eNOS: endothelial nitric oxide synthase
EPCs: endothelial progenitor cells
ERK-1/2: extracellular signal-regulated kinases 1 and 2
FGF: fibroblast growth factor

FGFR: fibroblast growth factor receptor

Fn: fibronectin

GAGs: glycosaminoglycans

GM: growth media

GM+AA: GM supplemented with AA

GPC: gel permeation chromatography

HA: hyaluronic acid

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hMSCs: human mesenchymal stem cells

HSPGs: heparan sulfate proteoglycans

HVTE: heart valve tissue engineering

JNK: c-Jun N-terminal protein kinase

LDL: low-density lipoprotein

MAPK: mitogen-activated protein kinase

MV: mitral valve

MW: molecular weight

MMPs: matrix metalloproteinases

NO: nitric oxide

NVP: N-vinylpyrrolidone
OCN: osteocalcin

Osteo M: osteogenic media

oxLDL: oxidatively modified LDL

PCL: poly(ε-caprolactone)

PDMS: polydimethylsiloxane

PDO: polydioxanone

PEG: polyethylene glycol

PEGDA: PEG diacrylate

PGA: polyglycolic acid

PGS: poly(glycerol sebacate)

PGs: proteoglycans

PHA: Poly(hydroxyalkanoate)

PHO: poly(hydroxyoctanoate)

PLGA: poly(L-lactic-co-glycolic acid)

PLLA: poly-L-lactic acid

PQ: GGGPQGIWGQGK

PV: pulmonary valve

PVA: polyvinyl alcohol

P4HB: poly(4-hydroxybutyrate)

SMCs: smooth muscle cells
TCPS: tissue culture polystyrene

TEA: triethyl amine

TEHVs: tissue-engineered heart valves

TEOA: triethanolamine

TGF-β1: transforming growth factor

TV: tricuspid valve

VAPG: GGGVAPGGG

VECs: valvular endothelial cells

VICs: Valvular interstitial cells

XO: xylenol orange

YIGSR: GGYIGSRG

2D: two-dimensional

3D: three-dimensional

67LR: 67 kDa laminin/elastin receptor
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Chapter 1 Introduction

1.1 Motivation

Pediatric cardiac disease remains a significant health problem and affects nearly 1% of all live births\textsuperscript{10}, with approximately 0.3% being congenital pulmonary or aortic valve abnormalities\textsuperscript{11,12}. Valve repair is recommended as the initial treatment when possible. When valve repair fails or is not feasible, valve replacement becomes inevitable. Despite significant advances in surgical techniques over the last 30 years, finding an ideal valve substitute remains a challenge. Current options for valve replacements generally enhance survival and quality of life. However, they have severe limitations and are especially problematic for pediatric patients: mechanical valves require lifelong anticoagulation therapy; bioprosthetic valves have limited durability due to rapid calcification in children (from months to years after implantation)\textsuperscript{11,13}. Moreover, both mechanical and bioprosthetic valves are non-viable and thus incapable of growth, which necessitates multiple valve replacement surgeries to implant larger valve substitutes as children grow, bringing significant dangers associated with repeat sternotomy\textsuperscript{14}. Analysis of the outcomes of aortic valve replacement (either mechanical or

bioprosthetic valves) in 160 children (≤18 years of age) showed that, within 10 years following replacement, 19% of patients died, 34% underwent a second replacement, and only 47% remained alive without need for repeated replacements\textsuperscript{12}. Reoperation is associated with significantly higher rates of mortality and mobility than first-time sternotomies\textsuperscript{10}. An ideal valve substitute for these patients should be non-immunogenic, non-thrombogenic and capable of adaptive growth with growing patients. This need has sparked interest in tissue engineering approaches where cells are seeded on a scaffold that supports tissue growth and remodeling. Heart valve leaflets, which are thin and avascular, seem to be a good candidate for tissue engineering strategies, where successes so far have been limited to thin and avascular tissues, such as bladder and skin\textsuperscript{15,16}, largely due to their simplicity in design without the requirement for vascularization\textsuperscript{17}.

Valvular interstitial cells (VICs), the predominant cell population in heart valve leaflets, are responsible for the maintenance and repair of the valve structure\textsuperscript{3,18}. VICs are highly heterogeneous and dynamic in phenotype, with the majority being quiescent fibroblasts in healthy adult valves\textsuperscript{1,2}. Under pathological injury or abnormal hemodynamic/mechanical conditions, VICs become activated possibly following activation of valvular endothelial cells (VECs), which may trigger inflammation and its associated cytokine and chemokine signals\textsuperscript{1,19}. Myofibroblast activation, characterized by the hallmark of smooth muscle α-actin (αSMA) expression and enhanced synthetic and
contractile properties, facilitates tissue remodeling and wound healing\textsuperscript{3,4}. After completion of remodeling activities, myofibroblasts are normally eliminated via apoptosis\textsuperscript{20}. When the myofibroblast life cycle is not regulated properly, its persistence with continued force generation and extracellular matrix (ECM) production results in pathological fibrosis and is believed to be associated with initiation of dystrophic calcification (calcification of necrotic tissue), which is found in approximately 83\% of calcified valves\textsuperscript{21,22}. Meanwhile, VICs may differentiate into an osteoblast-like phenotype and form calcified nodules via ossification\textsuperscript{5}. Therefore, to construct living valve substitutes that are free from fibrosis and calcification, it is critical to preserve the quiescent fibroblast phenotype of VICs.

Previous research has shown that VIC phenotype is highly sensitive to culture conditions. The most common method of \textit{in vitro} VIC culture is on two-dimensional (2D) stiff (plastic or glass) substrates, which are orders of magnitude stiffer than the valve tissue\textsuperscript{21,23–25}. On these substrates, VICs generally assume an activated myofibroblast phenotype that does not represent their primary state \textit{in vivo}\textsuperscript{23}. Culture on soft substrates (2D) reduce myofibroblast activation\textsuperscript{26,27} but can also impair cell adhesion and spreading\textsuperscript{28}. Hence, a three-dimensional (3D), culture platform that allows culture and characterization of VICs under conditions more reminiscent of native valves is highly desirable.
Moreover, as ECM is largely responsible for the unique mechanical properties of the valve tissue, to stimulate VIC-mediated ECM synthesis in vitro is important to the recapitulation of valve composition and function. A tissue engineering scaffold that supports and promotes VIC-mediated ECM remodeling while preserving their native, fibroblastic phenotype would be promising for constructing living valve substitutes. With this goal, the introduction chapter will provide a brief overview of heart valve composition, structure and function of the heart valves (with a focus on aortic valves) and review current progress in heart valve tissue engineering literature covering the cellular and scaffold choices.

1.2 Composition, Structure and Function of Heart Valves

The past 25 years have witnessed the major advances in understanding the structure, function, biology and pathobiology of heart valves. Heart valves are composed of organized and biomechanically responsive ECM and valvular cells, which maintain and actively regulate valve structure and function in both health and disease states.

1.2.1 Extracellular Matrix Components of Heart Valves

While all valves are important, the aortic valve is a critical hemodynamic nexus that separates the left ventricle from the aorta (Figure 1). Human valve leaflets are normally thinner than 1 mm and stratified into three layers that are histologically and
mechanically distinct (Figure 2). The upper (aortic side) fibrosa layer is dominated by circumferentially oriented collagen fibers (mainly type I and III) to withstand high pressure loads when the valve is shut\(^\text{31}\); the lower (ventricular side) ventricularis layer is composed of radially oriented elastin fibers to provide elasticity and preload for stretch and recoil\(^\text{31-33}\), the middle spongiosa layer mainly contains proteoglycans (PGs) and glycosaminoglycans (GAGs) to provide compression resistance and lubrication for rapid ECM reorganization when the valve opens and shuts. Such unique arrangements of ECM determine the anisotropic material properties of heart valves, \(\text{i.e., stiff in the circumferential direction while compliant in the radial direction, which provides sufficient mechanical strength as well as elasticity during valve opening and closing. Notably, the two sides of valve leaflets experience clearly different biomechanical forces due to the local patterns of blood flow (Figure 3)\(^\text{34}\). During systole, the valve cusps become relaxed and shorten due to elastin recoil and collagen crimping, leading to valve opening that allows blood to flow out; the ventricular side of the aortic valve leaflets is subjected to a unidirectional laminar flow that exerts shear stresses of up to 80 dyne/cm\(^2\) on the endothelium; in contrast, the fibrosa side of the leaflets experiences disturbed, oscillatory flow with shear stress ranging from -8 to +10 dyne/cm (Figure 1, Figure 3)\(^\text{29,34,35}\). During diastole, the backpressure from the blood in the aorta closes and stretches the valve tissue; collagen fibers un-crimp and align, and simultaneously elastin is
stretched, thereby locking the structure with the cusps apposed over a modest area of contact to avoid regurgitation (Figure 1, Figure 3)\textsuperscript{19,29,34,35}. Valve cusps during diastole are approximately 30\% larger in area than that during systole\textsuperscript{19}. The blood flow-induced shear stress has been recognized as an important stimulus for valve homeostasis and disease, probably through transducing into biomechanical signals to regulate valvular cell function.

**Figure 1. Aortic valve functional structure during cardiac cycles.** (A) Outflow aspect of aortic valve in open (top) and closed (bottom) configurations, corresponding to systole and diastole, respectively. (B) Schematic representation of architecture and configuration of aortic valve cusp in cross section and of collagen and elastin in systole and diastole. Adapted from Schoen 2012\textsuperscript{29}. 
1.2.2 Valvular Endothelial Cells (VECs)

The cellular components of valve leaflets include VECs that cover the blood-contacting surface of leaflets as a monolayer and VICs that are embedded in the ECM of all three layers of leaflets. VECs create a non-thrombogenic surface and control immune and inflammatory reactions\textsuperscript{37}. VECs may also regulate VIC function via paracrine signaling\textsuperscript{38}, such as nitric oxide release\textsuperscript{39-41}. VECs, have been compared with the well-characterized vascular endothelial cells (ECs) but are less understood. Although VECs
share some similarities with vascular ECs, hundreds of differently expressed genes have been identified between VECs and vascular ECs, suggesting that VECs are a distinct phenotype. Such phenotype difference between VECs and vascular ECs is also shown by reacting to mechanical stress differently. For example, VECs align perpendicularly to the blood flow whereas vascular ECs align in a parallel orientation.

1.2.3 Valvular Interstitial Cells (VICs)

VICs continually repair mechanically induced microdamage via frequent turnover of ECM components to guarantee long-term durability of valves. All mesenchymal cells within valve leaflets are classified as VICs. VICs are heterogeneous and dynamic in phenotype, designated as progenitor endothelial/mesenchymal cells, quiescent fibroblasts, activated myofibroblasts, progenitor VICs, and osteoblast-like cells (Table 1). The phenotypes have cellular functions essential to normal valve physiology and potentially contribute to pathologic conditions as well. Embryonic progenitor endothelial/mesenchymal cells undergo endothelial to mesenchymal transformation (EMT) to become VICs. This process is critical to the fetal development of heart valves where endocardial cells lining the surface of the heart tube become activated and invade into the cardiac jelly. Progenitor VICs consist of a heterogeneous population of progenitor cells that have the potential of self-renewal and multi-lineage differentiation, and may be important in valve repair. VIC phenotype switch occurs with valve
maturation and pathological progression. In fetal valves, a large fraction of VICs display a myofibroblast phenotype, with characteristics of both fibroblasts and smooth muscle cells, including both vimentin and αSMA expression\(^1\). Under equilibrium conditions in healthy adult valves, however, more than 95% VICs are categorized as quiescent fibroblastic cells, with vimentin but not αSMA expression\(^3\). Fibroblastic VICs maintain normal valve physiology. In valve injury or disease conditions, fibroblastic VICs may become myofibroblasts to activate valve repair processes, characterized by increased contractility and increased secretory properties including cytokines, chemokines, growth factors, ECM components, matrix metalloproteinases (MMPs) and their tissue inhibitors\(^4\). When equilibrium is restored, the cells should return to the quiescent state or be eliminated via apoptosis\(^2\). If not, myofibroblast persistence may cause valve fibrosis due to the increased but disorganized collagen fibers\(^3\). VICs may also undergo osteogenic differentiation to form osteoblast-like cells, indicated by the increased expression of alkaline phosphatase (ALP), osteocalcin (OCN), and core binding factor-α1 (CBF-α1), ultimately leading to valve calcification (the formation of calcified mineral deposits analogous to bone formation), which is the leading cause of aortic valve diseases\(^24,43,44\). It remains unclear whether myofibroblast activation is an intermediate stage for VICs to become osteoblast-like cells\(^45,46\). In addition, the heterogeneous nature of VICs also lies in their different characteristics depending on site of origin. For
example, VICs demonstrate age, valve-position and valve-region-specific responses to substrate stiffness and mechanical loading in terms of phenotype, ECM production and calcification.\textsuperscript{47-50}

**Table 1: Heterogeneous VIC phenotypes.** Adapted from Liu et al. 2007\textsuperscript{1} and Schoen et al. 2016\textsuperscript{19}.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Location</th>
<th>Markers</th>
<th>Function</th>
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<tbody>
<tr>
<td>Embryonic progenitor endothelial/mesenchymal</td>
<td>Embryonic cardiac cushions, during EMT</td>
<td>EMT (lose endothelial and gain mesenchymal</td>
<td>Give rise to resident quiescent VICs</td>
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<tr>
<td>Progenitor VICs</td>
<td>Bone marrow, circulation, cusp/leaflet</td>
<td>May be CD34-, CD133-, and/or S100-positive</td>
<td>Have potential for self-renewal and multi-lineage differentiation; provide activated VICs for valve repair</td>
</tr>
<tr>
<td>Quiescent fibroblasts</td>
<td>Valve cusp/leaflet</td>
<td>Vimentin positive; αSMA negative</td>
<td>Maintain physiologic valve structure and function; inhibit angiogenesis in the leaflets</td>
</tr>
<tr>
<td>Activated myofibroblasts</td>
<td>Valve cusp/leaflet</td>
<td>αSMA positive</td>
<td>Activate cellular repair processes (proliferation, migration, apoptosis, ECM remodeling, and TGF-β up-regulation)</td>
</tr>
<tr>
<td>Osteoblast-like VICs</td>
<td>Valve cusp/leaflet, bone marrow, circulation</td>
<td>ALP, OCN, CBF-α1</td>
<td>Serve as precursors that transform into osteogenic and chondrogenic phenotypes</td>
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1.3 Heart Valve Defects, Diseases and Replacements

Driven by the mechanical forces exerted by the heart and the inside blood, normal heart valves open and close about 40 million times a year with minimal obstruction and without regurgitation\(^1\). Heart valve diseases are commonly diagnosed clinically as stenosis (outflow obstruction due to incomplete openness), and regurgitation (backward flow resulting from inefficient closure).

1.3.1 Congenital Heart Valve Disease and Repair

Congenital heart valve disease is one of the most common malformations in children\(^5\). Valve defects are often not isolated, but associated with other structural defects, which may need extensive repair\(^5\). The most common symptoms associated with valve abnormalities in children include aortic stenosis, mitral stenosis, pulmonary stenosis, or regurgitation\(^5\). Mitral valve defects are primarily treated using reconstructive procedures to correct the abnormal anatomy\(^5\); aortic valve repair, on the other hand, is more complex and not as widely used\(^19,5^3\). Bicuspid aortic valve is a congenital abnormal morphology that occurs in 1-2% of the US population\(^19\). Most subjects do not develop complications (stenosis or incompetence) until after 40 years of age\(^5^4\) and thus are not usually treated as valve disease in pediatric populations (not usually counted in other statistics of congenital valve abnormalities). Congenital pulmonary defects are most often secondary to underdevelopment of portions or the
entire right ventricular outflow tract, including the pulmonary valve. If severe, placement of a conduit is required to facilitate blood flow from the right ventricle to the pulmonary arteries. Unfortunately, these conduits become dysfunction quickly, which requires multiple interventional and surgical procedures throughout patients’ lifetime. Freedom from conduit replacement at 5 and 15 years was 84% and 31% respectively. Isolated congenital pulmonary valve stenosis can sometimes be treated percutaneously with balloon valvuloplasty. However, this carries a risk of inducing regurgitation. Valvular regurgitation due to catheter-based or surgical intervention aimed at treating severe stenosis is the most common form of acquired valve defects in pediatric patients. When reconstruction fails or is not feasible, valve replacement is inevitable. The pulmonary valve is the most common valve replaced in the congenital population. Tricuspid valve abnormalities are fortunately uncommon.

1.3.2 Acquired Heart Valve Disease

1.3.2.1 Rheumatic Disease and Other Pathologies in Children

The etiology of valve disease varies geographically. Congenital heart disease is the most frequent in Europe and North America. Rheumatic fever, which is an inflammatory disease that occurs following pharyngitis due to group A streptococci, remains a major pathology of valve destruction in developing countries. Acute rheumatic fever may cause chronic rheumatic heart disease, which is characterized by
acute inflammation and subsequent scarring that manifest as chronic fibrotic valvular deformities (such as transmural, postinflammatory fibrosis, disruption of layer architecture, and neovascularization). Other less common pathologies include endocarditis, trauma and degenerative disorders.

1.3.2.2 Valve Stenosis and Calcification in Adults

Valve calcification has been identified as the leading cause of valve disease in adults. No longer considered as a passive consequence of aging, it is an active and progressive process that is intimately related to cellular changes, collagen degeneration, and accumulation of proteoglycans and lipids. Calcific aortic valve disease (CAVD) ranges from mild valve thickening, termed aortic sclerosis, to severe stenosis and calcification with obstruction of blood flow. The average time interval from a diagnosis of aortic sclerosis to progression to severe stenosis was 8 years. Approximately 25% of people over 65 years of age demonstrate aortic sclerosis to some degree. However, less than 15% of them progress to aortic stenosis over 2 to 7 years, though reasons for the differences in the patient population are not understood. Calcification can occur in patients with a normal tricuspid aortic valve, but approximately half of all aortic stenosis patients have a bicuspid aortic valve, emphasizing the vulnerability of this population with an underlying congenital abnormalities to calcification. Similar to
atherosclerosis, other risk factors for CAVD have been identified as older age, male gender, hypertension, smoking, hypercholesterolemia, and diabetes\textsuperscript{45}.

There are currently no medical treatments available for CAVD. The cellular mechanisms of valve calcification are of substantial interest as they may represent targets for prevention or treatment. VICs are considered central to this pathogenesis\textsuperscript{19}. Their normal function largely depends on the unique hemodynamic and biomechanical forces under normal valve physiological environments. Under abnormal hemodynamic/mechanical conditions or pathological injury, VICs become activated as a result of activation of VECs and by inflammation and associated cytokine and chemokine signals\textsuperscript{1,19}. Activated myofibroblastic VICs increase the synthesis of matrix proteins; up-regulate the expression of matrix remodeling enzymes; increase proliferation and apoptosis; as well as have the potential to undergo osteogenic differentiation\textsuperscript{1,19}. These processes are regulated by a variety of factors, several secreted by activated VICs. If activation persists, angiogenesis, chronic inflammation, fibrosis and calcification would follow, leading to progressive CAVD\textsuperscript{1,19}.

Studies on CAVD have revealed the side-specific susceptibility of valve cusps to calcification: the aortic side of the valve is more vulnerable to calcification than the ventricular side\textsuperscript{41}. This side-dependent vulnerability to calcification may be explained from several aspects. First, the two sides of valve leaflets experience clearly different
biomechanical forces, and host phenotypically different VECs\textsuperscript{19}. On the fibrosa side, there are more pro-calcification mediators, such as bone morphogenetic protein 4 (BMP4), and less inhibitors of fibrosis and calcification, such as osteoprotegerin, C-type natriuretic peptide, and chordin\textsuperscript{35}. In normal valves, this apparent vulnerability of the fibrosa side is balanced by an enhanced anti-oxidative state, which may protect against inflammation and lesion initiation\textsuperscript{35}. When the hemodynamic state is altered, VECs on the fibrosa side are more prone to increase expression of transforming growth factor (TGF-\(\beta\)1) and BMP4, which are able to elicit pathological differentiation of VICs and calcification of the valve\textsuperscript{60}. Sensitivity of VECs to mechanical stress may partially explain susceptibility to CAVD in patients with hypertension or bicuspid valves where hemodynamic forces are altered\textsuperscript{19}. Second, low-density lipoprotein (LDL) deposition, which is a biochemical stimulus of early CAVD, preferably occurs on the fibrosa side (Figure 4)\textsuperscript{35}. LDL tends to accumulate in the fibrosa, probably due to trapping in the proteoglycans that accumulate in early valve sclerosis. Oxidatively modified LDL (oxLDL) can inflame the endothelium to attract monocytes and active macrophages. Activated macrophages produce enzymes like MMPs and cathepsin S, which mediate ECM remodeling, and cytokines like TGF-\(\beta\)1, TNF-\(\alpha\), IL-1\(\beta\), and RANKL, all of which can potentiate pathological differentiation of VICs to myofibroblast and/or osteoblast lineages\textsuperscript{35}. Moreover, endothelium dysfunction further exacerbates this situation by the
enhanced permeability and apoptosis of VECs, leading to the diffusion of inflammatory cytokines into the tissue and decrease in the expression of protective paracrine signaling molecules\textsuperscript{35}. Additionally, VICs experience stiffer matrix in the fibrosa, which may contribute to the vulnerability of VICs to calcification (Figure 4)\textsuperscript{35}.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{Figure4.png}
\caption{Microenvironmental factors that potentially play a role in pathological differentiation of VICs and lesion formation in the fibrosa layer of the aortic valve. From Yip et al. 2011\textsuperscript{35}.}
\end{figure}

1.3.3 Current Options for Aortic Valve Replacements Are Not Ideal

Both congenital and acquired heart valve diseases can affect valve function, necessitating intervention. Patients with severe aortic stenosis have a mortality rate of
37% at one year after symptom onset without treatment\textsuperscript{61}. While percutaneous or surgical repair is recommended as initial management strategy, valve replacement may become necessary for patients with significant valve destruction and after repair or intervention failure\textsuperscript{2}. Aortic valve replacement has become the second most common cardiac operation in the world\textsuperscript{62}. The number of patients requiring valve replacements is expected to triple from annual \textasciitilde290,000 worldwide in 2003 to over 850,000 by 2050 due to the longer life expectancy and the wider access to diagnostic services\textsuperscript{63}.

Current options for valve replacements include mechanical valves and bioprosthetic valves (Figure 5)\textsuperscript{64}. They have greatly enhanced the survival and quality of life for adult patients. For example, without surgical treatment, a 60-year-old patient with severe aortic stenosis has a life expectancy of approximately 4 years, which increases to 13 years after aortic valve replacement\textsuperscript{63}. However, the common problem of current options is that both of them are non-viable, and thus lack the ability to grow and remodel. While this is not a problem for adult patients, it is a severe issue for children, where multiple valve replacement procedures are typically required through childhood to accommodate the growth of the heart and vasculature. Besides that, each option has their own advantages and problems as discussed below.
Figure 5: Mechanical valves (A) and bioprosthetic valves (B). From Neuenschwander et al. 2004.

Mechanical valves are generally composed of metals, pyrolytic carbon, and expanded poly(tetrafluoroethylene), with various models mainly including caged-ball, tilting disk and bileaflet. Although mechanical valves have evolved significantly toward better fluid mechanics and enhanced durability (>20 years), the risks of infections and thromboembolic complications after mechanical valve implantation are high. All mechanical valve recipients, therefore, must be managed on anticoagulant drugs for the rest of their lives, which induces a substantial risk of hemorrhage. Moreover, anticoagulation is especially incompatible with the active life style of children and significantly increases the risk of fatal complications.

Bioprosthetic valves from decellularized and glutaraldehyde-fixed biological valves (bovine pericardium being most common) do not require anticoagulation medication and allow more physiological hemodynamics. However, structural deterioration and extensive calcification are the major causes of their failure. Overall,
valve-related problems necessitated reoperation or caused death in approximately 60-70% of adult patients within 10-15 years after bioprosthetic valve replacement\textsuperscript{11,13,64}. The deterioration of bioprosthetic valves is closely associated with the chemical, mechanical and morphological changes induced during decellularization and chemical fixation\textsuperscript{29}. First of all, nonviable cells in biological valves are incapable of repairing the cumulative ECM damages, and their fragments cause calcification\textsuperscript{29}. It is hypothesized that phosphates on the cell membranes of devitalized cells can react with calcium in the surrounding fluid, inducing nucleation of calcium phosphate crystals\textsuperscript{19,29}. Second, chemical fixation increases the flexural rigidity of bioprostheses\textsuperscript{68} and locks them into one configuration, which limits the dynamic ECM arrangements (e.g. collagen crimp and unfolding during cardiac cycles) necessary for normal valve function\textsuperscript{29}. Specifically, the risk of valve failure is particularly high for pediatric patients due to accelerated tissue deterioration: the rate of bioprosthetic valve failure is 2.5 times faster in children than that in adults (approximately 10% in 10 years in elderly recipients versus 10% in 4 years in children)\textsuperscript{69}. As a result, bioprostheses have been largely abandoned for aortic valve replacement in children\textsuperscript{70}. As an alternative intervention is the Ross procedure, where patient’s own pulmonary valve (pulmonary autograft) replaces the diseased aortic valve, leaving the far less demanding pulmonary position to be replaced with a bioprosthetic valve\textsuperscript{2}. Pulmonary autograft has become the first choice of aortic valve
replacement in children and adolescents because of the excellent hemodynamic performance, superior longevity, freedom from anticoagulation and the potential for growth. Yet, the Ross procedure is technically more demanding and places two valves at risk for reoperation. The chance of valve failure of the pulmonary autograft (in the aortic position) and the bioprosthetic valve (in the pulmonary position) was 4% and 5% respectively at 10 years in children.

Considering the advantages and drawbacks of mechanical valves and bioprosthetic valves, the following conclusions can be made. Mechanical valves are more suitable for younger adult patients (17-59 years) with relatively higher physical activity levels and who generally tolerate anti-coagulant medication; bioprosthetic valves serve better for older patients (>60-65 years) who are relatively physically inactive; neither of them adequately meets the needs of pediatric and adolescent patients.

1.4 Heart Valve Tissue Engineering (HVTE)

As discussed above, there is no ideal valve substitute to date, particularly for pediatric patients. The difficulties associated with mechanical and bioprosthetic valve replacements (especially in children) have greatly motivated the development of tissue engineering approaches for valve replacement, the goal of which is to construct living valve substitutes, accommodating the requirements to grow, repair and remodel.
(especially in the pediatric population). Such substitutes seeded with living autologous cells are supposed to sense and respond to the changes in environments, and adapt themselves for optimal performance. Toward this goal, many studies have been performed over the past few decades to clarify the desirable characteristics of tissue-engineered heart valves (TEHVs) and to develop strategies for generating these valve substitutes. A widely accepted paradigm of tissue engineering comprises cells, which are seeded on a scaffold; the cell-laden scaffold is then incubated in a bioreactor to produce a more mature tissue construct. Following implantation of the construct, the in vivo stage of tissue growth and remodeling occurs, including cell proliferation and migration, ECM production and alignment, scaffold degradation and tissue remodeling.

This section will provide an overview of the research on the choices of cells and scaffolds for HVTE.

1.4.1 Choice of Cells for HVTE

As aforementioned, VICs are the predominant cell population in valve leaflets, and responsible for active ECM remodeling in valve repair as well as disease progression. To promote VIC-mediated ECM deposition in vitro may be a possible way to recapitulate the ECM components in the valve tissue in the development of living valve substitutes; to understand valve behaviors underlying valve diseases may represent targets for prevention or treatment. For these reasons, as well as their ease of
isolation, VICs have been widely used in the research of HVTE\textsuperscript{5,71–73}. VICs are commonly isolated from fresh porcine valve leaflets using sequential collagenase digestion of the leaflets and rubbing off VECs from the leaflet surfaces \textsuperscript{23,74–78}. After isolation, VICs are commonly plated onto tissue culture flasks for expansion. Due to the heterogeneity, source-dependence, and plasticity of these cells, it can be difficult to keep consistency between cell batches thus complicating the research. Hence, when VICs are used in experimental studies, they are often limited to early passages (P5 or earlier); pooling cells from different sources (different animals, different regions of the valve) may reduce batch-to-batch variation and enhance repeatability. More characterization is needed for a comprehensive understanding of VIC behavior and the molecular mechanisms underlying valve diseases, an understanding that will be beneficial to future HVTE designs.

Stem cells have increasingly been evaluated as potential cell sources for tissue engineering due to their potential to differentiate into various cell types and their self-renewal properties\textsuperscript{79,80}. Ongoing ethical concerns limit the use of embryonic stem cells in research and potential therapies. As an alternative, MSCs from bone marrow may serve as a clinically feasible cell source without raising ethical concerns\textsuperscript{80,81}. Studies have shown that at least 10% of VICs in human adult heart valves are bone marrow derived MSCs, although their role in aortic valve diseases remains unclear\textsuperscript{2}. A key consideration
in the generation of functional valve substitutes is the cellular responses to mechanical forces experienced by heart valves. Cells seeded into valve substitutes should mimic the functions of resident cells in valve leaflets. Studies show that MSCs can be induced to differentiate into a phenotype that resembles VICs\textsuperscript{72,82}. When exposed to the same stretching profile, MSCs demonstrated responses similar to VICs with regard to collagen synthesis\textsuperscript{72}. When implanted into the pulmonary position of sheep with established cardiopulmonary bypass, fibrous scaffolds of blended polyglycolic acid (PGA) and poly-\(L\)-lactic acid (PLLA) seeded with autologous MSCs functioned well for more than 4 months and underwent extensive cellular and ECM remodeling to resemble native valves\textsuperscript{80}. Besides expression of some surface markers (like cluster of differentiation CD73, CD90, CD105 and lack expression of CD14, CD34, CD45 and human leucocyte antigen-DR)\textsuperscript{83}, one critical characteristic of MSCs is the capability of osteogenic, adipogenic and chondrogenic differentiation\textsuperscript{84}. Their osteogenic potential may be a concern in valve calcification. Further characterization of the susceptibility to calcification of MSCs versus VICs under conditions mimicking valve \textit{in vivo} environment is needed.

Circulating endothelial progenitor cells also have the potential to differentiate into endothelial and interstitial-like cells\textsuperscript{85}. The option of creating valve constructs from autologous cells not only eliminates chances of immune rejections but also offers the potential to grow and remodel with recipients\textsuperscript{86}, which is especially important for the
pediatric population. Other autologous cell sources unique to the pediatric population include amniotic fluid, umbilical cord blood, placenta, and chorionic villi\textsuperscript{2,82,87-89}, which allow autologous replacements ready for use at or soon after birth of infant patients. Combined with the emergence of cell banking technology, these sources may have wider applications toward autologous cell based therapies.

\textbf{1.4.2 ECM Regulate VIC Phenotype and Function via Mechanical and Biochemical Stimuli}

ECM plays an important role in the dynamic regulation of VIC behaviors, such as proliferation, phenotype, \textit{de novo} matrix synthesis and force generation\textsuperscript{90}. For example, VIC-HA interactions are crucial in maintaining a healthy VIC phenotype\textsuperscript{5}; disruption of VIC-HA interactions upregulated markers of VIC disease and induced leaflet mineralization. However, the interactions between VICs and ECM are quite complex and not yet fully understood. Great efforts have been made to isolate factors from ECM to interpret roles of each factor. For example, extensive research shows that VIC phenotypes are sensitive to substrate stiffness\textsuperscript{26,27,50,91-94}. Culture of VICs on the surface of substrates of varying stiffness (0.15 kPa to 154 kPa to the order of GPa) suggest that αSMA expression and organization increases with substrate stiffness\textsuperscript{93}. Furthermore, reduction in substrate stiffness (E from ~32 kPa to ~7 kPa) could redirect VICs from activated myofibroblasts into a quiescent state\textsuperscript{26,27}. 

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ECM also interacts with cells via releasing biomolecules, such as growth factors and cytokines, upon cellular demand. For example, TGF-β1 stimulates myofibroblast activation and collagen synthesis by VICs in a dose-dependent manner\textsuperscript{21,95}. TGF-β1 also function synergistically with substrate stiffness and mechanical loading to enhance contractility of VICs\textsuperscript{21,74}. For example, VICs cultured with high levels of stiffness and TGF-β1 demonstrated continual maximal contraction whereas negligible residual tension was stored in the low stiffness and TGF-β1 groups\textsuperscript{74}. On the contrary, basic fibroblast growth factor (FGF) serves to repress myofibroblast activation via the activation of fibroblast growth factor receptor (FGFR) and co-receptor heparan sulfate proteoglycans (HSPGs)\textsuperscript{96}. As a competitive inhibitor of HSPGs, exogenous soluble or immobilized heparin glycosaminoglycan promoted myofibroblast activation and collagen synthesis of VICs\textsuperscript{96}. Further study showed that heparin oligomers could promote or inhibit VIC activation depending on the chain length of saccharides: heparin fragments long enough to extend over the FGF and FGFR promoted growth factor activity whereas sequences too short would bind the FGF, blocking the interaction with full-size heparin and inhibiting its activity\textsuperscript{97}.

1.4.3 Scaffolds for HVTE

Scaffold materials provide temporary structural support during tissue regeneration. Scaffolds for valve substitutes must possess desirable mechanical
properties and appropriate biodegradation, as well as supporting cell adhesion and migration\textsuperscript{19}. As they are replaced by the regenerating tissue, the scaffolds should degrade away via hydrolysis\textsuperscript{98,99} or by cell-secreted enzymes (e.g. MMPs, plasmin, and elastase)\textsuperscript{18,100–103}. The control of degradation rate and profile can be achieved by either manipulating the composition and number of degradable segments\textsuperscript{100–103} or regulating cellular activities\textsuperscript{18}. The balance between scaffold degradation and new ECM deposition is important for creating and maintaining sufficient mechanical properties of the cell-seeded constructs. Generally, scaffold degradation is a result of polymer chain scission and tends to decrease the mechanical properties of the scaffolds whereas new ECM synthesis by seeded cells might simultaneously enhance the mechanical properties\textsuperscript{104}. A more rapid degradation might impair the integrity and mechanical properties of constructs if the synthetic scaffold is lost before sufficient ECM deposition has occurred. On the contrary, a degradation that is too slow might impede tissue regeneration due to the mechanical barrier effect of the scaffolds\textsuperscript{105}. Thus, an ideal situation is that original scaffolds degrade away in balance with tissue generation, which requires the scaffold to possess a controlled degradation rate that matches the new ECM deposition and tissue regeneration\textsuperscript{104}. 
Biodegradable scaffolds that have been used in heart valve tissue engineering can be categorized into thermoplastic polymers (stiff) and hydrogels (soft), as summarized respectively in the following sections.

1.4.4 Application of Thermoplastic Polymers as Scaffolds in HVTE

Thermoplastic polymers have been tested in bioreactors and animal models more often than hydrogels due to their easy fabrication and excellent mechanical strength. The first polymer scaffold to be used as TEHVs was PGA, which is a hydrolytically degradable polyester. John Mayer and his protégés successfully replaced a single pulmonary valve leaflet with cell-seeded PGA scaffolds for 11 weeks in a lamb model\(^98,99\). A substantial limitation of the PGA-based scaffolds is their initial stiffness and thickness, making the creation of complex constructs, such as trileaflet heart valves, difficult. Mayer et al. subsequently evaluated poly(hydroxyoctanoate) (PHO), which is a strong, and flexible polymer. However, PHO has a prolonged biodegradation time, causing it to persist in vivo such that it was not sufficiently replaced by new tissue after 17 weeks\(^106\). Poly(hydroxyalkanoate) (PHA), was also evaluated but did not continue probably due to its low pliability (approximately 15 times stiffer than the tissue)\(^108-109\). Later, Mayer et al. shifted to a composite material composed of PGA coated with poly(4-hydroxybutyrate) (P4HB), which is a thermoplastic, strong, and flexible material with a more rapid biodegradation time than PHO\(^66\). This composite material combines the high
porosity of PGA mesh and the added favorable mechanical properties of P4HB\textsuperscript{86}, and turns out to be the most widely used scaffold material based on the number of publications in the literature (Table 2)\textsuperscript{3,85-87,110-116}. In the majority of these studies, cell-seeded scaffolds were incubated in a pulse duplicator (bioreactor) for a few weeks followed by \textit{in vivo} implantation into the pulmonary position of sheep. It was found that engineered valves grown in the bioreactor showed significantly higher deposition of matrix proteins, a more organized histological structure, and more favorable mechanical properties than did constructs grown under static culture conditions. Cells in the constructs underwent a reversible phenotype change from myofibroblasts in the bioreactor to fibroblasts in the implants after being implanted for 16-20 weeks, evidenced by a decrease in \(\alpha\text{SMA}\) expression; \textit{in vivo} monitoring showed leaflet coaptation, and explanted valves showed near-native trilaminar matrix striation with both endothelial-like and interstitial-like cell phenotypes; however, engineered cusps were still stiffer than the native valve tissues by approximately 3 folds, and there was mild to moderate valve regurgitation present at 16-20 weeks due to central malcoaptation, probably resulting from the shrinkage of the cuspal tissue during the process of scaffold degradation\textsuperscript{3,85,86,110}. Other biodegradable synthetic materials are also being evaluated for their efficacy as TEHVs, such as PLLA, poly(L-lactic-co-glycolic acid) (PLGA), poly(glycerol sebacate) (PGS), poly(\(\varepsilon\)-caprolactone) (PCL), polydioxanone
(PDO) and their composite, as summarized in Table 2. Although showing promise in HVTE, common problems of these organic solvent based acidic polymers (e.g. PGA, PGLA, PLLA, P4HB, and PHA) include that (1) cells cannot be seeded until all cytotoxic solvents are removed, (2) the end products from the degrading acidic polymers change local pH, causing undesired cell death and differentiation, (3) heterogeneous in vivo degradation process may be an issue for composite materials, (4) scaffolds are stiffer than soft tissues, and (5) cell infiltration into scaffolds is limited since they are usually seeded on the scaffold surface rather than the interior.

Table 2. Use of biodegradable, thermoplastic polymers for HVTE.

<table>
<thead>
<tr>
<th>Material</th>
<th>Cells seeded</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyglycolic-acid (PGA)&lt;sup&gt;98,99&lt;/sup&gt;</td>
<td>Fibroblasts and ECs</td>
<td>Increased collagen and elastin content on the TEHV leaflets implanted at the pulmonary position; allograft scaffolds were superior to allogenic scaffolds in terms of inflammation</td>
</tr>
<tr>
<td>Poly(hydroxyalkanoate) (PHA)&lt;sup&gt;107,108&lt;/sup&gt;</td>
<td>Vascular cells</td>
<td>Confluent cell layer on TEHV surface and minor cell ingrowth after incubation in a bioreactor for 8 days; proliferation and collagen production under pulsatile flow conditions</td>
</tr>
<tr>
<td>PGA/PHA&lt;sup&gt;118&lt;/sup&gt;</td>
<td>Vascular cells</td>
<td>Cell proliferation on TEHV surface; increased collagen production and cell alignment under pulsatile flow conditions</td>
</tr>
<tr>
<td>Poly(hydroxyoctanoate) (PHO)&lt;sup&gt;106&lt;/sup&gt;</td>
<td>Vascular cells</td>
<td>Minimal regurgitation of TEHV implanted at pulmonary position; GAGs and collagen deposition; PHO still in tissue by 17 weeks</td>
</tr>
<tr>
<td>Material</td>
<td>Cells and ECs</td>
<td>Description</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>--------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>PGA/PHO</td>
<td>Medial cells and ECs</td>
<td>No thrombus formation with mild regurgitation during 24-week implantation; increased cellular and ECM contents</td>
</tr>
<tr>
<td>PGA/ poly-4-hydroxybutyrate (P4HB)</td>
<td>Medial cells and ECs</td>
<td>αSMA-positive cells in TEHV constructs conditioned in a pulse duplicator for 14 days; fibroblastic cells in TEHV explants implanted for 16-20 weeks; ECM architecture reminiscent of native valves</td>
</tr>
<tr>
<td>PGA/P4HB</td>
<td>Myofibroblasts and ECs</td>
<td>Complete polymer degradation by 8 weeks; comparable mechanical properties to native tissue at 20 weeks; ECM content (collagen, GAGs, and elastin) and DNA content increased to levels of native tissue and higher; mild to moderate valve regurgitation</td>
</tr>
<tr>
<td>PGA/P4HB</td>
<td>VICs</td>
<td>αSMA-positive cells in the TEHVs significantly decreased from ~60% to ~5% after being implanted in the pulmonary position of lambs for 16-20 weeks</td>
</tr>
<tr>
<td>PGA/P4HB</td>
<td>EPCs</td>
<td>Stiffer than native valves; presence of both EC markers and αSMA</td>
</tr>
<tr>
<td>PGA/P4HB</td>
<td>Bone marrow-derived mononuclear cells</td>
<td>Cell-seeded scaffolds integrated into self-expanding nitinol stents were implanted at pulmonary valve position; substantial cellular remodeling and in-growth into the scaffold materials resulted in layered, endothelialized tissues</td>
</tr>
<tr>
<td>PGA/P4HB</td>
<td>Human amniotic progenitors</td>
<td>Amniotic progenitors demonstrated characteristics of mesenchymal progenitors and functional endothelial cells; engineered heart valve leaflets demonstrated endothelialized tissue formation and ECM production</td>
</tr>
</tbody>
</table>

30
<table>
<thead>
<tr>
<th>Material</th>
<th>Cell Type</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA/P4HB&lt;sup&gt;113–115&lt;/sup&gt;</td>
<td>Harvested from the human vena saphena magna</td>
<td>Mechanical loading led to collagen fiber architecture development toward native structural properties but the effect was small within tested range; scaffolds were stiffer than the native valves; superior tissue formation and mechanical properties in the strained valves than non-loaded controls; proper opening motion but suboptimal closure dynamics when exposed to physiological aortic valve flow</td>
</tr>
<tr>
<td>PGA/P4HB, and PGA/PLLA/P4HB&lt;sup&gt;116&lt;/sup&gt;</td>
<td>None</td>
<td>Dynamically flexed scaffolds were less stiff than static controls; directional anisotropy only in scaffolds with PLLA fibers</td>
</tr>
<tr>
<td>PGA/poly(L-lactic acid) (PLLA)&lt;sup&gt;120&lt;/sup&gt;</td>
<td>SMCs</td>
<td>Cyclic flexure increased the effective stiffness and collagen deposition of cell-seeded scaffolds</td>
</tr>
<tr>
<td>PGA/PLLA&lt;sup&gt;121&lt;/sup&gt;</td>
<td>MSCs</td>
<td>Both basic fibroblast growth factor supplementation and dynamic flexure and flow conditioning increased collagen production</td>
</tr>
<tr>
<td>PGA/PLLA&lt;sup&gt;122&lt;/sup&gt;</td>
<td>MSCs</td>
<td>Valve conduit cusps were increasingly attenuated and regurgitant after 6-week implantation at pulmonary position</td>
</tr>
<tr>
<td>PGA/PLLA&lt;sup&gt;80&lt;/sup&gt;</td>
<td>MSCs</td>
<td>Trivial or mild regurgitation at implantation</td>
</tr>
<tr>
<td>PGA/poly(L-lactic-co-glycolic acid) (PLGA)&lt;sup&gt;123&lt;/sup&gt;</td>
<td>Fibroblasts and ECs</td>
<td>A complete endothelial lining with a fibroblast-mesh core</td>
</tr>
<tr>
<td>Poly(glycerol sebacate) (PGS)&lt;sup&gt;124&lt;/sup&gt;</td>
<td>VICs</td>
<td>VIC-seeded PGS scaffolds either retained or exceeded initial mechanical properties, paralleling the accretion of collagen, whereas the mechanical properties of unseeded scaffolds progressively diminished with time</td>
</tr>
<tr>
<td>Poly(ε-caprolactone) (PCL)&lt;sup&gt;125&lt;/sup&gt;</td>
<td>None</td>
<td>Random fiber orientation with 90% porosity; slight rotation of leaflets during pulse duplicator testing</td>
</tr>
</tbody>
</table>
Mechanical properties of PGS/PCL scaffolds decreased gradually with no significant change for PCL scaffolds during accelerated degradation; VICs seeded on PGS/PCL scaffolds showed higher ECM secretion compared with PCL scaffolds.

Less fibrosis, less calcification and no thrombus compared with control PTFE patches; PDO scaffolds were degraded completely and replaced by an endothelialized tissue and ECM after implantation for 8 months.

<table>
<thead>
<tr>
<th>Material</th>
<th>Cells</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGS/PCL&lt;sup&gt;104&lt;/sup&gt;</td>
<td>VICs</td>
<td>Mechanical properties of PGS/PCL scaffolds decreased gradually with no significant change for PCL scaffolds during accelerated degradation; VICs seeded on PGS/PCL scaffolds showed higher ECM secretion compared with PCL scaffolds.</td>
</tr>
<tr>
<td>Polydioxanone (PDO)&lt;sup&gt;117&lt;/sup&gt;</td>
<td>MSCs</td>
<td>Less fibrosis, less calcification and no thrombus compared with control PTFE patches; PDO scaffolds were degraded completely and replaced by an endothelialized tissue and ECM after implantation for 8 months.</td>
</tr>
</tbody>
</table>

### 1.4.5 Application of Hydrogels in HVTE

Hydrogels, with hydrophilic and crosslinked structures, are attractive scaffold materials for HVTE because they provide a hydrated environment similar to soft tissues that allows the exchange of oxygen, nutrients and cellular wastes via diffusion<sup>105,126</sup>. Hydrogels can be made from either natural materials (such as collagen<sup>94,127–129</sup>, fibrin<sup>130–135</sup> and hyaluronic acid<sup>71,92,136</sup>), synthetic polymers (such as polyethylene glycol (PEG)<sup>105,137–140</sup>) or as composites, as summarized below.

#### 1.4.5.1 Natural Hydrogels

Natural hydrogels are composed of materials found in native ECM, such as collagen<sup>94,127–129</sup>, gelatin<sup>141</sup>, fibrin<sup>130–135</sup> and hyaluronic acid (HA)<sup>71,92,136</sup>. These materials have the advantage of being composed of the natural ECM that may carry abundant
adhesion and proteolytic degradation site, which may explain their widely use in tissue engineering.

Collagen is the most prevalent protein in tissue, constituting 25% of total protein mass found in the human body\textsuperscript{142}. Collagen is characteristically defined by the triple helix of three $\alpha$-chains. Each of the three chains is a repetitive series of the amino acid sequence (Gly-Xaa-Yaa) (Figure 6), where glycine allows the triple helix to pack tightly due to its small size, and Xaa and Yaa can be any amino acids, with proline or hydroxyproline occupying the position 20-22\% of the time\textsuperscript{142}. The hydroxyl groups on hydroxyproline allow for inter-chain hydrogen bonds that stabilize the triple helix\textsuperscript{143}. \textit{In vivo}, collagen molecules are synthesized as soluble precursors (procollagen), which are glycosylated and hydroxylated prior to self-assembly of the alpha chains. After secretion, the triple helix is cleaved by procollagen metalloproteinases. The collagen molecules can then self-assemble into periodic cross-striated fibrils, which are stabilized by the inter- and intra-molecular covalent bonds between collagen polypeptide chains\textsuperscript{144}. Collagen fibrils further aggregate into collagen fibers that assemble to form the macroscopic structures. Collagen hydrogels can be prepared from purified type I collagen extracted from skin or tendons. Human recombinant collagen is a possible alternative to that extracted from animal tissue, and the protein sequence can be designed based on the chemistry and structure of the collagen molecule\textsuperscript{145}. However, the
requirement for post-translational modification (such as proline hydroxylation) remains a significant obstacle in achieving large scale production\textsuperscript{146}. Collagen molecules (either from animal or synthesized) can be dissolved in acetic acid solution. When the acidic collagen solution is neutralized or warmed, fibrillogenesis and self-assembly of collagen fibrils occur, leading to formation of a hydrogel. The fibril diameter in the hydrogel, which is correlated with its mechanical properties, is influenced by solution pH, ionic strength, ion types, and gelling temperature\textsuperscript{147}. Because collagen is a predominant ECM component of the heart valve, its use has the potential to maintain distinct valvular mechanical properties and facilitate the interaction between valvular cells and the ECM. Collagen hydrogels have been shown to support valvular cell growth and proliferation\textsuperscript{94,127–129}. Cell alignment, proliferation, apoptosis, phenotype, and ECM production within collagen hydrogels can be regulated via applying mechanical loading (Table 3)\textsuperscript{127,129}. However, collagen gels are mechanically weaker than the native heart valve ECM for several reasons, such as low protein concentration, poor fibrillogenesis and fiber assembly, low crosslinking and lack of alignment. Higher protein concentration and the use of chemical crosslinking strategy result in higher strength collagen gels but also result in unfavorably dense microstructure, which restricts diffusion of oxygen and nutrients to cells\textsuperscript{2}.
Fibrin gels have also been used as scaffolds. Fibrin, the final product in the coagulation pathway, is converted from the plasma protein fibrinogen in the presence of the activated protease thrombin and calcium. An important advantage of fibrin use is that soluble fibrinogen in blood plasma can be collected from patients as an autologous source. Fibrinogen is made up of 6 paired polypeptide chains held together by disulfide bonds. Fibrin polymerization process is initiated by the thrombin cleavage of fibrinopeptides to produce fibrin monomer, which interacts to form oligomer and protofibrils via knob-hole interactions. When the twisted protofibrils are present in a sufficient concentration, they assemble into fibers that form the fibrin network. Calcium binding is important for fibrinogen stability and promotes polymerization.

Fibrin gels have been used for tissue engineering applications due to their availability as an autologous source and attractive bioactivity. Cells entrapped in fibrin gels were reported to have a higher proliferation and ECM production than those in collagen.
Furthermore, the structure, mechanical properties and degradation of fibrin hydrogels can be tuned by the initial fibrinogen, thrombin, and calcium formula and controlling the polymerization process\(^\text{150}\). Flanagan et al. prepared fibrin-based heart valve scaffolds using injection molding, where a mixture of cells, calcium and thrombin were injected into a mold consisting of a housing with silicone support, and vascular and ventricular stamp to form a heart-valve shape (Figure 7)\(^\text{151}\). The simultaneous addition of fibrinogen into the mold using a dual syringe system immediately initiated the fibrin polymerization process\(^\text{151}\). The molded valve conduits were then transferred to a bioreactor for mechanical conditioning. It was found that the dynamic conditioning increased cell attachment/alignment and expression of \(\alpha\)SMA as well as ECM deposition\(^\text{151}\). Implantation of the conditioned constructs in the pulmonary trunk in a sheep model for 3 months showed that fibrin scaffolds were completely absorbed and replaced with ECM proteins; a confluent monolayer of endothelial cells was present on the valve surface; however, leaflets demonstrated insufficiency because of scaffold compaction\(^\text{130}\), which is a common problem for collagen and fibrin scaffolds due to cell-mediated contraction. When active cell force is inhibited, total retraction (shrinking in size) is decreased by 80% in collagen gels and 75% in fibrin gels\(^\text{74}\). Tranquillo and colleagues attempted to overcome this problem by treating the fibrin-based TEHVs with blebbistatin (myosin II inhibitor) a few days prior to implantation at the end of a 3-week
static culture. The valves were initially functional but failed after four weeks due to tissue shrinkage that was only delayed, not prevented\textsuperscript{152}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Valve mold and fibrin-based valve conduit. (A) Valve mold. (B-C) Fibrin-based valves with (B) and without (C) silicon support. Adapted from Flanagan et al. 2007\textsuperscript{151}.
}
\end{figure}

HA (or hyaluronan) is a natural GAG that has been widely found in all tissues and body fluids of vertebrates as well as in some bacteria with high structural homology across the species, making it less immunogenic and relatively biocompatible\textsuperscript{153,154}. Distinct from other GAGs, HA cannot be sulfated, nor does it attach to a core protein to form a proteoglycan. This natural macromolecule consists of alternating D-glucuronic
acid and N-acetyl-D-glucosamine (Figure 8), and is characteristic of its big size, with a MW reaching the millions, a chain length up to 25,000 disaccharides\textsuperscript{153}. HA is hydrophilic and highly negatively charged that attracts cations and takes in a large amount of water, expanding as high as 1000 times its solid volume\textsuperscript{155}. In human heart valve leaflets, HA constitutes up to 60\% of the total GAGs\textsuperscript{156} and plays an essential role in valvular cell-matrix interactions via cell-membrane receptors CD44, ICAM-1 and RHAMM\textsuperscript{5,157}. Disruption of VIC-HA interactions upregulates markers of VIC disease and induces leaflet mineralization\textsuperscript{5}. It also provides compressive resistance in the cardiac cycle and serves as a lubricant for tissue motion as its viscosity is inversely proportional to shear rate (known as shear thinning)\textsuperscript{154}. HA can be quickly degraded \textit{in vivo} by hyaluronidases with a half-life ranging from less than one to several days\textsuperscript{153}. Generally, small HA fragments (~1,000 saccharides) are signals of inflammation, immune stimulation, and angiogenesis leading to the activation of specific cells, while large fragments (>1,000-5,000 saccharides) are involved in maintaining tissue structural integrity and cell quiescence\textsuperscript{158}. The use of HA in HVTE is summarized in Table 3. The MW and stiffness of HA hydrogels influence valvular cell spreading, proliferation, and ECM production.
Figure 8. Chemical structure of the disaccharide unit of HA.

<table>
<thead>
<tr>
<th>Material</th>
<th>Cells seeded</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen⁹⁴</td>
<td>VICs</td>
<td>Increasing anisotropy of biaxial strain significantly enhances VIC proliferation, apoptosis, and the expression of vimentin and αSMA; it also results in increased cellular orientation, followed by collagen fiber alignment, along the principal directions of strain</td>
</tr>
<tr>
<td>Collagen¹²⁷</td>
<td>VECs and VICs</td>
<td>αSMA expression of VICs increased by shear stress; VECs stimulated VIC differentiation to a more quiescent phenotype</td>
</tr>
<tr>
<td>Collagen¹²⁸</td>
<td>VICs or SMCs</td>
<td>VICs and SMCs compacted collagen gels similarly, and expressed similar levels of αSMA but differing amounts of desmin; VICs produced more protein and GAGs than SMCs</td>
</tr>
<tr>
<td>Collagen¹²⁹</td>
<td>VICs</td>
<td>Cyclic-stretch upregulated GAG production by VICs; mechanical stretch and relaxation reversibly regulate GAG and PG production</td>
</tr>
<tr>
<td>Fibrin¹³¹</td>
<td>Fibroblasts</td>
<td>Collagen fibers produced by cells coaligned with the fibrin; the resultant tensile mechanical properties of constructs were anisotropic</td>
</tr>
<tr>
<td>Fibrin¹³²</td>
<td>Fibroblasts or VICs</td>
<td>VICs remodeled fibrin faster than fibroblasts</td>
</tr>
<tr>
<td>Fibrin¹³⁴,¹³⁵</td>
<td>VICs or fibroblasts</td>
<td>Cyclic stretching of cell-laden constructs with an incremental strain amplitude leads to improved tensile properties compared to static controls, correlating with increased collagen deposition and maturation</td>
</tr>
<tr>
<td>Material</td>
<td>Cells/Proteins</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Fibrin 151</td>
<td>SMCs and fibroblasts</td>
<td>Valve leaflets conditioned in bioreactors showed reduced tissue shrinkage compared to stirred controls; dynamic conditioning increased cell attachment/alignment and expression of αSMA as well as ECM deposition</td>
</tr>
<tr>
<td>Fibrin 130</td>
<td>Myofibroblasts and ECs</td>
<td>After implantation for 3 months, fibrin scaffolds were completely absorbed and replaced with ECM proteins; a confluent monolayer of endothelial cells was present on the valve surface; however, leaflets demonstrated insufficiency because of tissue contraction</td>
</tr>
<tr>
<td>Fibrin 74</td>
<td>VICs</td>
<td>Transforming growth factor-β1 (TGF-β1) function synergistically with substrate stiffness to enhance contractility of VICs; VICs cultured with high levels of stiffness and TGF-β1 demonstrated continual maximal contraction whereas negligible residual tension was stored in the low stiffness and TGF-β1 groups</td>
</tr>
<tr>
<td>Fibrin 133</td>
<td>None</td>
<td>Fibrin-based constructs responded to biaxial tension differently than native valve tissue</td>
</tr>
<tr>
<td>HA 71</td>
<td>VICs</td>
<td>Low molecular weight HA (&lt;6700 Da) degradation products stimulated VIC proliferation and matrix production</td>
</tr>
<tr>
<td>HA 92</td>
<td>VICs</td>
<td>VICs encapsulated in HA hydrogels with lower mechanical stiffness showed a more spreading morphology compared to their sitter counterparts and dramatically up-regulated αSMA expression</td>
</tr>
<tr>
<td>HA 136</td>
<td>SMCs</td>
<td>SMCs on HA gels secreted more elastin and less collagen than that on plastic</td>
</tr>
</tbody>
</table>

### 1.4.5.2 Synthetic Hydrogels

Although naturally derived materials have been widely used and provided knowledge for HVTE, they carry risks of inducing immune rejection due to immunogenic remnants, and also suffer from poorly controlled cell-matrix interactions.
due to batch-to-batch variation and complex interactions with cells via coupling membrane receptors and sequestering growth factors and cytokines\cite{159,160}, which render it difficult to deconvolute the effects of specific factors on VIC behaviors. For example, the hyaluronan fragments released from hyaluronan scaffolds can influence collagen and elastin secretion in a molecular weight-dependent manner\cite{71,73}, which renders it difficult to separate the influence of the biomaterial scaffold degradation from other factors in culture.

What makes synthetic hydrogels attractive is that their mechanical and biochemical properties can be independently modulated at design, which provides fine control over the pericellular environment. First, the mechanical properties of hydrogels can be modulated via varying hydrogel composition and anisotropy, which can be used to direct cell behaviors\cite{161}. For example, hydrogel stiffness can be adjusted via varying polymer molecular weight\cite{162}, polymer weight percentage\cite{163}, and number of functional groups on each monomer or polymer precursor\cite{93}. Almost universally for most cell types, stiffer substrates facilitate cell adhesion, spread and proliferation on 2D surfaces\cite{164,165}. Many hydrogels can be formed under cytocompatible conditions that enable cell encapsulation in 3D, which immediately achieves even cell distribution with high viability within scaffolds. In 3D scaffolds, matrix stiffness affects cell behaviors in a more complex manner. For example, αSMA expression of VICs in stiffer PEG gels was less
than that in the soft ones, which is opposite to that seen in 2D. Substrate stiffness can also direct cell differentiation toward different lineages. Generally, gels mimicking the stiffness of target tissues are desirable to induce and maintain cell functions.

Moreover, anisotropy of hydrogel scaffolds can be induced by applying a controlled strain, creating patterned structure, or aligning fibers. Scaffold anisotropy not only enhances cellular and ECM alignment, but also influences cell phenotype, proliferation and apoptosis.

Second, these hydrogels are inherently bioinert due to their hydrophilic and neutral properties, serving as a blank slate, to which bioactive molecules can be added to control cell-matrix interactions. For example, the incorporation of cell adhesive ligands enhances cell attachment; the inclusion of MMP-sensitive peptides allows hydrogel degradation; these two modifications together allow cell spreading, migration and differentiation. In addition, the optical clarity of most hydrogels enables microscopy-based assays of cell function. This controlled, synthetic microenvironment can be used to achieve a better understanding of cell responses to specific cues, which in return can be applied to direct biomaterial design of tissue substitutes (Figure 9).
Figure 9. Application of bioactive hydrogels, with tunable biomechanical and biochemical properties to better understand and direct cell fate, which in return can be used to direct biomaterials design.

Synthetic hydrogels that have been used in HVTE include PEG\textsuperscript{26,27,39,40,73,95,166,173–177} and polyvinyl alcohol (PVA)\textsuperscript{178,179} hydrogels (Table 4). PEG is a polyether with repeating units of $\text{-(CH}_2\text{CH}_2\text{O)}$. PEG is commonly modified with reactive acrylate groups, which immediately form hydrogels in the presence of photoinitiators and proper light sources (UV or white light). The first trial using PEG for valvular cell study traces back to 2008, where Kristi Anseth and her protégés seeded VICs on the surfaces of PEG
hydrogels coated with various natural ECM proteins. They showed that PEG hydrogels repressed VIC calcification regardless of the surface chemistry compared to protein-coated tissue culture polystyrene (TCPS) plates\textsuperscript{23}, presumably due to the dramatic difference in stiffness. Later on, the Anseth group investigated VIC behaviors on the surfaces of PEG hydrogels functionalized with cell-adhesive ligands RGDS. They iterated that the quiescent, fibroblast phenotype of VICs was better preserved on the surfaces of soft PEG hydrogels whereas activation occurred with the increase in substrate stiffness\textsuperscript{26,27,176}, involving PI3K/AKT pathway\textsuperscript{176}. When PI3K or AKT was inhibited on stiff substrates, myofibroblast activation was blocked whereas when active PI3K was overexpressed, activation was promoted even on soft substrates, indicating that this pathway may be worthwhile target for treating valve fibrosis associated with tissue stiffening\textsuperscript{176}. Reduction in substrate stiffness (E from \textasciitilde32 kPa to \textasciitilde7 kPa) could redirect VICs from activated myofibroblasts into a quiescent state within 48 hours following substrate elasticity reduction (Figure 10)\textsuperscript{26,27}. Moreover, the de-activated fibroblasts were in a reversible state that could be re-activated to enter cell cycle in response to TGF-\textbeta\textsuperscript{177}. 3D culture guides cells to produce \textit{in vitro} responses that are more physiologically relevant than does planar 2D culture via affecting integrin ligation, cell contraction and associated intracellular signaling\textsuperscript{180}. The development of biodegradable PEG hydrogels via incorporation of MMP-sensitive peptides into the backbone of
hydrogels enabled the study of valvular cells within 3D materials better mimicking their in vivo environment. The formation of the cell-laden hydrogels using light-induced crosslinking is mild enough to allow cell encapsulation with high viability\textsuperscript{95}. Cell spreading, phenotype and ECM production of encapsulated VICs also depended on matrix stiffness as well as the type and density of adhesion ligands, however, in a quite different manner than did on 2D surface\textsuperscript{95,166,175}. VICs attained a spread morphology, proliferated and migrated within hydrogels\textsuperscript{95}. VIC process extension and integrin $\alpha_\nu\beta_3$ expression increased with RGD concentration within two days, but equalized between conditions at 10 days\textsuperscript{95}. The rate of VIC process extension also increased with decreasing the hydrogel matrix density presented to cells\textsuperscript{95}, indicating that stiffer substrates constrain VIC spreading, which is the opposite to that on 2D. Also in contrast to 2D, $\alpha$SMA expression of VICs decreased when substrate was stiffened (from 0.24 kPa to 1.2 kPa) via a second photoinitiated thiol-ene polymerization\textsuperscript{166}. 
Figure 10. Reduced myofibroblast activation of VICs in response to lowering substrate modulus. Blue = DAPI; green = αSMA. From Wang et al. 2012\textsuperscript{27}.

PVA, consisting of repeating units of $-\text{[CH}_2\text{CH(OH)]-}$, is bio inert and therefore, relatively biocompatible\textsuperscript{181}. Unlike PEG with only hydroxyl groups at the end of polymer chains, there are abundant hydroxyl groups on PVA that can be readily modified with biomolecules. While this provides ample sites for bioconjugation, it also raises concerns about complement activation via the alternative pathway \textit{in vivo}. PVA hydrogels can be fabricated via either chemical crosslinking\textsuperscript{181} or physical crosslinking\textsuperscript{178,179}. Similar to PEG, photoinitiators can be used for light-controlled chemical crosslinking\textsuperscript{181}. Hydrophobicity of hydrogels can be modulated via grafting hydrophobic segments,
such as PLA. VIC adhesion on hydrogels can be improved upon increasing hydrogel hydrophobicity or adding adhesive peptides.

Table 4. Use of synthetic hydrogels in heart valve tissue engineering.

<table>
<thead>
<tr>
<th>Material</th>
<th>Cells</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG&lt;sup&gt;23&lt;/sup&gt;</td>
<td>VICs</td>
<td>Calcification was repressed on PEG hydrogels regardless of the surface chemistry compared to TCPS</td>
</tr>
<tr>
<td>PEG&lt;sup&gt;174&lt;/sup&gt;</td>
<td>VICs</td>
<td>Manipulating the composition (e.g. molecular weight or weight fraction) of the hydrogels resulted in varied mesh sizes and swelling ratios and a flexural stiffness comparable with native tissues (15-220 kPa)</td>
</tr>
<tr>
<td>PEG&lt;sup&gt;95&lt;/sup&gt;</td>
<td>VICs</td>
<td>VIC process extension increased with the increase of RGD concentration and the decrease of hydrogel matrix density during the first few days of encapsulation within MMP-degradable PEG hydrogels; TGF-β1 increased expression of αSMA and collagen-I at both the mRNA and protein level</td>
</tr>
<tr>
<td>PEG&lt;sup&gt;175&lt;/sup&gt;</td>
<td>VICs</td>
<td>VICs were more elongated in RGDS-containing gels compared to VGVAPG or P15, correlating with MMP activity; αSMA positive VICs were higher in VGVAPG than RGDS and P15 group</td>
</tr>
<tr>
<td>PEG&lt;sup&gt;26,27&lt;/sup&gt;</td>
<td>VICs</td>
<td>Reduction in substrate stiffness (E from ~32 kPa to ~7 kPa) could redirect VICs from activated myofibroblasts into a quiescent state</td>
</tr>
<tr>
<td>PEG&lt;sup&gt;166&lt;/sup&gt;</td>
<td>VICs</td>
<td>Soft hydrogels preserved the quiescent fibroblast phenotype of VICs much better than stiff plastic plates; valvular fibroblasts were sensing the changes in matrix elasticity through the PI3K/AKT pathway</td>
</tr>
<tr>
<td>PEG&lt;sup&gt;166&lt;/sup&gt;</td>
<td>VICs</td>
<td>VICs spread in soft hydrogels (0.24 kPa) and were constrained in stiff hydrogels (12 kPa); αSMA expression of VICs within hydrogels decreased upon hydrogel stiffening</td>
</tr>
</tbody>
</table>
Anisotropic mechanical properties were established in PEG hydrogels by crosslinking stripes of low MW PEG within high MW PEG base hydrogels; the morphology and activation of VICs seeded atop PEG hydrogels could be modulated by controlling the concentration or micro-patterning profile of PEG-RGDS.

Co-culture with VECs decreased VIC myofibroblast activation via nitric oxide release, which was abolished by inhibiting cGMP pathway.

Spatially localized cell adhesive ligands bound in the scaffold promoted cell growth and organization of VICs and VECs in 3D co-culture; both cell types maintained phenotypes, homeostatic functions, and produced zonally localized extracellular matrix.

Tensile properties of PVA hydrogels can be optimized by controlling freeze-thaw cycles.

A trileaflet heart valves from PVA hydrogels were fabricated.

### 1.4.5.3 Composite Hydrogels

Various composite hydrogels have also been developed to mimic valve structure and properties, such as PEG/alginate composite, gelatin/alginate composite, collagen/polyurethane composite, and PVA/PLA composite. For example, PEG hydrogels blended with alginate can recapitulate the complex shape of heart valves with great fidelity using 3D bioprinting. Low mechanical strength is a common limitation for hydrogels in HVTE. In order to strengthen soft hydrogels and mimic the anisotropy in the valve tissue, stiff thermoplastic polymers have been used to make composites.
with hydrogels. For example, PEG/PCL composites showed enhanced mechanical properties and anisotropy with elastic moduli of ~3.79 MPa in the parallel direction and ~0.46 MPa in the perpendicular direction\textsuperscript{172}.

Researchers have also investigated valvular cell behaviors in response to hydrogel compositions (collagen/chondroitin sulfate composite\textsuperscript{184}, HA-gelatin hybrid\textsuperscript{92,185}, and HA-PEG hybrid\textsuperscript{73}). For example, compared to collagen alone hydrogels, the addition of chondroitin sulfate to collagen constructs increased matrix porosity, enhanced VEC surface coverage and its expression of eNOS, as well as ECM production by VICs including elastin and laminin\textsuperscript{184}.

### 1.5 Summary and Overview of Dissertation

In summary, the field of HVTE has seen many significant advances in the last few decades. The development of new biomaterials/culture platforms has enabled the investigation of valvular cell behaviors and cell-cell, cell-matrix interactions, progressing our understanding of the valvular diseases as well as the development of new treatments (medications or new living devices). However, significant hurdles still exist to construct a functional valve substitute largely due to the incomplete understanding and poor control over cellular behaviors (e.g. heterogeneous and dynamic phenotype, ECM remodeling and calcification). This dissertation outlines our efforts in understanding and regulating VIC behaviors within 3D, biomimetic hydrogels via either
free or immobilized biochemical cues, especially to promote VIC-mediated ECM remodeling without inducing detrimental differentiation or calcification. Chapter 2 describes the development of the cell-adhesive, proteolytically degradable, PEG-based hydrogels that was used for the 3D culture and study of VICs. In Chapter 3, in an effort to promote collagen deposition by VICs, a biomolecule, ascorbic acid was supplemented in the culture media to investigate its effects on VICs. Chapter 4 explore the use of different ECM-mimicking adhesive peptides with specific affinity to each receptor to regulate VIC phenotype and ECM production. Chapter 5 demonstrates the synergistic effects of AA supplementation and DGEA immobilization on the ECM deposition by VICs. Chapter 6 summarizes the work in this dissertation and suggests potential future research directions that may lead to a breakthrough in HVTE.
Chapter 2 A cell-adhesive, proteolytically degradable, 3D hydrogel platform for in vitro VIC culture and study toward the development of living valve substitutes†

2.1 Introduction

As discussed in Chapter 1, to date, there is no ideal valve substitute for pediatric and adolescent patients needing valve replacements, largely due to the lack of growth potential of current options to accommodate the somatic growth of patients\textsuperscript{11,13}. Tissue engineering may offer a better solution via regenerating a living valve substitute that would obviate the complications of conventional valve replacement devices and grow with patients. The substitute should contain the cellular and ECM components reminiscent of the valve tissue. The long-term success of the substitute will depend on the ability of its viable cellular components to assume normal function with the capacity to repair structural injury, remodel ECM, and potentially grow with patients\textsuperscript{19}. As aforementioned, VICs are the predominant population in the valve leaflets and responsible for ECM maintenance and remodeling during valve homeostasis as well as disease progression\textsuperscript{3,18}. VICs assume quiescent, fibroblastic phenotype during valve

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homeostasis, which, however, activate into myofibroblasts or osteoblast-like cells that are associated with disease progression (valve fibrosis and calcification)\textsuperscript{1-5}. To restore VIC quiescent, fibroblastic phenotype and function \textit{in vitro} is critical to the success of the valve substitute.

As discussed in Chapter 1, VIC culture on 2D substrates \textit{in vitro} fails to recapitulate VIC \textit{in vivo} phenotype in the valve tissue. A culture platform mimicking the characteristics of the native valve tissue to preserve VIC quiescent phenotype is needed. Compared to 2D, the 3D culture may promote cells to produce more physiologically relevant cellular responses \textit{in vitro}\textsuperscript{180}. Therefore, great efforts have been made to seed VICs into a 3D scaffold. In some attempts, VICs were seeded on top of preformed meshlike synthetic\textsuperscript{14} or natural\textsuperscript{186} matrix scaffolds and left to migrate to the interior. Since it may take several months to achieve complete cell infiltration, time delay and uneven cell distribution are an issue. Apart from the disadvantages of this approach, living cells can be homogeneously encapsulated inside some types of hydrogels with cytocompatible crosslinking techniques. Cell-laden hydrogels that have been studied in heart valve tissue engineering include naturally-derived molecules (\textit{i.e.}, collagen\textsuperscript{94}, HA\textsuperscript{5,71}, fibrin\textsuperscript{130,149}) and synthetic polymers (PEG\textsuperscript{73}). Natural materials have been the primary 3D matrices because of their advantages of being composed of the natural ECM that may carry abundant adhesion and proteolytic degradation sites. However, these
natural materials harbor batch-to-batch variability and interact with cells in a complex manner via coupling membrane receptors and sequestering growth factors and cytokines\textsuperscript{159,160}, which render it difficult to deconvolute the effects of specific factors on VIC behaviors. Synthetic PEG hydrogels are inherently biocompatible, bioinert and resist non-specific protein adsorption due to their hydrophilic and neutral properties\textsuperscript{161}. They provide a blank slate to which bioactive molecules can be added to control cell-material interactions. An advantage of PEG hydrogels is that the cell-matrix interactions are limited to the designed factors put into the system, excluding the confounding factors associated with the use of natural materials. For example, the inclusion of cell adhesive ligands and proteolytically degradable peptides allows control over cell attachment, spreading, and ECM remodeling\textsuperscript{137–139}. Previous work by Benton et al. showed that VICs could attain a spread morphology and proliferate in 3D PEG hydrogels that were functionalized with an adhesive peptide (RGD) and a MMP-sensitive peptide (GPQGIWGQ)\textsuperscript{95}. However, much remains to be learned regarding the VIC behaviors on 2D substrates versus inside 3D hydrogels.

This work aimed to develop a cell-adhesive, proteolytically degradable, PEG-based hydrogel platform for the 3D culture and study of VICs, and to investigate VIC responses on 2D stiff substrates versus inside hydrogels. We demonstrated that the soft PEG-based hydrogels developed in this work supported the long-term VIC growth and
ECM remodeling. As VIC quiescence and resistance to calcification are critical to the success of valve substitutes, we also tested VIC responses under the challenges of osteogenic media conditions in terms of osteogenic differentiation, indicated by the upregulation of ALP expression, and calcification, detected by a calcium-binding dye. We found that on 2D stiff substrates, VICs underwent osteogenic differentiation and showed extensive calcification, however, within 3D hydrogels, VIC quiescent phenotype was well preserved and little calcification was observed. A better understanding of VIC biology and pathology in biomimetic conditions not only is crucial to the development of living valve substitutes for pediatric and adolescent patients but also may lead to medical treatments against valvular diseases such as calcific aortic valve stenosis in the elderly.

2.2 Materials and Methods

All reagents were purchased from Sigma-Aldrich unless otherwise noted.

2.2.1 Cell Isolation and Culture

Primary VICs were harvested from aortic valve leaflets dissected out of fresh porcine hearts from a commercial abattoir (Fisher Ham and Meats, Spring, TX, USA). The leaflets were incubated with collagenase type II (550 U/mL, 30 min; Worthington Biochemical Corp, Lakewood, NJ) followed by manually scraping off endothelial cells and mincing the leaflets, which were then digested with collagenase type III (260 U/mL,
4 h; Worthington) in an incubated shaker. After excluding contaminations, isolations from six different animals were pooled together to compensate for biological variability and frozen down at passage two for storage. When needed, VICs were thawed and plated onto tissue culture polystyrene flasks until near confluency in growth media (GM) [1:1 DMEM (Corning, Tewksbury, MA, USA):F12 (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA) with 10% bovine growth serum (BGS; Hyclone), 1.6% 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Thermo Fisher Scientific, Waltham, MA, USA), and 1% antibiotic-antimycotic solution (Lonza, Walkerville, MD)] with media change every 2-3 days before being seeded on/in substrates. For osteogenic media treatment, VIC standard growth media was supplemented with 50 µg/mL ascorbic acid (AA), 10 mM β-glycerophosphate disodium and 10 nM dexamethasone (Osteogenic media; Osteo M).

Porcine aortic smooth muscle cells (SMCs) at passage one were purchased frozen from Cell Applications, Inc. (San Diego, CA, USA). Upon thawing, SMCs were plated onto tissue culture polystyrene flasks and cultured in SMC All-in-one Ready-to-use Growth Medium (Cell Applications, Inc.) with media changes every 2-3 days. These cells were used as a positive control for elastin secretion both on 2D glass substrates and within 3D PEG-PQ hydrogels, treated in the same manner as with VICs.
2.2.2 Cell Phenotype and Function Study on 2D Stiff Substrates

For studies on 2D stiff substrates, cells were seeded and maintained in culture on Chambered #1.0 Borosilicate Coverglass (Thermo Fisher Scientific), which is intended for cell culture applications employing inverted microscopes. The coverglass minimizes separation of the objective from the cells. For different marker assessments, the cell seeding densities and culture periods were listed in Table 5.

Table 5: Cell seeding densities and culture periods on 2D stiff substrates.

<table>
<thead>
<tr>
<th>Marker of study</th>
<th>Cell seeding density</th>
<th>Culture period</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ki67 (nuclear protein associated with cellular proliferation)</td>
<td>10,000 cells/cm²</td>
<td>5 days</td>
<td>Medium cell density and short culture period to reach near confluency at the end point</td>
</tr>
<tr>
<td>αSMA (hallmark of myofibroblast activation)</td>
<td>5,000 cells/cm²</td>
<td>7 days</td>
<td>Low cell density and long culture period to allow enough time for osteogenic differentiation</td>
</tr>
<tr>
<td>ALP (upregulation in osteogenic differentiation)</td>
<td>3,000 cells/cm²</td>
<td>14 days</td>
<td>High cell density until the observation of obvious nodule formation</td>
</tr>
<tr>
<td>Calcification (detrimental disease outcome)</td>
<td>50,000 cells/cm²</td>
<td>8 days</td>
<td>High cell density until the observation of obvious nodule formation</td>
</tr>
<tr>
<td>Elastin deposition (ECM components)</td>
<td>7,600 cells/cm²</td>
<td>10 days</td>
<td>Medium cell density and long culture period for sufficient ECM deposition</td>
</tr>
</tbody>
</table>

2.2.3 Synthesis and Purification of PEG-PQ-PEG and PEG-RGDS

PEG-PQ-PEG and PEG-RGDS were synthesized, purified and characterized as previously described\textsuperscript{17}. Briefly, the MMP-2 and -9 sensitive peptide GGGPQGIWGQGK
(PQ) was synthesized with a solid phase peptide synthesizer (Aapptec, Louisville, KY, USA) via standard Fmoc chemistry, and characterized with MALDI-TOF Mass Spectrometry (Applied Biosystems, Grand Island, NY, USA). This peptide was then reacted with heterobifunctional acrylate-PEG-succinimidyl valerate (PEG-SVA; Laysan Bio, Arab, AL, USA) at a molar ratio of 1:2 in 20 mM HEPES buffer (pH 8.0) (Figure 11a). To prepare PEG-PQ-PEG of different chain lengths, PEG-SVA of molecular weight (MW) 3400 Da and 8000 Da were used. MW 3400 was the standard MW that had been most commonly used in publications. Increasing the MW was expected to generate gels with a lower crosslinking density and a larger pore size. Unless specified, MW 3400 was used in most studies in this work. The resultant PEG-PQ-PEG was then dialyzed and lyophilized. PEG-RGDS was synthesized with a similar process by reacting the adhesive ligand RGDS (American Peptide, Sunnyvale, CA, USA) with PEG-SVA (MW 3400) in 20 mM HEPES buffer (pH 8.0) at a molar ratio of 1.2:1 (Figure 11b). Conjugation of peptides onto PEG chains was confirmed by gel permeation chromatography (GPC) equipped with UV and evaporative light-scattering detectors (Varian, Palo Alto, CA, USA).
**Figure 11: PEG-peptide conjugations.** Reaction scheme for PEG conjugation to (a) GGGPQGIWGQGK (PQ) and (b) RGDS. Heterobifunctional Acryl-PEG-SVA is reacted with the free amine on the N-terminus of a peptide (PQ or RGDS) and the primary amine on lysine (K) (PQ only), forming PEG-PQ-PEG and PEG-RGDS.

### 2.2.4 Cell Encapsulation in Hydrogels

Primary VICs at passage 3 or 4 were encapsulated within MMP-sensitive, cell-adhesive PEG hydrogels (at a seeding density of $1 \times 10^7$ cells/mL unless otherwise noted) (Figure 12). Briefly, hydrogel precursor solution was prepared with PEG-PQ-PEG (4
wt% unless otherwise noted), PEG-RGDS (5 mM unless otherwise noted), 10 µM eosin Y, 1.5% v/v triethanolamine (TEOA), and 3.5 µL/mL N-vinylpyrrolidone (NVP) in 10 mM HEPES buffered saline (pH 8.0). A 10 µL droplet of the precursor solution was sandwiched between a Sigmacote-treated glass slide, which is rendered hydrophobic, and a methacrylate-modified glass cover slip, which is modified with groups to which the hydrogel can covalently bind, with a polydimethylsiloxane (PDMS) spacer to control thickness. (Detailed methods of Sigmacote treatment of glass slides, methacrylation of coverslips, and PDMS spacer fabrication can be found in the Appendix.) The “sandwich” was exposed to white light for 25 s to crosslink (Fiber-Lite Series 180, 150 W halogen, Dolan Jenner, Dayton, Ohio, USA), forming “PEG-PQ” hydrogel disks (Figure 12). After encapsulation, cell-laden hydrogels were cultured in low-binding 24-well polystyrene plates and kept in a standard humidified incubator (37°C and 5% CO₂) with media changes every 2-3 days.
Figure 12: Schematic of cell encapsulation within PEG-based hydrogels. VICs were mixed with the hydrogel precursor solution containing PEG-PQ-PEG, PEG-RGDS and eosin Y photoinitiator in the aqueous state. Then the mixture was exposed to white light to induce photopolymerization, leading to VICs being encapsulated within 3D cell-adhesive, proteolytically degradable, PEG-based hydrogels.

2.2.5 Cell Viability Within Hydrogels

Cell viability within hydrogels was assessed by calcein AM and ethidium homodimer staining per manufacturer’s instructions (Live/Dead kit, Life Technologies, Grand Island, NY, USA) at day 3. This kit uses two fluorescent dyes to discriminate live from dead cells: green-fluorescent calcein-AM indicates intracellular esterase activity (live cells), and red-fluorescent ethidium homodimer-1 indicates loss of membrane integrity (dead cells). Briefly, cell-laden hydrogels were briefly rinsed with phosphate buffered saline (PBS) and then incubated with 2 µM calcein AM and 4 µM ethidium homodimer-1 in PBS for 30 min. (Detailed information of how the kit works can be
found in Appendix.) After incubation, hydrogels were rinsed with PBS and immediately imaged on a confocal microscope (Zeiss LSM 510, Jena, Germany). Based on the projections of z-stacks covering a depth of 90 µm beneath the hydrogel surfaces, the numbers of live and dead cells were counted using Image J. Viability was calculated with the following equation: viability = number of live cells/(number of live cells + number of dead cells)*100%.

**2.2.6 Zymography**

In order to characterize the MMP secretion by the encapsulated VICs, the conditioned media of cell-laden hydrogels was collected during one media change (day 1-3). Gelatin zymography was performed using 10% Ready Gel Zymogram Gel (Bio-Rad) per manufacturer’s instructions. Briefly, 12.5 µL media samples or protein standards (10-250 kDa, Bio-Rad) were equally mixed with the sample buffer (125 mM Tris-HCl, pH 6.8, 20% v/v glycerol, 4% w/v sodium dodecyl sulfate (SDS), 0.005% v/v bromophenol blue) and loaded into each well of a zymography gel for electrophoresis (100 V for 90 min, in 24 mM Tris base, 192 mM glycine, 0.1% w/v SDS). The gel was then soaked in the renaturing buffer (2.5% v/v Triton X-100 in water) for 30 min and in the developing buffer (10 mM Tris base, 40 mM Tris-HCl, 0.2 M NaCl, 5 mM CaCl₂, 0.02% v/v Brij 35) for at least 4 hr. After that, the gel was stained with 0.5% w/v Coomassie.
Brilliant Blue and destained in 10% v/v acetic acid and 20% v/v isopropanol in water before being imaged.

2.2.7 Cell Morphology Within Hydrogels

In the study investigating the influence of RGDS concentrations on cell spreading, VICs were encapsulated within PEG-PQ hydrogels modified with various concentrations of RGDS (0 mM, 1 mM and 4 mM). These concentrations were chosen based on the literature knowledge that cell adhesion increases with the concentrations of adhesion ligands within a range; 3-5 mM VAPG seem enough to saturate SMC adhesion; any increase in concentration beyond saturation would not further enhance cell adhesion and might impair cell migration\textsuperscript{188,189}. Bright-field images of cell-laden hydrogels were taken with a Zeiss Axiovert 135 inverted microscope at day 21.

In the study investigating the influences of PEG-PQ-PEG chain lengths and percentages on cell morphologies, VICs (1 million cells/mL) were encapsulated within PEG-PQ hydrogels made from various PEG chain lengths (PEG-SVA MW 3400 and 8000) and polymer percentages (4wt%, 5wt% and 8wt%). Instead of the 10 million cells/mL in most studies, the lower cell seeding density was chosen to avoid cell-cell contact in order to capture individual cell morphology accurately. PEG-PQ-PEG polymer percentages of 4 wt%, 5 wt% and 8 wt% were chosen based on the observations that ≤3 wt% led to inconsistent gelation whereas >8 wt% impaired cell spreading and
viability within gels. Cell-laden hydrogels were cultured for 1, 3, or 7 days and stained for 4',6-diamidino-2-phenylindole (DAPI, nuclei) and phalloidin (actin) to aid cell morphology analysis. Briefly, cell-laden hydrogels were incubated overnight in 1:100 dilution of Alexa Fluor 488 conjugated phalloidin (Life Technologies) in 2 µM DAPI and rinsed twice with PBS. The stained samples were then imaged with a confocal microscope (Zeiss LSM 510, Jena, Germany) to capture fluorescent z-stacks. Z-projections covering a depth of 80 µm beneath the hydrogel surfaces were obtained using Image J. Based on the phalloidin staining of the z-projections, cell outlines were extracted to calculate cell circularity using the following equation:

\[
\text{Circularity} = 4\pi \frac{\text{Area}}{\text{Perimeter}^2}.
\]  

Circularity values fall in the range of 0 to 1, where a value of 1 indicates a perfect circle and approaching 0 indicates an increasingly elongated polygon (Figure 13). Therefore, a lower circularity indicates a more spread morphology. In this work, the full range of circularity values were binned into four categories to reflect the degree of cell spreading: a range of <0.1 indicates well spread cells; a range of 0.1-0.2 indicates medium spread cells; a range of 0.2-0.4 indicates slightly spread cells; a range of >0.4 indicates balled-up cells.
2.2.8 Immunohistochemistry to Evaluate Various Marker Expressions

Cell-laden hydrogels were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), permeabilized with 0.25% v/v Triton X-100, and blocked with 3.5 wt% bovine serum albumin (BSA, Fisher Scientific, Loughborough, UK) in PBS (Corning, pH = 7.4). Samples were then incubated with primary antibodies including rabbit anti-ki67 (proliferation marker, day 5 on 2D substrates, day 14 in 3D hydrogels, 1:50, Abcam, Cambridge, MA, USA), mouse anti-αSMA (myofibroblast activation marker, day 7 on 2D substrates, day 1 and day 14 in 3D hydrogels, 1:100, Abcam), and rabbit anti-ALP (osteogenic differentiation marker, day 14 on 2D substrates, day 28 in 3D hydrogels, 1:100, Abcam) in PBS. After rinsing with 0.01% Tween in PBS three times with several hours per rinse, samples were incubated with secondary antibodies: anti-rabbit IgG and anti-mouse IgG antibodies conjugated with either Alexa Fluor 555 or Alexa Fluor 647 (Life Technologies). Finally, to aid
morphological analysis, actin and nuclei were stained with 1:100 dilution of Alexa Fluor 488 conjugated phalloidin (Life Technologies) in 2 µM DAPI. For collagen and elastin staining, the same procedure was followed except for the omission of the permeabilization step. The primary antibodies used were rabbit anti-collagen III (Col III, day 14, 21 and 30, 1:200, Abcam) and mouse anti-elastin (day 10 on 2D substrates, day 30 in 3D hydrogels, 1:50, Abcam). Fluorescent z-stacks were obtained on a confocal microscope (Zeiss LSM 510). For image analysis of cell-laden hydrogels, z-projections covering a depth of 80 µm or 20 µm beneath hydrogel surfaces were obtained via Image J. For image analysis on 2D substrates, z-projections of 12 µm covering the maximum fluorescence intensities on substrate surfaces were used. Relative fluorescence ratio was calculated by thresholding all channels uniformly and normalizing the mean fluorescent intensities of a specific channel to the corresponding DAPI channel using Image J.

In order to verify the proper function of the proliferation marker protein ki67, as well as to test VIC proliferation changes in response to serum content, VICs were also cultured in the low serum media (1% BGS serum, all other components were kept the same as that in GM) as a negative control. Proliferation ratios were calculated by dividing proliferating cell numbers (ki67 positive cells) to the total cell numbers (DAPI) using Image J “Adjust Threshold” and “Analyze Particles” function. 3-4 hydrogels were used for each group.
2.2.9 Assessment of Calcification

Xylenol orange (XO; excitation at 570 nm; emission at 610 nm) was used to detect calcium deposition by VICs. VICs seeded on 2D substrates or within 3D hydrogels were incubated in the media supplemented with 50 µg/mL XO for six hours and rinsed twice with PBS to remove unbound dye. Then, they were fixed with 4% paraformaldehyde and stained with DAPI and phalloidin as described above before being imaged with a confocal microscope (Zeiss LSM 510). For VICs encapsulated within 3D hydrogels under osteogenic conditions, two cell densities (1×10^7 cells/mL and 3×10^7 cells/mL) were tested as cell density may play an important role in the formation of cell-dense, nodule-like structures in osteogenic differentiation and calcification.

2.2.10 Hydrogel Mechanical Properties

To assess the compressive properties, cell-laden hydrogels with a thickness of 1 mm were prepared as aforementioned and tested with a Micro-strain Analyzer (TA Instruments RSA III, New Castle, DE, USA). Cell-laden hydrogels were cultured for 1 day and 28 days before testing. To evaluate the influence of cell tension on the compressive properties of hydrogels, a group of cell-laden hydrogels were treated with blebbistatin (a myosin II inhibitor to inhibit cell tension) before compression testing. Briefly, VIC-laden hydrogels were cultured in the standard growth media for 26 days and then cultured in media supplemented with 0.1 mg/mL blebbistatin for 2 days.
Compression tests were performed under the Transient, Multiple Extension Mode at a rate of 0.002 mm/s at room temperature. Compressive moduli were obtained from the linear part of the stress-strain curve ranging from 5% to 25% strain. 4-6 hydrogels were used for each group.

2.2.11 Statistical Analysis

Statistical analysis was performed using JMP 11 (SAS Institute Inc.). The statistical significance of differences was determined by two-tailed Students’ t-test or multi-factor ANOVA followed by post-hoc Tukey HSD. P-values less than 0.05 were considered statistically significant. Results are reported as the mean ± standard deviation. For fluorescence quantification, sample numbers were expressed as the numbers of hydrogels whereas images taken from the same hydrogels were considered as replicates of one sample.

2.3 Results

2.3.1 Material Characterization

Synthesis of the MMP-2 and -9 sensitive peptide, GGGPQGIWGQGK (PQ), was confirmed using MALDI-TOF Mass Spectrometry (Figure 14). The main peak at MW 1141 corresponds to the theoretical MW of the PQ peptide.
Figure 14: Mass spectrometry of GGGPQGIWGQGK (PQ) peptide. The main peak at MW 1141 corresponds to the expected MW of the PQ peptide.

Incorporation of the PQ peptide and the cell-adhesive RGDS peptide into the hydrogel scaffolds required the conjugation of these peptides to PEG-SVA. The successful conjugation was confirmed using gel permeation chromatography, demonstrated by the shift of the MW to a higher MW (to the left) compared to unconjugated PEG-SVA. The conjugation efficiency was calculated as the areas under
the left-shifted peak over the sum of the area under all peaks from the PEG-peptide curves. The conjugation efficiency was greater than 90% for all samples.

![Graphs A, B, and C with labels](image)

**Figure 15:** Representative gel permeation chromatography traces of PEG-PQ-PEG and PEG-RGDS. (A) PEG-SVA with a MW of 3400 Da and PEG-PQ-PEG resulted from it. Purple = PEG-PQ-PEG; white = PEG-SVA. (B) PEG-SVA with a MW of 8000 Da and PEG-PQ-PEG resulted from it. Purple = PEG-PQ-PEG; white = PEG-SVA. (C) PEG-SVA and PEG-RGDS. Red = PEG-RGDS; white = PEG-SVA. PEG-SVA of MW 3400 Da was used unless otherwise noted.
2.3.2 VICs Retain High Viability in 3D PEG-PQ Hydrogels

PEG-PQ-PEG and PEG-RGDS were used to prepare the hydrogel precursor solution, which was then mixed with VICs and photopolymerized to obtain cell-laden PEG-PQ hydrogels. Staining with calcein AM and ethidium homodimer of hydrogels showed that at day 3, VICs were evenly dispersed within hydrogels, and approximately 98% of the VICs remained viable (Figure 16).

Figure 16: Encapsulated VICs retained high viability within hydrogels at day 3. Representative live/dead images. Live = green; Dead = red. (A, calcein AM channel; B, ethidium homodimer channel; C, overlay). Scale bars = 50 µm.
2.3.3 Encapsulated VICs Secrete MMP-2

Scaffold degradability is critical to cell survival, spreading and migration. PEG-PQ hydrogels are designed to be degradable by MMP-2 and -9. Therefore, MMP-2 and -9 secretion by VICs encapsulated within hydrogels was evaluated. The conditioned media from the culture of encapsulated VICs was collected and analyzed with gelatin zymography. The zymography gel image showed clear bands at MW ~68kDa, corresponding to active MMP-2 (Figure 17). No appreciable MMP-9 (MW ~92 kDa) secretion was observed (Figure 17).

![Zymography image of conditioned media collected from encapsulated VIC culture. Clear bands correspond to active MMP-2.](image)

(Figure 17: Zymography image of conditioned media collected from encapsulated VIC culture. Clear bands correspond to active MMP-2.)
2.3.4 VIC Spreading Within Hydrogels is Tunable via Changing Hydrogel Compositions

With active MMP-2 section, VICs were able to degrade hydrogels, making room for cell spreading and migration. We next explored the influences of hydrogel compositions on VIC spreading. PEG-PQ hydrogels were composed of the MMP-sensitive PEG-PQ-PEG backbone and the pendent PEG-RGDS (integrin-binding adhesion ligands). Hydrogel compositions were changed by varying PEG-PQ-PEG monomer chain lengths and percentages to alter hydrogel stiffness as well as PEG-RGDS concentrations to alter the cell adhesivity. VIC spreading with time within hydrogels of different compositions was investigated.

VICs were first encapsulated within PEG-PQ hydrogels functionalized with different concentrations of PEG-RGDS (0 mM, 1 mM, or 4 mM) for 21 days. Results showed that without RGDS (0 mM), VICs did not spread within hydrogels (Figure 18 A). When RGDS was immobilized in the hydrogels, VICs spread and formed cellular networks within hydrogels. The networks became denser as the RGDS concentration increased from 1 mM to 4 mM (Figure 18 B, C).
Figure 18: Encapsulated VICs formed cellular networks within PEG-PQ hydrogels modified with RGDS but barely spread without RGDS. The networks became denser with the increase in RGDS concentration. Bright-field images of cell-laden hydrogels with different concentrations of RGDS at 21 days. Scale bars = 100 µm.

VICs were also encapsulated within PEG-PQ hydrogels made from different PEG-PQ-PEG chain lengths (PEG 3400 versus PEG 8000) and percentages (4 wt%, 5 wt% or 8 wt%). MW 3400 or 8000 denotes the MW of PEG-SVA that was used to prepare PEG-PQ-PEG, with the final MW being twice the PEG-MW plus 1140 Da for the PQ peptide. Cell circularity (value range 0-1), where a value of 1 indicates a perfect circle, and a lower circularity indicates a more spread cell morphology, was used to characterize VIC spreading within hydrogels at different time points (days 1, 3 and 7). Results showed that, VIC morphologies within hydrogels were a mixed population of well spread cells (circularity <0.1), medium spread cells (circularity 0.1-0.2), slightly spread cells (0.2-0.4) and balled-up cells (circularity >0.4) (Figure 19). At day 1, VICs spread more in PEG 8000 than those in PEG 3400. The trend maintained at day 3. At day
7, cell spreading in PEG 3400 had caught up with that in PEG 8000 (Figure 19). Also, VICs spread more with the decrease in polymer percentage in hydrogels, with the trend most obvious at day 3 (Figure 19). Moreover, VIC spreading increased with culture time in all conditions, with the average circularities fell in the range of 0.3-0.5 at day 1, and 0.1-0.2 at day 3 and around 0.1 at day 7, respectively (Figure 19).
Figure 19: VIC spreading within hydrogels increased with the decrease in polymer percentage and with culture time. Initially there was more cell spreading in PEG 8000, which was caught up by PEG 3400 during the culture period of 7 days. Blue = DAPI; green = phalloidin. Seeding cell density = 1×10⁶ cells/mL. Scale bars = 50 µm.
2.3.5 VICs Undergo Active Proliferation Both on 2D Glass Substrates and Within 3D Hydrogels

Being able to survive and spread, VICs were next evaluated for proliferation. VICs encapsulated within 3D hydrogels (4 wt% PEG-PQ-PEG and 5 mM PEG-RGDS) were stained for ki67 protein, which is a cellular proliferation marker that is present during active phases of the cell cycle (G1, S, G2 and mitosis), but is absent from resting cells (G0). Proliferation ratios (growth fractions) were defined as the ratio between the proliferating cell numbers (ki67 positive cells) and the total cell numbers (DAPI). VICs seeded on 2D glass substrates were used as a control. Results showed that VICs underwent active proliferations both on 2D glass substrates and within 3D hydrogels (Figure 20). In order to test VIC response to serum content and to verify the proper function of the proliferation marker protein (ki67), VICs were also cultured in low serum (1%) media as a negative control. Results showed that a higher percentage of VICs were positive for ki67 cultured in the standard growth media with 10% serum than those in the low 1% serum: (30 ± 6)% (high serum) versus (12 ± 7)% (low serum) on 2D glass substrates, and (59 ± 12)% (high serum) versus (10 ± 6)% (low serum) within 3D hydrogels (Figure 20).
Figure 20: VICs underwent active proliferation, and proliferated more in the standard growth media with 10% serum than those in low serum (1%) both on 2D glass substrates and within 3D hydrogels. For the culture on 2D glass substrates, VICs were seeded at 10,000 cells/cm² and stained at day 5. For the culture within 3D hydrogels, VICs were encapsulated at $1 \times 10^7$ cells/mL and stained at day 14. Blue = DAPI; red = ki67. Scale bars = 50 µm. Note that proliferation ratios on 2D and within 3D were not directly comparable due to the imaging and thresholding differences of the 2D and 3D systems.

2.3.6 VICs Assume Different and Reversible Phenotypes Depending on Culture Substrates

VIC phenotype plays a critical role in valve function and pathology. The expression of αSMA stress fibers was measured to characterize myofibroblast activation.
of VICs. When cultured on 2D glass substrates at 7 days, VICs were strongly positive for αSMA stress fibers (Figure 21 A), indicating an activated myofibroblast phenotype. However, upon encapsulation within PEG-PQ hydrogels for 1 day, the majority (86 ± 4)% of VICs lost their expression of αSMA (Figure 21 B), indicating the reversion to the quiescent, fibroblast phenotype. The VICs encapsulated within hydrogels remained negative for αSMA after culture of 14 days, indicating maintenance of the quiescent phenotype (Figure 21 C).

**Figure 21:** VICs assumed different and reversible phenotypes when cultured on/in various substrates. VICs cultured on 2D glass substrates for 7 days were strongly positive for αSMA stress fibers (A). However, upon encapsulation within PEG-PQ hydrogels in GM for 1 day, the majority (~86± 4%) of VICs lost their expression of αSMA (B). VICs inside hydrogels (80 µm z-stacks starting from 40 µm beneath the surfaces) all remained negative for αSMA during the culture period of 14 days (C). Blue = DAPI; red = αSMA. Scale bars = 50 µm.
2.3.7 VICs Express an Elevated Level of Osteogenic Marker (ALP) in Response to Osteogenic Media Treatment on 2D Glass Substrates but Not in 3D Hydrogels

ALP expression was measured to characterize the osteogenic differentiation of VICs in response to osteogenic media (Osteo M) treatment. Results showed that, compared to that in the standard growth media (GM), ALP expression of VICs on 2D glass substrates was doubled upon Osteo M treatment, indicating the initiation of osteogenic differentiation of VICs under these conditions. However, the ALP expression of VICs encapsulated within 3D hydrogels did not change upon Osteo M treatment.
2.3.8 VICs Form Calcified Nodules in Osteogenic Media on 2D Glass Substrates but Not Within 3D Hydrogels

In order to detect VIC calcification, the calcium binding dye, XO was used.

Results showed that in GM, no calcification was observed for VICs cultured on 2D
substrates or within 3D hydrogels (Figure 23 A, B). In Osteo M on 2D glass substrates, VICs formed calcified nodules (Figure 23 C). Yet, in Osteo M within 3D hydrogels, little calcification was observed (Figure 23 D). Cell density has been shown to correlate with VIC phenotype and calcification in prior studies191. Increasing cell density within hydrogels by three times (from 1×10^7 cells/mL to 3×10^7 cells/mL) generated slightly more calcification in Osteo M, but still very low when compared to the strong positive staining on 2D glass substrates (Figure 23 E). Encapsulated VICs did not form cell-dense, nodule-like structures in Osteo M even at the higher cell density (Figure 23 D, E).
Figure 23: Assessment of calcification of VICs cultured on 2D glass substrates or within 3D hydrogels in standard growth media or in osteogenic media. In growth media, no calcification was observed for VICs cultured on 2D glass substrates or within 3D hydrogels (A, B). In osteogenic media on 2D glass substrates, VICs formed obvious calcified nodules (C). However, in osteogenic media within 3D hydrogels, very little calcification (pointed by white arrows) was observed at both seeding densities (D, E).

Blue = DAPI; green = phalloidin; red = xylene orange. Scale bars = 50 µm.

2.3.9 VICs Deposit Collagen III Within Hydrogels

Collagen fibers (mainly collagens I and III) provide tensile properties of the valve tissue. Hence, as a representation, collagen III secretion by encapsulated VICs was characterized. Results showed that VICs secreted collagen III within hydrogels during the culture period of 30 days (Figure 24). The collagen III deposition remained
pericellular, and tended to increase and align along cell spreading directions with culture time (Figure 24).

![Image]

Figure 24: Collagen III secretion by VICs encapsulated within PEG-PQ hydrogels during the culture of 30 days. Pericellular collagen secretion tended to increase and align along cell spreading direction with culture time. Blue = DAPI; green = phalloidin; red = collagen III. A, B & C images (magnification of 20x) were from projections of z-stacks covering a depth of 80 µm whereas D images (magnification of 80x) were from z-stacks of 20 µm. Scale bars = 50 µm.

2.3.10 VICs Do Not Secrete Appreciable Elastin Within Hydrogels

Elastin provides elasticity and recoil of the valve tissue. Hence, elastin deposition by VICs was characterized. Results showed that appreciable elastin deposition by VICs was not observed on either 2D glass substrates (10 days) or within 3D PEG-PQ hydrogels (30 days) (Figure 25). The positive control, porcine aortic smooth muscle cells (SMCs), secreted slightly more, but still extremely low amounts of elastin under both conditions (Figure 25).
Figure 25: Elastin secretion by VICs and SMCs (positive control) on 2D glass substrates or within 3D hydrogels. For VIC culture either on 2D glass substrates at 10 days or within 3D PEG-PQ hydrogels at 30 days, no appreciable elastin deposition was observed. The positive control, porcine aortic smooth muscle cells (SMCs), secreted slightly more, but still quite low amounts of elastin under the same conditions. Blue = DAPI; green = phalloidin; red = elastin. Scale bars = 50 µm.

2.3.11 Mechanical Properties of Cell-laden Hydrogels

The mechanical properties of cell-laden constructs are an important consideration for valve substitutes. Cellular activities may influence the mechanical properties of scaffolds through several ways: cell tension, scaffold degradation and ECM deposition. The compressive properties of cell-laden hydrogels were characterized at day 1 (complete swelling of hydrogels without extensive cell spreading, scaffold degradation and ECM deposition) and day 28 (formation of cellular networks with considerable scaffold remodeling). Results showed that during the culture of 28 days, the compressive moduli of cell-laden hydrogels increased from ~4.3 kPa to ~6.0 kPa (p <
0.01; Figure 26). However, upon blebbistatin (a myosin II inhibitor) treatment for 2 days, the compressive moduli of cell-laden hydrogels decreased to a level similar to that at day 1 (p = 0.17).

![Graph showing compressive modulus changes](image)

Figure 26: Compressive moduli of cell-laden hydrogels increased during the culture of 28 days, which increase was counteracted upon blebbistatin treatment for two days (day 26-28). * indicates statistical significance: p < 0.01; n = 4-6 hydrogels.

### 2.4 Discussion

To understand VIC biology and pathology is critical to the success of developing an ideal valve substitute. It remains a challenge for scientists and engineers highly due
to the heterogeneous and dynamic nature of the cells, especially given the harvest and plating onto 2D stiff polystyrene substrates. This study aimed to develop a hydrogel platform for the 3D culture of VICs and to characterize and understand different VIC behaviors within 3D hydrogels versus on 2D stiff substrates. The bioinert nature of PEG with finely tunable bioactivity provides an optimal platform to control and reliably study cell-matrix interactions. The PEG-based hydrogels developed in this work serves as an \textit{in vitro} platform for 3D VIC culture and study, with cell behaviors and characteristics more similar to the valve tissue. First, superior to approaches where cells were seeded on a material’s surface and penetrated via migration\textsuperscript{3,186}, cell encapsulation within hydrogels using a cell-compatible crosslinking technique immediately achieves an even distribution of cells within the scaffolds (Figure 16). Second, as discussed in Chapter 1, the synthetic PEG-based hydrogel platform shows significant advantages over the widely used, naturally derived materials to provide controlled microenvironments. Synthetic PEG is inherently bio-inert (like a blank slate) and can be functionalized with bioactive molecules at design, which allows studying the variable of interest without introducing confounding factors. In this work, the key designs of PEG hydrogels are the incorporations of proteolytically degradable sequence in the hydrogel backbone and the pendant cell adhesive ligands, which are critical for the excellent cell compatibility. After encapsulation within functionalized PEG hydrogels, VICs retained
high viability (~98%) (Figure 16) and actively secreted MMP-2 to degrade hydrogels (Figure 17), making room for cell spreading and migration. The adhesive ligand RGDS was shown to be necessary for cell spreading whereas VICs barely spread on otherwise bioinert PEG hydrogels (Figure 18). Furthermore, the initial and subsequent cell spreading within hydrogels was tunable via changing the hydrogel compositions (Figure 19). Specifically, a higher monomer MW (PEG 8000) and a lower polymer percentage (4 wt%) (corresponding to a lower crosslinking density and a larger initial mesh size, i.e., a loose hydrogel network) facilitated the initial cell spreading up to day 3 (Figure 19). Cell spreading in all conditions increased (indicated by the decrease in cell circularity) with the culture time (Figure 19), suggesting the active degradation and remodeling of hydrogel scaffolds by VICs. At day 7, the initial more cell spreading in the loose hydrogel network was caught up by that in the dense, consistent with the fact that there were less cleavable sites available in the loose network. Hence, the degradation rate and profile of hydrogels can be modulated via tuning the availability of the cleavable sites, which can be used to control the progression of cell spreading. At 28 days, VICs spread extensively and formed cellular networks within hydrogels (Figure 24). The increased cell tension due to cell spreading caused an increase in the compressive moduli of cell-laden hydrogels (from ~4.3 kPa to ~6.0 kPa during the culture of 28 days), which was counteracted upon blebbistatin treatment (Figure 26),
suggesting the positive role of cell spreading and networking in the maintenance of mechanical properties of cell-containing constructs. Moreover, VICs not only survived and spread but also underwent active proliferation (ki67 positive) within hydrogels, in a similar manner as that on the 2D substrates -- VICs proliferated more in the standard media containing 10% serum than those in the low 1% serum media (Figure 20), suggesting that various growth factors in the serum may stimulate VIC proliferation. Additionally, VIC growth and proliferation were supported for up to 4 weeks in 3D hydrogels, showing the utility of these scaffold materials for long-term culture of VICs.

Another important finding of this study was that the soft hydrogels induced and preserved the quiescent phenotype of VICs as suggested by the low αSMA staining. A significant challenge of heart valve tissue engineering is to recapitulate VIC in vivo phenotype and function. In healthy adult valves, >95% of VICs are quiescent, fibroblast phenotype, without αSMA expression\(^3\). However, the most common method of culturing VICs on 2D stiff substrates led to marked VIC activation, evidenced by the expression of αSMA stress fibers (Figure 21). Stiffness of hydrogels in this work was designed to avoid VIC activation based on the literature knowledge that VIC activation tends to occur and increase with substrate stiffness\(^{26,27,93}\). Although there is some discrepancy among reported threshold stiffness for myofibroblast activation of VICs (3.93 kPa\(^92\) or 4.8-9.6 kPa\(^93\) or 7 kPa\(^{26,27}\)), VICs cultured on soft substrates mimicking
healthy valve fibrosa (0.5-5 kPa) maintained an un-activated phenotype. Therefore, in our study, hydrogel stiffness was designed to be slightly less than 5 kPa to minimize myofibroblast activation. Our results showed that upon encapsulation within 3D PEG hydrogels with a modulus of 4.3 kPa, the majority of VICs lost their expression of αSMA by day 1 (Figure 21), suggesting that the soft hydrogels quickly reverted VICs from myofibroblast activation to a quiescent state upon encapsulation. VICs that stayed inside of hydrogels remained negative for αSMA expression after being encapsulated for 14 days (Figure 21), demonstrating the advantage of this hydrogel platform to preserve the VIC quiescent phenotype during long-term culture and study. The studies of challenging VICs with osteogenic conditions on 2D glass substrates or within 3D hydrogels showed that VICs upregulated ALP expression and formed calcified nodules upon osteogenic media treatment on 2D glass substrates, but not within 3D hydrogels, suggesting that VICs were more resistant to detrimental osteogenic differentiation and calcification within 3D hydrogels (Figure 22 and Figure 23). Therefore, the 3D hydrogels developed in this work serve as a preferable platform for VIC culture and phenotype preservation.

Last but not least, VICs actively deposited collagen III within hydrogels, which is one of the two major types of collagens providing tensile strength of the valve tissue. It shows the potential of recapitulating the native ECM components of the valve tissue in
vitro via VIC-mediated ECM remodeling. The collagen deposition began to organize along cell spreading direction and tended to increase with culture time (Figure 24). ECM components are largely responsible for the mechanical properties of the valve tissue. Collagen deposition remained pericellular and was not quite connected yet in the hydrogels during the culture period of this study. This was probably why the collagen deposition did not cause an increase in the mechanical properties of hydrogels. Further enhancements in collagen deposition such that collagen occupies the vast blank space of the hydrogels may offer a significant increase in the mechanical properties. Besides, appreciable elastin deposition by VICs was not observed either on 2D substrates or within 3D hydrogels. The lack of elastin deposition was also reported by other research groups in the study of various cell types for heart valve tissue engineering, including human VICs, human mesenchymal stem cells, human marrow stromal cells, and ovine carotid artery-derived cells, despite of the obvious deposition of other ECM proteins, including collagen I and III, fibronectin, laminin and chondroitin sulphate. As a result, other strategies to incorporate elastin in tissue engineered heart valves need to be explored.

2.5 Conclusion
This study aimed to develop a cell-adhesive, proteolytically degradable, 3D hydrogel platform that is superior to the commonly used 2D substrates for in vitro VIC
culture and study. This platform could not only be used to understand VIC behaviors underlying valvular diseases but also to construct living valve substitutes that can grow and remodel with growing patients. We demonstrated that VICs retained high viability, spread, proliferated and deposited collagens within hydrogels, showing the potential of using functionalized PEG-based hydrogels as scaffold materials in HVTE. VICs also reverted from myofibroblast activation to a quiescent state upon encapsulation within hydrogels. Their healthy, quiescent phenotype was well maintained within hydrogels even with the osteogenic media challenge, suggesting that the hydrogel platform in this work is beneficial in the induction and preservation of the in vivo-like phenotype of VICs. Evaluation of VIC responses to synthetic mimics of the natural valve provides insights into VIC behaviors in both diseased and healthy states and will direct future heart valve tissue engineering research.
Chapter 3 Ascorbic Acid Promotes Valve Interstitial Cell Quiescence and Extracellular Matrix Deposition within 3D Hydrogel Scaffolds†

3.1 Introduction

In Chapter 2 of this thesis, a cell-adhesive, proteolytically degradable, 3D, PEG-based hydrogel platform was developed for *in vitro* VIC culture and study. When cultured in 3D within soft hydrogels, VICs quickly reverted from myofibroblast activation into a quiescent state, and the quiescence was well maintained for at least 14 days. VICs also secreted MMP-2 to degrade the hydrogels and deposited collagens within hydrogels, indicating active ECM remodeling. As ECM are responsible for the mechanical properties and the dynamic function of heart valves, to recapitulate the native ECM components in the valve tissue via VIC-mediated ECM remodeling *in vitro* is critical to the success of living valve substitutes. Therefore, in this work, to promote VIC-mediated ECM remodeling/deposition within hydrogels was a key goal.

Previous work in the literature showed that soluble transforming growth factor beta 1 (TGF-β1) treatment increased the mRNA levels for collagen I, but it also increased the expression of αSMA, indicating myofibroblast activation, which may cause fibrosis. In order to promote collagen production without causing activation, this work aimed to

† This section has been adapted from the publication: Wu Y, Puperi DS, Grande-Allen KJ, West JL. Ascorbic acid promotes extracellular matrix deposition while preserving valve interstitial cell quiescence within 3D hydrogel scaffolds. J Tissue Eng Regen Med 2015.
explore the potential of using a biochemical molecule, ascorbic acid (AA). AA, also known as vitamin C (Figure 27), is an essential nutrient acting as a co-factor of various enzymes. For example, AA is a cofactor of prolyl and lysyl hydroxylases. AA may promote the activity of hydroxylases via two mechanisms: (1) activation of enzymes from an inactive precursor to an active state, and (2) participation in hydroxylation by the active enzymes\textsuperscript{194}. During hydroxylation, AA acts as a reducing agent in iron cycling (Fe\textsuperscript{4+} \rightarrow Fe\textsuperscript{2+}) to restore the activity of hydroxylase enzymes during the uncoupled reaction cycles\textsuperscript{194}. Hydroxylation of proline and lysine is critical to the stability of the triple helical structure and formation of the intermolecular crosslinks in collagen\textsuperscript{9}. In AA deficiency, hydroxylation of collagen became negligible\textsuperscript{194}. Addition of AA into the culture of AA-deficient tissue stimulated collagen hydroxylation\textsuperscript{194}. AA also assists the hydroxylation of hypoxia-inducible factor 1α (HIF-1α), a transcription factor responsible for cellular responses to hypoxia (low oxygen)\textsuperscript{195}. Under hypoxic conditions, such as in fast growing tumors, hydroxylation of HIF-1α is repressed, thereby stabilizing HIF-1α and promoting tumor growth, which could be further exacerbated at low AA levels\textsuperscript{196}. Furthermore, AA serves as a free radical scavenger in the plasma, protecting cells from oxidative damage caused by reactive oxygen species\textsuperscript{195}. 

AA has been used as a supplement for culture of various cell types\textsuperscript{6–8,197}. For example, it has been reported to promote collagen secretion by smooth muscle cells and
fibroblasts\textsuperscript{7-9}, and to stimulate the proliferation of mesenchymal stem cells while preserving their differentiation ability\textsuperscript{6}. It also was shown to increase replicative lifespan of endothelial cells\textsuperscript{197}. Furthermore, administration of AA shows beneficial effects against arterial dysfunction\textsuperscript{198} and myocardial infarction\textsuperscript{199}. However, little work has been conducted to understand its effect on VICs. Therefore, the effects of AA treatment on VIC phenotype, proliferation, apoptosis, MMP secretion and ECM deposition were investigated. Findings demonstrated the promotion of VIC-mediated ECM remodeling while preserving quiescent fibroblastic phenotype, which can be beneficial in the development of tissue engineered heart valves.

![Formula of ascorbic acid (AA)](image)

**Figure 27:** Formulas of ascorbic acid (AA).

### 3.2 Materials and Methods

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise indicated.
3.2.1 Cell Isolation and Culture

Primary VICs were isolated, encapsulate in hydrogels and cultured as described in Chapter 2. After encapsulation, cell-laden hydrogels were cultured in GM for 1 day and changed to different media conditions including GM, GM supplemented with 50 µg/mL AA (GM+AA), or Osteo M.

3.2.2 Cell Encapsulation in Hydrogels

PEG-PQ-PEG and PEG-RGDS were synthesized, purified and characterized as described in Chapter 2. VICs between passage 2 and 4 were encapsulated within MMP-sensitive, cell-adhesive PEG hydrogels (4 wt% PEG-PQ-PEG and 5 mM PEG-RGDS) as described in Chapter 2 at a seeding density of 1×10⁷ cells/mL.

3.2.3 Cell Viability in Hydrogels

Cell viability within hydrogels was assessed by calcein AM and ethidium homodimer staining as described in Chapter 2.

3.2.4 Zymography

The conditioned media of cell-laden hydrogels (day 1-4) was collected, and gelatin zymography was performed as described in Chapter 2. Fresh media was used as a reference. Gel images were captured and band intensities were quantified using BioRad Image Lab software. 4-5 hydrogels were used for each group.
3.2.5 Immunohistochemistry

Immunohistochemistry was performed as described in Chapter 2. Antibodies used in this chapter included rabbit anti-ki67, rabbit anti-cleaved caspase-3 (apoptosis marker, 1:100, Cell Signaling Technology, Danvers, MA, USA), mouse anti-αSMA, rabbit anti-ALP, rabbit anti-collagen I (Col I, 1:200, Abcam), and rabbit anti-Col III. Fluorescent z-stacks were captured with a confocal microscope (Zeiss LSM 510). Z-projections covering a depth of 80 μm were obtained via Image J for visualization and quantitative analysis. Average z-projection area was calculated as the area covered by the z projections of phalloidin divided by cell numbers (DAPI-stained cell nuclei). Quantification of the expression level of a protein was normalized by that of cell nuclei (DAPI). Specifically, relative fluorescence was calculated by thresholding all channels uniformly and normalizing the mean fluorescent intensities of a specific channel to the corresponding DAPI channel using Image J. Eight z-stacks from 3-4 hydrogels per group were taken for quantification.

3.2.6 Cell Proliferation and Apoptosis

Cell proliferation was evaluated by measuring the change in cell number after encapsulation and culture. Cell-laden hydrogels were degraded using collagenase (250 U/ml, 10 min) and DNA content was measured using the Quant-iT PicoGreen dsDNA Kit (Life Technologies). DNA contents were translated into cell numbers using a
conversion factor of 5.6 pg DNA/cell (Appendix), which agrees closely with previously published data for VICs. Cell proliferation was also characterized by immunostaining for the proliferation marker ki67, as described in the above section. Proliferation ratios were calculated by dividing ki67+ cell numbers to the total cell numbers (counted from DAPI channels) using Image J “Adjust Threshold” and “Analyze Particles” function.

Cell apoptosis was assessed with Caspase-Glo 3/7 Assay Kit (Promega, Madison, WI, USA), and caspase content was normalized to DNA content as previously reported. Briefly, on defined days (7, 14, 21, 28), cell-laden hydrogels were degraded with collagenase (250 U/ml, 10 min). 100 µL of the degraded cell suspension were mixed with 100 µL Caspase-Glo reagent with PicoGreen dye at a ratio of 1:200, and transferred to a 96-well plate. After incubation at room temperature for 30 min, the total content of caspase 3 and 7 was determined by measuring luminescence and fluorescence (Promega, excitation 485 nm, emission 520 nm). Hydrogels without cells were used as the blank control in all of the assays. Caspase content was calculated as luminescence (from caspase 3/7 assay) divided by fluorescence (from PicoGreen DNA assay) and normalized to that of blank hydrogels at each time point. 3-4 hydrogels were used for each group.

3.2.7 Assessment of Calcification

Calcification was assessed with XO staining as described in Chapter 2.
3.2.8 Hydrogel Mechanical Properties

Hydrogel mechanical properties were characterized using compression testing as described in Chapter 2. 4-6 hydrogels were used for each group.

3.2.9 Statistical Analysis

Statistical analysis was performed using JMP 11 as described in Chapter 2. The statistical significance of differences was determined by two-tailed Students’ t-test without specifications. P-values less than 0.05 were considered statistically significant. The proliferation study and compression test study were assessed by multi-factor ANOVA followed by post-hoc Tukey HSD.

3.3 Results

3.3.1 AA Does Not Influence the High Viability of VICs in Hydrogels

VIC-laden hydrogels were cultured in GM or GM+AA for 3 days. Staining with calcein AM and ethidium homodimer showed that AA treatment did not significantly influence cell viability at day 3 (GM+AA 0.976 ± 0.012 versus GM 0.979 ± 0.006, n = 3, p = 0.81) (Figure 28).
Figure 28: VICs remained viable when encapsulated in PEG-PQ hydrogels for 3 days. Representative images of cell-laden hydrogels stained with calcein AM (green, live) and ethidium homodimer (red, dead) cultured in GM or GM+AA. Scale bars = 50 µm.

3.3.2 AA Promotes Cell Spreading and MMP-2 Secretion by VICs

One day after encapsulation within PEG-PQ hydrogels, the VICs started to demonstrate a spreading morphology in both GM and GM+AA groups (Figure 30 A-B) and were homogeneously distributed throughout the PEG hydrogel thickness (Figure 30 C-D). AA treatment increased the cell spread area within hydrogels by ~18% (Figure 30 E).
Figure 29: AA promotes cell spreading within MMP-labile PEG-PQ hydrogels. (A-D) Representative images of cell spreading within hydrogels after encapsulation for 1 day in GM (A, C) or GM+AA (B, D): Blue = DAPI; green = phalloidin. Top view (A, B) and side view (C, D). Scale bars = 50 µm. (E) Average z-projection area per cell of VIC spreading within hydrogels at day 1. * indicates statistical significance: p < 0.05; n = 3 hydrogels.

Increased cell spreading in AA treatment group was consistent with the enhanced MMP-2 secretion by VICs treated with AA. Zymography of cell culture supernatants (day 1-4) and the media control showed clear bands at MW ~68 kDa corresponding to MMP-2 (Figure 30 A). Quantitative analysis of band intensity showed that the bands from cell culture supernatants were 2-4 times brighter than those in the media control, confirming new MMP-2 secretion by encapsulated VICs (Figure 30 B). Also, bands at MW ~72 kDa appeared in cell culture supernatants, but not in the media control, indicating the secretion of pro-MMP-2 in cell culture (Figure 30 A). Moreover,
AA treatment enhanced MMP-2 secretion of encapsulated VICs by approximately 40% (Figure 30 B).

**Figure 30:** AA promotes MMP-2 secretion within MMP-labile PEG-PQ hydrogels. Zymography of fresh media control and conditioned media collected from encapsulated VIC culture (day 1-4): (A) Representative image of zymography with clear bands corresponding to MMP-2. (B) Quantitative analysis of band intensities relative to the media control. * indicates statistical significance: p < 0.01; n = 4-5.

### 3.3.3 AA Promotes VIC Growth and Inhibits Apoptosis

To evaluate the potential of cell-laden hydrogels to serve as a living tissue substitute and the effect of AA, cell proliferation and apoptosis of encapsulated VICs were assessed. Cell numbers increased by $33 \pm 16\%$ in GM and by $76 \pm 19\%$ in GM+AA after 28 days of culture. Quantification of ki67 staining revealed that proliferation ratios in GM+AA were higher than those in GM at 1 and 14 days ($20 \pm 6\%$ increase at day 1 and $19 \pm 8\%$ increase at day 14, n = 3, p < 0.05) (Figure 32 A-C). These results confirmed the
ongoing growth of the encapsulated VICs, which was further promoted by AA treatment.

**Figure 31:** AA treatment enhanced VIC proliferation in hydrogels. (A-B) Representative images of ki67 staining of cell-laden hydrogels in GM or GM+AA at 21 days: red (ki67), blue (DAPI, nuclei), green (phalloidin, actin). (C) Quantitative analysis of proliferation ratio. * indicates statistical significance: p < 0.05; n = 3 hydrogels.
Samples were immunostained for caspase-3 to estimate the cell population that underwent apoptosis. Only a small population (GM 3.3 ± 0.6% versus GM+AA 2.4 ± 0.6%, n = 3, p = 0.33) of encapsulated VICs underwent apoptosis (Figure 32 D and E). The caspase 3/7 content, normalized to DNA, was consistently lower for GM+AA than for GM (60 ± 3%, 43 ± 7%, 63 ± 3%, and 66 ± 8% decrease at day 7, 14, 21, 28 days respectively, n = 4, p < 0.01), indicating that AA inhibited apoptosis of encapsulated VICs (Figure 32 F).
Figure 32: AA treatment inhibited VIC apoptosis in hydrogels. (A-B) Representative images of caspase-3 staining of cell-laden hydrogels cultured in GM or GM+AA for 28 days: Blue = DAPI; red = caspase-3. Scale bars = 50 µm. (C) Relative caspase content of VICs cultured in GM and GM+AA normalized to that of blank gels without cells. * indicates statistical significance: p < 0.01; n = 4 hydrogels.
3.3.4 AA Treatment Enhances Collagens I and III Secretion by Encapsulated VICs

VICs actively secreted collagens (Col) I and III, which were mainly pericellular. Comparing collagen secretion at different time points, Col I and III secretion increased with culture time and began to organize into Col fibrils along the cell spreading direction (Figure 33). Moreover, AA treatment significantly increased Col I and III secretion by 113 ± 32% and 210 ± 18% respectively at day 28 (n = 3, p < 0.01).
Relative intensities to DAPI (RFU/RFU)
Figure 33: AA treatment enhanced Col I and III secretion by VICs encapsulated in PEG-PQ hydrogels for 28 days. (A-P) Representative fluorescent images of Col I and Col III staining at 14, 21 and 28 days, with or without AA treatment: Blue = DAPI; green = phalloidin; red = Col I or III. Scale bars = 50 µm. (Q) Quantitative analysis at 28 days based on relative fluorescence intensities of Col I, Col III and DAPI. * indicates statistical significance: p < 0.01; n = 3 hydrogels.

3.3.5 AA Treatment Does Not Affect the Expression of αSMA and ALP

The expression of αSMA and alkaline phosphatase (ALP) was measured to evaluate VIC phenotype. After culturing in GM or GM+AA within PEG-PQ hydrogels for 21 days, the majority of VICs remained negative for αSMA staining (Figure 34 A and B). ALP staining of VICs encapsulated within hydrogels for 28 days was diffuse and comparable in both groups (Figure 34 C and D). The results of αSMA and ALP staining of VICs encapsulated in PEG-PQ hydrogels also revealed that AA treatment did not significantly influence the expression of these phenotype markers (Figure 34 E, n = 3 hydrogels, p = 0.30).
Figure 34: AA treatment did not change the expression levels of αSMA and ALP of VICs encapsulated in PEG-PQ hydrogels. (A-D) Representative fluorescent images of αSMA or ALP staining of VICs cultured in GM or GM+AA: Blue = DAPI; green = phalloidin; red = αSMA or ALP. Scale bars = 50 µm. (E) Relative fluorescence intensities of αSMA, ALP and DAPI. No groups achieved statistical significance; n = 3 hydrogels.

3.3.6 AA Treatment Does Not Induce Calcification

XO (indicating calcification) was undetected in VIC-laden hydrogels cultured in GM or GM+AA for 28 days (Figure 35).
Figure 35: Xylenol orange (calcification) was undetected in cell-laden hydrogels cultured in GM or GM+AA for 28 days. Blue = DAPI; green = phalloidin; red = xylenol orange (calcium). Scale bars = 50 µm.

3.3.7 Mechanical Properties of Cell-laden Hydrogels Maintain and Enhance along with Culture

As seen from Figure 36, the compressive moduli of cell-laden hydrogels at day 1 were lower than those of blank hydrogels without cells. Upon culture for 21 or 28 days, the compressive moduli of cell-laden hydrogels increased significantly from the day 1
level (Figure 36). However, AA treatment did not significantly change the compressive moduli of cell-laden hydrogels (Figure 36).

![Graph showing compressive moduli of cell-laden PEG-PQ hydrogels throughout the culture period of 28 days.](image)

**Figure 36:** Compressive moduli of cell-laden PEG-PQ hydrogels throughout the culture period of 28 days. * indicates statistical significance: p < 0.01; n = 4-6 hydrogels.

### 3.4 Discussion

The main finding of this work was that AA significantly enhanced Col I and III secretion by VICs, promoting VIC-mediated ECM remodeling. Previous work showed that bundles of collagen fibers (mainly Col I and III) closely associate with VIC filopodia, forming a 3D superstructure in valve leaflets. These collagen fibers are circumferentially aligned to provide tensile strength to withstand high-pressure loads. Therefore, we sought to promote Col I and III deposition by VICs in order to
recapitulate the ECM components of the native valve tissue. Col I and III secretion by VICs remained pericellular and began to organize along cell spreading direction (Figure 33), which may represent the preliminary formation of fibrillar collagen. AA acts as a cofactor in the post-translational modification of collagen molecules, and increases collagen production. The collagen-promoting effect of AA on VICs in this work was consistent with its effects on other cell types, such as smooth muscle cells and fibroblasts.

AA also enhanced cell spreading and increased cell numbers. Cell adhesion and matrix degradation are necessary for cell spreading, migration and differentiation. The cell-adhesive ligand RGDS and an MMP-sensitive peptide were incorporated into PEG hydrogels to allow cell spreading and matrix degradation. Results showed that besides the deposition of native ECM components (Figure 33), VICs also remodeled their surrounding matrix via MMP-2 secretion (Figure 30). MMP-labile hydrogels, in this case, provided temporary structural support. AA treatment increased MMP-2 secretion of VICs and thus promoted cell spreading (Figure 30). Moreover, VICs cultured in GM+AA showed higher proliferation ratios and lower apoptosis compared to those in GM (Figure 32). A similar proliferation-promoting effect of AA on other mesenchymal cell types was previously reported at low concentrations of AA. However, high concentrations of AA or its derivatives may be cytotoxic and cause cell death. The
mechanisms by which AA affect cell proliferation and apoptosis are not clear. It may mediate through reducing intracellular oxidative stress and regulating the synthesis of proteins related to cell growth. In this work, the enhanced proliferation and reduced apoptosis upon AA treatment led to a greater total cell number in GM+AA than that in GM. The increased total cell number, together with increased cell spreading, promoted the formation of cellular networks by VICs, leading to increased cell-matrix and cell-cell contacts. This is of critical importance because cell-matrix and cell-cell communication determines cellular architectural organization, proliferation, differentiation and apoptosis. In native valves, VICs were recognized as a complex cellular network spanning the entire valve and comprising 30% of the valve’s volume. Among the cellular network, VICs were connected by adherent junctions. Although the physiological significance of these junctions remains unclear, there is a possibility that VIC cellular networks may facilitate valve function by sensing physical forces and transmitting the information among cellular networks.

As discussed in Chapter 1, VIC activation often occurs when cultured on stiff substrates. Therefore, in our work, hydrogel stiffness was designed to be close to 5 kPa to minimize myofibroblast activation. Results showed that the compressive moduli of cell-laden hydrogels were maintained throughout cell culture (E from ~4.3 kPa to ~6.0 kPa). Moreover, AA treatment did not significantly affect the compressive moduli of
hydrogels, indicating the balanced effect of AA on cell-mediated hydrogel degradation and ECM deposition. As shown in Chapter 2, the soft PEG hydrogels induced and preserved the quiescent phenotype of VICs. Findings in this chapter showed that VIC quiescent phenotype within hydrogels was well preserved even with AA treatment for 21 days, evidenced by the lack and unchanged expression of αSMA (Figure 34), resembling their phenotype in vivo. Unlike TGF-β1 treatment that is commonly used in literature, which causes myofibroblast activation while enhancing collagen secretion, AA treatment promoted collagen secretion without causing activation. The up-regulation of collagen secretion and ALP expression often coincide, leading to osteogenic differentiation and calcification. However, the up-regulation of ALP expression also requires the cessation of proliferation. In this work, enhanced ALP expression was not observed probably because VICs maintained proliferation throughout the culture time of 4 weeks, and AA treatment further stimulated cell proliferation. While the collagen-promoting effect is promising, phenotype preservation is also important because VIC phenotype changes could cause progression toward valve diseases. No calcification was observed in cell-laden hydrogels cultured for 28 days with or without AA (Figure 35), which was consistent with the healthy, fibroblastic phenotype of VICs. Therefore, in order to promote collagen deposition while preserving
the quiescent, fibroblastic phenotype of VICs, AA shows promise for applications in heart valve tissue engineering.

### 3.5 Conclusions

This work aimed to evaluate the effects of AA on VIC phenotype and function within biomimetic PEG-PQ hydrogels. PEG-PQ hydrogels nicely supported VIC adhesion, spreading, proliferation and ECM secretion for up to 4 weeks. AA promoted VIC growth, and accelerated VIC-mediated ECM remodeling without inducing detrimental differentiation, which is potentially beneficial for the formation of living valve substitutes. Future work will focus on promoting the maturation of the cell-laden hydrogels into a functional valve substitutes by optimizing the mechanical and biochemical cues from the hydrogels, such as substrate rigidity, anisotropy, and growth factor stimuli.
Chapter 4 Adhesive Peptide Sequences Regulate Valve Interstitial Cell Adhesion, Phenotype and Extracellular Matrix Deposition§

4.1 Introduction

As discussed in Chapter 1, VICs are responsible for ECM maintenance and remodeling in the valve tissue in a phenotype-dependent manner\(^1,3,5,11,24,38,43\). Reciprocally, VIC phenotype and function are also influenced by ECM motifs, largely via adhesion-mediated cell signaling (matricellular signaling)\(^38,90\). Signaling by ECM proteins in the valve has been investigated primarily in the context of tissue engineering, with the goal of identifying and developing substrates or scaffolds that dictate valve cell fate and regenerate valve tissue. In this context, VICs cultured on fibrin were shown to have greater expression of αSMA and ALP as well as calcification than those cultured on collagen and fibronectin\(^24\). The procalcific response on fibrin involves mitogen-activated protein kinase (MAPK) signaling\(^38\), as pharmacological inhibition of MAPK downstream (ERK-1/2) or upstream kinase (MEK) in VICs on fibrin reduces ALP activity and calcification\(^208\).

§ This section has been adapted from the publication: Wu Y, Grande-Allen KJ, West JL. Adhesive peptide sequences regulate valve interstitial cell adhesion, phenotype and extracellular matrix remodeling. Cell Mol Bioeng, 2016: 479-495.
However, the usage of natural ECM proteins to study the complex cell-matrix interplay within engineered tissue is limited by inherent drawbacks such as presence of multiple types of ligands, susceptibility to loss of bioactivity, batch-to-batch variation, and risk of inducing immune rejection. ECM-mimicking adhesive peptides, which are typically less than 10 amino acids and can be chemically synthesized, serve as a better substitute of whole ECM proteins to parse out specific cell-matrix interactions and regulate cell behaviors. Various peptides derived from different ECM molecules have been recognized as potential mediators of cell adhesion, and the number continues to increase.

The RGD sequence is by far the most effective and most often employed peptide sequence for inducing cell adhesion on otherwise non-adherent surfaces\textsuperscript{175,209–212}, largely due to its widespread distribution in ECM proteins and capability of binding to many types of receptors\textsuperscript{213}. Among these receptors, integrin family comprises the most numerous and versatile group. Integrins consist of two non-convalently associated transmembrane subunits, termed $\alpha$ and $\beta$. To date 18 $\alpha$ and 8 $\beta$ subunits are known, forming 24 different heterodimers\textsuperscript{213}. About half of the 24 integrins have been shown to bind to ECM molecules in a RGDS dependent manner: $\alpha\nu\beta\iota$, $\alpha\nu\beta\delta$, $\alpha\nu\beta\gamma$, $\alpha\nu\beta\kappa$, $\alpha\nu\beta\iota$, $\alpha\nu\beta\delta$, $\alpha\nu\beta\gamma$, $\alpha\nu\beta\kappa$, $\alpha\nu\beta\iota$, $\alpha\nu\beta\delta$, $\alpha\nu\beta\gamma$, $\alpha\nu\beta\kappa$, and $\alpha\nu\beta\iota$. The process of integrin mediated cell adhesion comprises a cascade of four events: cell attachment, spreading, organization of actin
cytoskeleton, and formation of focal adhesions (on 2D surfaces)\textsuperscript{214}. Cell attachment is the initial step that a cell physically contacts the substrate to withstand gentle shear forces\textsuperscript{214}. Spreading is noticed as a cell begins to flatten, and its plasma membrane spreads over the substrate. Next, actin organization into microfilament bundles occurs, referred to as stress fibers\textsuperscript{214}. Finally, focal adhesion is formed, linking the actin cytoskeleton to the ECM\textsuperscript{214}. This linkage permits bi-directional transmission of mechanical forces and chemical signals across the plasma membrane, which determine cellular responses such as survival, migration, proliferation, and differentiation\textsuperscript{215}.

However, since RGD binds to multiple integrin receptors\textsuperscript{213}, it is impossible to specifically regulate integrin-mediated cell signaling. Thus, adhesion ligands with specific affinity for single receptors may offer better control over cell-matrix interactions, which can be applied to guide cell behaviors. For example, integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are the major adhesion receptors for collagen. Both receptors bind to collagen using their $\alpha_1$ domains, however, with distinct binding preferences and signaling functions. Integrin $\alpha_2\beta_1$ preferably binds to fibrillar collagen (types I and III) over the network-forming collagens (types IV and VI) whereas integrin $\alpha_1\beta_1$ is the opposite\textsuperscript{216}. Generally, integrin $\alpha_1\beta_1$ may induce cell proliferation and reduce collagen synthesis, whereas integrin $\alpha_2\beta_1$ increases matrix remodeling by regulating collagen I and collagenase gene expression (Figure 37)\textsuperscript{217}. Therefore, collagen mimetics with affinity to integrin $\alpha_2\beta_1$ may facilitate
VIC-mediated ECM remodeling. Such peptides that have been reported include DGEA, GTPGPQGIAGQRGVV ("P15") and GFOGER with GPP repeats at the end. DGEA, is particularly interesting due to its simplicity and ability to enhance adhesion and influence differentiation of mesenchymal cells. DGEA-containing peptides have been shown to effectively inhibit $\alpha_2\beta_1$-mediated adhesion of platelets to collagen, but have no effect on $\alpha_5\beta_1$-mediated platelet adhesion to fibronectin or $\alpha_6\beta_1$-mediated platelet adhesion to laminin.

![Diagram of distinct signaling functions of collagen receptor integrins. Adapted from Heino et al. 2000.](image)

**Figure 37.** Distinct signaling functions of collagen receptor integrins. Adapted from Heino et al. 2000.
Non-integrin adhesion receptors also play important roles in regulating cell adhesion and adhesion-mediated cell signaling, so peptides targeting these receptors are also of great interest. For example, 67 kDa laminin/elastin receptor (67LR) expression/binding has been shown to influence a variety of disease progressions via MAPK pathway, such as valve calcification\textsuperscript{208} and cancer progression and metastasis\textsuperscript{222-224}. MAPKs become activated upon cellular recognition of various growth factors or upon cell adhesion to ECM proteins, and then their signaling diverges into three separate down-stream pathways: (1) extracellular signal-regulated kinases 1 and 2 (ERK-1/2), (2) c-Jun N-terminal protein kinase (JNK), which regulate cytokine expression; and (3) p38, which primarily affects apoptosis\textsuperscript{223}. Prolonged elevation of phosphorylated ERK-1/2 was found in calcifying VIC cultures\textsuperscript{208}. Anti-calcification requires a reduction in phosphorylation of ERK-1/2, which can be achieved via increasing 67LR expression/binding\textsuperscript{208,225}. Blocking 67LR significantly increased calcification\textsuperscript{25}. These findings suggest that 67LR activation protects VICs from calcification\textsuperscript{25}. Therefore, peptides targeting 67LR may be beneficial in HVTE to eliminate or reduce calcification. Such peptides that have been reported include YIGSR\textsuperscript{25,210,226-230}, which is derived from the \(\beta1\) chain of laminin, and VAPG\textsuperscript{175,188,189,209}, which is a hydrophobic sequence derived from elastin. Previous research in the literature showed that YIGSR adsorbed on substrates induced cell attachment but not spreading whereas that covalently attached
to a solid substrate (conformationally constrained) was sufficient to induce cell
spreading of a variety of cell types\(^{226}\); the cell-adhesive activity of YIGSR was abolished
by amino acid substitution or scrambling of the YIGSR sequence\(^{231}\); immobilized VAPG,
but not the scrambled VPAG control, induced adhesion of smooth muscle cells, but not
fibroblasts, endothelial cells or platelets\(^{180}\). Therefore, in the present study, the peptide
RGDS and adhesive peptides with affinity to specific receptors of potential beneficial
effects (DGEA, YIGSR and VAPG) (Table 6) were covalently bound to PEG diacrylate
(PEGDA) hydrogels to investigate their effects on VICs. We demonstrated how the
presence of these peptides alone or in combination influenced VIC adhesion, phenotype,
and ECM deposition on 2D hydrogel surfaces or within 3D environments. Regulation of
VIC adhesion, phenotype and ECM remodeling via adhesion ligands is crucial not only
to understanding the etiology of valvular diseases but also to constructing living valve
substitutes.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Source</th>
<th>Affinity</th>
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<tbody>
<tr>
<td>RGDS</td>
<td>Fibronectin</td>
<td>(\alpha_3\beta_1, \alpha_5\beta_1, \alpha_8\beta_1, \alpha_{IIb}\beta_3, \alpha_v\beta_1, \alpha_v\beta_3, \alpha_v\beta_5, \alpha_v\beta_6, \alpha_v\beta_8), and to some extent (\alpha_2\beta_1, \alpha_4\beta_1)</td>
</tr>
<tr>
<td>DGEA</td>
<td>Collagen I</td>
<td>Integrin (\alpha_2\beta_1)</td>
</tr>
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<td>YIGSR</td>
<td>Laminin</td>
<td>67LR (via residues 205-229)</td>
</tr>
<tr>
<td>VAPG</td>
<td>Elastin</td>
<td>67LR (via three separate sites)</td>
</tr>
</tbody>
</table>


4.2 Materials and Methods

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise indicated.

4.2.1 Cell Isolation and Culture

Primary VICs were isolated and cultured as described in Chapter 2.

4.2.2 Flow Cytometry

The expression of adhesion receptors on VIC surfaces was evaluated using flow cytometry. Briefly, VICs were treated with 0.25% trypsin-EDTA (Corning) for 7 min to detach from tissue culture flasks and then suspended into single cells in serum-free media. After that, VICs (2×10⁵) were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 10 min and permeabilized in 0.25% triton X-100 in PBS for 10 min. 1% BSA in PBS was used to block non-specific binding, followed by 20 min incubation with 100 μL primary antibody (mouse anti-integrin αv, Abcam, 3 μg/mL; rabbit anti-67LR, Abcam, 3 μg/mL; mouse anti-integrin α2β1, Abcam, 6 μg/mL). Samples were washed with 1% BSA in PBS to rinse off unbound primary antibodies followed by incubation with 100 μL secondary antibody (Alexa Fluor 488, 1:500, Life Technologies, Grand Island, NY, USA) for 20 min. Cells were then washed with 1% BSA in PBS to rinse off unbound secondary antibodies, and resuspended in PBS. Samples were spun down using an Eppendorf centrifuge (9000 rpm, 7 min) between rinsing and incubation steps.
Lastly, samples were transferred to BD Falcon tubes and tested using BD FACSCanto II (BD Biosciences, San Jose, CA, USA). Samples with secondary antibody incubation but without primary antibody incubation were used as the negative controls. A minimum of $1 \times 10^4$ events (single cells) were counted for each sample and three parallel samples were used for analysis of each receptor.

4.2.3 Synthesis and Purification of PEGDA, PEG-YIGSR, PEG-DGEA, PEG-VAPG, PEG-RGDS, and Degradable PEG-PQ-PEG

PEGDA was synthesized and purified as previously described. Briefly, PEG (MW 6kDa) was reacted with acryloyl chloride at a 1:4 molar ratio in anhydrous dichloromethane (DCM) with triethyl amine (TEA, PEG/TEA at a 1:2 molar ratio) overnight under argon at room temperature. PEGDA was purified via phase separation using 2 M K$_2$CO$_3$. The PEGDA-containing organic phase was dried using anhydrous MgSO$_4$ and filtered. PEGDA was precipitated with cold diethyl ether, filtered, and dried under vacuum. The resultant PEGDA was then characterized by $^1$NMR (Avance 400 MHz; Bruker, Billerica, MA, USA) to verify acrylation and stored at -20°C until use.
Figure 38: Reaction scheme for PEGDA synthesis. PEG is reacted with acryloyl chloride in anhydrous dichloromethane with triethyl amine under argon, forming PEGDA.

PEG-peptide and PEG-peptide-PEG were synthesized and purified as previously described\textsuperscript{17}. Briefly, the adhesive peptides GGGYIGSRG, GGGDGEAGG, GGGVAPGGG and MMP-2- and -9-sensitive peptide GGGPQGIWGQGK (PQ)\textsuperscript{17,161} were synthesized in the same manner as described in Chapter 2 and characterized with a Mass Selective Detector with an electrospray ionization source (Agilent Technologies Inc., Palo Alto, CA, USA) or MALDI-TOF Mass Spectrometry (Applied Biosystems). The above three adhesive peptides and RGDS (American Peptide, Sunnyvale, CA, USA) were reacted with PEG-SVA at a 1.2:1 molar ratio as described in Chapter 2, yielding PEG-X monoacrylate (X = YIGSR, DGEA, VAPG or RGDS). PEG-PQ-PEG was synthesized and characterized as described in Chapter 2.
4.2.4 PEGDA Hydrogel Polymerization and Cell Seeding

10 wt% PEGDA with specific concentrations of PEG-X was dissolved in 10 mM HBS (pH 8.3) with 1.5% v/v TEOA. 10 µM eosin Y and 3.5 µL/mL NVP were added to this hydrogel precursor solution. An 18 µL droplet of the precursor solution was crosslinked via white light exposure (at an intensity of 230 mW at 514 nm) to form PEGDA hydrogel disks in the same manner as PEG-PQ gels described in Chapter 2. VICs were seeded on hydrogel surfaces at $1\times10^4$ cells/cm$^2$ (for cell adhesion assay, evaluated at day 2), or $4\times10^3$ cells/cm$^2$ (for immunostaining, evaluated at day 5). n=6 hydrogels per group.
4.2.5 Cell Encapsulation in PEG-PQ Hydrogels

VICS were encapsulated within PEG-PQ hydrogels made from 4 wt% PEG-PQ-PEG with different types and concentrations of adhesive peptides (PEG-RGDS (1 mM or 4 mM) and PEG-DGEA (0 mM, 3 mM or 5 mM)), at a seeding density of $1.5 \times 10^7$ cells/ml as described in Chapter 2. $n=4$ hydrogels per group.

4.2.6 Cell Adhesion and Spreading Quantification

To assess the percentage of cell adhesion, VICS adhered to hydrogel surfaces were rinsed with PBS to remove non-adherent cells and harvested using 0.25% trypsin-EDTA solution after 2 days in culture. The number of VICS harvested from the surface was assessed via Quant-iT PicoGreen dsDNA Kit (Life Technologies) per manufacturer’s protocol using a conversion factor of 5.6 pg DNA/cell. The percentage of cell adhesion was calculated as the measured cell density divided by the seeding density.

To assess cell spreading, bright field images of VICS on peptide-functionalized hydrogels were taken with a Zeiss Axiovert 135 inverted microscope at day 2. Cell spread areas and aspect ratios were analyzed from cell outlines using Image J. Aspect ratio is defined as the ratio between the two principal (long/short) axes when the cell shape is fit to an ellipse. A minimum of 140 cells were analyzed for each group.
4.2.7 Immunohistochemistry

Immunohistochemistry was performed as described in Chapter 2. Primary antibodies used in this chapter included rabbit anti-ki67, mouse anti-vimentin (1:200, Abcam), mouse anti-αSMA, rabbit anti-ALP, rabbit anti-Col I, rabbit anti-Col III, and mouse anti-fibronectin (Fn, 1:400, Abcam). To aid morphological analysis, actin and nuclei were stained with DAPI and Alexa Fluor 488 conjugated phalloidin as described in Chapter 2. Image analysis and quantification were performed as described in Chapter 2.

4.2.8 Hydrogel Mechanical Properties

Hydrogel mechanical properties were characterized by compression test as described in Chapter 2. 3-5 hydrogels were used for each group.

4.2.9 Statistical Analysis

Statistical analysis was performed using JMP 11 as described in Chapter 2. The statistical significance of differences was determined by one- or two-way ANOVA followed by post-hoc Tukey HSD. For fluorescence quantification, sample numbers were expressed as the numbers of hydrogels (n = 6 for 2D study and n = 4 for 3D study) whereas images taken from the same hydrogels were considered as replicates of one sample.
4.3 Results and Discussion

4.3.1 Material Characterization

Synthesis of the adhesive peptides, GGGYIGSRG, GGGDGEAGG, and GGGVAPGGG were confirmed using a Mass Selective Detector. The main peaks at MW 676 (Figure 40) and 628 (Figure 42) correspond to the DGEA peptide or VAPG peptide carrying one proton respectively. The main peaks at MW 823 and 412 correspond to the YIGSR peptide carrying one or two protons respectively (Figure 41).

![Figure 40: Mass spectrometry of GGGDGEAGG (DGEA) peptide.](image)

The main peak at MW 676 corresponds to the DGEA peptide carrying one proton.
Figure 41: Mass spectrometry of GGGYIGSRG (YIGSR) peptide. The main two peaks at MW 412 and 823 correspond to the YIGSR peptide carrying two or one proton respectively.
Figure 42: Mass spectrometry of GGGVAPGG (VAPG) peptide. The main peak at MW 628 corresponds to the VAPG peptide carrying one proton.

The above three adhesive peptides and RGDS were conjugated to PEG-SVA in order to be immobilized onto PEG hydrogels. The successful conjugation was confirmed using gel permeation chromatography, demonstrated by the shift of the MW to a higher MW (to the left) compared to unconjugated PEG-SVA. The conjugation efficiency was greater than 90% for all samples.
Figure 43: Representative gel permeation chromatography traces of PEG-adhesive peptides. (A) PEG-RGDS and PEG-SVA. (B) PEG-DGEA and PEG-SVA. (C) PEG-YIGSR and PEG-SVA. (D) PEG-VAPG and PEG-SVA.

The mechanical properties of PEG hydrogels are highly tunable, and in this work were designed for physiological relevance to valve tissue. The tensile (Young’s) moduli of the valve tissue are on the order of MPa, i.e., ~1.6-7.5 MPa in the radial direction, and ~5.9-14.6 MPa in the circumferential direction from different investigators. To our best
knowledge, the compressive moduli of valve leaflets have not yet been reported largely due to their thin and region-dependent thickness, typically with a thickness <0.4 mm at the cusp coaptation region, and ~0.8 mm in cusp basal region. The compressive moduli of valve sinus, which is thicker, with a thickness of ~1.3-1.9 mm, were reported to be ~17 kPa. While these values are good indicators of the mechanical properties of the valve tissue materials, they may not represent the stiffness that cells actually sense. VICs have been shown to activate from a quiescent fibroblast phenotype to a myofibroblast phenotype on substrates above a threshold modulus in the range of 4-10 kPa, much lower than any of the values reported for the valve tissue. Activated VIC phenotype on stiff substrates (above the threshold stiffness) to some extent resemble their activation during valve diseases (fibrosis and calcification), which are accompanied by valve tissue stiffening. In this work, PEG-PQ hydrogels for 3D cultures were designed with a compressive modulus below the threshold (~2.1-4.7 kPa, Figure 44B) in order to preserve the quiescent, in vivo-like phenotype of VICs. Stiffer PEGDA hydrogels (~77-109 kPa, Figure 44A) were used as the cell culture substrates on 2D because they share similar chemical properties (except for the lack of proteolytically degradable sites) with PEG-PQ hydrogels but are easier to handle due to enhanced compliance.
Figure 44: Compressive moduli of PEGDA and PEG-PQ hydrogels functionalized with various peptides. (A) Compressive moduli of PEGDA (10 wt%) hydrogels covalently modified with 1 mM PEG-RGDS and 5 mM PEG-X’ (X’ = YIGSR, DGEA, VAPG or nothing). (B) Compressive moduli of PEG-PQ (4 wt%) hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM DGEA, or total 4 mM RGDS. Comparisons with different letters indicate statistical significance: p < 0.05; n = 3-5 hydrogels per group.

4.3.2 Expression of Target Adhesion Receptors by VICs

RGDS has affinity for approximately half of the 24 known integrins, including the integrin $\alpha_v$ family ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$). Hence, the expression of integrin $\alpha_v$ on VICs was analyzed via flow cytometry. Results showed that $98.4 \pm 0.2\%$ of VICs expressed integrin $\alpha_v$ (Figure 45 A), supporting the use of RGDS to induce VIC attachment on otherwise cell non-adherent PEG$_{95,96}$. Next, the expression of 67LR, which has affinity to YIGSR and VAPG, was evaluated, and $99.7 \pm 0.0\%$ of VICs expressed 67LR (Figure 45 B). To our knowledge, this was the first time that the presence of 67LR on VICs was characterized and confirmed. Lastly, integrin $\alpha_2\beta_1$, the target receptor of
DGEA, was characterized, and 75.0 ± 1.2% of VICs expressed integrin α2β1 (Figure 45 C).

The expression of target adhesion receptors by VICs supports the study of these selected peptides.

![Graphs showing expression of adhesion receptors](image)

<table>
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<th>Receptor</th>
<th>Integrin αv</th>
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<th>Integrin α2β1</th>
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<tr>
<td>Expression by % of VICs</td>
<td>98.4 ± 0.2%</td>
<td>99.7 ± 0.0%</td>
<td>75.0 ± 1.2%</td>
</tr>
<tr>
<td>Peptide Ligand</td>
<td>RGDS</td>
<td>YIGSR, VAPG</td>
<td>DGEA</td>
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</table>

**Figure 45**: Representative profiles of adhesion receptor expression of VICs tested via flow cytometry. (A) Integrin αv, with affinity to RGDS; (B) 67LR, with affinity to YIGSR and VAPG; (C) Integrin α2β1, with affinity to DGEA. Black fill = negative controls with secondary antibodies only; no fill = samples with primary antibodies against specific receptors and corresponding secondary antibodies. n = 3 runs with more than 1×10⁴ events (single cells) in each run.
4.3.3 VIC Adhesion and Proliferation on Hydrogels via RGDS, or 67LR- and Integrin α2β1- Specific Peptides (2D)

PEGDA hydrogels were used as substrates to study VIC adhesion, proliferation, phenotype, and ECM deposition on 2D. Cell adhesion was quantified by determining the number of adherent cells on the hydrogel surfaces using a DNA assay and comparing this to the total number of cells originally seeded on the surface. Results showed that RGDS induced the highest VIC adhesion (2 mM, 66 ± 27%; 5 mM, 94 ± 26%) whereas DGEA led to moderate adhesion (5 mM, 39 ± 10%), and YIGSR and VAPG induced low levels of cell adhesion (< 20%) (Figure 46 A). While VICs that adhered to RGDS proliferated rapidly and became confluent within a few days, those that adhered to the other three peptides alone generally detached after a few days. Previous work by the Anseth group used a basal level of 0.8 mM RGDS in conjunction with other small peptides to promote VIC adhesion\(^{209}\), but cell adhesion on these peptides alone without RGDS supplementation was not reported. Their inability to support stable cell adhesion might have been the reason. Similar phenomena were also reported for human mesenchymal stem cells (hMSCs)\(^{210}\). hMSCs attachment and viability upon binding to peptides RRETAWA, IKVAV or YIGSR could only be supported for less than 48 hr, so a minimum level of RGD had to be added together with these peptides\(^{210}\). Hence, supplementation of RGDS seems necessary to maintain cell adhesion for long-term cell culture. VIC adhesion on various concentrations of RGDS showed that 1 mM RGDS was...
able to induce 70 ± 13% cell adhesion and increasing RGDS concentration led to enhanced adhesion to different extents (2 mM, ~79%; 3.5 mM, ~91%; 5 mM, ~93%; Figure 46 B). As a result, 1 mM RGDS was supplemented with the other three peptides for the remaining studies to induce basal adhesion and to evaluate the effects of adding the other three peptides on VIC adhesion, phenotype, and ECM deposition. Although the addition of the other three peptides slightly reduced the compressive moduli of hydrogels compared to RGDS only group, the compressive moduli of hydrogels with any of these three peptides were not significantly different from each other (Figure 44). Therefore, the hydrogel stiffness should not be the factor influencing VIC behaviors discussed below.
Figure 46: VIC adhesion on the surfaces of peptide-functionalized hydrogels. (A) VIC adhesion on hydrogels covalently modified with 2 mM or 5 mM PEG-X (X = YIGSR, DGEA, VAPG or RGDS) at day 2. Two-way ANOVA identified both “peptides” and “concentrations” as significant factors affecting VIC adhesion, p < 0.05. Then, each pair of group was compared using post-hoc Tukey HSD. (B) VIC adhesion on hydrogels covalently modified with various concentrations of RGDS at day 2. Comparisons with different letters indicate statistical significance: p < 0.05; n=4-6 hydrogels per group.

When 1 mM RGDS was supplemented, the addition of 5 mM DGEA enhanced VIC adhesion by approximately 33% whereas YIGSR and VAPG did not significantly increase adhesion (Figure 47 B). VICs in all groups showed mixed morphologies of well-spread cells and more round cells at day 2 (Figure 47 A). Cell spread areas and aspect ratios were measured for over 140 cells/group using Image J. Compared to the RGDS only group, the addition of 5 mM DGEA increased the average cell spread area by 25% (Figure 47 C). The DGEA group also had higher aspect ratios (long/short axis) than YIGSR and VAPG groups although statistical significance between DGEA group and
RGDS only group was not achieved (Figure 47 D). Hence, VICs tended to spread more and adopt a more elongated morphology upon DGEA binding. On the contrary, VAPG seemed to decrease the cell spread area, which was not statistically significant at day 2 but became visually more obvious at day 5 (Figure 49 and Figure 50, not quantified due to intensive cell overlap at day 5).

The decreased spread area in VAPG group may correlate with its effects on interrupting actin stress fiber distribution (Figure 49) and cell proliferation (Figure 48). Previous work from Anseth group showed that the addition of peptide VGVAPG significantly reduced the elongation factor and metabolic activity of VICs175, which may indicate negative effects of VAPG on VIC spreading and growth. In our work, the addition of VAPG significantly reduced VIC proliferation (~25% of cells ki67+, p < 0.05) whereas YIGSR and DGEA did not significantly influence VIC proliferation ratio (~40% of cells ki67+) as shown by ki67 staining compared to DAPI (Figure 48). The cell densities from different peptide groups were again quantified at day 5. Indicative of active cell proliferation, there was a 2- to 11-fold increase in cell number (relative to the seeding density) during the five days of VIC culture on different peptide-functionalized hydrogels, with the highest to lowest rank as DGEA+ (11.1 fold) > RGDS only (7.7 fold) = YIGSR+ (7.1 fold) > VAPG+ (2.6 fold) (Figure 47 E). Together with the results from VIC adhesion and proliferation study, the finding of the highest cell density from DGEA+
group is likely due to the enhanced initial cell adhesion by DGEA; the finding of the lowest cell density from VAPG+ is likely due to the inhibitory effect of VAPG on cell proliferation; and YIGSR did not significantly influence either cell adhesion or proliferation. Therefore, VIC number (impacted by both adhesion and proliferation) can be regulated via the use of adhesive peptides. The use of specific targeting adhesive peptides also allows the investigation and regulation of VIC phenotype and functions, which is limited when using RGDS alone.
Figure 47: VIC adhesion and proliferation on the surfaces of hydrogels covalently modified with 1 mM PEG-RGDS and 5 mM PEG-\(X^\prime\) (\(X^\prime\) = YIGSR, DGEA, VAPG or nothing). VIC morphologies (A), spread areas (B) and aspect ratios (C) of VICs on hydrogels at day 2. (D) VIC adhesion ratios on hydrogels at day 2. (E) Significantly increased VIC cell density on hydrogels at day 5 compared to the seeding density (dashed line). Cell spread area and aspect ratio were quantified from cell outlines of more than 140 cells per group. Cell adhesion percent was calculated as the cell number quantified by PicoGreen dsDNA assay at day 2 divided by the seeding density. Comparisons with different letters indicate statistical significance: \(p < 0.05\); \(n=4-6\) hydrogels per group. Scale bar = 100 \(\mu\)m.
Figure 48: VIC proliferation on hydrogels covalently modified with 1 mM PEG-RGDS and 5 mM PEG-\(X'\) (\(X' = \text{YIGSR}, \text{DGEA}, \text{VAPG} \text{ or nothing})\) at day 2. Blue = DAPI; red = ki67. Proliferation ratio was defined as the ki67 positive cell numbers over the total cell numbers (DAPI). Comparisons with different letters indicate statistical significance: \(p < 0.05\); \(n = 6\) hydrogels per group. Scale bar = 50 \(\mu\)m.

4.3.4 Adhesive Peptides Regulate the Expression of Phenotype Markers of VICs Cultured on Hydrogels (2D)

The expression of VIC phenotype markers including vimentin (mesenchymal), \(\alpha\)SMA (myofibroblast) and ALP (osteogenic) were characterized after VICs were cultured on the surfaces of non-degradable PEGDA hydrogels for 5 days.

Vimentin, an intermediate filament expressed by mesenchymal cells, is commonly employed as a marker for fibroblasts, but not expressed exclusively by fibroblasts. VICs on hydrogel surfaces stained positively for vimentin and the expression
level of vimentin was not significantly different among groups at day 5 (Figure 49 A and D). Consistent with our findings here, previous studies have shown that almost all VICs stained positively for vimentin both in native valves and during culture 240-242, and vimentin expression was maintained at a similar level for VICs of different phenotypes (fibroblasts versus myofibroblasts) 27.

VIC differentiation into pathological myofibroblasts or osteoblast-like cells plays a crucial role in valve disease progression. As a result, inhibiting myofibroblast activation and osteogenic differentiation is critical for the success of developing new treatments for valve diseases. On surfaces of PEGDA hydrogels covalently modified with different peptides, VICs stained positively for αSMA at day 5, indicating an activated myofibroblastic phenotype for a portion of the cells (Figure 49 B). Compared to RGDS only, the addition of YIGSR and VAPG reduced αSMA expression (αSMA fluorescence normalized to DAPI fluorescence) by 22% and 33% respectively whereas DGEA showed no significant effect (Figure 49 E). Interestingly, the aforementioned work from Anseth group showed different effects of a peptide similar to VAPG on myofibroblast activation: the addition of 1.2 mM VGVAPG increased myofibroblast activation 175,209. This is probably due to the lack of cell spreading and growth in that work (with low concentrations of adhesion peptides, low serum (1%) medium) which are associated with pathological cell growth and differentiation 8,92. Previous work in the
literature showed that extensive cell spreading in 3D culture could better maintain a quiescent, fibroblast phenotype of VICs\textsuperscript{92}. Moreover, serum deprivation caused cell death, cessation of proliferation and osteogenic differentiation of fibroblasts\textsuperscript{8}. Overall, our results suggest that 67LR binding reduces myofibroblast activation since both YIGSR and VAPG target 67LR.

ALP upregulation is an important osteogenic differentiation marker and precedes the onset of calcification\textsuperscript{24,207}, which is the leading cause of aortic valve dysfunction\textsuperscript{5}. As a result, ALP expression was analyzed. Fluorescence quantification of ALP staining of VICs on hydrogel surfaces at 5 days showed that DGEA down-regulated ALP expression level by 23\% whereas VAPG and YIGSR showed no significant effect (Figure 49 C and F). Consistent with our work, previous literature investigating the effects of RGDS and DGEA on osteogenic differentiation of hMSCs showed that DGEA induced a lower level of ALP gene expression\textsuperscript{243} and ALP activity\textsuperscript{212} than RGDS, indicating the inhibitory effect of DGEA on osteogenic differentiation compared to RGDS. Thus, DGEA is potentially beneficial to inhibit pathological osteogenic differentiation which may lead to valve calcification. Moreover, the inhibition of myofibroblast activation or osteogenic differentiation based only on the presented ECM-mimicking ligands holds immense potential for \textit{in vivo} application by incorporating peptides into the valve replacements.
Figure 49: Phenotype marker expression of VICs cultured on the surfaces of peptide-functionalized hydrogels at day 5. Representative images and fluorescence intensity quantification of vimentin (A, D), αSMA (B, E) and ALP (C, F) of VICs on hydrogels covalently modified with 1 mM PEG-RGDS and 5 mM PEG-X’. Blue = DAPI; purple = vimentin; orange = αSMA; red = ALP. Comparisons with different letters indicate statistical significance: p < 0.05; n = 6 hydrogels per group. No statistical differences were observed for vimentin expression (D). Scale bar = 50 µm.
4.3.5 Adhesive Peptides Regulate ECM Deposition by VICs Cultured on Hydrogels (2D)

A significant challenge in heart valve tissue engineering is to recapitulate the ECM components of the native valve tissue, which provide the unique mechanical properties and dynamic function of heart valves. VICs are largely responsible for the ECM synthesis and maintenance in the valve tissue. Hence, strategies to promote VIC-mediated ECM remodeling and deposition hold great promise for future living valve replacement therapies. The ECM components of native valves consist of collagens, elastin, glycoproteins and GAGs. The glycoprotein fibronectin (Fn) serves as an intermediate protein, linking cells to other extracellular components, such as collagen and GAGs. Bundles of collagen fibers (mainly collagen I and III) associate closely with VIC filopodia and are circumferentially aligned to provide tensile strength to withstand high-pressure loads. Radially aligned elastin fibers provide flexibility that allows stretch and recoil during valve opening and closure. Elastin was not evaluated in this work because appreciable elastin deposition by cultured VICs was not observed in our previous work or in most published literature. Hence, deposition of collagen (Col) I, Col III, and Fn were investigated to evaluate VIC-mediated ECM remodeling in this work.

VICs cultured on PEGDA hydrogel surfaces for 5 days deposited Fn, Col I and Col III, which were mainly pericellular (Figure 50 A-C and Figure 51 A-B). Compared to
the RGDS only group, the addition of DGEA enhanced VIC-mediated Fn, Col I and Col III deposition by 48%, 20% and 19% respectively whereas YIGSR and VAPG had no significant effects (Figure 50 D-E and Figure 51 C). These results suggest that integrin \( \alpha_2\beta_1 \) binding via DGEA promotes VIC-mediated ECM remodeling. The up-regulation of collagen synthesis and MMP secretion via integrin \( \alpha_2\beta_1 \) binding has also been reported for human osteosarcoma cells, acting through the activation of p38 pathway\textsuperscript{217,244}. 
Figure 50: Collagen I and fibronectin deposition by VICs cultured on the surfaces of peptide-functionalized hydrogels at day 5. Representative images and fluorescence intensity quantification of Fn (B, D) and Col I (C, E) by VICs on hydrogels covalently modified with 1 mM PEG-RGDS and 5 mM PEG-X’. Blue = DAPI; green = phalloidin; yellow = fibronectin (Fn); red = collagen I (Col I). Comparisons with different letters indicate statistical significance: $p < 0.05$; $n = 6$ hydrogels per group. Scale bar = 50 µm.
Figure 51: Col III secretion by VICs on hydrogel surfaces at day 5.
Representative images (A, B) and fluorescence intensity quantification (C) of Col III by VICs on the surfaces of hydrogels covalently modified with 1 mM PEG-RGDS and 5 mM PEG-X’. Blue = DAPI; green = phalloidin; red = collagen III (Col III). Comparisons with different letters indicate statistical significance: p < 0.05; n = 6 hydrogels per group. Scale bar = 50 µm.
4.3.6 Combination of YIGSR and DGEA Inhibits the Expression of Pathological Differentiation Markers of VICs while Enhancing their ECM Deposition on Hydrogels (2D)

As discussed above, YIGSR and VAPG inhibited VIC myofibroblast activation whereas DGEA inhibited VIC osteogenic differentiation and promoted VIC-mediated ECM deposition on PEGDA hydrogels. As an effort to inhibit both myofibroblast activation and osteogenic differentiation at the same time and to promote ECM deposition, the combination of YIGSR and DGEA (along with 1 mM RGDS) was evaluated. VAPG was not chosen because it severely compromised cell proliferation whereas sufficient cell proliferation is generally desired in tissue engineering. As shown in Figure 52, the combination of 3 mM YIGSR, 3 mM DGEA and 1 mM RGDS [3 mM (Y+D)+] significantly reduced the expression of phenotype markers αSMA and ALP by 46% and 24% respectively on PEGDA hydrogel surfaces at 5 days. The combination also significantly enhanced the deposition of Fn, Col I and Col III by 38%, 25% and 37% respectively on hydrogel surfaces (Figure 53 and Figure 54). The higher dosage of 5 mM of each peptide was not significantly different from the 3 mM combination for each of the phenotype marker and ECM protein tested (Figure 52, Figure 53 and Figure 54).
Figure 52: Combination of YIGSR and DGEA significantly reduced the expression of phenotype markers αSMA and ALP of VICs cultured on the surfaces of hydrogels at day 5. Representative images and fluorescence intensity quantification of αSMA (A, C) and ALP (B, D) of VICs on the surfaces of hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM YIGSR and DGEA. Blue = DAPI; orange = αSMA; red = ALP. Comparisons with different letters indicate statistical significance: p < 0.05; n = 6 hydrogels per group. Scale bar = 50 µm.
**Figure 53: Combination of YIGSR and DGEA significantly enhanced collagen I and fibronectin deposition by VICs cultured on hydrogel surfaces at day 5.**

Representative images and fluorescence intensity quantification of Fn (B, D) and Col I (C, E) by VICs on the surfaces of hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM YIGSR and DGEA. Blue = DAPI; green = phalloidin; yellow = fibronectin (Fn); red = collagen I (Col I). Comparisons with different letters indicate statistical significance: p < 0.05; n = 6 hydrogels per group. Scale bar = 50 µm.
Figure 54: Combination of YIGSR and DGEA significantly enhanced collagen III deposition by VICs on hydrogel surfaces at day 5. Representative images (A, B) and fluorescence intensity quantification (C) of Col III by VICs on the surfaces of hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM YIGSR and DGEA. Blue = DAPI; green = phalloidin; red = collagen III (Col III). Comparisons with different letters indicate statistical significance: p < 0.05; n = 6 hydrogels per group. Scale bar = 50 µm.
4.3.7 DGEA Promotes ECM Deposition and Preserves Fibroblastic Phenotype of VICs within 3D Biomimetic Hydrogels (3D)

3D culture guides cells to produce in vitro responses that are more physiologically relevant than does planar 2D culture via affecting integrin ligation, cell contraction and associated intracellular signaling. Therefore, the effects of DGEA on VIC phenotype and ECM production were further characterized in 3D, proteolytically degradable PEG-PQ hydrogels. As demonstrated in Chapter 2, in contrast to the cell culture on 2D stiff substrates where myofibroblast activation occurs, most VICs lost their αSMA expression upon encapsulation in 3D soft hydrogels (Figure 21 A). The addition of DGEA did not significantly influence the expression of phenotype markers αSMA or ALP for VICs in 3D hydrogels at 28 days (Figure 55 A-D), presumably because expression of the myofibroblast and osteogenic markers were essentially nil in 3D cultures. This may result from several factors. First, 3D matrix adhesions may differ in molecular composition, localization, morphology and function from 2D focal adhesions. Relative to 2D focal adhesions, 3D matrix interactions have been shown to display narrowed integrin usage and enhanced cell biological activities, such as enhanced cell attachment and accelerated migration rates. 3D adhesions also lead to a characteristic in vivo-like spindle-shaped (long and slender) morphology of fibroblasts (19 µm long with a length/breadth axial ratio of 33, versus 1-3 µm long with an axial ratio of 3-7 for focal and fibrillar adhesions on 2D substrates). In addition, the PEG-PQ hydrogels
(~2.1-4.7 kPa) were ~30 times softer than PEGDA hydrogels (~77-109 kPa), leading to altered mechanotransduction. VICs cultured on the stiffer 2D hydrogels assumed activated myofibroblast phenotype, resembling that in the diseased condition, whereas those that cultured within the softer 3D hydrogels assumed quiescent fibroblast phenotype, resembling that in the healthy condition. Previous work investigating the effects of substrate stiffness on hMSCs showed that ALP activity of hMSCs on stiff substrates (>22 kPa) increased with time during 15 days of culture whereas activity on soft substrates (~3 kPa) was maintained at a baseline level 246, indicating that the upregulation of ALP activity is supported by substrate stiffness. The different responses of VICs within 3D versus on 2D hydrogels in this work may reflect their changes in baseline phenotype.

Similar to the effects of DGEA on ECM deposition in the 2D study, the addition of 3 mM DGEA significantly enhanced the deposition of Fn, Col I and Col III within hydrogels by 119%, 49% and 47% respectively at 28 days when compared to the 1 mM RGDS only group (Figure 56 and Figure 57). On the contrary, the addition of 3 mM RGDS (for a total of 4 mM RGDS) did not significantly influence the deposition of these ECM proteins (Figure 56 and Figure 57), indicating that it was not the availability of extra binding sites but rather the targeted binding to integrin α2β1 via DGEA that promoted the VIC-mediated ECM deposition. This is the first time reporting enhanced
ECM deposition by VICs via targeted binding of adhesive receptors, not only on 2D but also within more physiologically relevant 3D environments. This finding holds great promise to apply specific targeting cues in the design of valve scaffolds and in the treatment of valve disease.

Furthermore, two dosages of DGEA were tested in this study, i.e., 3 mM and 5 mM. These dosages were chosen based on the results of 2D cultures that 5 mM DGEA significantly increased cell adhesion whereas 2 mM was not enough (Figure 46). This choice was also based on the previous publication that a concentration of 2.8-5 mM VAPG induced full spreading of ~50-90% of smooth muscle cells whereas a lower concentration dramatically reduced cell adhesion and spreading. However, our results showed that these two dosages were not significantly different from each other in terms of influencing ECM deposition and phenotype marker expression of VICs (Figure 55, Figure 56 and Figure 57).
Figure 55: DGEA did not significantly change the expression of phenotype markers αSMA and ALP of VICs within 3D PEG-PQ hydrogels at day 28.

Representative images and fluorescence intensity quantification of αSMA (A, C) and ALP (B, D) of VICs within PEG-PQ hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM DGEA, or total 4 mM RGDS. Blue = DAPI; yellow = αSMA; red = ALP. No groups achieved statistical significance; n = 4 hydrogels per group. Scale bar = 50 μm.
Figure 56: DGEA promoted collagen I and fibronectin deposition by VICs within 3D PEG-PQ hydrogels at day 28. Representative images and fluorescence intensity quantification of Fn (B, D) and Col I (C, E) by VICs within PEG-PQ hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM DGEA, or total 4 mM RGDS. Blue = DAPI; green = phalloidin; yellow = fibronectin (Fn); red = collagen I (Col I). Comparisons with different letters indicate statistical significance: p < 0.05; n = 4 hydrogels per group. Scale bar = 50 µm.
Figure 57: DGEA promoted collagen III secretion by VICs within 3D PEG-PQ hydrogels at day 28. Representative images (A, B) and fluorescence intensity quantification (C) of Col III by VICs within PEG-PQ hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM DGEA, or total 4 mM RGDS. Blue = DAPI; green = phalloidin; red = collagen III (Col III). Comparisons with different letters indicate statistical significance: p < 0.05; n = 4 hydrogels per group. Scale bar = 50 µm.
4.3.8 Combination of YIGSR and DGEA [(Y+D)+] Promotes ECM Deposition and Preserves Fibroblastic Phenotype of VICs within 3D Biomimetic Hydrogels (3D)

The combined effects of YIGSR and DGEA [(Y+D)+] were also tested within 3D PEG-PQ hydrogels. The combination of YIGSR and DGEA significantly enhanced the deposition of Fn, Col I and Col III by 21%, 29% and 40% respectively within hydrogels at 28 days (Figure 58 and Figure 59). Similar to the effects of DGEA in 3D hydrogels, the combination of YIGSR and DGEA did not significantly influence the expression of phenotype markers αSMA and ALP, with the majority of VICs negative for αSMA (Figure 60), indicating a quiescent, fibroblastic phenotype. Comparing to the effects of DGEA (along with 1 mM RGDS) on VICs within 3D hydrogels, the addition of YIGSR did not bring any benefits in terms of phenotype preservation in the context of little activation within hydrogels. Moreover, the addition of YIGSR rendered the ECM-promoting effects of DGEA within hydrogels less pronounced (without YIGSR, 47-119% enhancements; with YIGSR, 21-40% enhancements), indicating potential competition between these two peptides mediated adhesion signaling in 3D culture.
Figure 58: Combination of YIGSR and DGEA significantly enhanced collagen I and fibronectin deposition by VICs within 3D PEG-PQ hydrogels at day 28.

Representative images and fluorescence intensity quantification of Fn (B, D) and Col I (C, E) by VICs within PEG-PQ hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM YIGSR and DGEA. Blue = DAPI; green = phalloidin; yellow = fibronectin (Fn); red = collagen I (Col I). Comparisons with different letters indicate statistical significance: p < 0.05; n = 4 hydrogels per group. Scale bar = 50 µm.
Figure 59: Combination of YIGSR and DGEA significantly enhanced Col III deposition by VICs within 3D PEG-PQ hydrogels at day 28. Representative images (A, B) and fluorescence intensity quantification (C) of Col III by VICs within PEG-PQ hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM YIGSR and DGEA. Blue = DAPI; green = phalloidin; red = collagen III (Col III). Comparisons with different letters indicate statistical significance: p < 0.05; n = 4 hydrogels per group. Scale bar = 50 µm.
Figure 60: Combination of YIGSR and DGEA did not significantly influence the expression of phenotype markers αSMA and ALP of VICs within 3D PEG-PQ hydrogels at day 28. Representative images and fluorescence intensity quantification of αSMA (A, C) and ALP (B, D) of VICs within PEG-PQ hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM YIGSR and DGEA. Blue = DAPI; yellow = αSMA; red = ALP. No groups achieved statistical significance; n = 4 hydrogels per group. Scale bar = 50 μm.
4.4 Conclusions

The cell adhesive peptides derived from ECM proteins circumvent limitations associated with animal-derived, full-length ECM proteins when considering biomaterials for tissue culture substrates and scaffolds that predictably guide cell fate. This work aimed to regulate VIC adhesion, phenotypes and ECM synthesis via adhesive peptides with affinity to specific adhesion receptors. We demonstrated that in combination with a basal level of RGDS, adhesive peptides YIGSR and VAPG (with affinity to 67LR) down-regulated myofibroblastic differentiation whereas DGEA (with affinity to integrin αβ1) promoted VIC adhesion and VIC-mediated ECM deposition and reduced osteogenic differentiation on 2D PEGDA hydrogel surfaces. The combination of YIGSR and DGEA [(Y+D)+] promoted ECM deposition and reduced both myofibroblastic and osteogenic differentiation on PEGDA hydrogels. However, within 3D PEG-PQ hydrogels, DGEA alone or the combination of YIGSR and DGEA promoted ECM deposition without significantly influencing VIC phenotype. This work not only shows potential in regulating VIC behavior via adhesion-mediated cell signaling, but also demonstrates different VIC responses to adhesion regulation on 2D surfaces and within 3D scaffolds, giving insights into VIC regulation in heart valve tissue engineering. Moreover, the knowledge of cell-matrix interactions via adhesion provides vital information for the future development of specific targeting drug carriers for valve
disease therapy. Yet, the use of these peptides with specific affinity is limited by the fact that they are not sufficient to support stable cell adhesion that is critical for VIC survival and function, requiring supplementation of a basal level of RGDS with affinity for multiple receptors, which renders the complete control over VIC adhesion impossible.
Chapter 5 Combination of AA and Adhesive Peptides for Greater Enhancements in VIC-mediated ECM Production

5.1 Introduction

As iterated in previous chapters, to promote VIC-mediated ECM remodeling in valve substitutes is critical to the recapitulation of valve composition and function. In Chapter 3 and Chapter 4, it was shown that ECM depositions by VICs within biomimetic PEG-based hydrogels could be enhanced via applying biochemical cues, including AA, which was freely supplemented in the cell culture media, and adhesive peptide DGEA, which was immobilized on hydrogels via covalent bonds. AA facilitates collagen synthesis via acting as a cofactor in the post-translational modification of collagen (hydroxylation of proline and lysine), which is critical to the stability of the triple helical structure and formation of the intermolecular crosslinks in collagen. DGEA increases collagen synthesis likely via activating the integrin α2β1 receptor to upregulate the gene expression of collagen. Taken together, DGEA promotes the upstream part of the bioprocess of collagen synthesis by VICs whereas AA facilitates the downstream part. Therefore, it is expected that the combination of DGEA and AA may promote collagen synthesis to a greater extent than either of them alone. In this chapter, in order to maximize ECM productions by VICs, AA supplementation and DGEA immobilization were combined for potential additive or synergistic effects on VIC-mediated ECM depositions.
5.2 Materials and Methods

5.2.1 Cell Isolation and Culture

Primary VICs were isolated, encapsulated and cultured as described in Chapter 2. VICs were cultured in tissue culture polystyrene flasks in the regular growth media (GM) before encapsulation. After being encapsulated, cell-laden hydrogels were cultured in GM or GM supplemented with 50 µg/mL AA (GM+AA) as specified.

5.2.2 Cell Encapsulation in PEG-PQ Hydrogels

Peptides and PEG-peptide conjugates were synthesized and purified as described in Chapter 2. VICs (1.5×10^7 cells/mL) were encapsulated within PEG-PQ hydrogels made from 4 wt% PEG-PQ-PEG with 1 mM RGDS only or 1 mM RGDS + 3 mM DGEA, as described in Chapter 2. Cell-laden hydrogels were cultured in GM or GM+AA for 28 days. In order to generate a more valve tissue-like structure and maximize ECM production by VICs, VICs were also encapsulated at a higher seeding density (4×10^7 cells/mL) within hydrogels made from 4 wt% PEG-PQ-PEG and 1 mM RGDS + 3 mM DGEA and cultured in GM+AA for up to 28 days.

5.2.3 Immunohistochemistry to Assess Cell Morphology and Collagen Deposition

Immunohistochemistry was performed as described in Chapter 2. Primary antibodies used in this chapter were rabbit anti-Col I. To aid morphological analysis, actin and nuclei were stained with DAPI and Alexa Fluor 488 conjugated phalloidin as
described in Chapter 2. Image analysis and quantification were performed using Image J as described in Chapter 2. The sample size was n=3 hydrogels per group.

5.2.4 Hydrogel Mechanical Properties

The mechanical properties of VIC-laden hydrogels (4×10⁷ cells/mL) were evaluated with compression testing as described in Chapter 2. As a reference, one test was performed 3 days after encapsulation when gels swelled to equilibrium without extensive degradation and ECM deposition. Another test was carried out after 28 days of encapsulation to evaluate the influence of cellular activities on the mechanical properties of cell-laden hydrogels during culture. In order to parse out the influence of cell tension on the mechanical properties of cell-laden hydrogels, a group of VIC-laden hydrogels were treated with 0.1 mg/mL blebbistatin (BS) for 24 hr before compression testing at 28 days. n = 4-6 hydrogels per group.

5.2.5 Statistical Analysis

Statistical analysis was performed using JMP 11 as described in Chapter 2.

5.3 Results
5.3.1 Synergistic Effects of DGEA and AA on VIC-mediated ECM Deposition

In order to test the potential additive or synergistic effects of DGEA and AA, VICs (1.5×10⁷ cells/mL) were encapsulated within PEG-PQ hydrogels functionalized with 1 mM RGDS or 1 mM RGDS + 3 mM DGEA and cultured in GM or GM+AA. After 28 days of culture, collagen I secretion by VICs was stained and quantified (Figure 61). It was found that, compared to the control group (1 mM RGDS, GM), AA supplementation significantly enhanced collagen I secretion by 92% (Figure 61 I). The mean value of normalized collagen I secretion of the DGEA group (in GM) was 25% higher than that of the control group, which, however, was not statistically significant (Figure 61 I; p=0.64). Notably, DGEA immobilization and AA supplementation together had a synergistic effect on collagen I secretion by VICs, with a 256% enhancement from the control (Figure 61 I).
**Figure 61: DGEA and AA had a synergistic effect on VIC-mediated ECM deposition.** (A-H) Representative images of VICs (1.5×10^7 cells/mL) encapsulated within PEG-PQ hydrogels functionalized with 1 mM RGDS (referred as “RGDS only”) or 1 mM RGDS + 3 mM DGEA (referred as “DGEA+”) cultured in GM or GM+AA for 28 days. Blue = DAPI; green = phalloidin; red = collagen I (Col I). Overlays (A-D), Col I channels (E-H) and quantitative analysis of fluorescence relative to that of DAPI (I). Comparisons with different letters indicate statistical significance: p < 0.05; n = 3 hydrogels per group. Scale bar = 50 µm.
5.3.2 Cellular Morphologies Within Hydrogels

To generate a more valve tissue-like structure, where 30% of its volume is occupied by cells\(^{20}\), VICs were encapsulated within hydrogels at a higher cell density (\(4 \times 10^7\) cells/mL). For maximal ECM production, cell-laden hydrogels (4 wt% PEG-PQ-PEG) were functionalized with 1 mM RGDS and 3 mM DGEA and cultured in GM+AA for 28 days. Cell-laden hydrogels were immunostained to characterize cell morphologies and ECM deposition within hydrogels. It was found that VICs spread extensively and formed dense cellular networks within hydrogels (Figure 62). Collagen I deposition by VICs occupied the vast blank space of hydrogels and aligned along cell spreading direction (Figure 62).
Figure 62: VICs formed dense cellular networks and deposited abundant collagen I within hydrogels functionalized with 1 mM RGDS + 3 mM DGEA cultured in GM+AA at 28 days. Seeding density = 4×10^7 cells/mL. Blue = DAPI; green = phalloidin; red = collagen I (Col I). Scale bar = 50 µm.
5.3.3 Mechanical Properties of Cell-laden Hydrogels Increase with Culture due to Cell Tension and ECM Deposition

Cell-laden hydrogels of the same conditions with Section 5.3.2 (VICs 4×10^7 cells/mL; hydrogels 4 wt% PEG-PQ-PEG, 1 mM RGDS + 3 mM DGEA; GM+AA) were also tested for mechanical properties to evaluate if the significantly enhanced ECM deposition would impact the mechanical properties of the cell-laden hydrogels. Compression test showed that the compressive moduli of cell-laden hydrogels were approximately 2.5 kPa at day 3 (Figure 63). After another 25 days of culture (i.e. 28 days after encapsulation), the compressive moduli of cell-laden hydrogels increased to ~17.4 kPa (~596% enhancement) (Figure 63). In order to isolate the contribution of cell tension on the enhancement of mechanical properties, a group of cell-laden hydrogels were treated with blebbistatin (Bleb.) (a myosin II inhibitor) for 24 hr to remove cell tension before compression testing at day 28. It was found that the compressive moduli of the cell-laden hydrogels decreased to ~12.8 kPa upon BS treatment, which was still significantly higher than that prior to the prolonged cell culture of 25 days (12.8 kPa versus 2.5 kPa) (Figure 63).
Figure 63: Compressive moduli of cell-laden PEG-PQ hydrogels with $4 \times 10^7$ cells/mL. Representative stress-strain curves (A) and compressive moduli (B) of cell-laden hydrogels at day 3 and day 28 with or without blebbistatin treatment. For maximal ECM production, PEG-PQ hydrogels were functionalized with 1 mM RGDS + 3 mM DGEA, and the cell-laden hydrogels were cultured in GM+AA. Comparisons with different letters indicate statistical significance: $p < 0.05$; $n = 4$-6 hydrogels/group.

5.4 Discussion

The main finding of this work was that DGEA and AA had a synergistic effect on ECM deposition by VICs within hydrogels, which led to a significant increase in the mechanical properties of cell-laden hydrogels. In Chapter 3, AA was shown to increase collagen production by VICs within hydrogels (Figure 33). Although there was an increase in the compressive moduli of cell-laden hydrogels along with culture, the increase was counteracted upon BS treatment (Figure 36), suggesting that it was the cell tension rather than collagen deposition that led to the increase. Collagen deposition did
not lead to mechanical enhancements in that work probably because collagen deposition remained pericellular and did not occupy the vast blank space in hydrogels. In Chapter 4, DGEA was identified as an effective adhesive peptide to promote VIC-mediated ECM deposition via specific targeting of integrin α2β1 (Figure 50, Figure 51, Figure 56 and Figure 57). Also shown in Chapter 4, the compressive moduli of PEG-PQ hydrogels functionalized with 1 mM RGDS or 1 mM RGDS + 3 mM DGEA were not significantly different from each other (Figure 44), excluding the possible influence of mechanical differences on the ECM deposition by VICs in the tested conditions. In this chapter, DGEA and AA were combined to test their potential additive or synergistic effects on ECM deposition by VICs. It was found that compared to the control (DGEA-, AA-), the combination led to a 256% increase in collagen deposition, which was significantly higher than AA (92%) or DGEA (non-significant 25%) alone, suggesting a synergistic effect of the two factors on ECM regulation (Figure 61). Similar additive or synergistic effects of AA with other factors have been reported in the literature\textsuperscript{247–250}. For example, AA has been reported to synergistically enhance sonic hedgehog (Shh)-induced osteoblast differentiation\textsuperscript{247}, and additively attenuate age-related skin atrophy together with collagen peptide (from hydrolysis of gelatin)\textsuperscript{248}. AA may facilitate the function of other factors via reducing oxidative stress, acting as a cofactor, or directly participating during these processes\textsuperscript{247–250}. Therefore, combination of AA and specific-targeting
peptides (DGEA) is beneficial in HVTE in terms of promoting cell-mediated ECM deposition.

In order to generate a more valve tissue-like structure, where VICs account for about 30% of its volume and reside in ECM proteins such as collagen, the cell density of encapsulation was increased to $4 \times 10^7$ cells/mL. Meanwhile, the combination of DGEA immobilization and AA supplementation was also applied to maximize ECM deposition. Histological analysis showed that the high density of VICs ($4 \times 10^7$ cells/mL) formed extensive cellular networks within hydrogels (Figure 62), which seemed denser than that from the lower cell density as expected. Collagen I deposition by VICs aligned along the cellular network and occupied the vast blank space of hydrogels (Figure 62), which seemed in higher amounts than that from the lower cell density. In order to evaluate if the significantly increased collagen deposition would lead to an improvement of the mechanical properties of cell-laden hydrogels along with culture, compression test was performed at the beginning (3 days after encapsulation when hydrogel swelling reaches equilibrium without extensive hydrogel degradation and ECM deposition) and after prolonged culture (28 days after encapsulation). It was found that the compressive moduli of cell-laden hydrogels were approximately 2.5 kPa at day 3 (Figure 63), which set the reference level of compressive moduli without extensive cellular activities. After culture of 4 weeks, the compressive moduli of cell-laden
hydrogels significantly increased compared to the reference level (~17.4 kPa, 596% enhancement) (Figure 63), suggesting that cellular activities strengthened hydrogels along with culture. In order to parse out the contributors in the enhancement of mechanical properties, a group of cell-laden hydrogels were treated with BS to remove cell tension. The compressive moduli decreased by 4.6 kPa upon BS treatment (Figure 63), suggesting the contribution of cell tension in compressive moduli. However, the decreased moduli were still significantly higher than the reference level (~12.8 kPa, 412% higher; Figure 63). These findings suggest that cellular activities including both cell tension and cell-mediated ECM deposition, could enhance the mechanical properties of cell-laden hydrogels along with culture. Compared to the results presented in Chapter 2, where cell tension but not collagen deposition increased the compressive moduli of cell-laden hydrogels by approximately 1.4 kPa during the culture of 28 days, the difference may result from several factors. First, DGEA immobilization and AA supplementation were added in this study to promote collagen deposition per cell by VICs. Second, the cell density of encapsulation in this study was four-fold of that in the previous study (4×10^7 cells/mL versus 1×10^7 cells/mL), which formed denser cellular networks and deposited more collagen in total. Compared to the low amounts of collagen deposition in that work, collagen deposition in this study was more and occupied a larger space of hydrogels. The increased collagen deposition per cell (due to DGEA and AA) and the
higher cell number may explain why collagen deposition in this work led to a significant enhancement in the mechanical properties of cell-laden hydrogels, which was not observed in the previous work. Moreover, cell tension contributed to ~4.6 kPa in the increase of compressive moduli in this work, which was significantly higher than the contribution of ~1.4 kPa in the previous work. For one thing, the increased cell tension may result from the denser cellular network. For another, AA may increase cell tension. Previous work in the literature showed that AA treatment improved the mechanical properties of SMC-seeded PEG-fibrinogen hydrogels due to the increased contractile properties of SMCs but not collagen deposition. Therefore, the mechanical properties of cell-laden hydrogels could be modulated via designing the original stiffness of hydrogels and regulating cellular activities.

A limitation of this study is that the contributions of different factors (including DGEA immobilization, AA supplementation and cell density) to the increase in mechanical properties were not individually studied. Following up experiments could include controls with changes of a single factor to parse out their contributions. Another limitation is that the influence of hydrogel degradation (due to MMP-2 secretion by VICs) on the mechanical properties of cell-laden hydrogels was not studied. Following experiments could be designed to quantify MMP-2 secretion by VICs and use cell-free hydrogels incubated in MMP-2 supplemented media as a control. Moreover, collagen
deposition by VICs were not aligned in a single direction as that in the native valve tissue. To further enhance collagen deposition and alignment, future work could apply mechanical stimuli, which has been shown to increase the ECM deposition and alignment by VICs as well the mechanical properties of cell-laden constructs \(^{94,129,134,135}\).

Combination of AA supplementation, DGEA immobilization, and mechanical stimulation represents a promising way to maximize ECM production and alignment by VICs toward a strengthened, mature valve substitutes.

**5.5 Conclusions**

DGEA immobilization and AA supplementation had a synergistic effect on promoting VIC-mediated collagen deposition within hydrogels. The increased collagen secretion that occupied the vast blank space of hydrogels, together with cell tension, led to a significant improvement in the mechanical properties of cell-laden hydrogels. Therefore, DGEA and AA could be co-applied in the design of TEHVs to promote the maturation of cell-laden constructs to a functional valve substitute with compositions and properties reminiscent of the valve tissue.
Chapter 6 Conclusions and Future Directions

6.1 Concluding Remarks

The work presented in this dissertation demonstrates regulation of VIC phenotype and function using mechanical and biochemical cues within synthetic, biomimetic hydrogels. The hydrogel platform used in this work enables fine control over cellular microenvironments, which allows reliable study of cell behaviors without introducing confounding factors. VICs assume a quiescent phenotype within the 3D hydrogel environments, which better represents their in vivo phenotype compared to most other studies where myofibroblast activation commonly occurs. The mechanical and biochemical factors that have been identified in this system to regulate VIC behaviors, especially phenotype and ECM deposition, could be applied in the future design of TEHVs.

6.2 Future Research Directions

The work presented in this dissertation builds on previous research and takes another step on the continuum toward development of living valve substitutes. In this work, great efforts have been made to preserve VIC phenotype, promote VIC-mediated ECM remodeling and enhance the mechanical properties of the cell-laden constructs via exploration of several mechanical and biochemical cues. Despite of the exciting prospects of living valve substitutes, immense challenges must be overcome before
translation to patients safely and effectively. Future research could focus on the optimization and maturation of the cell-laden constructs presented here and overcome the obstacles on the way to their clinical applications.

6.2.1 Characterization and Quality Control of VICs

As discussed above, VICs play critical roles in the long-term functionality of heart valves, however, the heterogeneous, dynamic and source-dependent nature of cell behaviors, the lack of characterization during isolation and culture, and the yet unknown pathology mechanisms make it difficult to reliably control and predict cell performances *in vitro*, not to mention *in vivo*, where interactions with other cells, such as circulating stem cells and immune cells, add to the complexity.

In this work, it was shown that VIC phenotype and function could be regulated via targeting specific adhesion receptors, such as integrin. However, integrin expression and turnover of VICs vary by sources (species and valve positions) and culture conditions. For example, previous work in the literature showed that bovine VICs expressed integrin $\alpha_9\beta_1$ and $\alpha_3\beta_3$ whereas baboon VICs expressed $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_5\beta_1$\textsuperscript{252}. Human VICs isolated from different types of valves (aortic valve (AV), mitral valve (MV), pulmonary valve (PV) and tricuspid valve (TV)) showed different expressions of integrin receptors and surface adhesion molecules, however, with high expressions (>50%) of $\alpha_2$, $\alpha_3$, $\alpha_5$, and $\beta_1$, and moderate expressions of $\alpha_1$ (14-25%) in common\textsuperscript{205}. 

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Interestingly, AV-derived and MV-derived VICs had the highest and the second highest expression of intercellular adhesion molecules-1 (AV, 29%; MV, 11%; PV, 2%; TV, 2%), which help to localize leukocytes to tissue injury\textsuperscript{205}. This difference may correlate with the susceptibility of AV and MV to inflammation during several valvular diseases\textsuperscript{29}. The integrin expression of human VICs were also compared with that of MSCs, with the major difference being the expression of integrin $\alpha_\beta_1$ (VICs, 81%; MSCs, 6% or 13% depending on sources)\textsuperscript{192}. Considering the fact that most VICs used in the literature were porcine due to their easy access, it would be interesting to characterize the integrin expression of porcine VICs and compare with human VICs, which is critical to the future translation to clinic. We performed a preliminary study on the integrin expression of porcine VICs. It was found that porcine VICs cultured on TCPS plates expressed an extremely high level of integrin $\alpha_1(\beta_1)$, which was several folds higher than any other integrin expression. Compared to TCPS cultures, VICs on PEGDA hydrogels decreased the expression of integrin $\alpha_1(\beta_1)$ whiling increasing the expression of integrin $\alpha_2(\beta_1)$ and $\alpha_5(\beta_1)$ (Appendix), suggesting active integrin turnover and level regulation of VICs in response to the changes in culture conditions. A correlation between culture substrate and integrin activity/expression was also reported in the literature: integrin $\alpha$ subunit activity of epithelial cells was 3 times higher when cultured on stiff plastic than on soft basement membrane matrix\textsuperscript{253}; integrin $\alpha_5\beta_3$ expression was found in osteoblasts
cultured *in vitro*, but not in osteoblasts in human tissue\(^{214}\). A better characterization of integrin expression and turnover in animal models versus in humans, *in vitro versus in vivo* is necessary, and it would be interesting to investigate how the integrin expression and turnover of VICs correlate with culture conditions (such as substrate stiffness and biochemistry) and influence VIC growth, phenotype and function.

Currently, all VIC cultures reported in the literature are without purification or sorting step largely due to the lack of markers for VIC characterization. Currently known markers are not sufficient to differentiate various cell populations obtained from isolation. For example, quiescent, fibroblastic VICs are characterized by the expression of vimentin but not \(\alpha\)SMA\(^3\). However, vimentin is not exclusively expressed by fibroblasts and still persist in activated myofibroblasts and osteoblast-like cells at a comparable level\(^{27}\). Almost all VICs stained positively for vimentin both in native valves and during culture\(^{240–242}\). Myofibroblasts are characterized by the expression of \(\alpha\)SMA, which is not expressed in fibroblasts. However, \(\alpha\)SMA expression also occurs in VICs undergoing osteogenic differentiation. Although there are changes in the expression levels of other genes/proteins during myofibroblast activation and osteogenic differentiation, such as collagen, MMPs, ALP, OCN and CBF-\(\alpha\)1, these changes are relative and there are no threshold values to set boundaries between different VIC populations\(^{4,24,43–46}\). A better characterization and understanding of VICs is needed for
the quality control of VICs, which would significantly influence the success of valve substitutes in vivo. To develop a disease model, such as a calcification model, using the PEG hydrogel platform presented in this work, may help the understanding of VIC behaviors under both healthy and diseased conditions.

Upon harvest, VICs are immediately plated onto 2D stiff TCPS flasks for in vitro expansion. However, in a recent study, it was shown that TCPS culture altered the transcriptional profile of VICs when compared to the freshly isolated VICs, with 2,173 gene probes up-regulated and 1,926 gene probes down-regulated. The most significantly up-regulated gene functions included cell cycle, cytoskeleton, and mitochondrion; the most significantly down-regulated gene functions were extracellular region, polysaccharide binding, inflammatory response, and regulation of transcription from RNA polymerase II promoter. Moreover, unlike the mainly quiescent, fibroblast phenotype in vivo (healthy adult valves), VICs cultured on TCPS assume an activated myofibroblast phenotype. These results suggest that conventional TCPS cultures would reprogram VICs and alter their behaviors, which may lead to compromised performance when these cells are put back in vivo. A culture platform that better preserves VIC in vivo-like behaviors is preferable for routine cultures of VICs. The PEG hydrogel platform in this work shows promise for this purpose because it better preserves the quiescent, fibroblast phenotype of VICs as presented in this work. Further
characterization and understanding of VIC behaviors on PEG hydrogels versus their \textit{in vivo} state are needed for evaluation.

\section*{6.2.2 Co-culture with VECs for VIC Regulation and Anti-thrombosis}

Recent findings have illuminated the importance of VIC-VEC interactions in maintaining valve homeostasis\textsuperscript{41}. VECs, but not VICs, express endothelial nitric oxide synthase (eNOS) in both porcine and human valves, which expression is reduced in calcified aortic valves in a side-specific manner\textsuperscript{41}. \textit{In vitro} studies have also showed beneficial effects of VECs in VIC phenotype preservation and anti-calcification\textsuperscript{39-41}. For example, in a study on TCPS plates, co-culture with VECs significantly attenuated VIC activation and calcification\textsuperscript{41}. To better mimic the physiological environment of VICs and VECs, advanced hydrogel platforms have been developed to study VIC-VEC interactions \textit{in vitro}\textsuperscript{39,40}. For example, in a study, VICs were cultured on the surfaces of PEG hydrogels covalently tethered in the transwell inserts whereas VECs lined the underside of the insert membrane\textsuperscript{39}. It was found that indirect co-culture with VECs significantly reduced VIC activation (\textasciitilde70\% decrease on soft 3 kPa gels and \textasciitilde20-30\% decrease on stiffer 27 kPa gels). This effect was prevented when nitric oxide (NO) release was inhibited with \textit{l}-NAME, suggesting that VECs inhibit VIC activation via NO release\textsuperscript{39}. In a more recent study from our collaborator (Grande-Allen lab at Rice University), a direct co-culture model of VECs and VICs in 3D was developed utilizing
PEG hydrogels with spatially organized adhesion ligands to mimic the spatial organization of VICs and VECs in vivo. In this model, RGDS peptide was incorporated into the hydrogel bulk whereas RKR peptide was tethered on the surface so that VICs were encapsulated throughout the interior of hydrogels whereas VECs formed a monolayer on the surface. It was found that both cell types maintained phenotypes, homeostatic functions, and produced zonally localized extracellular matrix (Figure 64). The addition of VEC monolayer on the surface reduced VIC activation and platelet adhesion to the underlying hydrogels. Co-culture with VECs represents an attractive method to preserve VIC phenotype, which also holds great significance in future transplantation of valve substitutes where endothelialization is desired for anti-thrombosis. Moreover, the co-culture platform may also be adapted as a disease model to study VIC-VEC interactions under stressed or diseased conditions to determine how VIC-VEC signaling may contribute to the onset and progression of valve disease, such as calcification.
Figure 64: VIC-VEC co-cultures maintain valve cell phenotypes. VECs on the surfaces of hydrogels formed a confluent monolayer and express CD31 (green), while encapsulated VICs had low levels of αSMA (red). Adapted from Puperi et al. 2015\(^40\). Scale bar = 50 µm.

6.2.4 Optimization of Scaffold Properties to Mimic the Heterogeneity of Valve Leaflets

As discussed in Chapter 1, valve leaflets are stratified into three layers with distinct ECM composition and alignment, which determine the unique valve mechanical properties and function during cardiac cycles. Circumferentially aligned collagen fibers in the fibrosa layer provide tensile strength to resist high pressure loads; elastin fibers in the radial direction in the ventricularis layer allow stretch and recoil; GAGs in the middle spongiosa layer provide compression resistance and lubrication\(^31-33\). PEG
hydrogels presented in this work are homogenous materials. Strategies need to be applied to create heterogeneity in order to mimic the layered, anisotropic structure of valve leaflets. Recent research has demonstrated that heterogeneous 3D scaffolds can be created using techniques such as photolithographic patterning\textsuperscript{139}, electrospinning\textsuperscript{125,254}, and layering\textsuperscript{162,172,255–258}, with each approach having their own advantages and shortcomings.

Photolithographic patterning could create mechanically or biochemically heterogeneous 3D features with high resolution. For example, anisotropic mechanical properties were established in PEG hydrogels via patterning stripes of low MW (3.4 kDa) PEG within high MW (20 kDa) PEG base gels\textsuperscript{171,177}. Furthermore, heterogeneous bioactive molecules could be patterned within PEG hydrogels with high resolution using two-photon absorption laser scanning lithography\textsuperscript{139}. A common problem of the patterning approaches is the compromise between resolution and timing, which limits the scale of the patterned features.

Electrospinning uses an electric field to disperse a solution and a mandrel to collect fibers\textsuperscript{125,254}. Electrospun fibers impart anisotropic mechanical behaviors with higher strength and stiffness in the fiber alignment direction. Limited by the small pore size, cells seeded on top of the electrospun fibers (2D) cannot populate the interior of the materials until fibers degrade\textsuperscript{125,254}. Electrospinning may also be combined with the
layering techniques, where layers of different material or cellular compositions can be sequentially added to create heterogeneous materials or tissues. For example, electrospun PCL or polyurethane urea fibers were sandwiched between PEG hydrogel layers to take advantage of the mechanical strength and anisotropic properties of electrospun fibers and the bioactivity of the hydrogels\textsuperscript{172,259}.

To generate a more complex structure with high resolution using the layering techniques, 3D printing employs robotic layer-by-layer additive fabrication technology\textsuperscript{260}. With computer-assisted design and manufacturing, specific types of cells and polymers can be placed into precise geometries that mimic actual tissue construction\textsuperscript{260}. For example, the Butcher lab have printed PEGDA/alginate hydrogels that recapitulated the complex geometry of aortic valve leaflets and roots with high fidelity\textsuperscript{162,182}.

A significant common limitation of these strategies is the compatibility with living cells in 3D. Maintenance of cell viability and function during these processes remains a challenge due to the harsh conditions (use of organic solvents or lasers, shear stress, lack of proper temperature, oxygen and nutrients) presented to cells. Enhancing cytocompatibility of these approaches or developing other innovative strategies to create heterogeneous structure, mechanical properties and cells may lead to breakthroughs in building viable tissues and organs.
6.2.3 Application of Mechanical Stimuli in Bioreactors

All of the work in this thesis was performed in static cell culture. Future work could explore the application of mechanical stimuli to regulate VIC behaviors and potentially improve the mechanical properties of cell-laden hydrogels. VICs respond to mechanical loading via varying phenotype, cell turnover rate, as well as matrix synthesis and reorganization. For example, ECM (e.g. collagen, GAG and PG) synthesis by VICs is dependent on the degree and duration of mechanical stretch and relaxation. Increasing anisotropy of biaxial strain or strain amplitude results in increased cellular orientation, proliferation, apoptosis, and αSMA expression, followed by increased collagen deposition and alignment along the principal directions of strain, leading to improved tensile properties of cell-laden, fibrin-based constructs. Overall, application of cyclic mechanical forces mimicking the stretch, flexure, and shear stress experienced by valves in vivo enhances the ECM deposition and alignment by VICs, which may lead to a significant improvement on the mechanical properties of cell-laden hydrogels.

The work presented in this dissertation has identified DGEA immobilization and AA supplementation as beneficial factors that can be applied in the future design of TEHVs to enhance the ECM deposition by VICs and thus the mechanical properties of cell-laden constructs. It would be interesting to investigate if these factors can be co-
applied together with mechanical stimuli for greater enhancements. Therefore, incubating cell-laden hydrogels functionalized with DGEA in AA supplemented media with applying mechanical loading in bioreactors before implantation may be a promising way to facilitate the maturation of engineered constructs for a desired in vivo performance. To our best knowledge, cell-laden PEG hydrogels have not yet been tested in bioreactors. Future research could focus on investigating how different types of mechanical forces influence the ECM remodeling and the mechanical properties of VIC-laden PEG hydrogels.

Moreover, the blood flow-induced shear stress has been recognized as an important stimulus for valve homeostasis and disease. Patients with hypertension or bicuspid valves, where hemodynamic forces are altered, have a higher risk of CAVD\(^9\). However, how hemodynamic and biomechanical forces are transduced into biomechanical signals that affect valvular cell function remains unknown. Discovery of the mechanisms may lead to targets for protection or treatment of CAVD associated with the biomechanics-related high risk factors.

6.2.5 Other Clinical Challenges (Fatigue Life Analysis, Animal Models, Safety and Efficacy, Patient Specificity, etc.)

Valve substitutes need to withstand billions of cycles without failure. After construction and maturation, they should be evaluated for long-term potential for fatigue. The international standard cardiac valve prostheses (ISO 5840) and the FDA
Draft replacement heart valve guidance documents require stress and fatigue life analysis for a prosthetic heart valve\textsuperscript{51}. During the analysis, valve substitutes are tested under various stress conditions for a certain number of cycles\textsuperscript{51}. To impart a significant degree of conservatism into the analysis, FDA requires that fatigue life analysis be applied to the “worst case”, which is the most severe situation where all the factors that could contribute to fatigue are combined\textsuperscript{51}.

Furthermore, valve substitutes need to be tested in animal models prior to clinical trials in humans. Sheep is the only FDA approved large animal model for heart valve replacement evaluation\textsuperscript{2}. However, sheep endothelialize cardiovascular grafts much more quickly and completely than do humans, which may artificially improve performance\textsuperscript{2}. The pig, as a more human-like animal model, poorly tolerates invasive surgery, creating too much risk for experimental variability and likelihood for prohibitive costs\textsuperscript{2}. An effective animal model that better represents human conditions remains to be developed. For more accurate prognosis in humans, future research could focus on modifying the sheep model to obtain a comparable endothelialization rate as in humans or improving the surgery tolerance of the pig model.

Moreover, there is a need to develop pre-clinical guidelines that specify evaluation standards of the safety (biocompatibility, durability, modes of failure and ease of monitoring) and efficacy (implantability, functionality and reliable long-term
performance) of TEHVs before implantation in humans\textsuperscript{11}. In addition, \textit{in vivo} remodeling of valve substitutes may display more variability among patients due to biological heterogeneity among individuals. A patient-specific scaffold design is demanded to match scaffold degradation with ECM deposition for optimal ECM remodeling. In order to gage the approximate rate of ECM remodeling of patients for customized design of scaffolds, future research may apply computational modeling to correlate ECM remodeling with characteristics of patients, such as gender, age, weight, health state and lifestyle.

6.3 Overall Summary

In this dissertation, we have demonstrated the use of a synthetic hydrogel platform to understand and regulate VIC behaviors in 3D, with a focus on cell phenotype and ECM deposition toward the development of living valve substitutes. This hydrogel platform represents an advanced, finely controlled system to reliably study and direct cell behaviors within a physiologically relevant microenvironment. Findings in this work advance our knowledge of VIC biology and pathology in response to mechanical and biochemical stimuli under both healthy and diseased conditions. This knowledge could not only be applied in the future design of TEHVs, which is the ultimate solution of valve replacements in pediatric and adolescent patients, but also may lead to discovery of novel therapeutic targets of valvular diseases in adults. To
summarize, the findings in this dissertation and their potential applications have widespread impact on heart valve treatment now and in the future.
Appendix: Supplementary Information

**Sigmacote-treatment of Glass Slides**

In order to create a hydrophobic surface from which PEG hydrogels can be easily detached, glass slides were treated with Sigmacote (Sigma). Briefly, glass slides were cleaned with acetone and baked at 90°C for 30 min to fully expose the surface silanol groups for reaction. After cooled down, the slides were submerged in Sigmacote solution (both sides) for 10 seconds, rinsed with diH2O, and wiped to dry. This submerging, rinsing and wiping processes were repeated once for complete coating. At last, the slides were baked at 90°C for 30 min to extend the durability of the treated slides. Treated slides without baking generally had a life time of approximately 2 weeks, which could be extended to over a year with baking.

**Methacrylation of Coverslips**

In order to immobilize PEG hydrogels onto coverslips for easy handling of hydrogels, coverslips were functionalized with methacrylate groups on the surface, which could covalently crosslink with the acrylate groups in PEG hydrogel precursor solution. Briefly, coverslips were cleaned with Piranha solution containing 30% (v/v) hydrogen peroxide and 70% sulfuric acid for 2 hours, followed by rinsing with diH2O and 95% ethanol three times respectively. Then methacrylation was performed via exposing to 2% 3-(trimethoxysilyl) propyl methacrylate in 95% ethanol overnight.
Modified cover slips were rinsed with 95% ethanol and baked at 90°C for 30 min before use.

**PDMS Spacer Fabrication**

In order to control the thickness of PEG hydrogels, PDMS spacers of the desired thickness were prepared using a Sylgard 184 Silicone Elastomer kit (Dow Corning, Auburn, MI, USA). Briefly, elastomer base (Part A) and curing agent (Part B) were mixed together in a petri dish at a 1:10 ratio (base: curing agent) using a plastic spatula. The mixture was degassed in a vacuum oven for 15 min and then carefully transferred to devices assembled with a Teflon spacer (400 µm or 1 mm thick) between two Sigracote-treated glass slides. The mixture was then baked at 60°C overnight to complete curing, forming polydimethylsiloxane (PDMS) slabs. The slabs were cut into strips or any designed shape to get PDMS spacers.

**Live/Dead Kit to Assess Cell Viability**

In order to assess cell viability, Live/Dead Viability Kit (Life Technologies) was used. This kit works based on the two distinguishing characteristics of live cells, i.e., the ubiquitous intracellular esterase activity and an intact plasma membrane. It quickly discriminates live from dead cells by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity. Cell viability can be
quantified using the number of live cells (positive for calcein AM) divided by the total number of cells (positive for either calcein AM or ethidium homodimer) *100%.

**Test of VIC DNA Content per Cell**

To determine VIC DNA content per cell, VICs cultured on TCPS plates were trypsinized and counted by hemocytometer. A total cell number of 0.22×10⁶ cells (n = 4) were taken to run PicoGreen DNA assay together with 6 standards per manufacturer’s instructions. A standard curve of absorbance-DNA content was generated from the standards and used to calculate the DNA content from cell samples. Conversion factor (DNA content per cell) was calculated by dividing the calculated DNA content by the cell number.

**Table 7: Test of VIC DNA content per cell using PicoGreen DNA assay and hemocytometer cell counting.**

<table>
<thead>
<tr>
<th>Hemocytometer</th>
<th>PicoGreen DNA assay</th>
<th>Calculated conversion factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell number</td>
<td>Absorbance (y) (n = 4)</td>
<td>Standard curve (n = 6)</td>
</tr>
<tr>
<td>0.22×10⁶</td>
<td>16895 ± 1394</td>
<td>y = 11141x + 3058, R² = 0.99</td>
</tr>
</tbody>
</table>
**α, β Integrin-mediated Cell Adhesion Array Kit to Assess Integrin Expression by VICs**

In order to assess the integrin expression by VICs, α, β Integrin-mediated Cell Adhesion Array Kit from EMD Millipore (Billerica, MA, USA) was used. In this kit, mouse monoclonal antibodies generated against human alpha (α1, α2, α3, α4, α5, αv and αvβ3) and beta (β1, β2, β3, β4, β5, αvβ5 and α5β1) integrins/subunits are immobilized onto a goat anti-mouse antibody coated microtiter plate. The plate is then used to capture cells expressing these integrins on their cell surface; goat anti-mouse antibody coated wells are provided as a negative assay control. Experimental cells are seeded onto the coated surface as single cell suspensions in Assay Buffer and incubated for 1-2 hr. Subsequently, unbound cells are washed away with Assay Buffer, and the adherent cells are lysed with Lysis Buffer and detected by the CyQuant GR dye (1:75 dilutions in Lysis Buffer). This green-fluorescent dye exhibits strong fluorescence enhancement when bound to cellular nucleic acids. Relative cell attachment is determined using a fluorescence plate reader.

Results showed that porcine VICs cultured on TCPS plates expressed an extremely high level of integrin α1(β1), which is several folds higher than any other integrin expression (Figure 65). A batch of VICs were harvested from TCPS and subsequently seeded on the surfaces of PEGDA hydrogels. After 2 days of culture, VICs were harvested from PEGDA hydrogels and evaluated for integrin expression. Results
showed that integrin $\alpha_1(\beta_1)$ still remained the highest expression in VICs on PEGDA hydrogels, but the fold differences over other integrins decreased (Figure 65). Meanwhile, there was an increase in the expression of integrin $\alpha_2(\beta_1)$ and $\alpha_5(\beta_1)$ (Figure 65). These changes suggest active integrin turnover and level regulation of VICs in response to the changes in culture conditions.
A: VICs harvested from culture on TCPS.

B: VICs harvested from culture on the surfaces of PEGDA hydrogels.

Figure 65: Integrin expression profiles of VICs cultured on tissue culture polystyrene plates or on the surfaces of PEGDA hydrogels. VICs were cultured on tissue culture polystyrene plates (TCPS) or on the surfaces of PEGDA gels prior to the assessment. PEGDA hydrogels were composed of 4 wt% PEGDA and 5 mM PEG-RGDS. VICs were harvested from culture using 0.05% trypsin-EDTA, and then assessed for integrin expression using \(\alpha, \beta\) Integrin-mediated Cell Adhesion Array Kit (EMD Millipore). Detailed information of this kit can be found in Appendix.
Application of Other Biochemical Cues for Better Preservation of VIC Phenotype

To mimic the NO regulation during VIC-VEC interactions, researchers have explored the use of NO donors\textsuperscript{41,261}, such as DETA-NONOate and sodium nitroprusside, and agents raising intracellular cyclic guanosine monophosphate (cGMP) levels, such as brain natriuretic peptide and C-type natriuretic peptide (CNP)\textsuperscript{261}, as a supplement in the culture of VICs. It has been shown that adding these drugs showed anti-calcification effects for VICs cultured on TCPS plates\textsuperscript{41,261}. As discussed in previous chapters, PEGDA hydrogels are more physiologically relevant than TCPS plates and show promise in the design of TEHVs. Therefore, in order to evaluate the potential of these drugs for future application in the context of TEHVs, we did preliminary study on the effects of NOC-18 (DETA-NONOate, with a half-life of 21 hr) and CNP on VICs seeded on PEGDA hydrogels. Cell-seeded hydrogels were cultured in low serum (1%) GM or Osteo M to minimize the influence of cell proliferation. Results showed that treatment of osteogenic components (50 \(\mu\)g/mL AA, 10 mM \(\beta\)-glycerophosphate disodium and 10 nM dexamethasone) caused an increase in the expression of \(\alpha\)SMA and ALP of VICs (78\% and 26\% increase respectively) (Figure 66). Supplementation of 20 \(\mu\)M NOC-18 in VIC culture media reduced the expression of \(\alpha\)SMA in the media conditions with or without osteogenic components (Figure 66, 33\% and 24\% decrease respectively). However, NOC-18 reduced the expression of ALP only for VICs cultured in GM (29\% decrease), but not
Osteo M (Figure 66), suggesting that NOC-18 was helpful to reduce Osteo M-induced myofibroblast activation (to a level that was still higher than that in GM) but not osteogenic differentiation in the tested conditions. Notably, NOC-18 seemed to cause a change in cell morphology (decreased cell spreading) and a decrease in cell numbers (Figure 66), which may correlate with concentration-dependent effects of NO on cell growth and proliferation\textsuperscript{262}. NO reacts with molecules such as oxygen, superoxide, nucleic acids and proteins\textsuperscript{262}. Several studies from the literature showed that relatively low concentrations of NO may promote cell proliferation and anti-apoptosis responses (mechanisms unclear) whereas higher levels of NO tend to induce cell cycle arrest, senescence or apoptosis (involving cGMP-mediated processes and nitrosative stress)\textsuperscript{262}. Evaluation of NO release using Griess assay showed that NOC-18 had a burst release of NO (40% in the first 6 min), followed by a slow release (approximately another 14% in the next 72 hr) (Figure 67). The burst release in the first few minutes mostly like had generated an overly high concentrations of NO that caused cell apoptosis; the extremely slow release during the majority of its lifetime could have compromised its anti-calcification effects. Strategies to generate a more stable production of NO over a relatively long period may provide enhanced performance of NO donors.
Figure 66: Nitric oxide release reduced the expression of αSMA and (or) ALP of VICs cultured on the surfaces of PEGDA hydrogels. VICs were seeded on PEGDA hydrogels at 10,000 cells/cm² and cultured in low serum (1%) media as specified for 6 days. NOC-18 (20 µM) supplemented media was prepared freshly for each media change (2 days per change). Blue = DAPI; yellow = αSMA; red = ALP. Comparisons with different letters indicate statistical significance: p < 0.05; n = 6 hydrogels per group. Scale bar = 50 µm.
Figure 67: Nitric oxide release profile from NOC-18 (DETA NONOate). Nitric oxide (NO) release from NOC-18 (Santa Cruz Biotech, with a half-life of 21 hr) was quantified using Griess assay. This assay measures the nitrite concentration in solution, which accounts for about 99% of NO release products. The release profile was plotted in both the standard and the log scale for better visualization of data at different time points.

CNP is one of the three natriuretic peptides (NPs) that are endogenous hormones released by the heart in response to myocardial stretch and overload\textsuperscript{263}. Besides regulating fluid and blood pressure, NPs have been implicated in the protection against atherosclerosis, thrombosis and myocardial ischemia, which can coexist and potentially lead to heart failure\textsuperscript{263,264}. Unlike A-type and B-type NPs which are expressed and stored in granules of atrial myocytes, CNP expression is mainly found in vascular endothelial cells and neurons\textsuperscript{263}. The precursor proCNP\textsubscript{1-103} is cleaved by furin into CNP\textsubscript{1-53} and processed further into CNP\textsubscript{1-22}, which is the main peptide form in circulating blood\textsuperscript{263}.  

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CNP elicits similar responses to NO via the induction of cGMP\textsuperscript{265}. In our study of CNP, it was found that CNP (100 nM) reverted the Osteo M-induced increase in the expression of αSMA and ALP in VICs, to a level that was comparable to that in GM (Figure 69). This significant effect may correlate with the extremely high expression of its receptor, natriuretic peptide receptor B (NPRB), on VICs (99.8%, Figure 68). Compared to VIC regulation using NO, which enters plasma membrane for function, one advantage of using CNP is that CNP binds to the extracellular parts of NPRB to activate the receptor to catalyze the conversion of intracellular guanosine triphosphate (GTP) to cGMP. It would be interesting to covalently conjugate CNP to scaffolds (via -NHS ester chemistry) to achieve prolong presentation of CNP at a constant and controlled concentration for optimal regulation of VIC phenotype.
Figure 68: Representative expression profile of natriuretic peptide receptor B on VICs tested via flow cytometry. Natriuretic peptide receptor B is a membrane receptor on VICs with extracellular binding sites for CNP. Black fill = negative controls with secondary antibodies only; no fill = samples with primary antibodies against specific receptors and corresponding secondary antibodies. n = 3 runs with more than $1 \times 10^4$ events (single cells) in each run.
Figure 69: CNP reversed the up-regulation of αSMA and ALP of VICs induced by Osteo M treatment. VICs were seeded on PEGDA hydrogels at 10,000 cells/cm² and cultured in low serum (1%) media as specified for 6 days. Blue = DAPI; yellow = αSMA; red = ALP. Comparisons with different letters indicate statistical significance: p < 0.05; n = 6 hydrogels per group. Scale bar = 50 µm.
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Biography

Yan Wu was born and grew up in China on March 12, 1986. She earned her B.A. and M.S. in Materials Science and Engineering from Beijing University of Chemical Technology in 2008 and 2011 respectively. She started her PhD study at Rice University in 2011 and transferred to Duke University at 2012. She has authored the following publications:


