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Evaluation of valvular endothelial cell hemostatic behavior in native valves and novel co-culture models

by

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Abstract

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The endothelial cell-mediated process of hemostasis is critical in all living heart valve tissues. As these tissues undergo changes with age and disease, the ability for valvular endothelial cells (VECs) to manage anti- and thrombotic mechanisms may also change. Furthermore, degeneration- and thrombosis-related failures in artificial valves emphasize the need to understand the anti-thrombotic mechanisms of VECs in order to develop effective strategies to endothelialize implants and tissue-engineered heart valves. Therefore, a study was performed to evaluate the regulation and function of von Willebrand Factor (VWF), ADAMTS-13 (VWF cleaving enzyme), and other thrombotic and anti-thrombotic mediators secreted from VECs from different aged valves. This work identified age-related differences in VEC hemostatic protein regulation, and an increased capacity of specific proteins to aggregate within regions of elderly valves, which are known to have age-associated loss of extracellular matrix (ECM) organization that are linked to calcific aortic valve disease. With the knowledge that ECM can influence hemostasis, we then studied changes in VEC hemostatic regulation using synthetic culture conditions that modulated substrate stiffness and adhesive ligands.

RKRLQVQLSIRT (RKR), a syndecan binding cell adhesive peptide derived from
laminin-α1 G-domain, was optimal for promoting strong VEC adhesion and balanced hemostatic function on hydrogel constructs of various stiffness in comparison to the commonly used integrin binding peptide RGDS. Next, to evaluate interactions between valve cells, magnetic levitation technology was used to co-culture VECs with valvular interstitial cells (VICs) in a 3D scaffoldless aortic valve co-culture (AVCC). The cell-based AVCC design allowed for production of multiple constructs within several hours. AVCCs had regional localization of CD31 positive VECs at the construct surface. Cells in the AVCC interior (including VECs) expressed low levels of α-smooth muscle actin (αSMA), suggesting maintenance of quiescent VIC phenotype, but potential endothelial to mesenchymal differentiation in the interior-localized VECs. In addition, AVCCs produced ECM and expressed hemostatic proteins such as endothelial nitric oxide synthase (eNOS) and VWF. In light of the VEC localization within the AVCC potentially affecting healthy phenotype, a more physiologically organized and customizable scaffold model was needed for further evaluation of direct interactions between VECs and VICs. Therefore, previous RKR-functionalization work was combined with strategies for VIC encapsulation in biofunctionalized-MMP degradable hydrogels to develop a 3D adhesive ligand localized hydrogel scaffold for an endothelialized aortic valve co-culture model. The resulting hydrogel-based endothelialized aortic valve model (HEAVM) promoted the formation of a stable VEC monolayer at the scaffold surface, and supported the maintenance of quiescent VIC phenotype within the scaffold, thereby mimicking physiological valve cell organization in aortic valves. Platelet adhesion and nitric oxide functional assays confirmed healthy VEC cell behavior, while immunohistochemistry and qRT-PCR were used to assess VIC and VEC phenotypes and extracellular matrix (ECM).
production. Overall, by utilizing principles from cell and extracellular matrix biology, biomechanics, and biomaterials, this work has improved the understanding of the VEC roles in valve homeostasis and the pathogenesis of valvular disease. Furthermore, new biomaterial-based models were designed to enhance the field’s understanding of VEC functions and communication with VICs. The knowledge learned from these models may be applied to future evaluation of various valve diseases, as well as endothelialization strategies for valve implants.
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Abbreviations

αSMA: Alpha-smooth muscle actin
ADAMTS 13: A Disintegrin And Metalloproteinase With A Thrombospondin Type 1 Repeat, Member 13
ADT: Adult sample group, 6 month old
AEC: Aortic endothelial cell
AFM: Atomic force microscopy
ANOVA: Analysis of variance
ARS: Alizarin Red S
AV: Aortic valve
aVIC: Activated valve interstitial cell
AVCC: Aortic valve co-culture
BSA: Bovine serum albumin
BGS: Bovine growth serum
CD31: Platelet endothelial cell adhesion molecule
Col I: Collagen type I
Col III: Collagen type III
Col IV: Collagen type IV
CAVD: Calcific aortic valve disease
CSVD: Congenital semilunar valve disease
CTRL: control group
DAB: 3,3’-Diaminobenzidine chromagen
Dil-Ac-LDL: Acetylated low density lipoprotein, labeled with 1,1'-dioctadecyl – 3,3,3’,3’-tetramethyl-indocarbocyanine perchlorate
DMEM: Dulbecco’s modified eagle medium
DMSO: Dimethyl sulfoxide
DSB: Donkey serum buffer
ECM: Extracellular matrix
EC: Endothelial cell
EGM: Endothelial cell growth medium
ELISA: Enzyme linked immunosorbent assay
EnMT: Endothelial to mesenchymal transdifferentiation
eNOS: Endothelial nitric oxide synthase
FN: Fibronectin
FBS: Fetal bovine serum
GF: growth factor
GAG: Glycosaminoglycan
HEMO: hemodynamically altered
HEMO/DYSP: hemodynamically dysplastic
hEGF: Human epidermal growth factor
hFGF-B: Human fibroblast growth factor-Basic
HEAVM: Hydrogel-based endothelialized aortic valve model
HUVEC: Human umbilical vein endothelial cell
I2959: Irgacure 2959
ICC: Immunocytochemistry
IHC: Immunohistochemistry
Lam: Laminin
LOX: Lysyl oxidase
LDL: Low density lipoprotein
Mag: Magnetic field
MMP: Matrix metalloproteinase
MOVAT: Movat’s Pentachrome stain
MW: Molecular weight
NS: Nanoshuttle
NO: Nitric Oxide
Notch-1: Notch homolog-1
obVIC: Osteoblastic valve interstitial cell
OLD: Old sample group, ≥2 years old
P/S: Penicillin/Streptomycin
PAI-1: Plasminogen activator inhibitor-1
PAVEC: Porcine aortic valve endothelial cell
PPAEC: Porcine pulmonary artery endothelial cell
P4H: Prolyl-4-hydroxyase
P4HB: Poly-4-hydroxybutyrate polymer
PBS: Phosphate buffered saline
PDMS: Polydimethylsiloxane
PEG: Poly (ethylene glycol)
PEGDA: Poly(ethylene glycol) diacrylate
PG: Proteoglycan
PGA: Polyglycolic Acid
PGI2: Prostacycllin
PLA: Polylactic Acid
Pln: Perlecán
PQ: GGGPQG↓IWGQGK, MMP-2 and 9 sensitive peptide
PRP: Platelet rich plasma
PV: Pulmonary valve
qRT-PCR: Quantitative real time, reverse-transcriptase polymerase chain reaction
qVIC: Quiescent VIC
R\textsuperscript{3}-IGF-1: Human recombinant analog of insulin-like growth factor-1
RGDS: Integrin binding, cell adhesive ligand
RKR: RKRLQVQLSIRT, syndecan-1 binding, laminin derived cell adhesive peptide
RT: Room temperature
SEM: Scanning electron microscopy
TEHVs: Tissue engineered heart valves
TEM: Transmission electron microscopy
TF: Tissue factor
TFPI: Tissue factor pathway inhibitor
TGF-B: Transforming growth factor-beta
tPA: Tissue plasminogen activator
TCPS: Tissue culture treated polystyrene
TTP: Thrombotic thrombocytopenic purpura
ULVWF: Ultra large von Willebrand factor
VEC: Valvular endothelial cell
VE-cad: Vascular endothelial cadherin
VEGF: Vascular endothelial growth factor
VIC: Valvular interstitial cell
VWF: von Willebrand Factor
YNG: Young sample group, 6 weeks old
Chapter 1: Introduction

Heart valves direct proper blood flow through the heart and experience a complex mechanical environment [1,2]. Dysfunctional valves require surgical replacement using mechanical or bioprosthetic valves that may themselves fail over time due to structural or thrombosis related problems [1,3]. Semilunar valve disease, particularly affecting aortic valves, causes >60% of valve disease mortality, and up to 50,000 valve procedures are performed per year in the US [4]. Semilunar valve dysfunction affects all ages, from congenital valve defects experienced by neonates and children to the calcified valves afflicting a growing number of elderly [4–8]. For pediatric patients, prosthetic implants that are unable to grow must be replaced periodically [3,7]. Despite high morbidity and mortality due to valve dysfunction, little is known about the underlying causes of disease in non-adult valve tissue. Furthermore, little progress has been made in the development of new therapeutic strategies that will overcome the limitations of current valve implants. These problems have generated interest in understanding how cells on the surface of valves, called valvular endothelial cells (VECs), regulate tissue homeostasis and pathogenesis in valvular disease, especially in regards to their natural prevention of thrombosis in healthy valves. Though work to characterize VEC phenotype and functions has grown within the past decade, many essential VEC homeostatic functions are not well understood. Furthermore, growing evidence suggest that VEC dysfunction likely to contributes to valve disease progression.

Therefore, the goal of this dissertation is to characterize VEC phenotype and regulation of hemostatic-related proteins. In addition, this work aimed to develop and implement biomaterial platforms to improve the capacity to study VEC responses from
changes in the environment and when in contact with valvular interstitial cells (VICs). By developing a greater understanding of VEC hemostatic function in native valves and designing tools to modulate those functions, the body of work in this thesis can be implemented into new endothelialization strategies for future valve implant designs. To achieve these goals the following specific aims were performed.

**Specific aim I: Evaluation of age-related changes in aortic valve hemostatic protein regulation**

Heart valves remodel throughout a person’s lifetime, resulting in changes in extracellular matrix (ECM) composition and tissue mechanical properties that may affect VEC hemostatic function. This work assessed VEC regulation of hemostasis *in situ* and *in vitro* as a function of specimen age. The increased capacity of specific proteins to aggregate within regions of elderly valves was found to be related to age-associated loss of ECM organization. *In vitro* calcific aortic valve disease model studies show that the presence of VWF significantly increases VIC formation of calcific nodules relative to baseline controls. Therefore, the hemostasis imbalance with aging and the accumulations of hemostatic proteins may contribute to the formation of calcific nodules, and warrants further investigation to determine the connection between VEC hemostatic mechanisms and the progression of valve disease. Furthermore, this works asserts the importance of selecting appropriate age- and species-based controls in future VEC and hemostatic work.

**Specific Aim II: Investigation of environmental stimuli effects on VECs and their hemostatic responses**
VECs have sensitive hemostatic responses affected by changes in valve tissues. Furthermore, little has been done to characterize how various environmental factors would affect VEC hemostatic function. In this study, a hydrogel based platform was used to evaluate the environmental effects of substrate stiffness and cell adhesive ligands on VEC phenotype and regulation of hemostatic functions. Poly(ethylene glycol) diacrylate (PEGDA) hydrogels of various molecular weights (MWs) were polymerized to apply different substrate rigidities in VEC culture. Thiol-ene reactions were used to covalently bind thiol-modified cell adhesive peptides to the acrylate groups on the hydrogel surfaces. RKRLQVQLSIRT (RKR) is a syndecan-1 binding ligand derived from the laminin-α1 G-domain. RGDS is an integrin binding peptide found in many ECM proteins including fibronectin. VECs adhered to and formed a stable monolayer on all RKR functionalized hydrogel-MW combinations. RGDS functionalized platforms supported VEC adhesion and growth on stiffer hydrogels. VECs cultured on softer RKR-coated hydrogel platforms had significantly higher and balanced gene expression for all anti-thrombotic and thrombotic proteins, as well as, promoted significant platelet adhesion when stimulated with histamine than VECs cultured on RGDS-coated hydrogels and tissue culture polystyrene controls. Therefore, utilization of non-integrin adhesive peptide sequences that are derived from basement membrane ECM may recapitulate balanced VEC function and may benefit endothelialization of valve implants.

Specific Aim III: Utilization of a 3D magnetically levitated VEC-VIC co-culture model to analyze valve cell phenotype and function
The aortic valve consists of VICs and VECs. While these cells work synergistically to maintain leaflet structure and valvular function, there are few co-culture models to evaluate interactions between these cells. In this study, aortic valve co-cultures (AVCCs) were assembled using magnetic levitation and cultured for 3 days. AVCCs stained positive for CD31 at the construct surface, and α-smooth muscle actin (αSMA) throughout the construct interior. AVCC had significantly lower αSMA activation and gene expression than 2D cultured VICs on tissue culture polystyrene, suggesting the maintenance of a quiescent VIC phenotype when in a 3D levitated state. However, colocalization of CD31 and αSMA within the AVCC interior suggested that endothelial mesenchymal transdifferentiation may be occurring in VECs localized within the AVCC. Nonetheless, the AVCC stained positively for functional markers endothelial nitric oxide synthase, VWF, and prolyl-4-hydroxylase; and produced ECM components collagen type I, laminin, and fibronectin in the scaffold-free constructs. Overall, a co-culture model of the aortic valve was designed, quickly fabricated, and can serve as a useful tool for future experiments in heart valve biology.

Specific Aim IV: Design of a novel adhesive ligand localized hydrogel scaffold for an endothelialized aortic valve co-culture model.

A better understanding of the underlying cellular mechanisms for valve disease may lead to preventative or early interventions. Although both VECs and valve VICs have been studied extensively, customizable 3D co-culture models are needed to expand the knowledge of valve cell behavior and interaction. Here, we used enzymatically degradable poly (ethylene glycol) (PEG) hydrogels functionalized with physiologically
relevant spatial arrangement of cell adhesive peptide sequences to emulate the valve structure. VICs were encapsulated in a 3D PEG hydrogel in which the integrin ligand, RGDS, was incorporated throughout the bulk of the scaffold. In addition, the syndecan binding peptide sequence, RKRLQVQLSIRT (RKR), was covalently bound to only the surface of the hydrogel, which promoted VEC cell adhesion and confluent monolayer formation on the surface. Platelet adhesion and nitric oxide functional assays confirmed healthy VEC cell behavior, while immunohistochemistry and qRT-PCR were used to assess VIC and VEC phenotype and extracellular matrix (ECM) production. Both cell types remained in a physiologic state, with VECs forming a stable monolayer on the surface and signaling VICs to express quiescent phenotype. Cells produced zonally localized ECM as basement membrane proteins were present on the scaffold surface and collagen type I, secreted by the VICs, was found throughout the hydrogel. These results demonstrate that the hydrogel-based endothelialized aortic valve model (HEAVM) can be used to investigate valve cell response to mechanical cues, matrix signaling, and nutrient and cytokine availability, as well as giving insight into the role of these factors in disease states.

The subsequent chapters in the dissertation will include: a comprehensive background of the heart valve field and VEC biology in Chapter 2; evaluation of age-related histological and hemostatic changes in different aged aortic valves in Chapter 3; evaluation of VEC hemostatic responses when cultured on syndecan-binding peptide coated hydrogels in Chapter 4; implementation of novel magnetically levitated or zonally organized hydrogel co-culture designs to characterize VEC-VIC interactions and
functions in Chapters 5 and 6, respectively; and a conclusion summarizing the impact of
this body of work in Chapter 7.
Chapter 2: Background and Significance

2.1 Heart Valves: Anatomy and Composition

Heart valves play an essential role in allowing the unidirectional flow of blood throughout the heart and into the body. These valves will open and close a billion times in an average person’s lifetime. Heart valve tissues experience a variety of biomechanical stimuli such as turbulent and high shear flow profiles, and mechanical bending and stretching, all while actively regulating blood flow through the heart’s compartments and preventing clotting or backflow [1,2]. Just from the blood flow alone, valves are exposed to more sizable shear stresses, pressure loads, and flexural deformations than any other tissues in the body [1,2].

Figure 2-1. Cross section of healthy heart with the 4 heart valves labeled in bold [9].

There are four heart valves: the tricuspid valve, the pulmonary valve, the mitral valve, and the aortic valve (Figure 2-1). The four valves can be paired into categories based on their similarity in structure and locations in the heart. The tricuspid and mitral
valves are often referred to as atrioventricular valves, as they are located between the atria and ventricles of the heart [10]. The tricuspid and mitral valves are composed of 3 and 2 leaflets respectively. Atrioventricular valves are unique in that they are anchored to the heart ventricle wall by chordae tendineae to create tension in order prevent the valve from prolapsing into the atria when closing [1,7].

The aortic and pulmonary valves are classified as semilunar valves, and are the valves that lead to arteries leaving the heart. Both composed of 3 leaflets, aortic and pulmonary valve leaflets are occasionally referred to as cusps that are semilunar in shape. The aortic valve is composed of 3 adjacent cusps attached at the aortic root, between the left ventricle of the heart and the aorta [1,7] (Figure 2-2). Behind each cusp are indented pockets called sinuses of Valsalva. Two of the pockets direct blood toward the coronary arteries of the heart, and allow for each cusp to be named with their associated sinus (i.e. right, left, and non-coronary cusp). At the free edge of each cusp is a fibrous thickening called the nodule of Arantius [1,7,10]. The pulmonary valve is located between the right ventricle and pulmonary artery and is similar structurally and functionally with the aortic valve, yet experiences lower pressures and fluid shear compared to the valves on the left side of the heart.

![Figure 2-2. View of left ventricular outflow tract (left) and aerial view (right) of a normal aortic valve. Adapted from Stephens et al. [11].](image)
Though valve leaflets vary depending on region and type, their thicknesses can be averaged to be less than 1 mm in thickness for an adult [7,12]. By being so thin, diffusion from passing blood is sufficient enough to provide the leaflets with oxygen and nutrients, such that healthy matured valve tissue have little to no vasculature [12]. Despite their small scale, these tissues are robust and can withstand a great deal of mechanical force due to their unique composition [10,13]. Though atrioventricular valves differ greatly from semilunar valves in regards to dimensions and anatomy, the overall matrix microstructure of all valves leaflets are similar. The tissue composition of heart valves can be described by identifying a distinct three layer pattern observed in the tissues. In semilunar valves, these layers are known as the fibrosa, spongiosa, and ventricularis. The fibrosa layer is found on the outflow side of valves, and is rich with collagen. The fibrosa collagen layer is often times found to be continuous with surrounding anchoring tissue and allows the leaflets to withstand mechanical forces, especially tension. The spongiosa is the central and thickest layer of valve leaflets, being composed of various proteoglycans (PGs) and glycosaminoglycans (GAGs). PGs and GAGs promote the formation of a loose matrix structure interwoven with surrounding collagen and elastin to act as a shock absorber for bearing compressive loads and facilitate matrix rearrangements as valves open and close. The ventricularis layer is known to be rich with elastin fibers and is found on the outflow side of semilunar valves. The ventricularis also allows for the valve to withstand large tensile forces and promotes leaflet recoil during diastole. Thus, each heart valve leaflet must be composed of this highly organized
layered structure to fulfill proper mechanical function and actively direct blood flow throughout the heart.

The cellular composition of heart valves is unique from other cardiovascular tissues as well. Each valve leaflet is composed of valvular interstitial cells throughout the valve extracellular matrix layers and is surrounded with an endothelial cell layer. Though valvular interstitial cells (VICs) have been reported to have contractile and relaxation responses to various vasoactive agents like vascular smooth muscle cells, VICs are believed to serve primarily regulatory and tissue homeostasis functions [1,14,15]. Healthy VICs are quiescent, and synthesize and remodel valve ECM components to maintain valve organization and structure [1,16,17]. The endothelial cells that surround the surfaces of the valves are believed to primarily serve an anti-thrombotic, anti-inflammatory role in valvular tissue, however, valvular endothelial cell (VEC) responses to stress transductions and modulating VIC function have been understudied relative to the extensive work done on VICs, and will be discussed in greater detail later. The regulation and health of valvular interstitial and endothelial cells translate to the maintenance of overall valve composition and function. Thus, dysfunction of these cells is believed to be the key mediators of valvular diseases.

2.2 Semilunar Valves

The remainder of this work will focus on the semilunar heart valves: the aortic valve (AV) and pulmonary valve (PV).

*Hemodynamics and Stresses in Semilunar Valves*
Unlike previously described atrioventricular valves, semilunar valves do not have chordae tendineae, but are more similar to valves found in veins in that they consists of 3 cusps that are driven by pressure changes in the heart chambers to open and close during systole and diastole. However, these semilunar valves will experience a tremendous amount more shear force than in vascular tissues, in addition to turbulent flow profiles, bending, and stretching [2,18].

Shear stresses experienced in blood vessels are known to have laminar flow profiles at shear stresses varying between 10-20 dyn/cm$^2$ depending on location in the vascular tree [18,19] (Figure 2-3). Hemodynamics through heart valves have been discovered to be much more complex, however, with notable differences in shear forces experienced on the different sides of the leaflets. When a semilunar valve is open, the inflow surface facing passing blood from the heart’s ventricles experience large magnitudes of unidirectional laminar shear stress, while the leaflet sides facing the outflow arteries (fibrosa layer) experiences disturbed flow due to blood recirculation. Furthermore, when the heart valve is closed, the fibrosa layer of the semilunar valves experience additional normal forces on the leaflet surfaces caused by the blood pressure, preventing any backflow into the ventricles [2,18]. The shear stresses experienced by semilunar valves have been more difficult to quantify due to the sudden changes in the leaflet surfaces and hemodynamic flow experienced as the valves open and close. Several studies suggest that the wall shear stresses experienced at the aortic valve wall range from 15-900 dyn/cm$^2$ [20,21]. Despite the wide ranges of stresses that have been reported, it can be concluded that larger forces are experienced by valve leaflet surfaces than can be found throughout the peripheral vasculature.
The strains experienced in the semilunar valve leaflets are greatly regulated by the differences in pressure gradients from the outflow arteries (aorta and pulmonary artery) and the heart ventricles. This causes the leaflets to be strained in a biaxial pattern, with peak radial and circumferential strains up to 40% and 10% respectively [18]. Furthermore, a large degree of bending is imposed on the leaflets as the valves open in systole. The bending will result in additional compression at the fibrosa, and tension in the ventricularis of the leaflets [1,2].

During valve morphogenesis and development, these 2 semilunar valves have been reported to be practically identical [22]. Work done by Stradins and associates compared the biomechanical and structural properties between human adult (20-50 years old) AVs and PVs by performing various tensile tests and scanning election microscopy (SEM) of tissue samples. They found that although no significant differences were observed in their biomechanical properties, the ultimate tensile stress of AVs were slightly larger than those of PVs [23]. The SEM images also confirmed similar collagen organization and tissue architecture between the two valve types. Though AVs and PVs
experience the same type of stresses and loads, reports indicate that the AVs experience larger shear forces from passing blood than PVs due to the increased blood flow out of the left ventricles into the aorta [7]. To compensate for the increased forces, aortic valve leaflets are thicker, with greater collagen content in the fibrosa layer. The overall similarities between the two valves has led to practice of the surgical valve transplant procedure known as the Ross procedure, where a patient’s diseased AV is replaced by his/her own PV and a pulmonary allograft is implanted into the AV position [24–26].

Cell Types in Semilunar Valves

The various stresses and strains experienced by semilunar valves are also sensed by and influence the cells that make-up and maintain the tissues. As previously mentioned, heart valve leaflets are composed of two types of cells: valvular endothelial cells (VECs) and valvular interstitial cells (VICs). Cardiac valvular cells were once believed to be the same type of cells found within the rest of the cardiovascular tissue. However, valvular cells have been proven to be unique in their own respect, expressing different phenotypes and roles from their vascular counterparts. These valvular cells are highly sensitive to biomechanical stimuli and play important roles in maintaining healthy valve tissue.

Valvular Interstitial Cells

Valvular interstitial cells make up the majority the cellular composition of valve tissue and play an essential role in valve remodeling, repair, and regulation of matrix composition and mechanical response. In cell culture, VICs present a fibroblast-like
phenotype with thin branched extensions and no specific organization. Findings show that VICs are a heterogeneous cell population that can be characterized based on phenotypic state and localization within the valve tissue [27,28]. Quiescent VICs (qVICs) are characterized as fibroblast-like cells in a resting state found in healthy valve tissue. qVICs typically do not express high levels of αSMA and MMPs. However, qVICs can become activated VICs (aVICs), that are αSMA positive and mediate matrix remodeling and adaptation (Figure 2-4). VICs can transition between quiescent and activated states depending on their biomechanical responses and needs of tissue maintenance. During development and early childhood, aVICs appear to more abundant, however, as a person reaches adulthood qVICs become the cellular majority in valve tissue. This indicates that most of the valvular tissues remodeling occurs at an early age, and transitions into a tissue maintenance role at adulthood.

Figure 2-4. Porcine aortic VICs displaying fibroblast like phenotype in cell culture.

As adults become older, some VICs can transition into an osteoblastic VIC (obVIC) phenotype that appear to promote calcification and disease in valve tissue
The mechanisms that initiate VICs into obVIC phenotype have become greatly studied, as calcified aortic valve stenosis has become a growing problem worldwide. Proteins such as TGF-β, BMPs, and low density-lipoproteins have been reported to play a role in promoting obVIC expression [2,29,30]. Other factors include the effects of the ECM on VICs, since valvular matrix composition has been reported to change in organization and material properties with age [31,32].

VIC dysfunction has also been linked to the onset of other valve diseases such as myxomatous heart valve disease and rheumatic heart valve disease. The onset of these valve diseases are also likely due on a combination of VIC responses to mechanical forces and the surrounding matrix environments.

Valvular Endothelial Cells

Endothelial cells from AV tissue have been the most studied in regards to VEC characterization. Though not nearly as much work has been done to investigate VECs as for VICs or vascular ECs, our understanding of these cells has grown tremendously within the past decade. Branching from the extensive work done to characterize vascular endothelial cells, it was originally believed that VECs shared the same phenotype and regulatory functions as vascular endothelial cells (Figure 2-5). In brief, vascular endothelial cells are known to be highly sensitive to mechanotransduction and play important roles in angiogenesis, regulating vascular tone, preventing platelet clotting and inflammation, and serving as the initial barrier to all blood contacting tissues. However, recent findings have begun to elucidate distinct differences between VECs and vascular ECs in regards to phenotype, biomechanical response, and gene expression.
One of the earliest observations about VECs that distinguished their unique behavior was when Deck and associates observed that the cells align across the valves, and not in the primary direction of flow [33] (Figure 2-6). This finding suggested that these cells are more greatly influenced by biaxial force from the leaflet deformation rather than shear flow. This finding was more recently supported by studies performed by Butcher et al., in which cultured VECs that were submitted to steady laminar flow aligned perpendicular to the flow direction, versus the parallel alignment to flow characteristic of vascular endothelial cells [34].

In comparing the influence of shear flow profiles on VEC and vascular EC gene transcriptional profiles, Butcher and colleagues found that both cell types express similar antioxidant and anti-inflammatory genes; however, VECs were found to express lower inflammatory related genes than did vascular ECs [34,35]. Another interesting finding from the study was that the VECs express more genes related to chondrogenesis, while vascular ECs expressed osteogenic genes. In cartilaginous tissues, chondrogenic cells
primarily produce collagen and PGs, and do not promote angiogenesis. However, chondrocytes have the potential to differentiate into osteogenic cells that make up bones tissues, and have a propensity for angiogenesis and tissue calcification [35]. This finding suggests that VECs initiate a protective role against calcification under shear environments, as well as identifies that VECs in mature valves do not readily express genes for angiogenesis.

Figure 2-6. Image for porcine aortic VECs (B, D, F, H) and aortic ECs (A, C, E, D) in static and flow environments. Top row imaged with phase microscopy, bottom row imaged with laser confocal microscopy and stained for f-actin (red) and cell nuclei (blue). Adapted from Butcher et al. [34].

The VECs on the two sides of valve leaflets also appear to have unique properties. Just from phenotypic observation alone, VECs lining the inflow ventricularis layer appear to have elongated phenotypes, whereas VECs on the outflow fibrosa surface have a cuboidal orientation [33,36]. Simmons and colleagues further investigated the side specific differences of VECs by performing RNA gene analysis on VECs from the separate sides of aortic valve leaflets [37]. They found that the aortic side of the leaflets
upregulate anti-oxidative, anti-inflammatory genes such as endothelial nitric oxide synthase and vascular adhesion molecule-1, but were also pro-osteogenic and more prone to calcification. This finding also supported the clinical finding that calcific aortic valve sclerosis is more prominent on the fibrosa layer of the valve leaflets, which has already been defined to be the region that experiences the greatest amount of turbulent flow.

Some of the regulatory roles of VECs in regards to tissue interactions have been evaluated as well. When the endothelium on whole aortic valve leaflets was stimulated by various neurostimulants such as serotonin and endothelin-1, noticeable changes in contraction and relaxation of the tissue resulted in differences in overall tissue mechanical properties [38]. An in vitro co-culture with VECs and VICs showed that the VECs play an important regulatory role of VIC phenotype and ECM maintenance [39]. Specifically, the presence of VECs in the culture reduced VIC proliferation and αSMA activation, representing a basal phenotype. Furthermore, the presence of VECs also reduced the degradation of PGs and the upregulation of various matrix proteins [39]. These findings indicate that the endothelium of heart valves do interact with the underlying interstitial cells and ECM to cause changes in the regulation of the tissue’s mechanical properties. Though the specific mechanisms of VEC feedback control over the underlying tissue has yet to be completely understood, the behavior does mirror that of vascular ECs’ mechanosensitive release of vasodilators to vascular smooth muscle cells and begs to be further investigated [2].

VECs have also exhibited unique qualities in in vitro culture. Though isolated VECs can organize into the typical EC cobblestone monolayer when confluent, the cells appear to be much more sensitive to the presence of various proteins. TGF-β, commonly
present in media serum supplements, was found to induce VECs to express a mesenchymal morphology, \( \alpha \text{SMA} \) (an interstitial marker), and decreased expression of CD31 \([40,41]\). The most widely used method of maintaining the traditional VEC phenotype has been to culture the cells in specific endothelial basal medium supplemented with various growth factors (hEGF, VEGF, hFGF-B). The capacity of VECs to present some mesenchymal-like phenotype has developed interest in their natural plasticity. Work done by Bischoff and Aikawa tested VEC plasticity by exposing them to combinations VEGF, TGF-\( \beta \_2 \), and Notch1. Their results show that VEGF could counteract TGF-\( \beta \) mediated transdifferentiation, but Notch1 was found to initiate transdifferentiation even with VEGF \([41–43]\). This finding only begins to elucidate the unique capabilities of VECs, and implicates VEC importance in valve tissue development and homeostasis, as their role in interstitial cell regulation and replenishment is not yet fully understood.

2.3 Extracellular Matrix

The ECM of semilunar valves has been heavily characterized with immunohistochemistry, biochemical assays, and proteomics \([13,17,44,45]\). As previously mentioned, the matrix composition of semilunar valves can be generalized by a tri-laminate architecture consisting of collagen, PGs and GAGs, and elastin \([11,17]\) (Figure 2-7).
Figure 2-7. MOVAT of porcine aortic valve leaflet displaying the tri-laminate fibrosa (F), spongiosa (S), ventricularis (V) layers. (Nuclei= purple, collagen=yellow, PGs and GAGs =blue, elastic fibers=black, fibrin=dark red, and muscle=red).

Not only is collagen the most abundant matrix component in valvular tissue, but also the most suited for load bearing. Collagen type I (Col I) is present throughout the valve tissue, but most abundant in the fibrosa layer [11]. Collagen type III (Col III) is the second most abundant type of collagen present in valvular tissue, and can be found evenly distributed throughout the leaflet layers [11]. The collagen fibers in the fibrosa are naturally crimped and allow for immediate and sensitive tissue response to various mechanical stimuli. The orientation and organization of these fibers in the valve tissue play an essential role in providing strength and stiffness to maintain valve coaptation during diastole. These fibers will apply compressive forces to the outflow tissue layer to aid in the retraction of the leaflets during valve opening during systole. During diastolic closing of the valve, the applied blood flow and pressure change forces the leaflets to become taut, and the collagen to uncrimp and to shift the load onto the valve hinges [1].
The redistribution of the stresses allows the leaflets to withstand the outflow of blood without prolapse and backflow.

The elastin-concentrated ventricularis layer provides tensile support for the crimped collagen bundles, and facilitates the collagen’s return to its crimped state when the valve is unstrained [1]. Recent work has identified elastic fibers present in the aortic valve spongiosa in distinct patterns at the various valve positions, implying their role in supporting region specific loading patterns [46]. For example, a subtype of thicker rectilinear elastin was found to be concentrated in the hinge and coaptation regions of the valve spongiosa, implicating elastin’s role in mediating flexure as well as preventing collagen in the fibrosa from buckling [46].

PGs are defined as core proteins with at least one covalently attached GAG chain. GAGs are long unbranched polysaccharides composed of repeating disaccharide units. There are various types of GAGs that can attach to protein cores to make up various types of PGs. The GAG chains are highly hydrophilic and promote the formation of a loose matrix network with PGs for cell migration, and integrin and growth factor deposition and diffusion [13]. Versican is one of the most abundant PGs in valve tissue. Versican is a large PG that is heavily hydrated with chondroitin sulfate GAGs. It has been reported to interact with hyaluronic acid (HA), the most abundant GAG type in valves that is not attached to a protein core [11,13,47]. Decorin and biglycan are small leucine rich proteoglycans also present throughout valvular tissue. Decorin is one of the smallest PGs, consisting of a 40 kDa core protein and only one chondroitin/dermatan sulfate side chain [48]. Despite being much smaller than versican, decorin and biglycan play important roles in collagen fibrillogenesis formation and elastogenesis, respectively [44].
Though a great deal of work has been done to characterize how VECs respond to various mechanical stimuli, there is a need to understand how their microenvironment provided by ECM affects VEC signaling and mechanotransduction. In a comparative study between porcine aortic EC and VEC adhesion properties, Cheung et al. cultured cells in Col I and fibronectin coated microfluidic channels and exposed the cells to various shear profiles [49]. They found that VECs and aortic ECs had different preferences in regards to the ECM substrates, with improved VEC spreading on fibronectin gels over the collagen gels and overall better adhesion strength to the fibronectin substrate when exposed to high shear profiles. Conversely, the aortic ECs had better adhesion and spreading on the collagen gels. This study is another example of how VECs are different from vascular ECs, and reiterates that cell behavior is dependent upon cell type and ECM.

Thus, there is a need to identify and characterize the native ECM environment at the outer VEC layers of heart valve leaflets. In particular, limited work has characterized the complete basement membrane composition in valve tissue relative to tissue ECM. In vascular tissue, the basement membrane is described as the ECM mesh that provides an anchor for ECs to the underlying ECM and tissues. The basement membrane is composed of laminin, Collagen type IV, nidogen/entactin, perlecan, and various integrin binding domains [48].

Laminins belongs to a family of heterotrimeric glycoproteins composed of combinations of α, β, and γ chains. Laminins in the basement membrane can average between 400-900 kDa in size, and play an integral role in the formation of the basement membrane network. The various regions of laminin have been reported to mediate
specific cell responses. For example, the G-domain of the laminin-α chains are usually associated with heparin binding and cell adhesion; whereas regions along laminin-β chains promote cell differentiation [50].

Collagen type IV (Col IV) is a non-fibrillar collagen that is an essential basement membrane network forming component, acting as the common linker to each of the other basement membrane components [48]. Only found in the basement membrane, Col IV molecules are approximately 400 kDa, and are composed of two α1, and 1 α2 chains [48]. The different regions of Col IV are also believed to influence specific cellular responses, as well as have specific affinities to other matrix components such as heparan. Dysfunctions in Col IV expression can be extremely detrimental and cause matrix disorders such as Alport syndrome or Goodpastures syndrome [7,51].

Nidogens/entactins are small glycoproteins (150kDa) that mediate laminin and Col IV interactions. The laminin-nidogen interaction is critical for basement membrane connection and stability. In a proteome analysis of semilunar valve ECM proteins, nidogen was identified to interact within a protein-protein network enriched with proteins essential for valve function that also begs to be investigated [45].

Perlecan (Pln), also known as low density heparin sulfate proteoglycan 2, is a large PG consisting of a 470 kDa core protein, and 2-3 heparan/chondroitin sulfate GAG chains at 70-100kDa each [48]. The PG is composed of 5 distinct domains that initiates various cellular functions and molecular attachment, some of which have structural similarities with other ECM molecules [48]. The core protein regions of Pln serve as sites for cell attachment and basement membrane assembly, while the side chains promote FGF binding. The cysteine-free domain I is the point of GAG chain attachments. Domain
II contains for LDL receptor-like cysteine repeats, whereas domain III is very similar to laminin structure. Domain IV contains neural cell adhesion molecule-like IgG repeats, and domain V is similar to the C-terminus of laminin with interspersed with EGF domains. Pln is also present in other regions of the body such as hyaline cartilage and bone marrow ECM. Pln not only mediates EC attachment, but also possess angiogenic and growth promoting attributes, acting as a co-receptor for fibroblast GFs [52]. Due to its complex size and various functions, there has been great interest in understanding the various mechanisms behind Pln functions relative to their domains. For example the Pln C-terminus in domain V, referred to as endorepellin, has anti-angiogenic activity and inhibits EC migration and tube formation [53–55]. The GAG-anchoring domain I of Pln promotes the accumulation of various growth factors and integrins to also promote cellular differentiation [55]. Domain IV has been reported to promote cell adhesion, spreading and focal adhesion kinase activation independent of integrin mechanisms [53].

The interactions of these components together result into a sheet-like layer that biologically and mechanically support ECs. However, the role of the basement membrane in valve tissue has been somewhat overshadowed by the extensive work done in characterizing the interstitial matrix. In an effort to understand matrix topography for tissue engineering heart valve applications, Brody and colleagues performed scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM) to characterize the basement membrane of aortic leaflets [56]. Though the study found no significant differences between the basement membrane topography of the ventricularis and fibrosa, it classifies as one of the first valve studies to acknowledge the importance of understanding the matrix region. Studying the basement

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membrane in valve tissue and their interactions with VECs and underlying tissues will provide a more comprehensive characterization of the VEC and ECM phenotypes, as well as identify matrix targets that can be utilized to therapeutics and pharmaceutical targets.

2.4 Valve disease and valvular endothelial cell dysfunction

Semilunar valve disease, particularly affecting AVs, makes up over 60% of valve disease mortality, and up to half of the 100,000 valve procedures performed per year in the United States [4]. AV and PV diseases and dysfunctions can affect all age populations, from semilunar congenital valve defects experienced with neonatal and pediatric patients to the growing number of calcified valves in the elderly populations [4].

Based on the current literature, the onset of valve disease is the result of VIC activation and increased ECM and tissue remodeling. However, the roles of VECs in valvular disease have yet to be understood. Insights in VEC mechanoregulation have provided some possible links to understanding valvular disease, particularly with links to aortic valve calcification and sclerosis.

In the vasculature, dysfunction of the endothelium is known to play a direct role in the onset of atherosclerotic plaque formation through inflammatory and angiogenic mechanisms. Calcific aortic valve disease (CAVD) is believed to have similarities to vascular atherosclerosis, and begs the need to understand how VEC dysfunction mediates valve calcification. Some hypotheses support the idea that VECs may undergo embryogenesis-like gene expression and enter endothelial to mesenchymal transdifferentiation (EnMT). This mature VEC EnMT would mimic the valvulogenesis
process in which the endothelial differentiated mesenchymal cells encourage the activation of VIC phenotypes, and possibly enhance the VIC mediated valvular degeneration [42]. Furthermore, there has been interest in how dysfunctional endothelium relates to the significant neovascularization observed in the diseased calcific and fibrotic valves. Healthy adult valves are typically devoid of vasculature beyond their attachment to the aortic or pulmonary roots, yet thickened vessels with inadequate oxygen transport are believed to initiate EC hypoxic responses to promote the angiogenesis seen in calcific valves [30,57]. The vascular network formation is also a characteristic of osteogenic behavior, which could be linked to the calcific deposition and bone formation in diseased valves.

Although there is a great need to understand the link between VEC dysfunction and valve calcification, there is also an equally great need to understand VEC roles in preventing and initiating other valvular conditions. Valvular endocarditis is believed to be greatly influenced by VEC mediated inflammatory response to endothelium disruption [6,51]. Furthermore, thrombotic vegetation on the ventricularis of the leaflets are commonly observed in the diseased tissues. Myxomatous heart valves are also prone to thrombosis and endocarditis [6].

Though a fair amount of work has been done to characterize the extracellular makeup and hemodynamics of congenital semilunar valve diseases (CSVDs), there is still many unanswered questions in regards to the underlying causes of CSVD and as well as insight into VEC response to the altered hemodynamics. Thus far, the published literature that characterize VECs have exposed their uniqueness in comparison to vascular ECs and importance in maintaining valve tissue homeostasis. These cells are highly
mechanosensitive and present potential plasticity and multi-functionality. However, we wish to understand VEC regulation of hemostasis and anti-clotting functions, and their behavior in diseased valves. Furthermore, the characterization of VEC anti-thrombotic mechanisms may provide a useful tool for identifying therapeutic targets or developing means of endothelialization of heart valve implants.

2.5 Thrombosis and Hemostasis

_Mechanisms of Coagulation and Thrombus Formation_

Blood coagulation and thrombus formation is the body’s response to endothelial damage as a result of mechanical damage, ECM degradation, and disease. The process involves platelet activation, adhesion, and aggregations, as well as the initiation of the coagulation cascade to further promote thrombus formation.

The coagulation cascade begins from the exposure of blood to tissue factor (TF) released by damaged cells (Figure 2-8). Thrombin generation then occurs by a series of activations of various coagulation factors. In brief, TF binds to factor VIIa to form a TF/VIIa complex that activates factors X and IX [58,59]. Activated factor IXa combines with factors VIIIa to convert factor X to Xa. Factor Xa then combines with factor Va and thrombin to initiate thrombin activation and interaction with fibrinogen ultimately to form fibrin clots that are then cross-linked by factor XIII to create blood clots [58,59].
Platelet thrombus formation also presents an issue in implants, such as mechanical heart valves, due to thrombus buildup on the material that eventually interferes with valve function and causes implant failure. Platelet activation can be caused by presence of Ca$^{2+}$, coagulation factors, or inflammatory cytokines [58,59]. Among one of the many pathways of platelet activation is the initiation of platelet adhesion and aggregation on damaged endothelial surfaces by von Willebrand Factor (VWF) (Figure 2-9).

Specifically, VWF binds to platelet membrane glycoprotein receptor Ib and tethers the platelets to the vessel wall. VWF, along with fibrinogen, binds to adhered platelets via integrin αIIbβ3 to cross-link additional activated platelets at the site of injury [58,60]. Prolonged presence of VWF at the site of the wound can therefore play a major part in the formations of platelet thrombi. However, VWF is only able to fully interact with platelets to promote thrombus formation when in the form of ultra large VWF (ULVWF) multimeric bodies that can have a mass up to 200,000 kDa [61]. During times without trauma and normal hemostasis, the ULVWFs are cleaved by a plasma metalloproteinase.
called ADAMTS-13 (a disintegrin and metalloproteinase with thrombospondin type 1 repeat), preventing the smaller cleaved VWF particles from forming large platelet thrombi. Studies have shown that monomeric VWF fragments have a much lower binding affinity to platelets, likely due to the drop in platelet binding sites as a result of their catalysis by ADAMTS-13 [62].

![Diagram of platelet interactions with extracellular factors to initiate platelet activation and adhesion](image)

**Figure 2-9. Diagram of platelet interactions with extracellular factors to initiate platelet activation and adhesion [63].**

Thus, thrombosis in the body is caused by the combination of platelets and blood coagulations with fibrin clots. The activation of these cascades often times initiates positive feedback loops leading to the release and activation of more platelets and coagulation factors.

**Endothelial Cell Roles in Thrombosis and Hemostasis**

The anti-clotting roles of endothelium in blood vessels have been well characterized. In addition to providing a physical barrier between blood and underlying
tissues, ECs have been discovered to express various proteins and molecules that regulate the balance of clotting and anti-clotting response. The anti-clotting roles of ECs can be summarized in the chart below.

Table 2-1. Summary of endothelial anti-clotting roles and hemostatic functions. Adapted from Vander's Human Physiology Textbook. [64]

<table>
<thead>
<tr>
<th>Action</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normally provide an intact barrier between the blood and sub-endothelium connective tissue</td>
<td>Platelet aggregation and formation of factor VIIIa complexes are not triggered</td>
</tr>
<tr>
<td>Synthesize and release PGI2 and nitric oxide</td>
<td>These inhibit platelet activation and aggregation</td>
</tr>
<tr>
<td>Secrete tissue factor pathway inhibitor</td>
<td>This inhibits the ability of tissue factor-factor VIIa complexes to generate factor Xa.</td>
</tr>
<tr>
<td>Bind thrombin (via thrombomodulin) which then activates protein C</td>
<td>Active protein C inactivated clotting factors VIIIa and Va.</td>
</tr>
<tr>
<td>Display heparin molecules on the surfaces of their plasma membranes</td>
<td>Heparin binds anti-thrombin III, and then inactivates thrombin and several other clotting factors</td>
</tr>
<tr>
<td>Secrete tissue plasminogen activator</td>
<td>Tissue plasminogen activator catalyzes the formation of plasmin, which dissolves clots</td>
</tr>
<tr>
<td>Secretes VWF and constitutively releases ADAMTS-13</td>
<td>Balance of constitutive VWF and ADAMTS-13 release prevents large platelet thrombi formations.</td>
</tr>
<tr>
<td>Stimulated ULVWF secretions by neighboring ECs as well as exposure of uncleaved VWF in the sub-endothelial ECM when endothelial layer is removed or damaged</td>
<td>Excess platelet thrombus formation in presence of ULVWF strings in plasma as a result from stimulated release. VWF flowed over collagen produces platelet thrombi.</td>
</tr>
</tbody>
</table>

However, the assessment of anti-clotting capacity in valvular endothelium has yet to be identified and elucidated. Specific proteins and factors speculated to be associated with VEC modulation of hemostasis and thrombosis are described in detail in the following sections.
VWF and ADAMTS-13

VWF is a glycoprotein constitutively produced by vascular endothelial cells and megakaryocytes that mediates the adhesion of platelets to sites of vascular injury as well as plays a general role in maintaining hemostasis [61,65] (Figure 2-10). A VWF monomer is composed of 4 repeated A, B, C, D domains that interact with numerous clotting components. For example, the VWF A1 domain binds to platelet GPIb receptor and heparin, while its D3 domain binds to inactivated factor VIII [61]. ULVWF multimers are formed when multiple VWF monomer subunits are linked via disulfide bonds, creating adhesive strings up to 20 MDa [65]. ULVWF multimers are typically formed in response to endothelium stimulation via inflammatory cytokines, hormones, toxins, and exposure to various levels of shear stresses [61,65,66]. The release of ULVWF multimers will promote the formation of platelet thrombi, as passing activated platelets in the blood are more likely to aggregate onto the many binding sites on the ULVWF string. Furthermore, additional research has shown that in addition to mediating platelet thrombus formation, VWF plays an important role in the exposure of P-selectin by platelets, which promotes the recruitment of leukocytes and eventually other inflammatory response reactions [67].

VWF is synthesized in the Weibel-Palade bodies of ECs and released in both basolateral directions of the cells [68]. Thus, VWF is not only released into the plasma, but the subendothelium as well. In fact, VWF interaction with collagen I via both VWF A1 and A3 domains plays an important role in platelet clot recruitment to the subendothelium when the endothelium is damaged [69,70]. Molecular studies have also identified VWF interaction with heparin via the A1 domain GPIb receptor [61].
Interestingly, VWF was also discovered to interact with the PG decorin via its GAG chain, such that the interaction blocked VWF binding with heparin [71]. However, the collagen and heparin binding A1 domain was not responsible for decorin binding, leaving the exact location of the VWF-decorin interaction unknown.

Though VWF is constitutively produced by ECs, VWF mediated thrombus formation is prevented by the constant multimer cleavage of the VWF cleaving enzyme, ADAMTS-13. ADAMTS-13 is a metalloproteinase composed of a catalytic metalloproteinase domain, a proximal, middle, and distal domains [65,66,72]. As with VWF, ADAMTS-13 is also constitutively produced and released by endothelial cells. ADAMTS-13 cleaves large VWF multimers specifically at the Tyr1605-Met1606 peptide bond in the VWF A2 domain, cutting the multimers into much smaller polymer strings and thereby limiting VWF adhesion to activated platelets [65,72]. A study by Moake and associates found that ADAMTS-13 release, unlike VWF release, is not modulated by histamine stimulation [65]. Consequently, the same basal concentration of ADAMTS-13 is unable to cleave the excess VWF strings produced, explaining the unbalanced VWF-ADAMTS-13 cleavage mechanism that occurs during endothelial damage resulting in platelet adhesion and activation. The disease thrombotic thrombocytopenic purpura (TTP) is caused by mutations or inhibitions of ADAMTS-13 by antibodies causing an accumulation of ULVWF eventually resulting in platelet-rich microthrombi in the microvasculature [73].
**Figure 2-10.** (A) ADAMTS-13 cleavage of VWF multimer strings at the exposed VWF A2 domain. (B) The promotion of platelet thrombus formation on long uncleaved VWF multimer strings. Adapted from Moake [73].

*eNOS and PGI₂*

Endothelial nitric oxide synthase (eNOS) is an enzyme that synthesizes nitric oxide (NO) from arginine in endothelial cells. Detection of NO has been difficult in vitro due to its ability to interact and be catabolized by neighboring molecules. Thus, eNOS provides a target to quantify the production of NO in the endothelium. NO serves many biological functions such as acting as vasodilator to relax smooth muscle cells in arteriole walls. In regards to hemostasis, NO also acts as an inhibitor to platelet adhesion, activation, and aggregation onto the endothelium [64]. NO is also known to mediate VEGF-induced angiogenesis in coronary vessels.

Another strong platelet inhibitor prostacyclin (PGI₂), also known as prostaglandin I₂, is an eicosanoid produced by ECs. Its anti-thrombotic mechanisms are to counteract the effects of thromboxane A₂ platelet activation. PGI₂ is also similar to NO, by acting as an effective vasodilator [64]. The continuous synthesis of NO and PGI₂ in adjacent undamaged ECs is important in the prevention of platelet thrombi from growing too large and spreading away from damaged endothelium.
The secretion of tissue plasminogen activator (tPA) by ECs is the last of the endothelial anti-clotting mechanisms. tPA is an enzyme part of the fibrinolytic system of the clotting cascade to dissolve platelet thrombi after they are formed. During clot formation, tPA and plasminogen are integrated into the clot by binding to fibrin [64]. tPA interaction with fibrin allow for both components’ enzymatic activation, increasing efficiency to generate plasmin from plasminogen [64]. Recombinant forms of tPA have been utilized in development of anti-clotting drugs to treat embolisms and thrombotic strokes [64].

Heparin

Heparin is a highly sulfated GAG present on EC surfaces. Acting as a natural anti-coagulant, heparin inhibits platelet function, and recruits and activates anti-thrombin III to the clot [64]. Heparin is one of the most commonly used anti-coagulant drugs and has been widely investigated for uses as cardiovascular implant coatings. However, excessive administration of heparin can cause heparin induced thrombocytopenia (types I and II), where a patient’s platelets are rapidly decreased or increased due to an immunological response [64,74].

2.6 Tissue Engineered Heart Valves

As the current understanding of valvular disease and treatments have been discussed, it is clear that there are currently no ideal treatment options for patients with valvular dysfunction other than valvular repair or replacements. The limited progress on
new therapeutic strategies can be contributed to our incomplete understanding of valvular biology and epidemiology, as well as the relative success of current mechanical and bioprosthetic valve implants. However, these treatments are unsuitable for the large pediatric populations that must undergo numerous valvular surgeries.

Thus, much interest in the field has focused on designing a better valvular implant that can overcome the many limitations of current implant design. These criteria include no initiation of inflammatory immunological responses, anti-thrombotic surfaces, the ability to provide normal structural and biological function of a patient’s lifetime, and the potential to grow and self-repair [1,3,75]. A customizable valve implant that can grow with a patient would make a tremendous improvement in the quality of life for patients suffering from CSVDs, as they will only have to undergo one valvular replacement surgery for their entire lives [1,3,75]. Thus, various research groups are working to develop tissue-engineered heart valves (TEHVs) that would satisfy all of these requirements.

Traditionally, the tissue engineering paradigm has involved using a scaffold with specific ECM and GFs to support the growth, and possibly differentiation, of cells in order to generate specific functional tissues. A majority of the work done for TEHVs has followed this approach while integrating the many unique properties of valve composition and mechanics into their designs. Given the appeal of biocompatibility, some TEHV approaches have utilized natural polymer scaffolds such as collagen and PG based materials [3,76]. However, these materials have vastly different mechanical properties from valve tissue. Use of decellularized valves is the most common and advanced approach to utilize natural scaffolds. Cells are removed from whole valve
tissues using a variety of detergents and enzymes, while keeping the native matrix composition intact to serve as a scaffold material. Though some clinical success has come with decellularized TEHVs, seeding cells onto the scaffolds have proven to be difficult and incomplete, resulting in formation of thromboembolisms and inflammation \[3,77\]. The adverse reaction and implant failure can be contributed to the fact that the decellularized valve matrix is highly thrombogenic, since the exposed collagen is a platelet activator. Furthermore, decellularized valves may also present toxic effects from residual detergents used from the decellularization process, and warrant further optimization. Other approaches to TEHVs involve the formation of synthetic polymer scaffolds. The appeal of synthetic scaffolds lies with the ability of customizing and producing material scaffolds for specific patient needs. Various materials have been investigated such as FDA approved polymers PGA and PLA. P4HB developed by Hoerstrup and colleagues have shown promising \textit{in vitro} culture results with the formation of ECM within the scaffolds after several days \textit{in vitro} culture \[3,78\].

Work in the Grande-Allen at Rice University has investigated the uses of PEG as a suitable scaffold material to mimic native valvular tissue mechanics and support valvular cell growth for TEHV approaches. PEGDA is a synthetic hydrogel that can be mechanically tuned by varying the concentrations and molecular weights used when gels are cured. PEGDA is an attractive material for tissue engineering due to its bioinert, nonimmunogenic properties, as well as natural prevention of protein absorption due to its highly hydrophilic properties. Integration of various biomimetic and enzymatically degradable peptides into 3D PEGDA scaffolds have shown to direct HUVEC growth and angiogenesis into the material \textit{in vitro} and \textit{in vivo} \[79,80\]. Furthermore, the material
properties of PEGDA of differing MW compositions can be combined in patterns which offer the potential for mimicking the unique biaxial, anisotropic qualities of native valve tissue [81].

There have been many advances made in TEHV design, yet none so far has completely met all the needs of a fully functional TEHV. In addition to the selection of an ideal valve scaffold, there is the question of the proper cell types and sources to be used, as well as the proper the application of various culture conditions such as substrate stiffness and ECM ligand presentation. As previously mentioned, biomechanical stimuli have been found to play an important role in ECM formation and cell function, thus various bioreactor designs have also been implemented into the TEHV process [82]. While many of these approaches are still being fine-tuned and developed, the identification and pursuits of the most feasible and practical of the design criteria may allow for improvements of current implant technologies.

2.7 Heart Valve Co-culture Models

While the development of an implantable TEHV would be an ideal treatment for valve dysfunctions in most patient populations, the field of tissue engineering is yet to develop a fully functional valve satisfying the required design criteria previously described. Therefore, noninvasive treatments for valve disease should also be explored. Ideally, preventing or treating valvular disease with noninvasive therapeutics and drugs would provide the best solution for patients suffering from valvular dysfunction by removing surgical risks presented with valve replacements. However, the current
understanding of valve biology and disease has not been revealing enough to develop successful drugs or therapies.

It is understood that both valve cell types and the interaction between the two are critical for valve homeostasis. However, much work characterizing VICs and VECs have been performed separately, and in non-physiological environments, typically on tissue culture polystyrene [34,35,83–85]. Though VECs form a complete 2D monolayer the line the surfaces of valve tissues, in vitro 2D cell culture environments are poor representations of the native 3D environment in which valvular cells reside and communicate through [7,16]. The inadequacy of 2D cultures for valvular research is highlighted by the varied results of studies on the effect of statins as treatments for calcific aortic valve disease. Early studies exposing VICs cultured in 2D polystyrene to statins showed significant reduction in calcific nodule size and αSMA expression [84,86–89]. However, no improvements in calcific aortic stenosis were seen in patients enrolled in clinical trials who took statins [90–94]. Following the failure of the clinical trials, subsequent studies of VICs in 3D collagen gels showed a smaller, conditional reduction in calcification in comparison to 2D cultures [88].

These results demonstrate the enormous gap in complexity between simple 2D cell culture models and the human body, and necessitate the development clinically relevant and physiologically representative 3D co-culture models of the aortic valve. Among the oldest co-culture approaches include the use of Transwell inserts to culture VECs and VICs within the same well, but separate direct contact between the cells via the Transwell membrane [95,96]. This approach allowed for investigation of paracrine signaling between VECs and VICs and showed that the nitric oxide produced VECs
reduced VIC αSMA activation and calcific nodule formation [95]. However, the
Transwell co-culture approach is limited by preventing direct cell-cell contact between
the VECs and VICs, and may result in overlooking many important VEC-VIC responses
such as the initiation of pathogenic angiogenesis and directed cell migration and
organization. The most common valve cell co-culture approaches in the field include the
encapsulation of VICs within a biological scaffold such as collagen-based hydrogels, and
seeding VECs directly on the surface of the scaffold [39,76,97]. Studies using these
platforms were successful in showing important interplay between valve cells to maintain
quiescent cell phenotypes, especially in regards to VIC activation. However, biological
scaffolds are limited by the overall lack of scaffold design flexibility in regards to
mechanical strength, and control of the biological cues presented to the cells. Despite the
limitations of these co-culture systems, it is clear that co-culturing of valve cells in
physiologically relevant 3D states influences cell function, and can allow the study of
more physiological cell responses in vitro.
Chapter 3: Age-related changes in aortic valve hemostatic protein regulation

3.1 Introduction

Semilunar valve diseases, particularly those affecting the aortic valve (AV), cause >60% of valve disease mortality, and approximately 50,000 procedures to replace or repair semilunar valves are performed each year in the US [4]. Semilunar valve dysfunction affects all ages, from congenital valve defects experienced by neonates and children to the growing number of calcified valves in the elderly [1,4–7,98]. These dysfunctional heart valves most often require surgical replacement using mechanical or bioprosthetic valves that may themselves fail over time due to structural or thrombosis related problems [1,3].

In order to understand valvular disease and develop non-invasive therapeutic solutions, it is necessary to improve fundamental knowledge about the response of valve cells and extracellular matrix (ECM) to surrounding environments in various physiological states. The characterization of changes in valvular biology with respect to aging has become especially important, as the occurrence of aortic valve sclerosis and calcification continues to rise in elderly populations [7,98,99].

While most studies of valvular biology employ AV tissues and cells from adults (either animal models or human specimens resected at surgery), it is clear that consideration of specimen age is necessary to evaluate age-specific conditions and

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1 This work has been published in LR Balaoing, AD Post, K Minn, KJ Grande-Allen. Age-related changes in aortic valve hemostatic regulation. Arteriosclerosis, Thrombosis, and Vascular Biology. 2014.
pathologies. Previous studies from our research group and others have identified numerous age-related changes in the ECM composition and mechanical properties of the AV, as well as in the valve cell phenotypes [1,100–103]. Considerable tissue remodeling and growth occurs in AV tissues prior to adulthood [7,44,102]. During fetal development, AV tissues have not yet formed tri-laminate ECM structures or high degree of elastin and collagen alignment that is apparent in adult valves [1,44,102]. To mediate this microstructural organization of the mature AV, the developing AV also demonstrates cellular activation of both valvular interstitial cells (VICs) and valvular endothelial cells (VECs) [1,102,103].

As subjects reach adulthood, valve cells become quiescent and maintain homeostasis of the highly organized valve leaflet structure and function [1]. However, the AV continues to change with aging. Older valves have increased thickness and increased levels of collagen type III interspersed with proteoglycans and glycosaminoglycans throughout the leaflets [101,104–106]. Corresponding to the altered ECM organization in older valves, the stiffness in the circumferential direction increases with age as well [101]. Several studies have suggested that the remodeling occurring in elderly AV tissues is mediated by the activation of VICs, which can lead to osteogenic differentiation and ultimately valve calcification [1,5,29,101]. However, additional factors including VEC dysfunction, changes in hemodynamics and valve mechanics, and ECM remodeling may also influence calcific formation in valve tissues. Thus, as valves remodel throughout a lifetime, the resulting changes in ECM composition and tissue mechanical properties are likely to affect cellular behavior and increase the risk of valvular diseases.
VECs have been identified to play an important role in maintaining valve homeostasis as well as affect the onset of valvular disease. However, many aspects of VEC function and behavior remain unknown. Recent work has shown that VECs have distinct phenotypes compared to vascular and cardiac endothelial cells, e.g., VECs align perpendicular to the direction of fluid flow, unlike vascular ECs, and have different mechanotransduction pathways and gene expression, likely due to the unique flow and mechanics to which VECs are subjected [34,35,39]. VECs have been observed to have strong interactions with VICs and ECM, and appear to be highly sensitive to their surrounding environments [37,39,49]. These cells can undergo endothelial to mesenchymal transdifferentiation, and thus play an important role in valvulogenesis during fetal development [36,43]. Furthermore, previous studies have suggested that VEC dysfunction may play a role in initiating valvular diseases [2,16,36,38].

Despite these links between VECs and valve diseases, little has been done to characterize the fundamental hemostatic behavior of VECs and the relation between this behavior and the valvular ECM in health, disease, and aging conditions. It is well-known that vascular endothelial cells perform substantial anti-thrombotic roles via production and release of tissue plasminogen activator (tPA), tissue factor pathway inhibitor (TFPI), VWF cleaving enzyme (ADAMTS-13), nitric oxide, and heparin [64,107,108]. Endothelial cells are also responsible for the production and release of thrombotic protein mediators such as plasminogen activator inhibitor (PAI-1), tissue factor (TF), von Willebrand factor (VWF), and P-selectin [64,107,108]. Under homeostatic conditions, vascular endothelial cells constitutively express and produce both anti-thrombotic and thrombotic mediators to balance hemostatic effects and prevent thrombus formation.
However, blood coagulation and thrombus formation are quickly initiated by endothelial cells in response to mechanical damage, ECM degradation, and disease [64,107,108]. The production and regulation of these hemostatic factors have not been well characterized in VECs.

3.2 Objectives

While valvular endothelial cells (VECs) have unique responses compared to vascular ECs, valvular regulation of hemostasis is not well understood. Heart valves remodel throughout a person’s lifetime, resulting in changes in extracellular matrix (ECM) composition and tissue mechanical properties that may affect VEC hemostatic function. This work assesses VEC regulation of hemostasis in situ and in vitro as a function of specimen age. We hypothesize that aging affects VEC regulation of hemostatic proteins and their interaction with valve ECM components, resulting in an unbalanced regulation of anti-thrombotic and thrombotic proteins within the tissues. Thus, the evaluation of AV tissues and VEC function with respect to anti-thrombotic and thrombotic functions in aging valves will provide insight into the specific factors and conditions that affect VEC hemostatic regulation. This information is also relevant to the development of valve replacement devices, since the breakdown and thrombosis formation associated with mechanical and decellularized valve implants has generated interest in understanding how the natural anti-thrombotic behavior of VECs can be integrated into age-specific tissue engineered heart valves. Overall, there is a critical need to understand the hemostatic behavior of VECs.
3.3 Materials and Methods

Preparation of sample groups

Aortic valve leaflets were dissected from fresh porcine hearts acquired from commercial abattoirs (Fisher Ham and Meats, Spring, TX; Animal Technologies, Tyler, TX), and assigned into one of three age groups: young (YNG=6 week old), adult (ADT=6 month old), or older (OLD=2 years old). Aortic valve leaflet tissues were either dehydrated, processed in paraffin and radially sectioned for *in situ* analysis, or enzymatically digested in a solution of DMEM containing dispase (2 U/mL) and collagenase II (60 U/mL) to isolate porcine aortic valvular endothelial cells (PAVECs) from the valve surfaces for cell culture following previously described methods [34,109]. At first passage, the VECs were purified using CD31 antibody conjugated-CELLection magnetic sorting beads (Invitrogen, Carlsbad, CA). Porcine pulmonary artery endothelial cells (PPAECs) and human umbilical vein endothelial cells (HUVECs) were used as baseline controls for *in vitro* experiments. PPAECs were isolated from the lumen of fresh porcine pulmonary artery tissue following the same procedures described for the PAVEC isolations. HUVECs were isolated from umbilical cord tissues acquired from St. Luke’s Episcopal Hospital, Houston, TX, following previously described methods [65]. All cells were cultured on tissue culture plastic previously coated with a 2.5% gelatin solution and supplemented with specialized EGM-2 medium (Lonza, Walkersville, MD) with 2% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in an incubator (37°C, 5% CO₂, 95% humidity). Cell culture medium was changed every 2-3 days, with cell passaging when confluence reached 85%.
Investigated hemostatic mediators

Known vascular EC-expressed thrombotic proteins were used to assess the hemostatic capacity of VECs from different aged specimens (see Table 3-1). Antibodies against thrombotic proteins (von Willebrand Factor [VWF] (Abcam, Cambridge, MA), tissue factor [TF], and plasminogen activator inhibitor-1 [PAI-1]) as well as anti-thrombotic proteins (VWF cleaving enzyme [ADAMTS-13] (Bethyl Laboratories, Montgomery, TX), tissue plasminogen activator [tPA] (Bioss Laboratories, Woburn, MA), and tissue factor pathways inhibitor [TFPI]) were used for immunohistochemistry and immunofluorescence to assess mediator localization and production. All antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA), unless otherwise noted.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thrombotic proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Von Willebrand factor (VWF)</td>
<td>Binds platelets to form platelet thrombi when in ultra-large multimer form</td>
<td>1:200&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tissue Factor (TF)</td>
<td>Surface protein expressed by activated endothelial cells to initiate coagulation cascade</td>
<td>1:50&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor (PAI-1)</td>
<td>tPA inhibitor</td>
<td>1:100&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Anti-thrombotic proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A disintegrin and metalloproteinase with a thrombospondin type I motif, member 13 (ADAMTS-13)</td>
<td>VWF cleaving enzyme</td>
<td>1:500&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tissue factor pathway inhibitor (TFPI)</td>
<td>Major inhibitor of TF, Factor Xa, and thrombin</td>
<td>1:100&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tissue plasminogen activator (tPA)</td>
<td>Regulator of fibrinolysis</td>
<td>1:50&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Phenotypic markers</strong></td>
<td></td>
<td></td>
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<tr>
<td>CD31</td>
<td>Endothelial cell membrane protein</td>
<td>1:50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Extracellular Matrix Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen type I</td>
<td>Fibrillar collagen concentrated at fibrosa of AV leaflet; most abundant AV ECM component</td>
<td>1:50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>Fibrillar collagen found mainly in fibrosa of AV leaflet</td>
<td>1:500&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Elastin</td>
<td>Highly elastic ECM component concentrated at ventricularis of AV leaflet</td>
<td>1:50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Histology and Immunohistochemistry**

Paraffin embedded and sectioned valve tissue samples were stained with Movat’s pentachrome (MOVAT) to identify differences in ECM organization and composition between the three age groups. The MOVAT used a series of tissue processing and...
chemical stains to dye cell nuclei purple, collagen yellow, proteoglycans and glycosaminoglycans blue, elastic fibers black, fibrin dark red, and muscle red. To localize the endothelial cell produced-hemostatic mediators in valve tissues, immunohistochemistry (IHC) was performed using the primary antibodies listed in Table 3-1 and biotinylated secondary antibodies (Jackson Immunoresearch, West Grove, PA) and visualized using a 3,3'-Diaminobenzidine (DAB) chromagen reaction (Vector Laboratories, Burlingame, CA) with a hematoxylin-2 counterstain for cell nuclei. All immunostained tissue specimens were pretreated with Citrate Buffer Antigen Decloaker (Biocare Medical, Concord, CA) for 30 min at 80°C, and blocked with 1% donkey serum buffer (DSB) (GeneTex, Irvine, CA) for 1 hr at room temperature. A negative control for each stained section remained incubated in DSB, while primary antibodies were incubated overnight at 4°C. Biotinylated secondary antibodies were incubated on all samples, including negative controls, for 1 hr at room temperature prior to Vectastain and DAB treatments. Stained sections were compared with parallel tissue sections immunostained for the ECM components collagen type I, collagen type III, and elastin (Abcam). Whole tissue images, as well as magnified images of the midleaflet region of each tissue specimen, were generated using a PathScan Enabler slide scanner (Meyer Instruments, Houston, TX) and Leica DMLS upright light microscope (Buffalo Grove, IL), respectively.

Semi-quantitative analysis of the DAB chromagen levels was performed at the midleaflet region of each tissue (n=3-6 valves per age group). All histological and immunohistochemical images that were analyzed and compared with one another were captured on the same microscope, at the same magnifications and exposure, with white
balancing performed on only the first sample of the set. Grading of DAB staining intensity was performed using ImageJ Software (NIH, Bethesda, MD). A background subtraction of 150 pixel rolling ball radius was applied to an image, then the image was processed using a Color Deconvolution plugin developed by A.C. Ruifrok to separate hematoxylin channels (nuclear counterstain) from the antigen-positive DAB stain channels. A binary mask was created by taking the threshold of the DAB channel images at the minimum threshold intensity measured in the negative control tissue samples. The DAB intensity and associated areas of the antigen-positive regions were quantified by applying the binary mask to the original sample image and analyzing particles such that the positive areas stained were recorded in pixels. The output data was used to quantify the proportion of tissue area stained as: \[ \frac{\sum(a_i)}{A} \], where \( a_i \) was the measured section of stained area, and \( A \) was the total sample area in the image field of view.

**Immunocytochemistry**

Hemostatic mediator antibodies (at the same dilution used in IHC) were used with fluorescent Alexa-fluor secondary antibodies (Invitrogen) to localize and verify PAVEC production of the above noted hemostatic proteins *in vitro*. PAVECs were fixed with 2% paraformaldehyde for 10 min after 5-7 days in culture on gelatin coated chamber slides. The fixed cells were permeabilized with 0.2% Triton-X for 10 min, then blocked with DSB for 1 hr prior to the addition of primary and secondary antibodies. Cells were also immunostained for the endothelial marker CD31 (Abcam) to ensure that the PAVECs had not undergone any change in cell phenotype. Fluorescence imaging was performed using a Zeiss LSM 5Live Confocal Microscope (Zeiss, Oberkochen, Germany).
Quantitative RT-PCR

RNA from the cell cultures was extracted using Trizol Reagent (Invitrogen) and a series of ethanol centrifugations. The mRNA was reverse transcribed into cDNA using Primescript 1st Strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan). Quantitative RT-PCR (qRT-PCR) was performed on the cDNA using 2X QuantiTect Sybr Green PCR Master Mix (Clontech, Mountainview, CA) with a Mastercycle ep Realplex (Eppendorf, Hamburg, Germany) to measure differences in gene expression levels for hemostatic mediator proteins between the YNG, ADT, and OLD PAVECs, PPAECs, and HUVECs (n=5-6 samples per cell type). qRT-PCR was performed in each sample in triplicates. The GAPDH gene was used as a housekeeping gene, and sample group protein gene expression was normalized to the corresponding PPAECs gene expression levels following the mathematical model for relative qRT-PCR [111]. All DNA primers were purchased from Integrated DNA Technologies (Coralville, IA; see Table 3-2 for DNA primer sequences).
<table>
<thead>
<tr>
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<th>5’→ DNA Sequence → 3’</th>
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<tr>
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**Table 3-2. Summary of hemostatic protein DNA primer sequences used for qRT-PCR.**

**VWF release and cleavage assay**

Previous work has shown that the addition of histamine to HUVEC cultures *in vitro* effectively initiates endothelial cell secretion of hyper-thrombotic ultra-large VWF multimer chains previously stored in Weibel-Palade bodies at the cell membrane, while leaving EC expression and release levels of the VWF cleaving enzyme ADAMTS-13 unchanged [65,112]. The quantities of total VWF protein or inactivated, cleaved VWF fragments in the solution were measured using enzyme linked immunosorbent assays (ELISAs) to assess the functionality and capacity of ADAMTS-13 enzyme to cleave VWF.
PAVECs, PPAECs, and HUVECs (n=6) were incubated with PAVEC stimulation medium consisting of serum-free EGM-2 media (with 1% v/v of insulin-transferring selenium A (Sigma-Aldrich, St. Louis, MO) and 1% (w/v) BSA (Sigma-Aldrich)) containing 100 μM of histamine at 37°C for 10 min. Next, the culture medium was collected with 10 mM EDTA) and stored at -20°C until use. Maxisorb 96-well plates (Thermo Scientific, Waltham, MA) were coated with 1 μg/mL of polyclonal rabbit anti-porcine VWF antibody (Abcam) in a Coating Solution buffer (KPL, Gaithersburg, MD) overnight at 4°C. The wells were then blocked with 1% w/v BSA/PBS solution for 1hr at 37°C. Samples of cell media supernatant or pooled human plasma were diluted with 1% BSA/PBS solution and incubated in the wells for 1 hr at 37°C. After washing, wells were incubated with 1 μg/mL of detection antibody for mouse anti-porcine full length VWF protein monoclonal antibody (2Q2134, Abcam) or mouse anti-human VWF 140-kDa fragment antibody (amino acids L^{1591}-Y^{1605}, Bethyl Laboratories) for 1 hr at 37°C.[65,113] The mouse detection antibodies were tagged with 1 μg/mL of peroxidase-labeled anti-mouse IgG (KPL) and then incubated with SureBlue Reserve TMB peroxidase solution (KPL) to expose the peroxidases on the bound detection antibodies. The reaction was stopped with the addition of TMB Stop Solution (KPL), and the 450 nm absorbance of each well was read using a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA). Serial dilutions of human plasma were used to create a standard curve, with the assumption of 10 μg/mL of VWF present per mL of pooled plasma [114].
**Calcific nodule formation assay**

To assess whether VEC-released VWF has a role in the development of CAVD, a CAVD *in vitro* model in which primary porcine aortic valvular interstitial cells (PAVICs) were cultured with various conditioned culture mediums to observe calcific nodule formation was performed [32,85]. Following the VWF release protocol used above, 5 mL of PAVEC stimulation medium supernatant (from histamine stimulated PAVECs in T-75 culture flasks) were collected after 10 min of incubation at 37°C. The collected PAVEC treated medium for each age group was mixed at 3% (v/v) with low serum PAVIC culture medium (48% DMEM, 49% F12, 1% HEPES, 1% ABAM, 1% BGS), aliquoted and frozen at -20°C until use.

Concentrations of VWF protein from each conditioned medium group were quantified using VWF sandwich ELISA. Histamine is an inflammatory mediator that is found in almost all tissues and can affect PAVIC proliferation and nodule formation *in vitro* at high concentrations (unpublished observations). Therefore, to provide an appropriate control, the levels of residual histamine within the conditioned PAVEC culture mediums were measured using a histamine ELISA (Genway Biotech, San Diego, CA). The average residual histamine levels from the different aged PAVEC conditioned mediums was found to be 8.4 nM.

Three control groups were prepared. First, low serum PAVIC culture medium served as a baseline condition for calcific nodule formation based on previous work [85]. Second, low serum PAVIC culture medium mixed with 3% (v/v) fresh PAVEC stimulation medium without histamine served as an additional control group. Third, to ensure the addition of residual histamine was not the cause of PAVIC nodule formation,
another group of PAVICs were treated with low serum PAVIC culture medium mixed with 3% (v/v) fresh PAVEC stimulation medium with 8.4 nM of histamine was used.

PAVICs here harvested from porcine aortic valve leaflets and cultured in PAVIC growth medium (10% BGS) following previously described methods [115]. At P2-P3 PAVICs were seeded into 24 well plates at 50,000 cells/cm². PAVICs groups were cultured at 37°C, 5% CO₂. The culture mediums were replenished every other day. At day 10, the cultured PAVICs were fixed with 4% paraformaldehyde, and stained with 40mM Alizarin Red S (ARS) for 30 min. After the dye was removed and rinsed, it could be observed that the calcific nodules were stained red. A photomask consisting of a circle divided into four quadrants was applied to each well of the culture plate in a way that excluded the outer 2.25mm edges of the well. Each well was imaged in quadrants using a Zeiss Stemi 200C stereoscope with a SPOT camera (Diagnostic Instruments Inc., Sterling Heights, MI). Using image J software threshold and particle count functions, the total nodule count and respective area was quantified for two quadrants of each well and averaged per culture condition to quantify average total calcified area per well.

Statistical Analysis

One way analysis of variance (ANOVA) statistics and Tukey post hoc tests were performed to compare quantified values between different aged tissue and PAVEC groups, and PPAEC and HUVEC control groups. ANOVA and Tukey post hoc tests were also performed to compare calcified values between PAVIC groups treated with different aged-conditioned mediums and the control culture medium groups. P-values <0.05 were considered as significant for all studies.
3.4 Results

*Hemostatic proteins distribute differently within aortic valve tissues*

Immunostaining showed that endothelial cell-mediated anti-thrombotic and thrombotic proteins were present in each of the different aged AV leaflet groups (Figure 3-1). The proteins were often localized with the endothelium at the edges of each leaflet. Many of these hemostatic mediators are actually secreted by endothelial cells, and correspondingly were also found within the tissue interior to varying degrees [108].

![Figure 3-1 MOVAT stains of aortic valve leaflet sections and higher magnification views of the midleaflet regions (indicated by the black box on MOVAT) for a disintegrin and metalloproteinase with a thrombospondin type I motif, member 13 (ADAMTS-13), tissue factor pathway inhibitor (TFPI), tissue plasminogen activator (tPA), von Willebrand factor (VWF), tissue factor (TF), and plasminogen activator inhibitor 1 (PAI-1). ADT indicates 6-month old porcine aortic valve sample; YNG, 6-week-old porcine aortic valve sample, OLD, 2-year-old porcine aortic valve sample; f, fibrosa; s, spongiosa; and v, ventricularis.](image)
There were no significant differences in the proportions of tissues stained between these proteins within YNG and OLD samples. The proportion of VWF in ADT tissue was significantly higher than the proportions of all other proteins except for ADAMTS-13 (Figure 3-2). However, the proportion of tissue stained for VWF was significantly higher in OLD tissue than in YNG tissues (p<0.05). There were trends of higher proportions of OLD valve tissues stained for PAI-1 (p=0.06), and TFPI (0.07) than in the corresponding portions of staining of YNG tissues, and a trend of higher proportion of TFPI staining in OLD tissues than in ADT tissues as well (p=0.09). The proportions of the valve tissues stained for PAI-1, TF, and tPA were low (< 10%) among all age groups in comparison to the other proteins evaluated (up to 40%) (Figure 3-2), and these less abundant proteins were localized primarily in the endothelium (Figure 3-1).

Figure 3-2. Semi-quantitative analysis results of the proportion of assessed tissue stained for the hemostatic proteins in the differently aged aortic valve leaflets. *= p<0.05 between noted age groups for that protein.
PAVEC hemostatic protein gene regulation changes with age

Gene expression of the anti-thrombotic and thrombotic proteins was quantified with qRT-PCR (Figure 3-3). PAVEC gene expression in all age groups was quantified relative to PPAECs (denoted by dashed lines in Figure 3-3). Gene expression for VWF in YNG PAVECs was significantly higher than in OLD PAVECs (48x, p<0.005). Similarly, gene expression for the anti-thrombotic proteins TFPI and tPA was three-fold higher in YNG PAVECs than in OLD PAVECs (p<0.05 for each). Compared to the PPAEC control group, the YNG PAVEC gene expression for was significantly higher for VWF (58x, p<0.0005), TFPI (3x, p<0.05), and tPA (30x, p<0.05).

There were no significant differences between ADT PAVECs and the other two age groups for all proteins. However, ADT PAVECs did have significantly higher gene expression of TFPI (2.5x, p<0.05) compared to the PPAEC controls.

The only protein that had the highest expression in the OLD PAVECs was the thrombotic protein PAI-1, which was two-fold higher than in both YNG and ADT PAVECs (p<0.05). There were no significant differences in the gene expression for ADAMTS-13 and TF between the PAVEC groups and PPAEC controls. Furthermore, all PAVEC and PPAEC groups had approximately 1000x higher gene expression levels for all hemostatic proteins than did HUVECs (data not shown).
Figure 3-3. PAVEC gene expression of antithrombotic (A–C) and thrombotic (D–F) proteins relative to porcine pulmonary artery endothelial cells (PPAECs). ^p<0.05 between noted PAVEC age group vs. the relative PPAEC gene expression (indicated by dashed line). *p<0.05 between noted age groups for that protein.

**PAVECs synthesize hemostatic mediators in vitro**

Primary PAVECs harvested from different aged AV tissues maintained their endothelial phenotype and stained positively for CD31. ADAMTS-13 and VWF were generally co-localized in all age groups, though there was a greater abundance of VWF
staining overall (Figure 3-4, first column). However, VWF levels in the OLD PAVECs appeared more profuse than in the YNG PAVEC cultures.

PAVECs positively stained for low levels of TFPI and TF (Figure 3-4, second column). TFPI staining in YNG and ADT PAVECs was primarily localized intracellularly with some punctate staining outside of the cells; TFPI staining in OLD PAVECs was low, with punctate staining outside of the cells. For the YNG PAVEC cultures, there appeared to be higher levels of TFPI than of TF. Conversely, TF was more abundant than TFPI in OLD cultures.

PAI-1 and tPA were also detected in PAVEC cultures (Figure 3-4, third column). There was intracellular staining for PAI-1 in all PAVEC age groups, although punctate extracellular staining of PAI-1 was present in the OLD PAVEC cultures. Staining for tPA appeared to be more abundant in YNG and ADT PAVEC cultures than in OLD PAVEC cultures. Although the stains for PAI-1 and tPA were generally less abundant in the OLD PAVEC cultures, the complimentary proteins appeared to be co-localized with slightly higher levels of PAI-1.
**PAVECs can mediate functional VWF release and cleavage**

Histamine stimulation of PAVECs *in vitro* initiated the rapid release of thrombogenic VWF multimer proteins into the culture medium. For the comparison of PAVEC age groups, VWF protein release paralleled the VWF gene expression trend in that YNG PAVECs released a significantly higher concentration of VWF protein than did OLD PAVECs (p<0.05) (Figure 3-5A). VWF release by the PPAECs and ADT PAVECs was not significantly different from that released by the YNG and OLD PAVECs. HUVECs released significantly higher amounts of VWF protein than all porcine derived PAVEC and PPAEC groups (p<0.0001).

The fraction of cleaved VWF-140 fragments was measured using ELISA assays with a specific monoclonal antibody for cleaved VWF proteins. There was no statistically significant difference in the fraction of VWF-140 fragments between porcine derived
PAVEC and PPAECs (Figure 3-5B). For the HUVECs, approximately 10% of the VWF protein was cleaved into VWF-140 fragments, which was a significantly lower cleavage product fraction compared to the OLD PAVECs (p<0.05).

![Graph A](image)

**Figure 3-5.** Quantification of rapidly released von Willebrand factor (VWF) protein from PAVEC, HUVEC, and PPAEC groups after 10 minutes incubation with histamine stimulation solution (A) and the fraction of cleaved VWF-140 fragments in solution (B). ^p<0.05 between noted age groups. *p<0.001 between noted age group vs. all groups.

**PAVEC released VWF increases PAVIC calcific nodule formation in vitro**

PAVICs were cultured with various culture medium conditions to assess how the addition of VEC released VWF affect calcific nodule formation *in vitro*. Measured levels of VWF protein present in the low serum PAVIC medium mixed with 3% (v/v) conditioned culture mediums collected from YNG, ADT, and OLD PAVEC groups were
257 pg/mL, 130 pg/mL, and 90 pg/mL, respectively. No levels of VWF protein were detected in any of the three control conditions.

Within 5 days of culture, nodule formation was visible in PAVIC cultures treated with each of the conditioned mediums from the different aged PAVECs. At the end of the 10 day culture period, there were no significant differences in average number of nodules per well, average nodule size, or total calcified area between the control groups of low serum PAVIC medium, 3% PAVEC stimulation medium without histamine, and 3% PAVEC stimulation medium with histamine (Figure 3-6). The total calcified area in PAVICs treated with the conditioned mediums from YNG, ADT, and OLD PAVEC supernatants ($2.0-2.4 \times 10^6 \mu m^2$) were all significantly greater than all three control groups ($1.2-3.2 \times 10^4 \mu m^2$, $p<0.001$) (Figure 3-6). PAVICs in both YNG (40 nodules) and ADT (45 nodules) conditioned medium groups had significantly more nodules per well than the three control groups (9-13 nodules, $p<0.001$, Figure 3-7A). Furthermore, the average nodule size in PAVICs treated with one of the three conditioned mediums ($52-78 \times 10^3 \mu m^2$) were significantly larger that each of the three control conditions ($13-23 \times 10^3 \mu m^2$, $p<0.001$) (Figure 3-7B).
Figure 3-6. Schematic of *in vitro* calcific nodule experiment to test the effects of VEC–released VWF on PAVIC calcification (A). Conditioned culture mediums consisted of VIC low-serum medium with VWF-enriched media supernatant from histamine stimulated PAVECs from each age group. PAVICs were cultured in conditioned culture medium and control mediums for 10 days. Calcified nodules were stained red with Alizarin Red S (ARS), and the average total calcified area per culture condition was quantified (B). Representative images of PAVIC calcific nodules formed in low-serum VIC medium (C) and conditioned OLD medium (D). Bar=100 μm.
Figure 3-7. Quantification of the average PAVIC nodules per well (A) and average PAVIC calcified nodule size (B) for the different aged PAVEC-released VWF conditioned mediums and the three control groups. * = p-value < 0.001, ^ = p-value < 0.01.
3.5 Discussion

The endothelial cell-mediated process of hemostasis is essential for the function of all living heart valve tissues. As these tissues undergo remodeling with age and disease, VEC regulation of hemostatic proteins also change. This study is the first to examine the production and expression of numerous hemostatic proteins in AV tissues and \textit{in vitro} PAVEC cultures from three distinct age groups.

There are several age-related differences in the abundance and localization of thrombotic and anti-thrombotic proteins within the aortic valve. As expected, all examined hemostatic proteins were strongly present at the leaflet edges in each age group. However, many of these soluble components, namely ADAMTS-13, TFPI, VWF, TF, and PAI-1, were found throughout the interior of the valves as well. These proteins were primarily localized in the ventricularis layer of the AV in YNG and ADT tissues. Conversely, they were distributed more evenly across the valve layers in OLD AV samples. Due to their interior location, it is possible that these hemostatic components interact with sub-endothelial extracellular matrix components such as elastin and collagen type I. The ventricularis layer contains densely packed elastin, which may sequester the soluble hemostatic proteins that have permeated into the sub-endothelium. As elastin becomes more disperse throughout the OLD AV tissues [101], the hemostatic proteins may be able to permeate throughout the entire interior of the AV.

These proteins may also interact with collagen type I, as it is most abundant valve ECM protein and is present throughout the leaflet. Previous work investigating fiber alignment in articular cartilage tissues suggest that highly organized collagen alignment limits diffusion of proteins through anisotropic tissues [116,117]. Similarly, the lower
amount of hemostatic proteins within the fibrosa layer of the YNG and ADT AV tissues may be due to the highly organized and aligned collagen fibrils serving as a barrier to limit the diffusion of hemostatic proteins into the aortic side of the AV leaflet. Given the age-associated collagen remodeling and turnover observed in older tissues [104], this barrier function of the fibrosa may be attenuated with age and result in the increased permeation of VEC-secreted hemostatic proteins throughout the valve layers.

In addition, qRT-PCR analyses showed highly significant changes in gene expression of many hemostatic proteins with age in PAVEC cultures. PAI-1, the protein inhibitor for tPA in the fibrinolysis pathways, was significantly elevated with age as shown by mRNA analysis, immunocytochemistry, and a trend in the proportion of tissue stained. The increased accumulation of PAI-1 in older valves suggests that this smaller molecular weight protein can easily permeate through the remodeled ECM architectures in OLD valves. Previous studies have shown that inflammatory or cytokine stimuli can increase endothelial secretion of PAI-1 while not affecting tPA secretion [108,118]. It seems likely that elderly patients with higher levels of inflammation, cholesterol, and tissue remodeling, will also experience more pro-coagulant states, in which PAI-1 levels in the blood and ECM overcome the basally secreted levels of tPA [118–120]. Interestingly, there were no differences in tPA accumulation in AV tissues despite the significant drop in tPA gene expression in OLD PAVECs relative to YNG PAVECs. This may suggest that the expressed tPA is released more into the bloodstream as opposed to accumulating within valve tissues. The reduction in PAVEC tPA expression also reflects the hemostatic imbalance between tPA and PAI-1 associated with age.
PAVEC expression and tissue accumulation of TFPI and VWF were affected by age-related changes as well. In both cases, YNG PAVECs had significantly higher levels of TFPI and VWF gene expression than did OLD PAVECs. Consistent with qRT-PCR results, stains for TFPI in YNG PAVECs appeared to be more abundant than in OLD PAVEC cultures. Conversely, OLD PAVEC cultures had slightly higher levels of VWF staining than in YNG PAVEC cultures. However, the polyclonal anti-VWF antibody used in the stains binds to both cleaved and uncleaved VWF proteins. Therefore, the increased staining observed in the OLD PAVEC cultures is likely from the higher proportion of cleaved VWF proteins in the condition, as observed in the ELISA experiments. Furthermore, YNG valves showed trends of lower proportions of tissue stained for both TFPI and VWF than in OLD tissues. This discrepancy could be a result of the retention of these basally secreted proteins within the disorganized OLD tissue ECM through interactions with heparin and collagen type I [70,108].

PAVEC expression of ADAMTS-13 and TF was not affected by age for gene expression or the proportion of tissue stained. Thus, these specific hemostatic proteins seem less likely to be direct players in causing age-related valve disease. These results, however, suggest that the valvular hemostasis may become unbalanced over a lifetime, as many of the associated counteracting hemostatic proteins (VWF for ADAMTS-13, TFPI for TF) were shown to be affected by age. This imbalance was also observed in the immunostaining of PAVECs, as co-localization of the associated hemostatic component pairs were less present in the OLD PAVEC cultures.

Functional testing of the fragmentation of histamine-stimulated VWF confirmed that PAVECs secrete ADAMTS-13 to cleave VWF multimers. The total amount of VWF
protein released by OLD PAVECs was significantly lower than by YNG PAVECs, which was consistent with the gene expression results. The fractions of cleaved VWF (140 kDa fragments) were not significantly different between all porcine derived endothelial cell groups, which supports the previous finding that ADAMTS-13 expression and function is not affected by age [65]. On the contrary, since VWF expression and release is affected by age, with the elderly age group having a drop in VWF expression and production, there will be lower proportions of complete VWF proteins present in patient plasma that can mediate coagulation. This phenomenon is consistent with clinical reports that elderly patients require lower doses of anti-coagulants after heart valve surgeries than do adult patients, due to lower clearance kinetics of the drugs and decreased clotting ability in elderly patients [121].

The changes in hemostatic protein regulation by VECs from our results matches with the age-related hemostatic protein plasma levels previously reported [118–121]. We believe that these age-related imbalances in hemostatic protein regulation and production by VECs are not likely to cause spontaneous development of thrombus. However, when injury or insult occur at the valve endothelium, the aging-induced imbalance in the expression of anti-thrombotic and thrombotic proteins by VECs will lower the VEC’s capability to maintain hemostasis; thus causing AV tissues to be more susceptible to thrombotic complications. Also, injury to the endothelium exposes the underlying tissue, and therefore, the accumulation of these proteins in valve tissues along with the structural changes can induce further thrombotic events.

Aside from intravascular complications, accumulation of these proteins in valve tissues along with the structural changes can cause tissue level progression of age related
diseases such as CAVD. Utilizing an *in vitro* CAVD model, PAVIC nodule formation experiments showed that the presence of VEC-released VWF significantly increased the total number of nodules, nodule area, and total calcified area in PAVICs relative to control groups. Having no differences in nodule formation between the three control culture conditions confirmed that the significant increase in calcific nodule formation by PAVICs was not due to the addition of PAVEC stimulation medium or residual levels of histamine in solution, but due to the presence of VEC-released proteins including VWF, after histamine stimulation. Therefore, the secretion and accumulation of VWF and other hemostatic proteins within the valve tissues affect the development and progression of CAVD and other acquired valvular diseases.

Though there were no significant differences in PAVIC nodule formation between the different aged PAVEC-based conditions, the significant increase in VWF present within OLD valve tissues in conjunction with age-associated ECM disorganization seen in the histology slides suggest that calcification could result from increased pro-osteogenic VWF-VIC interactions, which correlates with the classical clinical presentation of CAVD in the elderly. Although VWF is the main component of histamine stimulated PAVEC supernatant, other factors in the supernatant (e.g. prostacyclin, platelet activating factor, angiopoietin-2, and interleukin-8) may also intensify PAVIC nodule formation, and warrants further investigation into how other hemostatic proteins within valve tissues can affect AV calcification [122–124]. Nonetheless, our studies have shown that PAVECs will constitutively secrete VWF and other hemostatic factors, which will result in their accumulation of these proteins within the valve sub-endothelium. As ECM organization changes with age and the valve hemostatic protein regulation becomes
more imbalanced, there is potential that VWF and some of these proteins may play a substantial role in the progression of age-related valve disease.

This study was also the first to perform an extensive characterization of PAVEC hemostatic protein expression and production relative to vascular endothelial cell types PPAECs and HUVECs. PPAECs have been documented to produce significant levels of VWF and were chosen as a porcine vascular endothelial cell control group [125]. Previous findings have found that porcine endothelial cultures isolated from the thoracic aorta expressed low levels of VWF mRNA and did not contain Weibel Palade bodies, indicating that porcine endothelial cells have different regional capacities for hemostatic regulation [125,126]. The gene expression of TFPI in YNG and ADT PAVECs, and of tPA and VWF in YNG PAVECs were significantly higher than in the baseline PPAECs, suggesting that PAVECs have distinct hemostatic regulation activity. Gene expression levels differed greatly between PAVECs and HUVECs, with expression of the investigated proteins being approximately three orders of magnitude higher in the PAVECs. HUVECs have been well documented to produce and release VWF in vitro [108,112], and thus are frequently used in endothelial cell studies and hemostatic experiments. However, the concentration of VWF protein released from histamine stimulated HUVECs was significantly higher than all porcine derived endothelial cell groups, suggesting that HUVECs are capable of storing more VWF in their Weibel Palade bodies than are porcine endothelial cells. Therefore, future studies regarding PAVEC expression and mediated protein functions should consider using porcine derived vascular endothelial cells as baseline controls.
Limitations of this study include the inherent variability in the semi-quantitative analysis of immunohistochemistry, which reduces the ability to distinguish between age groups. A larger sample size may help in confirming the promising trends. Still, this study examined several key endothelial cell-mediated hemostatic proteins. Additional factors such as ectonucleotidases, prostacyclin, and thrombomodulin have yet to be studied with respect to PAVEC hemostatic regulation. Future studies using a gene array for a wider panel of thrombotic and anti-thrombotic proteins in conjunction with ECM components and inflammatory markers may be informative in elucidating the relationship between the VECs regulation of their environment and how the protein accumulation influences the biology and pathology of the AV. Furthermore, while the interaction and balance of thrombotic and anti-thrombotic proteins is well understood at the apical side of the endothelium, further studies to investigate the interactions of each protein with sub-endothelial ECM and cells may be important in understanding the mechanisms that can promote the onset of valvular dysfunction. Lastly, further investigation into the molecular mechanisms behind the age-related imbalances in PAVEC hemostatic protein regulation will greatly enhance our knowledge of these changes and may provide potential targets for the prevention and treatment of acquired valve abnormalities.
3.6 Conclusions

In conclusion, it is well understood that valve tissues experience different environments with respect to age as a result of changes in valve mechanics, hemodynamics, and matrix composition. This study has identified age-related differences in PAVEC hemostatic protein regulation and the ability of basally secreted proteins to aggregate within different aged AV tissues. In addition, we have verified that PAVECs express many of the same thrombotic and anti-thrombotic proteins as vascular endothelial cells. Though there was not one pattern for all hemostatic protein expression and aggregation with respect to age, we demonstrated age-related differences in the overall expression and localization of tPA, PAI-1, TFPI, and VWF in AV tissues and cells. Though thrombosis does not commonly occur as an age-associated dysfunction in valve tissues, we observed that thickened elderly valve tissues with ubiquitous distributions of elastin and collagen sequester high levels of the hemostatic proteins in the sub-endothelium, which warrants further investigation into potential roles for these aggregated proteins in the formation of calcific nodules that are so prevalent in older AVs [1,98]. Our in vitro PAVIC studies suggest that the presence of these VWF promotes PAVIC calcification, and that VWF and other hemostatic proteins within the tissue interior may have a potential role in the development of CAVD and other acquired abnormalities as age-related valve tissue ECM remodeling occurs. Thus, future studies regarding valve biology, pathology, and tissue engineering require consideration of environments that reflect the specific age group in question, including the necessary hemostatic framework.
Chapter 4: Investigation of environmental stimuli effects on VECs and their hemostatic responses\(^2\)

4.1 Introduction

The endothelium plays an essential role in maintaining homeostasis in cardiovascular tissue. These cell monolayers act as a barrier between the circulating blood and the underlying tissue, produce and release various growth and vasomotor factors, and initiate inflammatory and clotting mechanisms in response to injury and disease [108,127]. Although the function of vascular endothelial cells has been thoroughly characterized, recent work has shown that cardiac valve endothelial cells (VECs) have distinct phenotypes and distinct gene and protein expression compared to vascular endothelial cells [34,35,128]. Additionally, we have shown from results described in Chapter 3 that VECs produce anti-thrombotic and thrombotic proteins differently from vascular endothelial cells, and can be affected by environmental changes in aortic valve tissue with respect to aging [128]. However, VECs still maintain characteristic endothelial functions, including signaling to the underlying valve interstitial cells (VICs) with vasoactive factors, and reducing extracellular matrix (ECM) degradation in the valve [13,39].

\(^2\) The work in this chapter was prepared as a manuscript: L.R. Balaoing, A.D. Post, A.Y. Lin, H. Tseng, J.L Moake, and K. J. Grande-Allen. Laminin peptide-immobilized hydrogels modulate valve endothelial cell hemostatic regulation.
Given the interplay between VECs, VICs, and ECM, VECs and their regulation of valve homeostasis are highly sensitive to surrounding stimuli. For example, the presence of TGF-β and Notch-1 has been shown to promote VIC calcification, as well as to induce endothelial to mesenchymal transdifferentiation and influence VEC plasticity [42,129,130]. VEC dysfunction through pathogenic angiogenesis and imbalanced inflammatory and thrombotic protein regulation contribute to the progression of calcific aortic valve disease (CAVD) [5,29,57,128]. Myxomatous diseased valves have also been linked to thrombosis and endocarditis from disrupted valve endothelium [6,131]. Thus, the preservation of a healthy, functional valve endothelium is necessary for valve homeostasis and prevention of valve disease.

Various strategies have been investigated to promote the endothelialization of tissue-engineered heart valve scaffolds and implants to reduce thrombosis-related failures in vivo. However, heart valves are particularly challenging to endothelialize due to the high hemodynamic and mechanical forces experienced by valve tissues. Most approaches have had mixed success using vascular endothelial cells in combination with mechanical stimulation to promote endothelialization of decellularized valve tissues or mechanical valves [132–135]. Other studies have attempted to drive endothelial progenitor cells and mesenchymal stem cells to differentiate into endothelial cell lineage for endothelialization purposes [82,136–138]. However, little work has been done to evaluate differences or similarities in how these various cell types regulate hemostasis and perform their anti-clotting roles relative to native VECs [138,139].
4.2 Objective

This work investigates the thrombotic and anti-thrombotic behavior of primary porcine aortic VECs when cultured in the presence of various environmental stimuli. We have developed a platform utilizing poly(ethylene glycol) diacrylate (PEGDA) hydrogels and specific cell adhesive peptides to modulate the substrate stiffness and ECM presented on the surface to which the VECs are seeded. PEGDA-based hydrogels are bioinert polymers that can have tunable mechanical properties based on the molecular weight of the PEGDA used [81,140,141]. The acrylates on the surface of the gel are free to interact with desired adhesive peptides containing a thiol group [142,143]. Furthermore, VECs have been shown to have protein-dependent adhesion in previous studies [49]. To investigate the effects of ECM on VEC phenotype and hemostatic function, peptide sequences derived from laminin and fibronectin proteins were used. Laminin belongs to a family of heterotrimeric glycoproteins composed of combinations of α, β, and γ chains. Laminins in the basement membrane play an integral role in the formation of the basement membrane network [144]. The peptide motif RKRLQVQLSIRT (RKR) is found in the laminin-α1 heparin binding G-domain, and has previously been shown to promote strong cellular attachment activity [145,146]. The cell adhesive peptide RGDS is found in fibronectin and other ECM proteins, and is one of the most commonly used peptide sequences to promote cellular adhesion [142,147,148]. These studies will provide information about VEC hemostatic capacity and conditions necessary to maintain essential VEC functions in vitro, and potential strategies for endothelialization of cardiovascular implants.
4.3 Materials and Methods

**PEGDA synthesis and hydrogel characterization**

PEGDA hydrogels are non-immunogenic, mechanically tunable, and naturally prevent protein adsorption and cell adhesion unless they are chemically modified [81,141,147]. To control scaffold substrate rigidity, PEGDA hydrogels of molecular weights (MWs) 3.4, 8, or 20 kDa were used.

PEGDA was prepared following previously described methods [140,141]. Briefly, PEG powder with molecular weight of either 3.4, 8, or 20 kDa was acrylated by mixing 0.4 mmol of PEG (Sigma-Aldrich) with 0.016 mol of acryloyl chloride and 0.8 mmol of triethylamine in anhydrous dichloromethane (DCM) under argon gas overnight. The PEGDA solution was washed and mixed with 2M K$_2$CO$_3$ and phase separated into aqueous phase, and dried with anhydrous MgSO$_4$ to remove any residual solution. Next, the MgSO$_4$ was filtered from solution, and PEGDA precipitated from the DCM and filtered with cold diethyl ether. The resulting PEGDA powder was tested with $^1$H-NMR to verify acrylation of PEG chains. PEGDA samples were stored at -20°C until use.

PEGDA hydrogels were polymerized by dissolving PEGDA powder of a particular MW in deionized H$_2$O at 10% (w/v) with 45 mM of the photoinitiator, Irgacure 2959 (Ciba, Basel, Switzerland), and exposed to UV light for 5 min. on each side (365 nm, 10 mW/cm$^2$). Once polymerized, the hydrogels were soaked in phosphate buffered saline (PBS, pH 7.4) at room temperature overnight to swell and remove excess photoinitiator. Hydrogels used for experiments were approximately 0.5-1.5 mm thick.
after swelling. Disks 22 mm in diameter were punched from the bulk hydrogels, placed into 12-well tissue culture plates, and seeded with VECs for experiments.

Compressive mechanical testing was performed on 22 mm diameter, 5 mm thick hydrogel disks made of each PEGDA MW using a Bose ElectroForce ELF 3200 (Eden Prairie, MN) system using a 1000 gram load cell (Bose). Each sample was compressed to 30% strain, and the resulting load was measured. The stress and strain was calculated at each time point, and plotted against each another. The compressive elastic modulus of each PEGDA sample was calculated as the slope of the linearly elastic stress-strain curve using Microsoft Excel (n=5).

**ECM Adhesive Peptide Motifs**

Both RK and RGDS peptides were modified to include a cysteine at the N-terminus of each sequence, resulting in peptide sequences CRKRLQVQLSIRT and CRGDS. The cysteine introduced a thiol group at the end of each peptide chain that could react with the acrylate groups on the surface of the PEGDA hydrogels. The custom modified RKR and RGDS peptides were synthesized by American Peptide Company (Vista, CA), reconstituted in sterile dimethyl sulfoxide, and stored at -80°C until use.

To immobilize the peptide motifs onto the PEGDA hydrogels, 3 mM peptide solutions were added to the hydrogel surface and exposed to 5 min. of UV light and 45mM I2959 to initiate a thiol-ene reaction, covalently binding the desired peptide to the gel via click reaction [142,149]. The hydrogels were vigorously washed with 0.05% Tween-20 detergent and PBS to remove unbound peptides and solvent solution. Thiol-PEG-fluorescein (FITC, NanoCS, New York, NY) served as a negative adhesive substrate control capable of undergoing the thiol-ene reaction with hydrogels. Peptide
immobilization and surface saturation was verified by measuring fluorescent signal (418 nm) from increasing concentrations (0-15 mM) of thiol-PEG-FITC immobilized onto 3.4 and 20 kDa hydrogels using a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA) (n=3 per concentration) (Figure 4-1).

![Saturation curve of immobilized thiol-PEG-FITC on PEGDA hydrogels. Fluorescent intensity of immobilized thiol-PEG-FITC with increasing initial concentrations added. Saturation occurs at initial thiol-PEG-FITC concentrations of 3 mM on 20 kDa PEGDA hydrogels and 5 mM on 3.4 kDa hydrogels.](image)

**Valvular Endothelial Cells**

VECs were harvested from fresh heart aortic valves dissected from 6-month old porcine hearts purchased from a local commercial abattoir (Fisher Ham and Meats, Spring, TX). VEC isolation and purification procedures followed a combination of previously described methods [34, 109, 128]. Briefly, each dissected aortic leaflet was rinsed in sterile 5% P/S in PBS, and soaked in an enzyme digestion composed of 2 U/mL of dispase (Stem Cell, Vancouver, Canada), and 60 U/mL of collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ) at 37°C for 1 hr. After the enzyme digestion, the VECs were gently scraped off each leaflet surface by rolling a swab tip across both sides of the leaflets. The cells were rinsed off the swab with media,
centrifuged, and then resuspended in media and plated. All VEC cultures used EGM-2 media supplemented with growth factor bullet kit (hydrocortisone, FBS, VEGF, hEGF, hFGF-B, R3-IGF-1, ascorbic acid) and 1% v/v P/S solution (PenStrep, Lonza, Walkerville, MD). After the initial passage, VECs were purified from any contaminant interstitial cells via magnetic bead cell isolation (CELLection Pan Mouse IgG Kit, Invitrogen, Carlsbad, CA) using mouse anti-porcine CD31 antibody (AbD Serotec, Oxford, UK). VECs were cultured in a humidified incubator (37°, 5% CO2) until they reached 90% confluence, with media changed every 2-3 days. VECs from passages P2-P4 were used in the following experiments.

**Experimental Groups**

To assess the effects of substrate stiffness and ECM on VEC phenotype and behavior, VEC were seeded at a cell density of 250 cells/mm² onto combinations of the 10% w/v PEGDA hydrogels composed of three different PEGDA MWs (3.4, 8, and 20 kDa) and immobilized with either modified ECM adhesive peptides (RKR or RGDS). Thus, six microenvironment culture conditions were examined. Uncoated PEGDA hydrogels of each PEGDA MW were used as negative controls, whereas tissue culture treated polystyrene (TCPS) dishes were used as baseline controls for VEC attachment. Cells were cultured for 5-8 days on the peptide-immobilized gels until 90% confluence was reached, and then evaluated as described below. VEC adhesion and proliferation was monitored daily with light microscopy.
**Immunofluorescence and Quantitative RT-PCR**

Immunofluorescence was performed on cells fixed in 4% paraformaldehyde. Mouse anti-CD31 (LCI-4, AbD Serotec), and mouse anti-α-smooth muscle actin (αSMA) (1A4, Abcam, Cambridge, MA) antibodies were used to verify that the VEC cultures demonstrated the endothelial phenotype with no interstitial cell contamination. Rabbit anti-laminin (Abcam) and mouse anti-fibronectin (A17, Abcam) were used to observe ECM production by VECs. Rabbit anti-porcine von Willebrand factor (VWF, Abcam) was used to visualize VWF produced by VECs. Secondary antibodies conjugated with Alexa Fluor (Invitrogen) were used after primary antibody incubation. Fluorescent imaging was performed using a Zeiss LSM 5Live Confocal Microscope (Zeiss, Oberkochen, Germany).

Once confluent, VECs were trypsinized and scraped from their substrates, centrifuged, and lysed with Trizol Reagent (Invitrogen). Using a series of ethanol washes and centrifugations, mRNA from the VEC samples were collected. The mRNA was reverse transcribed into cDNA using PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan). The cDNA samples were stored at -80°C until use. Using the 2X QuantiTect SYBR Green PCR Master Mix (Clontech, Mountain View, CA), quantitative RT-PCR (qRT-PCR) on the cDNA was performed to assess differences in gene expression levels for the above mentioned anti-thrombotic and pro-thrombotic proteins between VECs cultured in the various platforms (n=3-4). GAPDH gene was used as the housekeeping gene, and sample group gene expression levels were normalized to the corresponding expression levels of the TCPS seeded VECs (See Table 4-1 for primer sequences).
Table 4-1. Summary of hemostatic protein DNA primer sequences used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>5'→ DNA Sequence → 3'</th>
<th>Product Size</th>
<th>Accession Number</th>
</tr>
</thead>
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<tr>
<td>ADAMTS-13</td>
<td>Forward CTCTGTTTCCCTGGGGGATG</td>
<td>97</td>
<td>Q76LX8</td>
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<tr>
<td></td>
<td>Reverse CAAGTGCTGGCAGAGGTACAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TFPI</td>
<td>Forward GACCTCTTACTGCTATTC</td>
<td>138</td>
<td>EU090729</td>
</tr>
<tr>
<td></td>
<td>Reverse ATCTCTTCTATGCTATTC</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Forward GGGACAGAAGCTGGGATA</td>
<td>136</td>
<td>Y11347</td>
</tr>
<tr>
<td></td>
<td>Reverse GTCTAAGAGGCGAGTTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWF</td>
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<td>S78431</td>
</tr>
<tr>
<td></td>
<td>Reverse ATCACCTCCTCACAAAC</td>
<td></td>
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<tr>
<td>TF</td>
<td>Forward TACAAGAGTAGAATCCAT</td>
<td>107</td>
<td>AY50424</td>
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<tr>
<td></td>
<td>Reverse AAGTGTCTAATGCTAATG</td>
<td></td>
<td></td>
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<tr>
<td>tPA</td>
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<td>102</td>
<td>BK007995</td>
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<tr>
<td></td>
<td>Reverse AAGCCTTCTCCATCA</td>
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<tr>
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<td>AY266137</td>
</tr>
<tr>
<td></td>
<td>Reverse TATTGAGCGGATTTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward CATTGACCTCCTACAT</td>
<td>119</td>
<td>AF017079</td>
</tr>
<tr>
<td></td>
<td>Reverse AGATGATGGGAGATTC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**VEC Histamine Stimulation**

Previous work has shown that the addition of histamine to human umbilical vein EC cultures in vitro effectively initiates EC secretion and anchorage of hyper-thrombotic ultra-large VWF (ULVWF) multimer chains previously stored in Weibel-Palade bodies at the cell membrane, while not affecting EC expression and release levels of the VWF cleaving enzyme ADAMTS-13 [65,112]. The measurable quantities of total VWF protein or inactivated, cleaved VWF fragments (VWF 140-kDa or VWF 176-kDa) in the solution are commonly used to quantify the functionality and capacity of ADAMTS-13 enzymatic cleavage of VWF. VECs were incubated with serum-free EGM-2 media (with 1% v/v of insulin-transferring selenium A (Sigma-Aldrich) and 1% BSA w/v) containing 100 μM of
histamine at 37°C for 2 minutes, and then rinsed with PBS. After, cells were incubated in complete EGM-2 media for 10 min. Then, media supernatants were collected into 10 mM of EDTA and analyzed by sandwich ELISA for either total VWF antigen or VWF 140-kDa fragments.

**Measurement of total VWF and VWF 140-kDa fragments**

A sandwich ELISA was performed to measure cleaved and uncleaved VWF from histamine stimulated VECs (n=3) following previously described methods [65,128]. Maxisorb 96-well plates (Nunc, Penfield, NY) were coated with 1 μg/mL of polyclonal rabbit anti-porcine VWF antibody (Abcam) in a Coating Solution buffer (KPL, Gathersburg, MD) overnight at 4°C. The wells were then blocked with 1% w/v BSA/PBS solution for 1 hr at 37°C. Samples of cell media supernatant or porcine plasma were diluted with 1% BSA/PBS solution and incubated in the wells for 1 hr at 37°C. Next, the wells were washed 3x with 1x Washing Solution (KPL), and incubated with 1 μg/mL of detection antibody for mouse anti-porcine full length VWF protein monoclonal antibody (2Q2134, Abcam) or mouse anti-human VWF 140-kDa fragment antibody (amino acids L<sup>1591</sup>-Y<sup>1605</sup>) for 1 hr at 37°C [65,113]. Afterwards, the wells were again washed 3X with Washing Solution, and incubated with 1 μg/mL of peroxidase-labeled anti-mouse IgG (KPL) for 1hr at RT. The wells were washed 4x with Washing Solution and then incubated with SureBlue Reserve TMB peroxidase solution to expose the peroxidases on the bound VWF-detection antibodies. The reaction was stopped with the addition of TMB Stop Solution (KPL), and the 450 nm absorbance of each well was read using a spectrophotometer. Serial dilutions of porcine plasma (Animal Technologies, Tyler, TX)
was used to create a standard curve, with the assumption of 10 µg/mL of VWF present per mL of pooled plasma [114].

*Platelet Adhesion Assay*

To assess how the microenvironmental conditions affect VEC capacity to activate and adhere live platelets, dyed platelets were added to VEC seeded constructs and quantified following previously described methods by Xu et al [150]. Platelet rich plasma (PRP) was isolated from whole blood drawn from healthy, adult volunteers following a protocol approved by the Institutional Review Board at Rice University. The PRP was treated with prostacyclin (0.5 µg/mL of PRP) to prevent platelet activation during subsequent dyeing and washing steps. The platelets were isolated from the PRP, resuspended in Tyrode’s buffer, and dyed with Sudan B Black solution (5% w/v) for 1 hour. The dyed platelets were washed 3x with PBS.

The VECs seeded on hydrogel constructs and TCPS (n=3) were gently washed with PBS to remove residual media. Next, 4x10⁶ platelets were added to each sample and allowed to incubated at 37°C for 30 minutes. The constructs were then moved to a new well plate to remove any unadhered platelets on the bottom of the gel and then gently washed 3x with PBS. Next, 400 µL of DMSO was added to each well to lyse the bound platelets and release the SBB dye. The absorbance of the supernatant for each sample was read at 595 nm using a spectrophotometer. The total number of adhered platelets was calculated based on a standard curve made from serial dilutions of the prepared dyed platelet stock solution. This platelet adhesion assay was subsequently performed on VECs stimulated with histamine (as described above) to assess the thrombogenicity of activated VECs and their released VWF.
Statistics

Data are presented as means with standard errors of means (SEM). ANOVA statistics and Tukey *post hoc* tests were performed using JMP statistical software (SAS, Cary, NC) to compare the differences between the peptide-gels used as platforms for VEC cultures. P-values < 0.05 were considered statistically significant.
4.4 Results

*PEGDA hydrogels have tunable mechanical properties*

The compressive moduli for the 3.4, 8, and 20 kDa 10% w/v hydrogels were 132.0 ± 5.9 kPa, 34.6 ± 3.2 kPa, and 7.3 ± 1.1 kPa, respectively. Increasing the MW of PEGDA hydrogels resulted in a decrease of the bulk stiffness of the gels.

*ECM peptides influence VEC adhesion and proliferation on different rigidities*

VECs seeded onto the laminin derived RKR coated constructs were found to adhere onto all hydrogel rigidities by 24 hrs. The cells proliferated on all the RKR samples, reaching 100% confluence within 3 days on the RKR-8 kDa PEGDA hydrogels. Within 7 days of culture, VEC monolayers were 100% confluent on the RKR-3.4 kDa and RKR-8 kDa hydrogels, whereas VECs on the RKR-20 kDa gels were at approximately 90% confluence (Figure 4-2).

![Figure 4-2. VECs adhere onto hydrogel scaffolds functionalized with extracellular matrix derived peptides. Representative images of valve endothelial cell (VEC) adhesion and growth when seeded on combinations of 3, 8, or 20 kDa molecular weight PEGDA hydrogels with immobilized CRKRLQVQLSIRT (RKR) (first panel) or CRGDS (RGDS) (second panel) and on tissue culture treated polystyrene (TCPS) on days 1 and 7. Scale bar = 100 µm.](image)

Although VECs adhered to the RGDS coated hydrogels within 24 hrs, the cells were observed to spread and proliferated slower compared to VECs on the TCPS control
and RKR based hydrogel samples. After 7 days of culture, a 100% confluent monolayer was present on the RGDS-3.4 kDa gels, whereas the VECs on the RGDS-8 kDa gels were only at ~85-90% confluence. The VECs on the RGDS-20 kDa hydrogels, however, did not proliferate and were only weakly adherent on the constructs. VEC samples seeded on the soft RGDS-20 kDa hydrogels were therefore excluded from the following experiments.

**Peptide-hydrogel platforms support VEC monolayer formation and ECM production**

VEC cultures on all substrate combinations maintained their cobblestone morphology (Figure 4-2), stained positively for CD31 and VWF (Figure 4-5A), and were negative for VIC marker αSMA. After 7 days of culture, VEC samples produced their own ECM, specifically laminin and fibronectin proteins (Figure 4-3).

![Image](image.png)

**Figure 4-3.** VECs on peptide coated hydrogel produce extracellular matrix proteins. Extracellular matrix proteins fibronectin (FN, red) and laminin (Lam, green) present throughout the cell layer of VECs cultured on RKR-8 kDa scaffolds after 7 days. Scale bar = 50 µm.

**VEC gene expression of hemostatic proteins affected by microenvironment**

Quantitative RT-PCR (qRT-PCR) analysis was used to assess the VEC gene expression of various anti-thrombotic and thrombotic proteins. With the gene expression
levels for each protein normalized relative to the TCPS VEC control, VECs cultured on RKR-8 kDa and RKR-20 kDa hydrogels had increased gene expression for all tested proteins relative to other groups (Figure 4-4). In assessing gene expression for antithrombotic proteins, there were no differences in gene expression for ADAMTS-13 between VECs seeded on the RKR-8 kDa and RKR-20 kDa hydrogels; however, these cultures expressed significantly more ADAMTS-13 than VECs on RKR-3.4 kDa hydrogels, RGDS-3.4 kDa and RGDS-8 kDa hydrogels, and TCPS (80x v. the other groups, p<0.05). Similarly, the tPA expression of VECs on RKR-8 kDa and RKR-20 kDa constructs were greater than for VECs on all other groups (~75x v. the other groups, p<0.05). Furthermore, the gene levels for TFPI of VECs on RKR-20 kDa gels were greater than from those on RGDS-3.4 kDa and RGDS-8 kDa gels, RKR-3.4 kDa gels, and TCPS (p<0.05).

Microenvironmental effects on VEC gene expression for thrombotic proteins were also assessed. The VECs seeded onto RKR-8 kDa and RKR-20 kDa hydrogels had significantly higher expression of VWF than the remaining groups (10x v. RKR-3.4 kDa; 1000x v. all RGDS groups, p<0.05). Immunofluorescent stains confirmed lower levels of VWF in VECs on RGDS-3.4 kDa gels than in VECs on RKR-3.4 kDa samples as well (Figure 4-5A). TF gene expression for the VEC group on RKR-20 kDa gels was greater than the RKR-3.4 kDa VEC group (15x, p<0.05); furthermore, neither of the VECs on RGDS-3.4 kDa and RGDS-8 kDa groups had any detectable levels of TF expression, and were excluded from statistical analysis for that gene. Gene expression for P-selectin was significantly higher in VECs on RKR-8 kDa gels than in VECs on the RGDS-3.4 kDa, RGDS-8 kDa samples, RKR-3.4 kDa gels, and TCPS (12x v. the other groups, p<0.05).
Figure 4-4. VEC hemostatic-related protein gene expression. VEC gene expression of anti-thrombotic related proteins [A disintegrin and metalloproteinase with a thrombospondin type I motif, member 13 (ADAMTS-13), tissue factor pathway inhibitor (TFPI), and tissue plasminogen activation (tPA)] and thrombotic related proteins [von Willebrand factor (VWF), tissue factor (TF), and P-selectin)] when cultured on combinations of different molecular weight formulations (3.4, 8, and 20 kDa) of hydrogels and adhesive ligands (RKR or RGDS) relative to VECs cultures on TCPS (indicated by dashed line. * p < 0.05.

Adhesive ligands influence VWF protein release, but do not affect ADAMTS-13 activity

VWF release and cleavage assays were performed on VECs cultured on each microenvironment combination and TCPS. Consistent with the qRT-PCR expression data, VECs cultured on RKR-8 kDa and RKR-20 kDa gels had the highest levels of VWF protein release among the tested microenvironments, though the RKR coated groups overall showed no statistically significant difference in VWF protein release compared to TCPS (Figure 4-5B). The VECs on RGDS-3.4 kDa and RGDS-8 kDa groups had significantly lower VWF release than did VECs on the RKR-8 kDa and RKR-20 kDa gels and TCPS control (p<0.05).

To evaluate the VWF cleavage activity of ADAMT-13, ELISAs were performed to quantify the ULVVF cleavage byproduct, VWF-140 fragments (Figure 4-5C). There
was no difference in the fraction of cleaved VWF-140 fragments between VECs cultured RKR-3.4, RKR-8 kDa, RGDS-3.4 kDa, and RGDS-8 kDa gels. However, the fraction of VWF-140 measured for the VECs on RKR-20 kDa samples was significantly lower than for the VECs on RKR-3.4 kDa and both RGDS coated groups. The fraction of VWF-140 fragments by VECs on TCPS was significantly less than in all hydrogel based groups.

Figure 4-5. VECs produce, release, and actively cleave von Willebrand factor. (A) VECs seeded on hydrogels (3.4 kDa) functionalized with RKR (left) or RGDS (right) stained for CD31 (red) and VWF (green). Nucleus was stained with DAPI (blue). Scale bar = 50 µm. (B) Quantification of rapidly released VWF and (C) the fraction of cleaved VWF-140 fragments from histamine-stimulated VECs cultured on different molecular weight hydrogels functionalized with RKR or RGDS, or TCPS. * p < 0.05.
**VECs on hydrogels maintain capacity to adhere platelets when stimulated**

The total number of adhered platelets on each VEC seeded hydrogel construct ranged between 0.5-1x10^6 platelets (Figure 4-6). However, the VEC seeded scaffolds had significantly less platelet adhesion on their respective blank peptide-hydrogel combination (Figure 4-6A) (p<0.05). The ratio of adhered platelets on cell-free RKR-coated hydrogels to VEC-seeded RKR coated hydrogels increases with increasing MW of the hydrogel used (1.6x for 3.4 kDa, 2.8x for 8 kDa, and 3.0x for 20 kDa). As VECs seeded on RGDS-8 kDa had a tendency to lift off the hydrogel after histamine stimulation, and could not maintain adhesion throughout the platelet adhesion assay, the RGDS-8 kDa group was excluded from this experiment.

When VECs cultured on the hydrogel-peptide platform combinations and TCPS were stimulated with histamine, a significantly greater amount of platelets adhered to the stimulated VECs compared to their respective non-stimulated states (Figure 4-6B) (p<0.05). Dyed platelets were observed to adhere onto anchored ULVWF strings of stimulated VECs under light microscopy (Figure 4-6C). However, there were significantly less adhered platelets on the stimulated VECs on RGDS-3.4 kDa hydrogels than on all other conditions (0.5x v. RKR and TCPS groups, p<0.05).
Figure 4-6. Histamine stimulated VECs on hydrogel based platforms are capable of promoting platelet adhesion. (A) Endothelialized peptide-immobilized hydrogel platforms significantly reduce platelet adhesion. Platelet adhesion on hydrogel scaffolds increases with increasing molecular weight PEGDA hydrogel. Platelet adhesion is significantly reduced on VEC seeded peptide-coated hydrogels (gray bars) when compared to acellular peptide-coated constructs (white bars). * p < 0.05. (B) Total adhered platelets onto stable and stimulated VECs cultured on TCPS and hydrogels with immobilized RKR or RGDS. Histamine stimulated VECs (black bars) of all culture condition promoted significantly more platelet adhesion when compared to the non-stimulated VECs cultured on the same condition (gray bars). * p < 0.05. Platelet adhesions were significantly lower for the stimulated VECs on RGDS gels than for all other stimulated conditions. # p < 0.05. (B) Dyed platelets adhered onto anchored ultra-large VWF strings (yellow arrows) on the surface of histamine stimulated VECs cultured on a RKR-3.4 kDa hydrogel. Scale bar = 50 µm.
4.5 Discussion

VECs play important roles in maintaining valve tissue health. Due to their unique endothelial sub-phenotype, VECs have slightly different regulation of homeostatic functions than do vascular endothelial cells [35]. Among the many essential functions, the balance in VEC production of anti-thrombotic and thrombotic proteins is lost with age associated remodeling in the native valve environment [128]. Under pathological conditions, VEC expression for anti-thrombotic proteins is altered, and the ability to respond to injury is decreased [6,128,131]. The sensitivity of VEC hemostatic responses may, therefore, be important in designing optimal endothelialization strategies specifically for tissue engineered heart valves that can withstand physiological mechanical loads while protecting underlying cells and matrix from injury. This study investigated the effects of microenvironmental factors, specifically substrate stiffness and adhesive cell ligands, on VEC stability and hemostatic function.

The adhesive cell ligands immobilized in each culture environment affected VEC adhesion and hemostatic regulation. VECs seeded on the modified RKR ligands had significantly higher levels of hemostatic protein expression than did VECs seeded on RGDS, both for thrombotic and anti-thrombotic proteins, exhibiting increased cellular activity and hemostatic balance. For example, VWF is a hyper-adhesive hemostatic protein secreted by stimulated or injured endothelial cells that mediates platelet adhesion to sites of endothelial injury when released in an activated ULVWF form [65,151]. Results from VWF ELISAs and immunohistological stains showed that RKR coated gels with seeded VECs produced and released high levels of antigenic VWF. Furthermore, ADAMTS-13 enzymatic cleavage activity of VWF remained intact, reflecting the
balanced regulation of VEC anti-thrombotic and thrombotic proteins. After introducing platelets to histamine stimulated VECs on RKR scaffolds, the platelets adhered to the VEC secreted/anchored ULVWF strings. In comparison, VECs grown on RGDS coated scaffolds expressed and produced hemostatic proteins, but the mRNA expression levels for thrombotic proteins VWF and TF were significantly lower than for VECs grown on RKR and TCPS groups. The fraction of cleaved VWF by RGDS seeded VECs was consistent with the RKR groups, however, suggesting that overall ADAMTS-13 cleavage activity and VWF/ADAMTS-13 ratio is not influenced by adhesive ligand composition. Furthermore, the decrease in VWF expression and release by the VECs on RGDS functionalized gels explains the significantly lower number of platelets adherent to the histamine stimulated VECs on RGDS-3.4 kDa constructs in comparison to RKR and TCPS groups. These findings are relevant because healthy endothelium maintains a balanced production of various hemostatic molecules important in mediating blood clotting and platelet adhesion [108,127]. When endothelial damage occurs, simulated by the addition of exogenous histamine, the balance of hemostatic protein expression shifts to a more pro-thrombotic phenotypes (i.e., increased VWF release and platelet adhesion) [65,108,127]. Both the balance of hemostasis during stable conditions and the pro-thrombotic shift during injury represent the expected physiologic VEC behavior as observed with the cells seeded on the RKR-immobilized hydrogels. However, VECs cultured on RGDS-functionalized hydrogels had unbalanced gene expression levels between anti-thrombotic and thrombotic during stable culture conditions (leaning toward anti-thrombotic), as well as significantly lower levels of released VWF and adhered
platelets when injured/histamine stimulated, suggesting an overall less healthy phenotype.

The pronounced effects of the adhesive ligands on VEC culture may be attributed to the specific adhesion receptors being engaged. RKR is a syndecan-1 and heparin binding ligand derived from the laminin-α1 globular G-domain [146,152]. In comparison, RGDS is a ubiquitous cell adhesive domain that is present in many ECM proteins, that promotes cell attachment via α\(_v\)β\(_3\) or α\(_2\)β\(_3\) integrin binding. Syndecan-1 is one of the major components of the endothelial glycocalyx [153], and maybe, therefore, highly abundant on VEC surfaces. Activation of synedcan-1 via ligand binding, such as RKR, primes the syndecan-1 ectodomain to couple with the β subunit of integrins, which promote a high affinity ligand binding state [154,155]. This conformational change may be sufficient for integrin activation [154], and may provide an explanation for the rapid adherence of VECs to RKR coated gels while maintaining stable phenotype in comparison to the VECs less confluent adhesion to RGDS coated gels. Though both syndecans and integrins have been shown to influence cell adhesion and function, our data indicates that VEC adhesion and microenvironmental responses could be more sensitive to syndecan-mediated binding and signaling, and warrants further investigation.

In addition to comparing VEC responses to specific adhesive ligands, we examined effects of substrate rigidity on VEC hemostatic functions. In vivo, the mechanical environment experienced by valves is sensed by and influences the resident cells that maintain the tissues. Moreover, previous work has shown that the mechanical support, modulated by the rigidity of ECM, strongly influences cellular behavior and plasticity [156]. Using hydrogels made from three different MWs provided a wide range
of substrate stiffness to test VEC cultures and to compare with the commonly used stiffer materials such as TCPS (~50-90 GPa). RKR provided an advantage in promoting VEC adhesion to all substrate stiffness, including the softest 20 kDa (7 kPa) hydrogels. VECs on all RKR hydrogel compositions were able to form a confluent monolayer within a few days of culture, similar to VECs seeded on TCPS. However, VECs on the softer 8 kDa (35 kPa) and 20 kDa (7 kPa) RKR hydrogels had significantly more gene expression (>50x) for all hemostatic-related proteins relative to VECs on TCPS, which suggests that softer substrates in combination with appropriate cell adhesive ligands promotes a robust, balanced VEC hemostatic capacity. In contrast, the RGDS ligand supported VEC attachment on hydrogels that were soft (compressive modulus 35 kPa), but overall had reduced hemostatic response levels than all RKR coated gel conditions, which suggests that VEC adhesion and activation of the $\alpha_i\beta_3$ and other integrins alone may be insufficient to support healthy VEC hemostatic regulation. Thus, it appears necessary to develop modulating microenvironments with appropriate adhesive ligands and substrate rigidity when attempting to develop a functional anti-thrombotic endothelium on cardiovascular implants.

In this study, we developed a simple, yet highly adaptable biomaterial platform to promote successfully stable VEC monolayer formation in vitro and characterize microenvironmental effects on VEC hemostatic regulation. PEGDA hydrogels are biocompatible, have easily tunable mechanical properties, and do not allow non-specific protein and cell adsorption. In addition to providing a modifiable substrate for cell culture experiments, PEGDA-based scaffolds are attractive biomaterials for tissue engineering and surface modification applications. Exploiting the available acrylate groups also
allows simple covalent immobilization of thiolated adhesive ligands throughout the entire hydrogel surface. These methods and surface modification techniques can, therefore, be integrated into more complex 3D tissue engineered heart valve or cardiovascular endothelialization designs. Furthermore, adjustment of this culture platform could allow for *in vitro* modeling of valve disease states, especially in regards to mimicking pathological changes in environmental rigidity (e.g., fibrotic valves are stiffer and myxomatous valves become more compressible) [6,157].
4.6 Conclusions

In summary, after exposing VECs to various substrate stiffness and adhesive ligand combinations, we show that VEC stability and hemostatic protein expression is modulated by environmental factors such as specific ECM-substrate rigidity combinations. To our knowledge, this is the first report of the use of the RKR peptide sequence in combination with a PEGylated platform to promote stable endothelium formation. The efficiency of syndecan-1 binding to RKR ligands promoted VEC adhesion and proliferation on all substrates rigidities. The balanced and reactive nature of VECs seeded on the RKR immobilized hydrogels suggests preservation of a physiological and functional phenotype in vitro. Combinations of integrin (RGDS) and syndecan (RKR) mediated cell adhesion ligands on this platform may activate synergistic aspects of both pathways. Consideration of additional adhesive ligand domains from regional ECM proteins from non-integrin pathways warrants further investigation. Utilization of basement membrane derived adhesion peptides along with softer substrate stiffnesses demonstrated the importance of environmental factors influencing phenotype of VECs, and may be important in valve implant endothelialization strategies.
Chapter 5: Utilization of 3D magnetically levitated VEC-VIC co-culture model to analyze valve cell phenotype and function\textsuperscript{3}

5.1 Introduction

The aortic valve, located between the left ventricle and the aorta, regulates unidirectional blood flow from the heart to the systemic circulation. The valve consists of three leaflets, each of which is comprised of two cell types: valvular interstitial cells (VIC) that populate the interior of the leaflet, and valvular endothelial cells (VEC) that cover the surface of the leaflet. VICs are a heterogeneous group of cells with fibroblast and smooth muscle cell phenotypes that maintains the extracellular matrix (ECM) of the leaflet [158–160]. VECs regulate the transfer of signals in the bloodstream to the valve interior, mediate inflammatory and hemostatic responses, and have a phenotype similar to vascular endothelial cells, but demonstrate major differences in alignment to flow and mechanobiology [34,35]. Together, both VICs and VECs work to maintain the leaflet structure and valve function.

The interactions between these two cell types are critical for normal valve function. The presence of VECs has been shown to keep VICs in a quiescent state, as defined by the low expression of $\alpha$-smooth muscle actin ($\alpha$SMA) [39]. The key role that their interaction plays in valve function is also demonstrated by the finding that

dysfunction of both cell types appear in calcific aortic valve disease (CAVD) [161]. Injury to the valvular endothelium leads to thrombosis, inflammation, and lipid accumulation [162–167]. These factors lead to the activation of VICs, or increased expression of αSMA, matrix remodeling, and their progression towards an osteoblastic phenotype, which ultimately result in calcification and stenosis [27,157,168–170].

Despite the well-established notion that both cell types and the interaction between the two are critical to valve maintenance, function, and disease, there are few co-culture models of VECs and VICs in literature. The lack of co-culture models of the aortic valve can partly be attributed to the inability to produce such models using traditional 2D cell culture techniques, with which the majority of research on valvular cells is conducted. 2D environments are poor representations of the native 3D environment in which valvular cells reside [171–173]. The inadequacy of 2D cultures for valvular research is highlighted by the varied results of studies on the effect of statins as treatments for calcific aortic valve disease. Statins in 2D in vitro cultures of VICs significantly reduced calcific nodule size and area, as well as αSMA expression [84,86–89], yet, clinical trials did not show any improvement in outcome for patients with calcific aortic stenosis who took statins [90–94]. Indeed, studies of the effect of statins on VICs in 3D collagen gels showed a smaller, conditional reduction in calcification in comparison to 2D cultures [88]. These results demonstrate the enormous gap in complexity and fidelity between simple 2D cell culture models and the human body, and necessitate the development of cost-effective, clinically relevant, and representative 3D co-culture models of the aortic valve.
To that end, this study used a magnetic levitation method with magnetic nanoparticles to assemble 3D co-cultures of VECs and VICs. In this method, cells are incubated with a nanoparticle assembly consisting of poly-L-lysine, magnetic iron oxide (MIO; Fe$_3$O$_4$, magnetite), and gold nanoparticles that form a gel via electrostatic interactions [174–177]. The uptake of this gel by cells renders them magnetic and allows for their manipulation, specifically by levitating the cells off the surface into the media, where the cells aggregate and interact to form larger 3D structures. This method has previously been used to create 3D cultures of glioblastomas, smooth muscle cells, adipose stem cells, and pulmonary cells [177–181]. Magnetically levitated human glioblastoma cells demonstrated greater proliferation and more *in vivo*-like protein expression in comparison to 2D cultures [177].

In addition, this method has previously been used to create co-cultures. Layered co-cultures of the lung were sequentially assembled using epithelial cells, smooth muscle cells, fibroblasts, and endothelial cells within 8 hours [181]. After 2 days of culture, ECM (collagen type I, fibronectin, laminin) was formed and organized, and the phenotypes of all four cell types were maintained. After 7 days, epithelial cell function and phenotype were still present [181]. In comparison, previously reported co-culture models of the valve leaflet consisted of collagen-based gels which were embedded and contracted with VICs, and then seeded with VECs on their surfaces in a process that took between 4-8 days to assemble [39,76]. As a result, magnetic levitation is a quick alternative method for assembling co-culture models.
5.2 Objective

Based on the success of the magnetically levitated co-culture of the bronchiole, this study used magnetic levitation to create 3D co-cultures of aortic valve cells. VICs and VECs were sequentially assembled into layered co-cultures. Immunohistochemistry (IHC) was used to verify the phenotype and function of both VECs and VICs, and assess ECM formation within the co-culture. Unlike previous studies using magnetic levitation, this study is the first to analyze the gene expression profiles of these cultures using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). In addition, the effects of the magnetic nanoparticles and exposure to the magnetic field on cell proliferation were investigated. As a result, this study builds upon work from my previous specific aims by evaluating VEC functional responses when directly co-cultured with VICs in a 3D scaffold-free environment.
5.3 Materials and Methods

Cell Isolation and Culture

Fresh porcine hearts were obtained from a local commercial abattoir (Fisher Ham and Meats, Spring, TX), from which the aortic valves were extracted. Aortic VECs and VICs were harvested as previously described [109,115]. Both cell types were cultured in an incubator (37°C, 5% CO$_2$, 95% humidity) with medium changes every other day.

VECs were isolated from the leaflets via digestion using collagenase II (60 U/mL) and dispase (2U/mL) [109]. VECs were seeded on flasks or glass slides coated with 2.5% gelatin in 1:1 H$_2$O:phosphate buffered saline (PBS, pH~7.4) [182], and cultured in specialized medium (EGM-2, Lonza Biosciences, Walkersville, MD) with 2% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). At the first passage, magnetic cell sorting was used to purify the VECs for CD31+ cells (Mouse monoclonal anti-CD31 antibody TLD-3A12, Millipore, Billerica, MA) [109]. VECs were used at their third passage.

VICs were isolated from the leaflets with a multistep digestion using collagenase II (~450 U/mL), hyaluronidase (~50 U/mL), and collagenase III (~350 U/mL) [115]. VICs were seeded on uncoated flasks or glass slides in DMEM/F12 medium supplemented with 10% bovine growth serum (BGS, Hyclone, Logan, UT), 1% HEPES buffer, and 1% P/S. VICs were used at their third passage.

Magnetic Levitation

Magnetic levitation using the Bio-Assembler Kit (Nano3D Biosciences, Houston, TX) was employed to create 3D cultures (Figure 5-1B) [177]. Confluent flasks of cells
were treated with a magnetic nanoparticle assembly (8 μL/cm² of cell culture surface area or 50 μL/mL medium, NanoShuttle, NS, Nano3D Biosciences) for overnight incubation to allow for cell binding to the nanoparticles. NS was fabricated as previously described, by mixing Au nanoparticles prepared by citrate reduction, poly-L-lysine, and iron oxide. [174,175,177] Treated cells were then detached with trypsin and resuspended in a low attachment 24-well plate with 400 μL of medium. A magnetic driver of 24 neodymium magnets (field strength = 50 G) designed for 24-well plates and a plastic lid insert were placed atop the well plate to levitate the cells to the air-liquid interface.

Cell Metabolism

The effects of both the NS and magnetic field on cell metabolism over 8 days were measured using an MTT assay. Briefly, MTT reagent (0.5 mg/mL in medium, thiazolyl blue tetrazolium bromide, Sigma-Aldrich, St. Louis, MO) was added to each well. After 3-4 hours of incubation, the medium was aspirated to yield the formazan blue crystals at the bottom of the well. Acidified isopropanol (0.1 N HCl in isopropanol) was added to dissolve the formazan blue crystals, and the absorbance of the resulting solution was read in triplicate on a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA) at 570 nm with background subtraction at 690 nm. All measurements were normalized to day 0 results.

To test the effects of adding NS (+NS) or exposure to a magnetic field (+mag) to VIC and VEC proliferation, 25,000 cells were seeded in 24-well plates. For +NS wells, NS was added to the wells (8 μL/cm²) on day 0 for incubation overnight. For +mag wells, neodymium magnets were placed 1 mm underneath each well (field strength = 300 G).
Medium was changed at day 1 and every other day after that for the duration of the study. Cell metabolic activities were measured on days 2, 4, 6, and 8 (n=9).

Co-Culture Assembly

Magnetic levitation was used to create co-cultures of VICs and VECs (Figure 5-2A) [181]. VICs and VECs were incubated overnight with NS, and then levitated into 3D cultures of 500,000 cells each. After 4 hours of levitation, a 0.1875” OD Teflon pen housing a neodymium magnetic rod was used to sequentially pick up a 3D culture of each cell type to assemble the co-culture: first VECs, then VICs. The still attached co-culture was then submerged in 150 μL VEC medium in a 96-well plate for 4 hours. The co-culture was then put back into a 24-well plate by first filling a well with 400 μL of VEC medium, then removing the rod magnet from the Teflon pen, and placing the magnetic driver underneath the well to attract the co-culture into the well. Once detached, the magnetic driver was moved to the top of the well and the co-culture was levitated again. These co-cultures are heretofore referred to as the aortic valve co-culture (AVCC).

Immunohistochemistry

IHC was used to verify the maintenance of phenotype and function, and the formation of ECM. AVCCs were fixed in 4% paraformaldehyde for at least 5 hours, then dehydrated, embedded in paraffin, and sectioned according to standard procedures. To preserve the structure of the AVCC, the Teflon pen used for co-culture assembly was used to hold the co-culture down and maintain its structure during processing. For 2D
immunocytochemistry (ICC), VICs and VECs were seeded on glass chamber slides at 50,000 cells/well. The next day, cells were fixed with 4% paraformaldehyde.

Sections to be stained were rehydrated and underwent antigen retrieval using a citrate buffer solution (Antigen Decloaker, Biocare Medical, Concord, CA) at 80°C for 30 min. For intracellular antigens, sections were then permeabilized using 0.2% Triton X-100 for 15 min. All sections were then washed and blocked using 1% donkey serum (GeneTex, Irvine, CA) in PBS for 1 hour at room temperature. Experimental sections were then incubated overnight at 4°C with the primary antibody of interest at the manufacturer’s recommended dilution in PBS with 1% bovine serum albumin. Negative controls were left incubating with donkey serum. The next day, all sections were washed and incubated with a fluorescent secondary antibody (AlexaFluor 488/633, Invitrogen, Carlsbad, CA) for 1 hour at room temperature and counterstained with DAPI (KPL, Gaithersburg, MD) for 15 min. All slides were then washed, mounted, and imaged. 2D ICC slides were stained similarly to 3D cultures after permeabilization with Triton X-100. Images were captured on a confocal microscope (LSM 510 META NLO, Zeiss).

The antigens stained for in this study included: αSMA (Abcam, Cambridge, MA) for VIC phenotype; CD31 (Abcam) for VEC phenotype; collagen type I (Col I, Abcam), laminin (Lam, Abcam), and fibronectin (FN, Abcam) for ECM; prolyl 4-hydroxylase (P4H, Bioss, Woburn, MA) for collagen synthesis; endothelial nitric oxide synthase (eNOS, Santa Cruz Biotechnology, Santa Cruz, CA) and von Willebrand factor (VWF, Abcam) for endothelial function; and VE-cadherin (VE-cad, Cell Signaling Technology, Danvers, MA) and N-cadherin (N-cad, Invitrogen) for cell-cell interactions (Table 5-1). For each antigen, the AVCC, 3D and 2D cultures were stained at the same time.
Table 5-1. Summary of antibodies used for immunohistochemistry and immunocytochemistry.

<table>
<thead>
<tr>
<th>Antibody (clone)</th>
<th>Manufacturer</th>
<th>Catalog #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31 (poly)</td>
<td>Abcam</td>
<td>ab28364</td>
<td>1:50</td>
</tr>
<tr>
<td>αSMA (1A4)</td>
<td>Abcam</td>
<td>ab7817</td>
<td>1:50</td>
</tr>
<tr>
<td><strong>Functional</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWF (poly)</td>
<td>Abcam</td>
<td>ab6994</td>
<td>1:200</td>
</tr>
<tr>
<td>P4H (poly)</td>
<td>Bioss</td>
<td>bs-5090R</td>
<td>1:100</td>
</tr>
<tr>
<td>VE-cadherin (D87F2)</td>
<td>Cell Signaling Technologies</td>
<td>2500</td>
<td></td>
</tr>
<tr>
<td>N-Cadherin (3B9)</td>
<td>Invitrogen</td>
<td>33-3900</td>
<td>1:100</td>
</tr>
<tr>
<td>eNOS (H-159)</td>
<td>Santa Cruz</td>
<td>sc-8311</td>
<td>1:50</td>
</tr>
<tr>
<td><strong>ECM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen I (poly)</td>
<td>Abcam</td>
<td>ab34710</td>
<td>1:50</td>
</tr>
<tr>
<td>Fibronectin (A17)</td>
<td>Abcam</td>
<td>ab26245</td>
<td>1:50</td>
</tr>
<tr>
<td>Laminin (poly)</td>
<td>Abcam</td>
<td>ab23753</td>
<td>1:50</td>
</tr>
</tbody>
</table>

Quantitative real-time polymerase chain reaction

Sample mRNA was extracted using Trizol Reagent (Invitrogen) mediated lysis and a series of ethanol washes and centrifugations. The mRNA was reverse-transcribed into cDNA using Primescript 1st strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan). The cDNA samples were stored at -20°C until use. Using the 2X QuantiTect SYBR Green PCR Master Mix (Clontech Laboratories, Mountain View, CA), qRT-PCR on the
cDNA was performed (Mastercycler ep realplex, Eppendorf, Hamburg, Germany) to assess differences in gene expression levels between the AVCC, 3D VEC and VIC cultures, and 2D VEC and VIC cultures. For quantification, the GAPDH gene (Integrated DNA Technologies, Coralville, IA) was used as the housekeeping gene, and sample group gene expression levels were normalized to the corresponding expression levels of the AVCC. The AVCC was used as the standard for the purposes of ease of analysis and comparison, given that some markers are specific to either VICs or VECs.

qRT-PCR was performed (n=3-5) to measure the positive gene expression of: αSMA for VIC phenotype; CD31 for VEC phenotype; COL1A1, FN, Lam-β1 for ECM; lysyl oxidase (LOX) for collagen fibrillogenesis; and eNOS and VWF for endothelial function (Integrated DNA Technologies) (Table 5-2).
Table 5-2. Summary of the DNA primer sequences used to assess sample gene expression using quantitative RT-PCR. DNA primer sequences were manufactured by Integrated DNA Technologies, Coralville, IA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’← DNA Sequence → 3’</th>
<th>Product Size</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTA2 (αSMA)</td>
<td>Forward: AATAGAACACGGCATCATC</td>
<td>77</td>
<td>FJ547477</td>
</tr>
<tr>
<td></td>
<td>Reverse: CACGAAGCTCATTGTGAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PECAM1 (CD31)</td>
<td>Forward: ACTGCTAACAACCAGAATT</td>
<td>80</td>
<td>X98505</td>
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<tr>
<td></td>
<td>Reverse: GCTTGACAGAGAATAATATAAC</td>
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<td></td>
</tr>
<tr>
<td>COL1A1</td>
<td>Forward: AGTTGTCTTATGGCTATGAGG</td>
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<td>XM_003483014</td>
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<tr>
<td></td>
<td>Reverse: GACCACGAGGACAGAAGCA</td>
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<td></td>
</tr>
<tr>
<td>FN1</td>
<td>Forward: CACTATTACTGTGTCTGGA</td>
<td>75</td>
<td>AY839862</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCACTCTCTGATTGTTCTTT</td>
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<td></td>
</tr>
<tr>
<td>LAMB1</td>
<td>Forward: CACCACGGATTCCAACAG</td>
<td>75</td>
<td>AF329358</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGCTCCAACATCAAAGTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOX</td>
<td>Forward: CAGTGGATTTGATATTACAG</td>
<td>99</td>
<td>NM_001206403</td>
</tr>
<tr>
<td></td>
<td>Reverse: ATTTTGGGAAATAGTCTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWF</td>
<td>Forward: CGAACCCAAGAAGAAGAAT</td>
<td>108</td>
<td>S78431</td>
</tr>
<tr>
<td></td>
<td>Reverse: ATCACTTCCCTCCACAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOS3 (eNOS)</td>
<td>Forward: AGAGAATGGAGAGAGTTT</td>
<td>104</td>
<td>AY266137</td>
</tr>
<tr>
<td></td>
<td>Reverse: ATTTGAGCGGATTGTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: CATTGACCTCCACTACAT</td>
<td>119</td>
<td>AF017079</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGATGGTGATGGAGTTT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Statistical Analysis**

One-way ANOVAs were performed on the data (JMP, SAS, Cary, NC), with significance defined as p<0.05. If a significant effect was observed, post-hoc Tukey’s testing was used to observe pairwise comparisons. Data is presented as mean ± standard error of the mean.
5.4 Results

*Magnetic Levitation*

Both VICs and VECs were successfully levitated into 3D cultures. Both VICs and VECs were able to bind with the NanoShuttle without grossly affecting morphology (Fig. 5-1A). Neither incubation with NanoShuttle nor exposure to the magnetic field significantly affected the metabolism of VICs and VECs (Figure 5-1C-D).

![Image showing VICs and VECs before and after incubation with NanoShuttle](image)

**Figure 5-1:** (A) VICs and VECs before and after incubation with NanoShuttle. Note the maintenance of morphology after incubation, and the localization of the magnetic nanoparticles (indicated by red arrow) with the cells. Scale bar = 100 μm. (B) Schematic of magnetic levitation. A confluent flask of cells is incubated with NanoShuttle overnight to allow for cell binding. The next day, the cells are detached from the surface and resuspended in 400 μL of medium in a 24-well plate. A magnetic driver and plastic lid insert is placed atop the well plate to levitate the cell constructs to the air-liquid interface.
interface. Metabolic activity of VICs (C) and VECs (D) with incubation with NanoShuttle and exposure to the magnet over 8 days (n=9). There was no significant difference found between these groups for both cell types. Error bars represent standard error of the mean. (E) 3D cultures (indicated by yellow arrow) levitating in a 24-well plate as seen from below the plate.

Co-Culture Assembly

AVCCs were successfully assembled and maintained for 3 days. At day 3, the AVCC maintained its competent structure (Figure 5-2B). After 12 hours of levitation, the planar size of these co-cultures decreased significantly, but did not change for the remaining 60 hours (Figure 5-2C). The AVCC was approximately 500 μm thick after culture. Hematoxylin and eosin staining showed two color stains, demonstrating the presence of two distinct cell types (Figure 5-3).

Figure 5-2: (A) Schematic of co-culture assembly using magnetic levitation. 3D cultures of VECs (yellow) and VICs (green) were separately levitated for 4 hours. A Teflon pen was used to then sequentially assemble the co-culture together, VECs then VICs. The co-culture is submerged in medium, still attached to the Teflon pen, for 4 hours. The co-culture is then detached off the Teflon and levitated for 3 days in VEC medium. (B) Schematic of the co-culture, within which the VEC
layer sits atop the VIC layer. (C) A resulting VIC-VEC culture after 3 days of levitation as seen from above the plate. Scale bar = 5 mm. (D) The planar size of the co-culture over 3 days (n=9). The co-culture starts close to the size of the Teflon pen used to assemble the co-culture. By 12 hours, the co-culture significantly shrinks in size and maintains that size over the next 60 hours. *: p<0.05 versus the rest.

Figure 5-3: Hematoxylin & eosin (H&E) stains of 3D VIC and VEC constructs and AVCC after 3 days of culture. There are two distinct colors in the stain (yellow arrows), relating to two different cell types producing two different types of matrices. There is also a notable difference in magnetic nanoparticle (brown) density between the two layers (green arrow). Scale bar = 50 µm.

Phenotypic Markers

Immunohistochemistry demonstrated the presence of VICs and VECs within the AVCC by positive staining for αSMA and CD31 (Figure 5-4). Both 3D and 2D VIC cultures stained positively for αSMA. Similarly, both 3D and 2D VEC cultures stained positively for CD31, and both 3D monotype cultures stained positively for αSMA. qRT-PCR confirmed IHC results, as AVCCs expressed both αSMA and CD31 mRNA (Figure 5-4B). There was no difference in αSMA gene expression between the AVCC and 3D monotype cultures, but these cultures expressed significantly less αSMA mRNA than 2D VIC cultures (652.078x v. AVCC, p<0.05). CD31 gene expression in the AVCC was
statistically similar to that in 3D monotype cultures and 2D VEC cultures. There was virtually no CD31 expression by 3D VIC cultures (0.007x v. AVCC), and CD31 expression by 3D VEC cultures was significantly higher than that in 3D VIC cultures (472x v. 3D VIC, p<0.05), whereas CD31 expression by 2D VEC cultures trended higher than 3D VIC cultures (427x v. 3D VIC, p=0.055).

Figure 5-4: (A) IHC stains for the phenotypic markers αSMA (red) and CD31 (green) in the AVCC, 3D VIC and VEC cultures, and 2D VIC and VEC cultures (insets). Nuclei are counterstained using DAPI (blue). The AVCC stained positively for both phenotypic markers, indicating the presence of
both VICs and VECs. CD31 was limited to the outer edges of the AVCC, while αSMA was distributed throughout. Scale bar = 50 μm. (B) qRT-PCR results for the phenotypic markers αSMA and CD31 (n=3-5). αSMA gene expression for the AVCC and 3D monotype cultures was significantly lower than 2D VIC cultures. CD31 gene expression is non-existent in 3D VIC cultures. *: p<0.05 v. other groups. ^: p<0.05 within bracket. Error bars represent standard error of the mean.

Functional Markers

All functional markers of interest stained positively in the AVCC (Figure 5-5A). VE-cad was limited to the outer edges of the AVCC and co-localized with CD31. Similarly, VWF was located towards the edge of the AVCC. N-cad, P4H, and eNOS all stained positively throughout the AVCC. qRT-PCR demonstrated that LOX gene expression was significantly less in the AVCC than in both 2D VIC (15x v. AVCC, p<0.005) and VEC (11.3x v. AVCC, p<0.05) cultures, but similar to both 3D VIC and VEC cultures (Figure 5-5B). VWF gene expression in 2D VEC cultures was the highest, and significantly higher than 3D monotype cultures and the AVCC (48.6x v. AVCC, p<0.05). Similarly, eNOS gene expression was highest in 3D VEC cultures, where it was significantly higher than 3D VIC cultures, 2D VEC cultures, and the AVCC (5.6x v. AVCC, p<0.005).
Figure 5-5: (A) IHC stains (green) for the functional markers N-cad, P4H, VWF, eNOS, and VE-cad (double-stained with CD31 in red) in the AVCC. Nuclei are counterstained using DAPI (blue). The AVCC stained positively for all these functional markers. VE-cad and VWF were localized to the outer edges of the AVCC, while N-cad, P4H, and eNOS were distributed evenly throughout. Scale bar = 100 μm. (B) qRT-PCR results for the functional markers LOX, VWF, and eNOS (n=3-5). Gene expression for LOX and VWF was significantly lower in the AVCC in comparison to 2D VIC and VEC cultures, respectively, while for eNOS, expression in the AVCC was significantly lower than expression in 3D VEC cultures. *: p<0.05 v. other groups. ^: p<0.05 within bracket. Error bars represent standard error of the mean.

**Extracellular Matrix**

With regards to the ECM components of interest, Col I, FN, and Lam stained positively, and evenly distributed within IHC stains of the AVCC (Figure 5-6A). In 2D cultures, Col I and FN stained more intensely and prominently in 2D VIC cultures than
2D VEC cultures, while Lam stain intensity was higher in 2D VEC cultures. Gene expression for COL1A1 was the highest in 2D VIC cultures, where it was also significantly higher than the other groups (98.1x v. AVCC, p<0.0001) (Fig. 5-6B). There was a significant difference in FN mRNA expression between 2D cultures and all 3D cultures (95-113x v. AVCC, p<0.01). 2D VEC cultures expressed significantly higher amounts of Lam-β1 mRNA than 3D monotype cultures (2.8-5.7x v. 3D cultures, p<0.05), but statistically similar amounts to 2D VIC cultures and the AVCC (2.2x v. AVCC, p=0.095).

![Figure 5-6: (A) IHC stains (green) for the ECM components Col I, FN, and Lam in the AVCC and 2D VIC and VEC cultures. Nuclei are counterstained using DAPI (blue). These ECM components are present and distributed throughout. In 2D cultures, Col I and FN are more prominent with VICs, while Lam is more prominent with VECs. Scale bar = 50 μm. (B) qRT-PCR results for the ECM markers COL1A1, FN, and Lam-β1. AVCC expressions of COL1A1 and FN were significantly less than 2D VIC cultures. Lam-β1 for all groups was found to be statistically similar with expression in the AVCC, but was significantly larger in 2D VEC cultures, than 3D monotype cultures. *: p<0.05 v. other groups. ^: p<0.05 within bracket. #: p<0.05 v. 3D monotype cultures.]
5.4 Discussion

This study used magnetic levitation to construct a layered co-culture of the aortic valve, the AVCC. This is the first study to both magnetically levitate aortic valve cells, and then use this technique to assemble such co-cultures. AVCCs were assembled and cultured for 3 days in VEC medium. The size of the AVCC decreased by 12 hours of levitation, likely due to cell contraction, then was held constant for the remaining 60 hours. Immunohistochemistry was used to stain for markers of cellular phenotype, function, and ECM. qRT-PCR was used to quantitatively measure the gene expression for these same markers in these 3D cultures, the first time it has been used on magnetically levitated cultures. Positive stains and gene expression for αSMA and CD31 verified VIC and VEC phenotype. The presence of P4H, LOX, VWF, and eNOS indicated the preservation of function by both cell types. ECM formation in the form of Col I, FN, and Lam was demonstrated. The positive stain for N-cad affirmed the cell-cell interactions within the AVCC, while the positive stain for VE-cad showed that VECs in the AVCCs form tight junctions. This study also affirmed the use of magnetic levitation as a simple method to create 3D co-cultures.

The AVCC and 3D VIC cultures were found to have significantly reduced gene expression of αSMA, as well as COL1A1, FN, and P4H, than 2D VIC cultures, suggesting that magnetically levitated VICs in the AVCC are in a quiescent state [103,158,183]. This quiescence could be a result of the difference in substrate stiffness between 2D cultures on glass, and 3D self-aggregated cultures. Higher stiffnesses have previously been shown to increase αSMA expression and the formation of stress fibers [184]. In addition, these cells reverted to a quiescent state from an activated state when
levitated from 2D to 3D, confirming previous results that VIC activation is both plastic and reversible [103,185]. The presence of VECs could also have reduced VIC activation, as VICs in a collagen-gel based co-culture with VECs demonstrated reduced αSMA expression.[39] This role of VECs is supported by the fact that the gene expressions of COL1A1 and FN were reduced by more than half in AVCCs compared to 3D VIC cultures, even though VICs made up half of the AVCC. As an activated VIC state is commonly associated with matrix remodeling and the onset of CAVD [27,130,168], the quiescent state of VICs suggests that the AVCC can be used to study the activation of VICs under various environmental conditions. Further research is required to understand VIC quiescence and activation within the AVCC.

Interestingly, while VIC phenotype was found to be quiescent and ECM gene expression was significantly less in the AVCC compared to 2D cultures, the AVCC stained positively for ECM components, such as Col I, FN, and Lam. The formation and organization of ECM in the AVCC within 3 days would presumably require VIC activation for matrix remodeling [157]. IHC and qRT-PCR were conducted after 3 days of culture to find a quiescent state, but activation and matrix remodeling could have occurred earlier, yielding the ECM that was observed. Col I was found to have formed in the AVCC as early as 2 days of levitation (data not shown), while magnetically levitated pulmonary fibroblasts and tracheal smooth muscle cells have been demonstrated to produce and extrude Lam into the extracellular space within 6 hours [186]. As with other studies in heart valve biology, αSMA was used alone as a marker of VIC activation [184,187] and to distinguish VICs from VECs within the AVCC, but other markers for VIC activation, including non-muscle myosin heavy chain (NMM) and embryonic
smooth-muscle myosin heavy chain (SMemb) [183,188], should be explored in future studies. Future studies using this model will also require time-based studies to further characterize VIC activation and ECM formation within the AVCC.

The positive stains for CD31, VWF, eNOS, Lam, and VE-cad verify that VEC phenotype and function is maintained within the AVCC and competent endothelium is being formed. Unexpectedly, on the edges of AVCCs and throughout 3D VEC cultures, CD31 was co-localized with αSMA. In addition, gene expression for eNOS, which catalyzes nitric oxide in endothelial cells and has previously been used as a functional marker for VECs for its important role in vasoactivity and resistance to oxidative stress [18,37,131], in the AVCC was less than half that of 3D VEC cultures, while consisting of half VECs. Gene expression for CD31, VWF, and Lam in the AVCC also trended similarly to eNOS. Together, these results suggest the possibility of endothelial to mesenchymal transdifferentiation (EnMT) occurring within the AVCC. VECs commonly differentiate into myofibroblast phenotypes in 2D in vitro culture [109], but the fact that the AVCC was cultured in VEC media and without a stiff substrate suggest that this result is unique. VECs have been shown to undergo EnMT in vitro in a manner stimulated by TGF-β1 and inhibited by vascular endothelial growth factor (VEGF) [42,189]. EnMT has been implicated in valvular disease and mechanically regulated [190], but the finding that the AVCC was positive for VE-cad, and its VICs were in a quiescent state, would suggest that EnMT is not occurring as a response to injury or endothelial dysfunction within the AVCC. Rather, VECs could possibly be undergoing EnMT to replenish or supplement VICs, a role previously suggested and investigated as a possible source for progenitor cells in engineered heart valves [43,137]. Further research
using the AVCC will require a deeper look into EnMT within the model, and could provide further understanding of EnMT and its utility.

An interesting result of this study was that 3D VEC cultures showed significantly reduced expression of VWF, but increased expression of eNOS, relative to 2D VEC cultures. As VWF and eNOS are both VEC-mediated proteins essential in facilitating endothelial pro- [191–193] and anti-thrombotic responses [194,195], respectively, these results suggest the possibility that 3D VEC cultures are in a more stable, anti-thrombotic state as opposed to 2D cultures. The expression of these proteins is highly influenced by the surrounding environment. Previous studies have shown that when subjected to physiological shear flow conditions, vascular endothelial cells produce less pro-thrombogenic components such as VWF [196,197]. Thus, the changes in VWF and eNOS gene expression between 2D and 3D are possibly a result of the lower stiffness and higher cell density of the 3D environment compared to 2D. Therefore, culturing valvular cells in 3D culture environments may not only maintain cell phenotype and function, but possibly enhance overall homeostatic cell function relative to 2D cultures. Further studies of hemostatic factors within the AVCC are necessary to understand its anti-thrombotic potential.

A major concern for this study was the choice of medium for co-culture, which was the medium used to culture VECs. In the study using magnetic levitation to assemble co-cultures of the bronchiole, epithelial medium was used to maintain the co-cultures [186]. The rationale behind that choice was that epithelial cells were the definitive cell type of the bronchiole, and the most sensitive to serum due to fears of epithelial-mesenchymal transdifferentiation. As such, in that study, those co-cultures were cultured
in epithelial medium with and without 1% FBS, and both cases showed the maintenance of epithelial phenotype and cell survival after 7 days [186]. Similarly, in this study VEC medium, which contained 2% FBS, was chosen over VIC medium, which contained 10% BGS, in order to preserve VEC phenotype against the possibility of EnMT. The results showed VEC phenotype was eventually maintained, as confirmed by positive stains and gene expression for CD31, VWF, and eNOS, although co-localization with αSMA was present. Further research using this model should look at further optimization of the medium used for co-culture.
5.5 Conclusions

Magnetic levitation was successfully used to assemble co-cultures using VICs and VECs. Immunostaining and gene expression analysis were used to verify that the phenotype and functions of both cell types were maintained, and relevant ECM was formed. The result is a model that maintains phenotypes and induces ECM formation with a faster assembly time compared to other co-cultures. The AVCC could in the future be used for a wide variety of experiments, such as those involving mechanobiology, or the progression of CAVD. This study also affirms the versatility and utility of magnetic levitation to create representative 3D cell culture models.
Chapter 6: Design of a novel adhesive ligand localized hydrogel scaffold for an endothelialized aortic valve co-culture model

6.1 Introduction

Cardiac valve leaflets are heterogeneous tissues containing regionally varying extracellular matrix (ECM) that function to direct unidirectional blood flow through the heart. Diseased aortic valves, however, show disorganized ECM, resulting in fibrotic or calcific hardening [198]. Mitral valves can also become myxomatous, a condition in which the valve ECM is remodeled to become less stiff, resulting in regurgitation [199]. In the United States the overall prevalence of valve disease is approximately 2.5% of the population, while calcified aortic valves constitute 82% of the all aortic stenosis cases [200]. However, there are no non-surgical treatments available for valve diseases because researchers have only recently begun learning how valve cells interact with the ECM to maintain tissue homeostasis and valve function and how those interactions contribute to the onset and progression of these diseases.

Although native valves are populated by two distinct cell types, there are very few studies of how the two essential cell types interact. Valvular interstitial cells (VICs) are fibroblast-like cells that occupy the interior of the valve and are responsible for ECM maintenance [201]. The valvular endothelial cells (VECs) line that the exterior surfaces

4 The work in this chapter was prepared as a manuscript: L.R. Balaoing*, D.S. Puperi*, R.W. O’Connell, J.L. West, and K.J. Grande-Allen. Novel design of 3-dimensional spatially organized PEG-based hydrogels as an aortic valve co-culture model. *Denotes equal contribution to this work.
of valves regulate tissue inflammatory and thrombotic responses, as well as underlying VICs and ECM [6,128]. Both cell types have been implicated in valve disease and understanding how the cells contribute to the disease state may help the development of a non-invasive treatment option. During valve disease and remodeling, VICs transition from a quiescent state to an activated or myofibroblast phenotype demonstrating distinct αSMA stress fibers throughout the cells [202]. In the activated phenotype, VICs can cause disorganization of the ECM via overexpression of matrix metalloproteinases in diseased valves [103,203]. VEC dysfunction is also believed to cause the onset of valve disease, by locally influencing VIC behavior [16], losing the ability to regulate hemostatic or inflammatory responses, or invading the interior of the valve or participating in neoangiogenesis [5,30,204]. In vitro co-culture models can be used in order to study the roles of and the communication between the two cell types, but the complex interactions between cells and their substrate needs to be controlled so it does not obfuscate the results of such studies. Therefore a tunable synthetic co-culture model is needed to give the researcher control over parameters such as ligand presentation and organization and mechanical properties of the scaffold to provide cell-type specific cues and thereby more accurately mimic natural ECM presentation to the cells.
6.2 Objective

To create a physiologically relevant valve co-culture model in a synthetic material platform, we have developed the hydrogel-based endothelialized aortic valve model (HEAVM). Co-culture models have primarily been used in order to investigate VIC-VEC interactions, but this model is the first to have zonally organized ECM ligand presentation to facilitate cell specific binding to the scaffold. Previous reports have demonstrated zonally organized VIC and VEC placement in collagen gels [39,76] and through magnetic levitation [205]. Valve cell interactions have also been studied in non-contact co-cultures, with cells separated by either a membrane [206] or Transwell insert [95]. These studies have all demonstrated that VIC-VEC communication is essential for maintaining valve homeostasis, and specifically that the presence of VECs reduces the activation of VICs. The HEAVM was developed to expand on previous studies and investigate some additional questions about valve cell interactions, including how VECs respond to the presence of VICs in the scaffold and which external cues from the ECM and surroundings are necessary to maintain valve homeostasis. In answering these questions, we will lay a foundation for future research using this model to investigate onset, propagation, and possible treatments of valve disease. Furthermore, the method described here can also be adapted to study healthy and diseased states of many other endothelialized or epithelialized tissues.
6.3 Materials and Methods

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

Valve cell isolation and culture

Aortic valve leaflets were dissected from fresh young adult (3-6 month old) porcine hearts acquired from a commercial abattoir (Fisher Ham and Meats, Spring TX). Aortic valve endothelial cells (VECs) and valve interstitial cells (VICs) were harvested following previously described methods [34,109,128,207]. Dissected aortic valve leaflets were enzymatically digested in a collagenase II/dispase solution for 1 hour at 37°C. Next, VECs on the surface of the leaflets were isolated via gentle scraping using sterile swabs. The VECs were cultured on tissue culture plastic (TCPS) coated with 2.5% gelatin in EGM-2 medium and growth factor bullet kit (Lonza, Walkerville, MD) with 2% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin in an incubator (37°C, 5% CO₂, 95% humidity). At first cell passage, CD31 positive VECs were purified using anti-CD31 (Millipore, Billerica, MA) antibody-conjugated CELLection magnetic sorting beads (Invitrogen, Carlsbad, CA). If VECs were after freeze, a second bead sort purification was performed. VECs were used between passages 2 to 5.

VICs were harvested by mincing aortic valve leaflets which had been denuded of their endothelium. The minced leaflets were digested in a collagenase III buffer for 4 hours. The cells were isolated by straining the digested tissue solution through a 70 micron cell strainer and then seeded onto TCPS flasks. VICs were cultured in a standard humidified incubator at 37°C with 5% CO₂ in 50:50 DMEM:F12 (Corning, Tewksbury, MA) media with 1% FBS (Lonza) and 1% penicillin/streptomycin/amphotericin (Lonza)
and buffered with 1% 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Thermo Fisher Scientific, Waltham, MA). VICs were used between passages 1 to 3.

Porcine aortic endothelial cells (AECs) were harvested from fresh porcine aortic tissues (Fisher Ham and Meats) following the same methods as VEC isolation, and cultured using the same culture conditions and medium as VECs. AECs were also purified at passage 1 for CD31 positive expression using magnetic bead sorting. AECs were used between passages 2 to 5.

Flow cytometry for CD31 expression using mouse anti-porcine CD31 antibody (AbD serotec, Oxford, United Kingdom) was performed to verify endothelial cell phenotype. Culture media for all cell cultures were changed every 2-3 days. All cell cultures in subsequent hydrogel scaffold based experiments used a 50:50 ratio of VIC:VEC culture medium based on the above formulations.

**Peptides**

MMP-2 and 9 sensitive peptide sequence GGGPQG↓IWGQGK (hereafter identified as PQ) was synthesized using solid phase synthesis (APEX 396, Aapptec, Louisville, KY). Fibronectin-derived integrin binding peptide sequence RGDS and laminin-derived syndecan binding peptide CRKRLQVQLSIRT (hereafter identified as RKR) were purchased from American Peptide Company (Vista, CA). RKR was dissolved in DMSO at a concentration of 15 mM and stored at -80°C in aliquots.
**PEG-peptide conjugation**

PEG-RGDS and PEG-PQ-PEG was synthesized as previously described [208]. 3.4 kDa monoacrylated PEG succinimidyl valerate (Laysan Bio, Arab, AL) was reacted at a molar ratio of 1.2:1 RGDS: PEG-SVA or 1:2.1 PQ: PEG-SVA overnight in HEPBS buffer (Santa Cruz Biotechnology, Dallas, TX) at pH 8.0. The PEGylated peptide solution was dialyzed against pure water in a 3500 molecular weight cutoff dialysis membrane (SpectrumLabs, Rancho Dominguez, CA) for 3 days to remove unreacted precursors. After dialysis, the solutions were sterile filtered and kept sterile throughout freezing, lyophilization, and experimental use. Gel permeation chromatography was used to confirm PEG-peptide conjugation and proton NMR was used to ensure the acrylate groups remained intact.

**Co-culture construction**

The hydrogel-based endothelialized aortic valve model (HEAVM) was constructed over 2 days as depicted in Figure 1A. First a 500 µm thick, 12 µm diameter base gel of 6 kDa PEGDA was made in a silicone mold. PEGDA was synthesized from 6kDa PEG (Sigma) using acryloyl chloride (Sigma) as previously described [140]. The base gel was used in order to be able to manipulate the cell-encapsulated HEAVM gel without direct contact thereby preventing damage. The base gel was blotted dry using sterilized Kimwipes and a 250 µm thick square of PDMS with a 5 mm diameter cut out was placed on the base gel to be used as a mold for the HEAVM gel. VICs were released from a TCPS flask using 0.25% Trypsin in EDTA (Corning), diluted in media, and counted. Six gels were made simultaneously to minimize the time that VICs were in the
photoinitiator solution, so 900,000 VICs were centrifuged at 750x g and resuspended in 60 µL of 4% w/v PEG-PQ-PEG and 2 mM PEG-RGDS photoinitiator solution (15x10^6 cells/ml). An established white light photoinitiator system[209] consisting of 1.5% triethanolamine (TEOA), 10 µM Eosin Y, and 0.35% (v/v) 1-vinyl-2-pyrillidinone (NVP) in HEPES buffered saline was used to crosslink hydrogels. 9 µL of the cell suspension was pipetted into each PDMS mold and photo-crosslinked under 160 kLux of white light (UltraTow LED Floodlight, Northern Tool and Equipment, Burnsville, MN) for 35 seconds. The PDMS mold was removed, and VIC encapsulated gels were put in an incubator overnight in VIC media to wash out unreacted photo-initiator and allow VICs to grow in the scaffold.

After overnight incubation, the VIC encapsulated gels were washed in sterile PBS and blotted dry with sterilized Kimwipes. The 15 mM RKR in DMSO was diluted to 3 mM using Eosin Y photoinitiator solution. 10 µL of the 3mM RKR solution was pipetted onto the top of the hydrogel. The gels were placed under white light for 1 minute to initiate a free-radical thiol-ene reaction between the cysteine of the RKR peptide and unreacted vinyl groups on the surface of the VIC encapsulated hydrogel. The gels were then washed extensively to remove unbound RKR peptide. Sterile PBS was pipetted onto the surface of the hydrogel to physically dislodge unbound RKR and then the gels were placed in VIC media in an incubator for 10 minutes to allow for diffusion of unbound RKR and photoinitiator solution. This process was repeated 3x to ensure only covalently bound RKR was left on the surface while keeping the encapsulated VICs viable. VECs were released from TCPS flasks using 0.25% Trypsin in EDTA, counted, and resuspended in VEC media at a concentration of 200,000 cells/ml.
cloning columns were used to restrict VEC seeding to the HEAVM surface and 150 µL of VEC cell suspension was added in the cloning columns to seed 30,000 cells per gel. The outside of the cloning columns was flooded with 50:50 VIC:VEC media and the cloning columns were removed after 1 hour in culture. The HEAVM gels were cultured for 7 days.

In experiments where VIC-only and VEC-only controls were compared to the HEAVM co-culture, the same procedure was followed. VIC-only scaffolds were constructed by encapsulating VICs in 4% PEG-PQ-PEG hydrogels with 2 mM PEG-RGDS. The gels were incubated in VIC media overnight and the next day RKR was crosslinked to the surface, but no VECs were seeded onto the gels. VEC-only scaffolds were constructed by forming acellular 4% PEG-PQ-PEG hydrogels with 2 mM PEG-RGDS, incubating them in VIC media overnight, and crosslinking RKR peptide to the surface followed by seeding VECs on the surface as detailed above.

Fluorescently Labeled Peptides

RKR and PEG-RGDS were fluorescently tagged with AlexaFluor 532 or AlexaFluor 488 succinimidyl ester, respectively, following the manufacturer’s protocol (Invitrogen, Carlsbad, CA). PEG-RGDS was dissolved at 20 mg/mL in 0.1 M sodium carbonate buffer and RKR was first dissolved in DMSO at 30 mM and then adjusted to 10 mg/ml in 0.1 M sodium carbonate buffer. The AlexaFluor succinimidyl ester was dissolved at 10 mg/mL, added dropwise to the peptide solutions, and vortexed lightly for 1 hour. The solution was dialyzed against pure water and lyophilized before use. PEG-RGDS-AF488 was incorporated into a 4% PEG-PQ-PEG hydrogel and RKR-AF532 was covalently bound on top of the hydrogel in the same manner as described above for
unlabeled peptides. 500 µm thick sections were sliced with a razor blade and laid on their side to image the cross-section of the hydrogel using a confocal microscope (LSM 510 Live 5, Zeiss, Oberkochen, Germany).

**Mechanical characterization of hydrogels**

HEAVM scaffold stiffness was characterized through compression testing. Hydrogels consisting of 4% PEG-PQ-PEG and 2 mM PEG-RGDS were crosslinked under white light in cylindrical PDMS molds 6 mm in diameter by 0.8 mm thick for 35 seconds. The hydrogels were soaked in PBS overnight and subject to unconfined compression at a rate of 0.02 mm/sec using a Bose Electroforce ELF 3200 mechanical testing machine equipped with a 1000 g load cell (Bose Electroforce, Eden Prairie, MN). After this non-destructive testing, RKR was added to the surface of each gel as described in the previous section and crosslinked under white light for 1 minute. Unreacted RKR was washed off and the gels were left to soak in PBS overnight. Hydrogels were mechanically tested again using the same parameters to determine how the bulk stiffness of the hydrogel changed with additional crosslinking. For all tests, the compressive modulus was calculated as the least-squares linear fit slope of the stress/strain curve between 5% and 15% strain. Volumetric swelling was calculated by using cross-sectional area and thickness measurements.

**2D cell adhesion and phenotype verification**

To verify the ability of valve cells to adhere to adhesive peptides RKR and RGDS, VECs and VICs were seeded onto the surface of PEG-PQ-PEG scaffolds that had either: 3 mM of RKR conjugated only on the hydrogel surface, 2 mM PEG-RGDS conjugated throughout the bulk hydrogel, or combined RKR (on the surface) - RGDS.
(bulk) conjugated hydrogels. AECs were used as a reference endothelial population, and were also seeded onto all hydrogel scaffold combinations. After 7 days of culture, the cells were imaged using brightfield microscopy to visualize cell morphology, and then immunostained for CD31 and αSMA.

Cytotoxicity assessment

The ability of the cells to survive the encapsulation and seeding process was assessed using the Live/Dead viability kit (Invitrogen, Carlsbad, CA) following manufacturer’s instructions. After 3 days in culture, HEAVM constructs were incubated in 2 µM of calcein AM and 4 µM of ethidium homodimer-1 in culture media for 30 minutes at room temperature. The gels were washed once in PBS and immediately imaged with confocal microscopy (A1-Rsi, Nikon, Tokyo, Japan). Cells were counted using the ImageJ 3D Objects Counter plug-in.

Immunohistochemistry

Cells cultured on all hydrogel scaffold combinations were fixed in 4% paraformaldehyde for 45 minutes and permeabilized with 0.25% Triton-X for 15 minutes at RT. Next, samples were blocked with PBS with 3.5% bovine serum albumin (BSA) at 4°C overnight. The samples were incubated with primary antibodies of interest at manufacturer recommended dilutions in PBS and 0.35% BSA overnight at 4°C. Negative controls were left incubating in PBS with 0.35% BSA. The next day, samples were incubated with fluorescent secondary antibodies (AlexaFluor 488/555/633; Pacific orange, Invitrogen) overnight at 4°C. After washing, samples were counterstained with
DAPI and phalloidin for 1 hour prior to imaging with confocal microscopy (Nikon A1-Rsi or Zeiss LSM 510).

Antigens investigated in this study included the endothelial marker CD31 (Abcam, Cambridge, MA., ab28364, 1:50); VIC activation marker αSMA (Abcam, ab7817, 1:50); eNOS (BD biosciences, BD610296, 1:100); and extracellular proteins laminin (Lam, Abcam, ab14055), collagen type IV (Col IV, Abcam ab6586, 1:500), perlecan (Pln, Abcam, ab26265, 1:1000), collagen type I (Col I, Abcam, ab34710, 1:50) and fibronectin (FN, abcam, ab6328, 1:100).

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed to visualize VEC monolayer morphology on the surface of HEAVM scaffolds that had been in culture for 28 days and to visualize adhered platelets. The gels were fixed with 4% paraformaldehyde for 45 minutes at RT, dehydrated through a serial ethanol gradient (35-100%) and dried in a critical point dryer (EMS 850, Electron Microscopy Sciences). Before drying, the VEC monolayer and associated basement membrane were carefully peeled back from one HEAVM sample to visualize the underlying VICs. The dried samples were sputter coated with 10 nm of gold (Desk V, Denton Vacuum, Moorestown, NJ) and imaged using an FEI Quanta 400 ESEM microscope (FEI, Hillsboro, OR) in high vacuum mode at 15 kEV.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was used to quantify the relative gene expression of αSMA, CD31 and eNOS between all 3D scaffold culture groups (n=4–6).
After 7 days in culture, cell seeded hydrogels were homogenized and mRNA was extracted and purified using Quick-RNA MiniPrep Kit (Zymo Research, Irving, CA). The purified mRNA was reverse-transcribed into cDNA (1st stand cDNA synthesis kit, Takara Bio, Otsu, Japan). All cDNA samples were stored at -20°C until use. qRT-PCR was performed using QuantiTect SYBR Green PCR Master Mix (Clontech Laboratories, Mountain View, CA) in a Mastercycler ep realplex qRT-PCR system (Eppendorf, Hamburg, Germany). For HEAVM scaffolds, the housekeeping gene, GAPDH, was adjusted to account for average ratio of VIC:VEC cell numbers. Relative expression ratios were calculated using the REST 2009 program [210] using the pairwise fixed reallocation randomization test for statistical significance, which was considered p-value less than 0.05. Relative gene expression of αSMA was normalized to VIC-only seeded scaffolds; and CD31 and eNOS gene expression were normalized to VECs cultured on TCPS. All primers used (Integrated DNA Technologies, Coralville, Iowa) are listed in table 6-1.

Table 6-1. Summary of hemostatic protein DNA primer sequences used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’← DNA Sequence → 3’</th>
<th>Product Size</th>
<th>Accession Number</th>
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<tbody>
<tr>
<td>CD31</td>
<td>Forward: TGCTGTCTATAAGGATG&lt;br&gt;Reverse: CTTGGTGTTCAAGATCAC</td>
<td>131</td>
<td>X98505</td>
</tr>
<tr>
<td>αSMA</td>
<td>Forward: GGAATCCTGACCTTGAAG&lt;br&gt;Reverse: CACGAAGCTCATGTAAG</td>
<td>100</td>
<td>FJ547477</td>
</tr>
<tr>
<td>eNOS</td>
<td>Forward: AGAGAATGGGAGAGAGTTT&lt;br&gt;Reverse: TATTGAAGCGGATTTTGT</td>
<td>104</td>
<td>AY266137</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: CATTGACCTCCACTACAT&lt;br&gt;Reverse: AGATGGTGATGGGATTTTTC</td>
<td>119</td>
<td>AF017079</td>
</tr>
</tbody>
</table>
Nitric Oxide Production

A total nitric oxide (NO) detection kit (Enzo Life Sciences, Farmingdale, NY) was used to measure the sum of nitrites ($\text{NO}_2^-$) and nitrates ($\text{NO}_3^-$) in collected media samples to determine the levels of nitric oxide produced by HEAVM co-cultures. Because nitric oxide is quickly degraded into nitrates and nitrites in culture, the kit converts the collected NO breakdown products into nitrites that can be colorimetrically detected when reacted with Greiss reagents.

Fresh culture media samples were collected from HEAVM co-culture, VEC-only, and VIC-only scaffolds after 3 days in since last medium change, on day 5 of culture (n=5). Fresh 50:50 VIC-VEC culture medium was used as a baseline control. Following the kit protocol, media samples were diluted 1:2 with NO kit reaction buffer and ultrafiltered through a 10,000 molecular weight cut off filter (Millipore). Next, 50 µL of each sample was incubated in a microtiter plate with NADH and nitrate reductase enzyme for 30 min. at 37°C. The mixtures were then reacted with Greiss reagents for 10 min. at room temp. The peak absorbance of each of the solutions were measured between 400-600nm with a spectrophotometer (Cary 60 UV-Vis Spectrophotometer, Agilent Technologies, Santa Clara, CA) and the total nitrite concentration was calculated using a standard curve produced using serial dilutions of a nitrate standard. The nitrite concentration from the fresh 50:50 culture medium controls were used as a baseline and subtracted from the nitrite concentrations measured in the cell seeded hydrogel scaffolds.
Platelet adhesion assay

A quantitative platelet adhesion assay adapted from Xu et al. was performed to assess the HEAVM co-cultures’ ability to prevent platelet activation and adhesion [150]. Whole blood was collected from consented healthy adults using a Rice University IRB approved protocol and centrifuged to isolate platelet rich plasma (PRP). The PRP was then treated with prostacyclin (0.5 µg/mL of PRP), washed 2x with CGS buffer (12.9 mM sodium citrate/33.33 mM glucose/123.2 mM NaCl, pH 7) to remove remaining plasma. The platelets were resuspended in calcium free Tyrode’s buffer at half the original volume of collected PRP and were dyed with Sudan B Black (SBB) solution (5% w/v in 70% ethanol) for 2 hours at RT. After staining, the platelets were washed 3x with PBS to remove excess dye, counted using a hemocytometer, and resuspended to a concentration of 10x10^6 platelets/mL in PBS. Next, 5x10^6 platelets were added to HEAVM co-culture, VEC-only, VIC-only, and blank hydrogel scaffolds and incubated for 1 hour in shaking incubator at 37°C. All constructs were then washed 3x with PBS to remove unbound platelets. Next, each scaffold was carefully removed from the PEG base gel and placed in a microcentrifuge tube. DMSO was added to each sample to lyse the bound platelets and release the SBB dye into solution. Afterwards equal volume of PBS was added to the lysed platelet suspension in order to normalize to the PBS measured in the standard curve. The solutions for each sample were collected into cuvettes and the peak absorbance were measured at 614 nm using a Cary 60 UV-Vis spectrophotometer. The total adhered platelets were calculated based on a standard curve made from serial dilutions of the dyed platelet stock solution, and then normalized to the amount of adhered platelets on the blank scaffolds.
To visualize SBB dyed adhered platelets, a sample of blank, VIC-only, VEC-only, and HEAVM were fixed in 4% PFA for 30 minutes. Gels were washed 3x in PBS and counter stained with DAPI for 1 hour before being imaged on Nikon A1-Rsi confocal microscope. Each of the fixed samples were incubated with 15 µg/mL of fluorescent acetylated low-density lipoprotein complexes (Dil-AcLDL, Invitrogen) 4 hours prior to the addition of the platelets. Dil-AcLDL is commonly used to identify endothelial cells in culture by their ability to take up the fluorescent AcLDL complexes. Fluorescent and brightfield images were overlaid to identify areas of the hydrogels where activated platelets adhered.

Statistics

Unless otherwise noted, data are presented as means with standard errors of means (SEM). All data (except for qRT-PCR) were analyzed using statistical software (JMP, SAS, Cary, NC) and compared by a two-tailed paired Student’s t-test. Significance was defined as p-values < 0.05.
6.4 Results

*Constructed hydrogel-based aortic valve model scaffolds have relevant spatial arrangement of cell adhesive peptides*

HEAVM scaffolds were constructed over a 2 day period (Figure 6-1A). A 12mm diameter by 0.5 mm thick 10% (w/v) 6 kDa PEGDA base gel was used to facilitate moving the HEAVM scaffold with tools with minimal damage. The base gel was cross-linked for 35 seconds to match the swelling ratio of the HEAVM scaffold, while leaving enough free acrylates on the surface for the HEAVM gel to allow covalent binding. The VIC encapsulated PEG-PQ-PEG gel was shaped by a PDMS mold 5 mm diameter by 250 µm thick. The resulting co-culture system consisted of a VEC endothelialized-VIC encapsulated scaffold on top of the PEGDA base gel (Figure 6-1B).

Peptides were successfully incorporated into zones of the hydrogel using fluorophore labeled RGDS and RK R peptides (Figure 6-1C). PEG-RGDS+AlexaFluor 488 was incorporated into the bulk of the PEG-PQ-PEG gel (Figures 6-1C-2 and 6-1C-4) while RKR+AlexaFluor 532 was crosslinked to the surface (Figure 6-1C-1). Some excitement of the AF488 fluorophore due to the 532 nm laser was observed, but there is a distinct difference between the fluorescent signal on the surface with RKR (Figures 6-1C-1 and 6-1C-2) compared to the surface without RKR (Figure 6-1C-3 and 6-1C-4). These results indicate that the RKR peptide was able to attach to the gel covalently even after it had been incubating in cell culture media overnight.

The mechanical properties of the HEAVM scaffold were characterized by the bulk compressive modulus of the hydrogel. Thick (~0.8 mm) cylindrical acellular scaffolds of 4% (w/v) PEG-PQ-PEG with 2mM PEG-RGDS were made and swollen in
PBS overnight in order to perform the compression tests. The volumetric swelling ratio of $22.9 \pm 1.5\%$ was calculated by comparing the initially cross-linked volume to the fully swollen volume of the hydrogel. The bulk compressive modulus of the swollen hydrogels was $5.95 \pm 0.20$ kPa. After compressive testing, RKR was cross-linked to the surface of the hydrogels, which were then left overnight in PBS. There was not a significant difference in swelling after this secondary crosslinking, but the compressive modulus increased to $10.25 \pm 0.98$ kPa ($p = 0.02$), suggesting that photo-initiated free-radical polymerization continued throughout the bulk of the scaffold with additional light exposure.

Figure 6-1. Hydrogel-based endothelialized aortic valve model (HEAVM) scaffold has spatial arrangement of cell adhesive peptides. (A) Schematic of HEAVM co-culture construction. (B) HEAVM scaffold on base gel. (C) Fluorescently tagged RKR+AF532 (red) on the surface of RKR conjugated scaffold (left only). PEG-RGDS+AF488 (green) present throughout the hydrogel. Scale bars = 100 µm.
**Combined RKR-RGDS conjugated scaffolds support VEC monolayer formation**

VECs, VICs, and aortic endothelial cells (AECs) were seeded onto PEG-PQ-PEG scaffolds with different combinations of functionalized RGDS and RKR ligands to observe the cell adhesion behavior. VECs adhered onto the surface of RGDS-only, RKR-only, and combined RGDS-RKR conjugated scaffolds, and maintained characteristic cobblestone morphology as well as positive CD31 expression atop all conditions (Figure 6-2, middle row). There was no positive VEC expression of \( \alpha \)SMA on any of the scaffold combinations. However, VECs did not form continuous monolayers on the RGDS-only gels, whereas VECs on the RKR-only and the RGDS-RKR scaffolds formed continuous, tightly packed monolayers. No VEC invasion into the PQ-degradable scaffold was observed in any of the scaffold conditions.

AECs served as reference endothelial cell population. Similar to VECs, AECs adhered onto the surface of all hydrogel scaffold combinations, and maintained their cobblestone morphology and positive CD31 expression (Figure 6-2, top row). No \( \alpha \)SMA expression was observed in any AEC culture. However, AECs seeded on RGDS-only and RKR-only did not form continuous monolayers on the scaffold surface. In contrast, AECs on the RGDS-RKR scaffold formed continuous, tightly packed endothelial monolayers.

2D cultures of VICs adhered and spread onto both RGDS-only and RGDS-RKR conjugated scaffolds. VICs adhered onto-RKR only scaffolds, but aggregated with surrounding VICs rather than spreading and proliferating throughout the hydrogel (Figure 6-2, bottom row). VICs on all hydrogel conditions stained moderately positive for the VIC activation marker \( \alpha \)SMA, and were negative for CD31. However, VICs cultured in
2D atop he RGDS-only and RGDS-RKR conditions showed invasion into the hydrogel scaffold and stained strongly for αSMA.

**Figure 6-2.** Aortic endothelial cells (AECs), valve endothelial cells (VECs) and valve interstitial cells (VICs) cultured on the surface of PEG-PQ scaffolds immobilized with +RGDS/-RKR, -RGDS/+RKR, or +RKR/+RGDS. (A) Brightfield images showed that cell adhesion and proliferation were affected by adhesive ligand combination. Black scale bars = 100 µm. (B) Confluent regions of VECs and AECs stained for CD31 (green) and no αSMA (red) on all scaffold combinations. VICs stained for αSMA on all scaffold combinations, but had limited spreading on the -RGDS/+RKR scaffold. White scale bars = 50 µm.

**HEAVM co-cultures maintain valve cell phenotypes and physiologically relevant localization**

Live/Dead stains of cell seeded HEAVMs after 3 days in culture showed that most of the VECs (88%) seeded on the surface remained viable and stained positively for calcein (Figure 6-3). While ethidium homodimer-1 stain showed very little VEC death on the surface of the gels, the encapsulated VICs showed lower survival with only 66% of cells staining positive for calcein.
After 7 days in culture, immunohistological stains of HEAVM co-cultures showed that cells localized on the surface formed a monolayer and were positive for endothelial marker CD31, while only cells within the hydrogel had low, diffuse expression of VIC activation marker αSMA (Figure 6-4A). SEM pictographs of the HEAVM confirmed the formation of a tight, continuous VEC monolayer on the scaffold surface (Figures 6-5A and 6-5B), as well as showed 3-dimensional spreading by VICs within the hydrogel scaffold (Figure 6-5C).
Figure 6-4. HEAVM co-cultures maintain valve cell phenotypes and physiological relevant localization. (A) After 7 days in culture, VECs on the surface of the gel formed a confluent monolayer and express CD31 (green), whereas encapsulated cells had low levels of aSMA (scale bars = 50 µm). (B) HEAVM co-cultures, VEC-only scaffolds, and VECs on TCPS all expressed significantly less aSMA mRNA relative to VIC-only scaffolds. (C) All VEC-based cultures had similar levels of CD31 gene expression, whereas VIC-only scaffolds expressed significantly less CD31 than all other groups. *p<0.05 between noted samples for that protein. n=4.
VEC-only surface-seeded scaffolds stained positive for CD31 and negative for αSMA, and did not invade into the PQ-degradable hydrogel. 3D encapsulated VIC-only scaffolds stained negative for CD31, but stained positive for αSMA when localized closer to the bottom of the scaffold (Figure 6-6).

Quantitative real time-polymerase chain reaction (qRT-PCR) confirmed that HEAVM co-culture and VEC-only scaffolds, as well as VECs cultured atop TCPS had significantly lower levels of αSMA mRNA expression versus VIC-only encapsulated scaffolds (0.05x for HEAVM, p<0.005; 0.005x for VEC-only, p<0.01; and 0.12x vs VEC TCPS, p<0.05) (Figure 6-4B). Conversely, VIC-only scaffolds had one-thousand times less CD31 mRNA expression than the baseline VEC TCPS group (p<0.05) (Figure 6-4C). HEAVM and VEC-only scaffolds had no significant differences in CD31 gene expression relative to VEC TCPS.
VICs stained for low levels of αSMA (red) throughout the VIC-only scaffold, but increased in αSMA levels towards the bottom of the scaffold (left). VECs on the VEC-only scaffold formed a continuous monolayer on the hydrogel surface and stained positive for CD31 (green) and negative for αSMA (right). Scale bar = 50 μm

HEAVM co-cultures synthesize and release nitric oxide

HEAVM co-cultures stained for endothelial nitric oxide synthase (eNOS) co-localized with CD31-positive VECs at the surface of the scaffold co-culture (Figure 6-7). HEAVM co-culture (p=0.14) and VEC-only (p=0.06) scaffolds expressed two times greater eNOS mRNA than VECs on TCPS (Figure 6-7B). However, VIC-only scaffolds expressed significantly lower levels of eNOS mRNA than VECs on TCPS (0.001x, p<0.05).

To quantify the levels of nitric oxide (NO) produced by the cells, the NO byproducts released into culture medium were converted into nitrates and measured
(Figure 6-7C). Less than 1 µM of nitrate was measured in VIC-only scaffolds. The VEC-only and HEAVM scaffolds, however, had significantly higher nitrate concentrations than the VIC-only scaffolds (~5.5x, p<0.05).

Figure 6-7. HEAVM co-cultures synthesize and release nitric oxide. (A) HEAVM co-cultures stained for eNOS (yellow) co-localized with CD31 positive (green) VECs at the surface of the scaffold (Scale bars = 50 µm). (B) HEAVM co-cultures had similar levels of eNOS gene expression as VEC-only scaffolds and VECs on TCPS, and all had significantly higher eNOS gene expression than VIC-only scaffolds. n=4 (C) VEC-only and HEAVM scaffolds released significantly higher concentrations of
nitric oxide byproducts (that were converted into nitrites) into the culture medium than VIC-only scaffolds. n=5. *p<0.05 between noted samples.

VEC monolayer reduces platelet adhesion to underlying HEAVM scaffold

Platelet adhesion on the hydrogel constructs was quantified through the measured absorbance of lysed adhered platelets that were pre-dyed with Sudan B Black dye. Relative to acellular, blank HEAVM hydrogels, VEC-only scaffolds had 0.33% less adhered platelets (p = 0.26), while HEAVM scaffolds had two times more adhered platelets (p = 0.06) (Figure 6-8A). HEAVM co-cultures, however, had significantly greater platelet adhesion than was found on VEC-only scaffolds (p<0.005). VIC-only scaffolds promoted significantly greater platelet adhesion than all other groups, including ten times more adhered platelets than found on VEC-only scaffolds (p<0.005).

Examination of VEC-only scaffolds showed the formation of a continuous endothelial monolayer that was stained positively for dil-AcLDL with minimal platelet adhesion throughout the surfaces of the hydrogel (Figure 6-8B). Large quantities of platelet clumps adhered throughout the surface of VIC-only scaffolds, especially localizing at regions where VICs appeared to have migrated to the surface of the hydrogel (Figure 6-8C). Acellular blank gels also had regions of adhered platelet clumps scattered throughout the hydrogel surface (Figure 6-8D). The majority of HEAVM co-culture scaffolds had a dil-AcLDL positive endothelial monolayer free from adhered platelets on the scaffold surface (Figure 6-8E). However, platelet clumps could be seen on small regions on the HEAVM scaffold with dil-AcLDL negative cells, blank regions, and areas where there were disruptions in the endothelial monolayer (Figure 6-8F-G).
Figure 6-8. VEC endothelialized monolayers reduce platelet adhesion to underlying HEAVM scaffolds. (A) Adhered platelets on VEC-only, VIC-only, and HEAVM scaffolds relative to the number of adhered platelets on acellular, blank scaffolds. HEAVM co-cultures had significantly less platelet adhesion than VIC-only scaffolds, but significantly more adhered platelets than VEC-only cultures. VIC-only was significantly higher than all other cases. HEAVM was significantly greater than VEC-only, significantly less than VIC-only, but not significantly different from blank gel, n=6-8. Bars that are not connected by the same letter are significantly different, p<0.05. (B) VEC-only scaffolds stained for Dil-AcLDL and had minimal visible platelet adhesion across the hydrogel surface. (C). Large platelet clumps adhered to regions where VICs were present on the surface on VIC-only scaffolds. (D) Platelets adhered to acellular, blank HEAVM scaffolds. (E) Most HEAVM co-culture scaffolds stained for Dil-AcLDL on the surface coated monolayer and had minimal platelet adhesion. (F) Blank regions and (G) cells negative for Dil-AcLDL uptake on HEAVM co-cultures promoted platelet clump adhesion. Scale bars = 50 μm.
Long-term HEAVM co-cultures produce zonally localized extracellular matrix

HEAVM co-cultures cultured for 28 days produced an abundance of basement membrane proteins laminin, perlecan, and collagen type IV on the surface of hydrogel scaffold (Figure 6-9A). Within the scaffold, aligned collagen type I fibers were present throughout the hydrogels. In addition, fibronectin, a ubiquitous ECM protein, was also present throughout the HEAVM scaffold (Figure 6-9B).

Figure 6-9. HEAVM co-culture produce zonally localized ECM. (A) HEAVM co-cultures secreted basement membrane components laminin (red), perlecan (green), and Col IV (purple) at the surface of the scaffold within 28 days of culture. (B) Fibronectin (FN) (red) was present throughout the surface (top) and within the HEAVM scaffold, while aligned Col I fibers (green) were present beginning 10 µm below the scaffold surface (bottom). Scale bars = 50 µm.
6.5 Discussion

Aortic valve cells, both VECs and VICs, play a major role in the maintenance of valve tissue homeostasis, as well the progression of valve dysfunction. However, our understanding of VIC-VEC communication and regulation of valve functions is not well characterized and warrants the use of co-culture strategies to study the interplays of valve cells, their responses to environmental stimuli, and their actions in the initiation and progression of valve pathology. In this study, we present a novel hydrogel-based endothelialized aortic valve model (HEAVM) construct to co-culture VICs and VECs. This platform was designed to allow for precise control and independent modulation of various factors that can influence cell behavior, including mechanical properties and cell ligand concentration and arrangement. Therefore, PEG was chosen as the scaffold material due to its innate property to resist non-specific protein adsorption, and ability to incorporate covalently bound cell signaling molecules, as well as its tunable material properties and ligand arrangement through several different available crosslinking mechanisms [211]. Previous studies have successfully supported VIC viability and function in long-term 3D culture using PEG hydrogels with PQ MMP degradable peptide sequences and RGDS adhesion ligands [212,213]. VEC cultures, on the other hand, have been shown to maintain healthy phenotype and monolayer formation when cultured on RKR peptide-functionalized PEGDA hydrogels, but lose stability and hemostatic balance when cultured on RGDS-functionalized hydrogels (Balaoing, Chapter 4). Thus, the HEAVM platform was constructed by combining the strategies to encapsulate VICs in 3D throughout the interior of PEG-PQ-PEG/PEG-RGDS hydrogel, and to functionalize the surface with RKR adhesive ligands to support VEC monolayer growth (Figure 6-1).
This design mimics the natural arrangement of heart valves with VICs throughout the middle of the valve and VECs lining the blood-contacting surfaces [7,13]. Furthermore, VECs seeded onto the RKR functionalized surfaces (both RKR only and the RKR-RGDS combination) quickly formed stable monolayers and did not invade into the MMP-2 degradable gel (Figure 6-2). These results are in agreement with published data which has demonstrated the synergistic effects of integrin and syndecan in the binding of endothelial cells to their substrate [155]. Conversely, VICs did not spread on the RKR only hydrogels, but retained cell spreading and proliferation when cultured on the RGDS only and combined RKR-RGDS scaffolds (Figure 6-2). These results suggest that syndecan binding alone is insufficient to support VIC spreading and proliferation, whereas the addition of integrin binding may be necessary. Therefore, using a tunable scaffold with regionally localized syndecan binding (RKR) at the surface, and integrin binding (RGDS) throughout the hydrogel was ideal in promoting the corresponding cell growth of VECs at the scaffold surface and VICs within (Figure 6-4A).

The novel HEAVM scaffold design is the first reported direct contact co-culture model that utilizes synthetic constructs to distribute multiple cell adhesive peptides in a spatial manner in order to support layer specific cell growth as a means to mimic physiological cell organization. Previous valve cell co-culture models using collagen gels [39,76] achieved VEC monolayer formation with VIC encapsulation, but collagen gels are limited for customization of specific ligand presentation and localization. While specific scaffold functionalization can be applied in non-contact co-culture models, the elimination of valve cell contact limits the evaluation of key aspects of disease progression, such as VEC invasion or VIC disruption of the endothelial layer [95,206].
Even in the broader field of vascular research, there are many examples [214] of vascular endothelial cell / smooth muscle cell co-culture platforms using collagen [215], fibrin [216], polytetrafluoroethylene (PTFE) [217], and poly-glycolic acid (PGA) + poly-4-hydroxybutyrate (P4HB) [218] scaffold materials; none of these studies, however, incorporated spatially organized cues into the scaffold designs. The HEAVM concept can also be applied to the vascular field to study atherosclerosis, intimal hyperplasia, and cancer metastasis in vitro. Our model improves upon the limitations of previously reported co-culture platforms by providing customizable control of localized ligand presentation and mechanical properties, as well as mediating physiologically relevant cell localization to improve the in vitro study of normal and pathological cell functions.

In addition to supporting cell adhesion to physiologically relevant locations within the hydrogel scaffolds, the long term maintenance of valve cell phenotypes are essential in developing a healthy aortic valve model. Previous studies of valve cell co-cultures have shown to greatly influence both VIC and VEC phenotypes. Specifically, the presence of VECs in co-cultures reduced VIC proliferation and αSMA activation, and diminished the VIC’s capacity to form calcific nodules [39,76,97,205]. Conversely, a recent direct contact co-culture study using magnetically levitated valve cell constructs resulted in decreased VEC expression of endothelial markers (CD31, VE-cadherin, eNOS, von Willebrand factor) and increased αSMA activation within the construct when surrounded by VICs [205]. However, the cells on the surface of the magnetically levitated construct were positive for VEC markers, suggesting that physiological localization of VECs when co-cultured with VICs is critical for the maintenance of VEC phenotype. In HEAVM co-cultures, VECs were seeded onto the surface of the scaffold,
promoting their attachment to the functionalized RKR-RGDS hydrogel surface. Within 7 days in culture, the VECs on the HEAVM scaffold surface proliferated to form a continuous CD31 positive monolayer with limited αSMA activation (Figure 6-4A). Similar VEC monolayer formation and gene expression levels for CD31 were observed in VEC-only scaffold and TCPS groups, suggesting that organized placement of VECs relative to VICs on the HEAVM co-culture supported the maintenance of healthy VEC phenotype (Figure 6-4C). Consistent with previously reported valve co-culture systems, the HEAVM co-culture conditions significantly reduced αSMA activation levels in encapsulated VICs relative to VIC-only scaffolds, and promoted VIC quiescence while providing an organized platform for a healthy VEC layer (Figure 6-4B).

Furthermore, the long-term culture (28 days) of VECs and VICs in the HEAVM construct resulted in cell secretion of ECM corresponding to their location within the valve. In vivo, VECs are adhered to basement membrane composed primarily of laminin, perlecan, collagen type IV, and nidogen proteins [219]. Though initially supported only by RKR and RGDS ligands, VECs on the HEAVM constructs secreted abundant quantities of laminin, perlecan, and collagen type IV proteins localized at the scaffold surface, resulting in an interwoven basement membrane to support long term endothelial monolayer growth (Figure 6-9A). Within the scaffold, encapsulated VICs produced aligned collagen fibers and fibronectin proteins throughout the hydrogel interior, both primary components of the leaflet fibrosa layer [13,219,220], suggesting the potential to produce other ECM present in mature valve tissues in future optimized conditions (Figure 6-9B). While the simplicity of the HEAVM design provided sufficient cell adhesion through the presentation of ECM-derived peptide ligands, long term co-culture of the
valve cells in this scaffold promoted regional production and organization of full ECM proteins, mimicking a native valve composition.

Since the HEAVM design was able to maintain valve cells in stable phenotypes and promote production of physiologically appropriate ECM, the model provided a platform for physiologically relevant studies of valve cell interactions and functions. For example, NO, a vasodilator, has been shown to lower VIC αSMA activation in vitro, and may be an important VEC signaling molecule in maintaining VIC quiescence and valve tissue health [95,97]. VECs on HEAVM co-culture scaffolds had similar eNOS expression, and thus, similar NO production levels in comparison to VEC-only scaffolds (Figure 6-7B-C). Therefore, NO produced by VECs was likely a major factor in overall reduction of VIC activation as seen by the lower αSMA levels of VICs in HEAVM co-cultures compared to VIC-only scaffolds. In addition to influencing VIC phenotype, VECs also play a critical role in regulating thrombotic and inflammatory events at the tissue surface [5,128,221]. In homeostatic states, endothelial cells prevent platelet adhesion and activation, as depicted by the minimal platelet adhesion measured and observed on VEC-only scaffolds (Figure 6-8A-B). On the contrary, VIC-only scaffolds promoted significantly more platelet adhesion than all other groups, with large platelet clumps adhered to regions where VICs have migrated through to the hydrogel surface (Figure 5C). HEAVM co-cultures promoted more platelet adhesion than the VEC-only scaffolds, but significantly less than VIC-only scaffolds. Under closer inspection with confocal microscopy, the majority of the HEAVM surface was covered by dil-AcLDL-positive VEC monolayers, which had minimal platelet adhesion similar to VEC-only scaffolds (Figure 6-8E). However, platelet adhesion was visible on regions on the
HEAVM co-culture surface that had VIC infiltration (as seen by dil-AcLDL-negative cells), blank regions (lack of VEC adhesion), and disruptions in the endothelial monolayer (caused by handling of the hydrogels) (Figure 6-8F-G). Nevertheless, most of the hydrogel regions with continuous VEC monolayer formation reduced platelet adhesion, acting as a barrier to underlying ECM and VICs, and confirming that the endothelialized hydrogel-based scaffold can maintain essential anti-thrombotic functions.

There are still improvements that can be made in this novel co-culture construct. After the secondary crosslinking step in HEAVM construction, the viability of encapsulated VICs decreased, which may have been due to an increase in scaffold stiffness and decrease in pore size or caused by photo-initiated free-radicals harming the cells during the second crosslinking. The surviving VICs maintained their quiescent phenotype and produced organized ECM; regardless, further optimization in the HEAVM synthesis protocol will be explored to improve encapsulated cell viability in future studies. Secondly, the mechanical properties of the current HEAVM design are not representative of a physiologic valve. However, because PEG-hydrogels can be mechanically tuned, further work can be performed by optimizing polymer concentration or crosslinking time to match native valve tissue properties and withstand the mechanical loads experienced at the aortic valve.
6.6 Conclusions

In conclusion, we developed a novel and physiologically relevant HEAVM co-culture scaffold that was used to evaluate VIC-VEC maintenance of quiescent cell phenotypes, production of ECM and NO, and prevention of platelet adhesion. This system utilized the tunable characteristics of PEG-based hydrogels to localize adhesive ligands shown to support cellular adhesion of specific cell types to spatially organized regions. As a result, the HEAVM co-culture platforms preserved both VIC and VEC phenotypes and basic functional responses to develop a healthy aortic valve model. Though valve cells cultured in the HEAVM model produced organized ECM within the initially simple construct, developing an aortic valve model with the valve’s complex ECM composition and layered structure would likely require additional approaches to recapitulate physiological organization. Additional strategies such as 2-photon photolithography and or embedding layered biomaterials into the constructs may be integrated to increase scaffold design complexity of future studies [140,222]. Furthermore, optimization and combinations varying the cell seeding density, adhesive ligands, and growth factors can be implemented to investigate injured and diseased valve states. The application of these proposed improvements to this fully synthetic, non-animal derived scaffold could be considered in future tissue engineering applications. The PEG based VIC-VEC co-culture model presented here is a first step in designing a system to improve the field’s understanding of how arrangement and communication between VICs and VECs affect the onset and progression of valve disease.
Chapter 7: Conclusions

Heart valve disease is a global problem that affects people from every socioeconomic class. Currently, there is no sufficient medical management for diseased valves, just use of surgical replacements prone to mechanical or clotting related failures. These surgical replacements are suboptimal treatment solutions for pediatric patients with valvular disease, as the implants will have to be replaced more frequently and are not suitable for children’s lifestyle and blood composition. In order to improve on these devices and develop noninvasive solutions, there is still a great need to understand the biology and natural disease progression of these unique tissues.

In specific aim I, we performed the first extensive age-dependent characterization of hemostatic proteins in valves and VEC hemostatic regulation compared to vascular endothelial cells. Increased capacity of specific proteins that aggregate within regions of elderly valves were identified and correlated with areas with age-associated loss of ECM organization. The results also showed age-related differences in VEC hemostatic protein regulation. The reported hemostasis imbalance with aging and the accumulation of hemostatic proteins may contribute to the progression of CAVD, and reemphasizes the need to continue investigations of VEC hemostatic mechanisms and their roles in valve pathogenesis.

In specific aim II, we altered biomaterial variables to characterize the effects of environmental factors such as substrate stiffness and adhesive ligands on VEC phenotype and hemostatic responses in vitro. The laminin-derived adhesive ligand RKRLQVQSIRT (RKR) promoted the most stable VEC adhesion and balanced hemostatic responses on soft hydrogels relative to commonly used RGDS ligands and TCPS. This is the first
report to use the RKR peptide in combination with a PEGDA hydrogels to promote stable endothelial monolayer formation. These findings suggest that syndecan-1 mediated cell adhesion is the optimal mechanism to support VEC adhesion and function in comparison to integrin pathways. Thus, strategies using RKR have the potential to be applied for valve implant endothelialization designs.

Next, we implemented two unique co-culture systems to evaluate VEC phenotype and functional responses when in direct contact with VICs using a rapid cell-assembled model, and a more sophisticated zonally organized hydrogel model. In specific aim III, we utilized nanoparticle-based magnetic levitation technology to rapidly construct layered aortic valve co-cultures (AVCCs). The AVCC system allowed for observation of valve cell responses when direct co-culture including ECM production, functional protein expression, and overall reduction in αSMA activation within 3 days in culture. However, the scaffold-free approach may have also resulted in initiation of VEC EnMT when VECs were not localized at the AVCC surface. Nonetheless, the AVCCs provide a potential tool to study direct cell interactions for valvular development, pathogenesis of disease, and drug screening studies.

In specific aim IV, we created a novel, sophisticated hydrogel-based endothelialized aortic valve model (HEAVM) by implementing the RKR surface modifications developed from specific aim II to promote VEC monolayers on the surface of MMP degradable/RGDS functionalized PEG scaffolds. The HEAVM construct is the first co-culture model to implement spatial localization of specific ligands on a mechanically tunable synthetic scaffold, and successfully promote physiologically relevant regional growth of valve cells. The resulting co-culture maintained quiescent
VIC phenotype, VEC stability and CD31 expression, as well as produced organized ECM. Furthermore, the VECs on the HEAVM surface maintained their capacity to produce nitric oxide and prevent platelet adhesion, demonstrating the HEAVM’s ability to recapitulate healthy valve functions. Thus, the success of this design shows great potential to be applied to future valve disease models and tissue engineering applications.

Future studies can increase our understanding of VEC homeostatic functions, environmental responses, and roles in valve disease progression. Such studies include the evaluation of VEC secreted hemostatic protein localization in pathological human valves. In Appendix 1, preliminary histological analysis of surgically resected congenital valve tissues shows potential in understanding hemostatic proteins regulation within diseased valve tissues. Furthermore, because VECs have unique phenotypic responses compared to vascular endothelial cells, there is a need to characterize where VECs fit in the spectrum of endothelial cell subpopulations. Work characterizing VEC expression of vascular and lymph endothelial cell markers are discussed in Appendix 2. Finally, although several biomaterial strategies that can be used to promote endothelialization \textit{in vitro} were discussed here, there is still a need to identify a clinically applicable cell type that can recapitulate native VEC functions and an anti-thrombotic endothelial coating for future heart valve implants. Endothelial progenitor cells may be an attractive cell source to investigate for future valve endothelialization applications as they can be harvested from patient or donor blood, and be differentiated to have VEC phenotypic and hemostatic responses without mounting a graft versus host response. Thus, future directions of this work would benefit in the optimization of these biomaterial and surface
modification approaches to promote endothelial progenitor cell recruitment and differentiation for valve implant endothelialization.

In conclusion, the work in this thesis was completed with the implementation of knowledge from the fields of cell and ECM biology, cardiovascular biology, biomaterials, and biomechanics. Furthermore, various technical methods were developed and established to complete these studies including isolation and purification of VECs from fresh valves, automated quantification of DAB-based immunohistological stains, customized ELISAs, and characterization of hemostatic functions of VECs. The overall impact of this dissertation can be summarized by: (1) the first characterization of VEC hemostatic responses in aortic valves tissues, (2) optimization of environmental culture conditions to maintain healthy VEC functions in vitro, (3) co-culture models of the aortic valve that provide complexity of 3D culture environments, and (4) biomaterial surface coating strategies that can be translated to implant endothelialization or TEHV designs.
Appendix 1: Immunohistological evaluation of congenital heart valves

Introduction and Motivation

Although endothelial dysfunction has been strongly linked with adult semilunar valve diseases, little has been done to characterize the relationship between VEC hemostatic function and changes in valve tissue ECM in health and disease. The altered hemodynamics and malformations in leaflet anatomy and matrix composition in diseased congenital semilunar valves are also likely to influence the onset of VEC dysfunction. Work from Chapter 3 evaluating porcine AVs of various ages showed that young valve tissues can accumulate various hemostatic proteins, and that VECs from young AVs had significantly higher mRNA expression for hemostatic proteins VWF, TFPI, and tPA than elderly valves. Thus, the evaluation of the endothelium and the VEC interactions with ECM with respect to anti-thrombotic functions in congenitally diseased valves may provide insight into the specific factors and conditions that cause VEC hemostatic dysfunction. Here, we evaluated localization of VEC hemostatic-related proteins in normal and diseased pediatric semilunar valves in order to gain insight into the VEC roles in the pathology and epidemiology of these tissues.
Summary of Materials and Methods

Our lab, in collaboration with Drs. Kearney and Fraser from Texas Children’s Hospital, has collected human pediatric valves that were surgically resected. Children involved in this study demonstrate a broad range of ethnicities and socioeconomic status. Collected tissues were paraffin embedded, sectioned, and stained with MOVAT to demonstrate the various ECM components and the distinct valve layered structure. Valves will be classified as normal/control (CTRL), hemodynamically altered (HEMO), and/or dysplastic (HEMO/DYSP) based on gross and microscopic appearance, and clinical pathology as previously described. Representative MOVATs for each group are shown in Figure A1-1 [223].

Figure A1-1. MOVATs of representative CTRL (A, B), HEMO (C, D), and HEMO/DYSP (E, F) tissue samples.
Samples were continuously collected and classified until at least 10 samples were to be collected per group. IHC for various EC (CD31), hemostasis (VWF, ADAMTS-13, eNOS, tPA, PGI2), and ECM (fibronectin, laminin, perlecan, collagens IV, I, III, and decorin) related antigens were stained. The stained tissues were scanned and the colorimetric antigen intensity per stain was calculated and compared to the negative controls. Double immunohistological staining was performed to co-localize multiple antigens with unique or unexpected expression patterns as needed.
Preliminary Results and Discussion

Thus far, we have performed various DAB based-IHC on semilunar valve tissue slides previously collected from Texas Children’s Hospital, Houston, TX. In this preliminary study, 15 different patient semilunar valve samples (average age of 12 years old) were assessed following the classification criteria described, with 5 samples in the CTRL group, 6 samples in HEMO group, and 3 samples in HEMO/DYSP group. IHC stains were performed for antigens CD31, VWF, ADAMTS-13, fibronectin, laminin, perlecan, collagen types IV, I, and II, and decorin. Representative images of the IHC stains are shown in figure A1-2 below.

Figure A1-2. IHC stains for ADAMTS-13, CD31, COL III, decorin, and VWF in CTRL, HEMO, and HEMO/DYSP tissues.

An unexpected finding from gross observation was that both VWF and ADAMTS-13 stained very strongly and profusely in the valve tissues, even in the subendothelium and deeper into the valve interior. VWF appears to localize in regions that are also decorin-rich in normal tissues. This finding has led us to conclude that VECs
produce regulatory thrombotic-related proteins from both the apical and the basal sides, and motivated us to explore the hemostatic regulation of VECs relative to vascular ECs.

The stains also showed the different overall localization and intensity of CD31 and VWF stains. Though both antigens are commonly used EC markers, CD31 was localized at the leaflet edges (as expected based on EC location), whereas VWF was present at the leaflet edges, as well as, in the subendothelium, specifically at the ventricularis layer. Profuse VWF expression in the subendothelial region is likely due to the dual-sided release of VWF in by ECs [68]. Through semiquantitative analysis, we saw that the proportion of area stained for CD31 is lower than the proportion positively stained for VWF, confirming that VWF accumulates within valve ECM, and may not serve as a specific VEC marker.

Semi-quantitative analysis of the antigen stains for the study groups thus far indicate that the proportion of ADAMTS-13, COLs I and III, and VWF significantly decreased in HEMO/DYSP valves, however the proportion of decorin increased. The increased distribution of decorin is a characteristic of myxomatous hemodysplastic valves [11,223]. However, the overall intensity of the decorin staining was consistent in all disease states. These findings suggest that the changes in ADAMTS-13 and VWF presence and intensity in normal and decorin-rich myxoid HEMO/DYSP valves may have a potential molecular interaction with increased decorin present in the leaflets.
Since performing the preliminary immunohistochemical stains and analysis on previously collected tissue samples, we have collected 9 additional human valve samples from our collaborators at TCH, to have a total of 24 pediatric valve specimens. However, we have not yet acquired the necessary sample size of n=10 per group to complete the proposed work. The preliminary results are significant and interesting, and future studies are warranted. We expect to collect several more samples within the next few years to allow for the continuation of this work by another lab member.
Appendix 2: Assessment of valve endothelial cell sub-phenotype

Introduction

Valvular endothelial cells (VECs) been identified to have distinct differences in biomechanical responses and gene expression in comparison to endothelial cells found in the vascular system. Among the earliest observations that distinguish the biomechanical responses of VECs from that of vascular endothelial cells (ECs) was their perpendicular alignment to the direction of flow, contrary to the parallel cell alignment characteristic of vascular ECs [33,34]. While both cell types express similar antioxidant and anti-inflammatory genes, VECs were found to demonstrate lower expression of inflammatory-related genes than did vascular ECs [34,35]. Additionally, VECs expressed more genes related to cartilage formation, whereas vascular ECs had greater expression of genes involved in bone formation [35]. One of the key differences between cartilage and bony tissues is their vascularization; cartilage tissues are primarily avascular, whereas bone tissues are highly vascularized and in fact require angiogenesis for bone formation. These comparative results suggest that VECs perform a protective role against calcification, even within their dynamic shear stress environment, and that they may not readily express genes for angiogenesis.

Furthermore, even though confluent VEC cultures can demonstrate the cobblestone appearance typical of EC monolayers, VECs appear to be much more

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5 The work in this section was prepared as a manuscript: A.A. Blancas, L.R. Balaoing, F. Acosta, K.J Grande-Allen. Identifying behavioral phenotypes and heterogeneity in heart valve surface endothelium.
sensitive to the presence of various proteins than would be observed in other EC cultures. For example, chemokines transforming growth factor β and tissue necrosis factor α (TNF-α) have both been shown to induce VECs to express a mesenchymal morphology, to express αSMA, an interstitial marker, and to reduce VEC expression of CD31 [41,189,224,225]. Therefore, this particular process results in the VECs taking on traits characteristic of valve interstitial cells (VICs), which are located in the interior of the valve. This complex biochemical process has been studied in detail, with studies describing some heterogeneity in VEC transdifferentiation responses to various chemokines [19,41,189,225]. Previous gene analysis comparisons of VECs isolated from the outflow aortic side to the inflow side of valve leaflets have identified side specific differences in VEC expression for various calcification inhibitors, suggesting that there is heterogeneity in VEC phenotype [37]. However, this is not the phenotypical heterogeneity discussed and addressed in this study. Despite the observed phenotypic differences between VECs and vascular ECs, little work has been done to characterize where VECs fit in the spectrum of known endothelial cell behavioral subpopulations. Comparing VECs to known EC phenotypes serves to provide a frame of reference for potential differentiation studies needed in the future to provide an EC population similar to VECs for tissue engineering applications.

Vascular ECs can be generally categorized into three phenotypes: tip, stalk, and phalanx. Here, these populations will be referred to as angiogenic ECs and non-angiogenic ECs. Angiogenic ECs (tip/stalk) are stimulated by a chemical signal, usually vascular endothelial growth factor (VEGF) [226], to begin migration, proliferation, and sprout formation towards the source of the signal. However, the more recently identified
non-angiogenic ECs (phalanx) do not respond in the same manner, preferring to remain in the lumen of the parent vessel [227,228]. Since the VEC layer in normal valves also appears to be generally non-migratory [57], the potential for similarity between VECs and non-angiogenic ECs merits investigation.

Previous studies have shown that the ECs located in the interior of normal heart valves contain a population of lymphatic endothelial cells (LECs) [229,230]. In pathological conditions, there is an increase of angiogenesis as well as lymphangiogenesis within the valves, possibly in response to a variety of inflammatory signals [230]. However, it is unknown whether the LECs are restricted to the interior of the valve, or if there is a population on the outside of the valve leaflets as well.

Objective

Therefore, the purpose of this study was to characterize the population of VECs cultured from the surface of aortic valves with respect to a broad range of EC phenotypes, including angiogenic, non-angiogenic, and lymphatic. Determining a more precise profile of the makeup of the VEC population on the outer layer of heart valves would facilitate not only the development of a proper seeding population for tissue engineered transplants, but also further elucidate the mechanisms of valvular disease and dysfunction.
Materials and Methods

Cell isolation and culture

VECs were harvested from aortic valve leaflets and AECs from aortic root tissues dissected from fresh porcine adult hearts. Tissues were purchased from commercial abattoirs Fisher Ham and Meats (Spring, TX) and Animal Technologies (Tyler, TX). After dissection, tissues were digested in a collagenase II/dispase solution for 1 hr at 37°C to loosen the ECs. Next, VECs and AECs were gently scraped from the surface of valve leaflets and the lumen of aortic tissues, respectively. The collected cells were then cultured on gelatin-coated flasks in EGM-2 basal growth media with bullet kit (Lonza, Walkersville, MD) plus 1% penicillin/streptomycin (Lonza). At passage P1, isolated endothelial cells were purified for CD31+ populations using CD31 antibody (Millipore, Temecula, CA) conjugated-CELLection magnetic sorting beads (Invitrogen/Life Technologies, Carlsbad, CA). Cells were sorted once when freshly isolated, and once more if thawed from frozen stock. VECs were used between passages P1-P5, and AECs were used between passages P1-P6.

Flow Cytometry Analysis

VECs and AECs were analyzed via flow cytometry for various EC and LEC markers. Cells were harvested using Cell Dissociation Buffer (LifeTechnologies) and fixed in 4% formaldehyde for 1 hr at 4°C. For intracellular staining, cells were additionally treated with ice-cold methanol (-20°C) for 1 hr at 4°C. Primary antibodies CD31 (MCA1746 clone LCI-1, AbD serotec, Raleigh, NC), Flt-1 (sc-31173 clone N-16, Santa Cruz, Dallas, TX), CXCR4 (ab2074, Abcam, Cambridge, MA), DLL4 (ab7280,
Abcam), Prox1 (ab33219 clone 5G10, Abcam), and LYVE1 (ab33682, Abcam) were all used at a concentration of 1 µg per million cells in a staining buffer containing 1% bovine serum albumen (BSA) in phosphate buffered saline (PBS). Primary antibody incubation occurred at RT for 30 min, with agitation using a vortex at 15 min. Secondary antibodies conjugated with AlexaFluor 488/647 (Invitrogen/Life Technologies and Abcam) were also used at 1 µg per million cells in a staining buffer containing 1% BSA in PBS. Secondary antibody incubation occurred at RT for 30 min, with agitation using a vortex at 15 min. All samples were run on a BD FACSCantoII (Franklin Lakes, NJ) and analyzed using FlowJo software (FlowJo, Ashland, OR).

**Transwell Migration Assay**

Migration toward an angiogenic stimulus was evaluated by the use of a transwell migration assay. Fluoroblok 8 µm transwell inserts (Corning, Corning, NY) were used in a 24-well plate. Inside the transwell were seeded with 2x10^5 cells, (either AECs or VECs) in 300 µl of EGM-2 (Lonza). The well underneath the transwell insert contained 500 µL of EGM-2 with an additional 50 ng/mL of VEGF (R&D Systems, Minneapolis, MN). After cells were incubated for 24 hrs, the transwells were fixed in 4% formaldehyde and stained with DAPI. The undersides of the inserts were then imaged using a Zeiss LSM 510 LIV5 Confocal Microscope (Germany). Image field was 968 µm x 968 µm. Intact nuclei were counted and presented as means ± standard error of mean (n=12-16). JMP Statistical software (SAS, Cary, NC) was used to compare difference between cell counts using a two-tailed Student’s t-test. P-values <0.05 were considered statistically significant.
**Immunofluorescence**

To assess the *in situ* expression of EC and LEC markers, VECs and AECs cultured on gelatin coated chamber well slides were fixed with 4% paraformaldehyde for 30 min at RT and blocked with 2% FBS in PBS overnight at 4°C. Primary antibodies for CD31 (ab28364, Abcam), Flt-1 (sc-31173, Santa Cruz), CXCR4 (ab2074, Abcam), and LYVE1 (ab33682, Abcam) were used at 1:100 dilutions in PBS and incubated onto samples overnight. Secondary antibodies conjugated with AlexaFluor 488/555/633 (Invitrogen/Life Technologies) were used after primary antibody incubation. Fluorescent imaging was performed using a Zeiss LIVE5 Confocal Microscope.
Results

Flow cytometry analysis demonstrated that VECs expressed almost double the amount of Flt-1 compared to AECs, 74% vs. 38% respectively (Figure A2-1A). Angiogenic EC markers CXCR4 and DLL4 both showed over 90% expression on AECs whereas VECs showed much lower levels, with only 35% CXCR4+ and 47% DLL4+, indicating a less angiogenic phenotype. Immunofluorescent staining also verified high expression of CXCR4 and low expression of Flt-1 in AECs and the opposite result in VECs (Figure A2-1B). Pan-endothelial marker CD31 expression was consistently high (90% +) in both cell populations, indicating the high level of EC purity post isolation and sorting.
Figure A2-1: VECs display fewer pro-angiogenic markers than AECs. (A) Flow cytometry analysis of both cell populations demonstrates that AECs express higher levels of pro-angiogenic tip EC markers CXCR4 and DLL4 than VECs. Additionally, VECs express higher levels of Flt-1, which is common in non-angiogenic/phalanx EC as it dampens the migratory signal of VEGF. CD31 is used as a pan-endothelial check to ensure both populations consist of only ECs. (B) Immunofluorescence comparison of VEC and AEC expression of CD31, Flt-1, and CXCR4. Results confirm flow cytometry results. Scale bars = 50 µm.
To compare the migratory potential of the AECs and VECs, a transwell assay was performed utilizing VEGF as the migratory signal (Figure A2-2). After 24 hrs, the AECs demonstrated significantly greater migration with a mean of 71.5 ± 11.0 cells per image field while the VECs mean per image field was 30.0 ± 15.3 with p=0.032. The behavioral difference between the two populations corresponded to the high expression levels of angiogenic/tip ECs markers found on the AECs and the lower levels of the same markers, as well as the high expression of Flt-1, on the VECs.

Figure A2-2: VECs display largely non-migratory behavior. (A) AECs demonstrated a significantly higher migration towards an angiogenic stimulus (EGM-2 plus an additional 50ng/ml of VEGF). p<0.05 (n=12-16) Error bars represent standard error of the mean (SEM). (B) Representative images of AECs and VECs that have migrated through the transwell. Scale bar = 200 µm.
For further characterization of the molecular and behavioral phenotype of VECs, the possibility of EC heterogeneity was considered. Both cell types were examined for expression of lymphatic EC markers LYVE-1 and Prox1 (Figure A2-3A). An appreciable population of VECs, approximately 30%, were LYVE1+/Prox1+; whereas these markers were not present in AECs. Immunofluorescent staining verified the difference in LYVE1 expression between VECs and AECs (Figure A2-3B). Another well-established lymphatic marker, Flt-4/VEGFR3, was not expressed on either the VECs and AECs (data not shown), but it is likely that the lack of expression in this study was due to culture conditions not conducive to its expression. Flt-4 is a receptor for isoforms VEGFC and VEGFD [231] and EGM-2 medium only contains VEGFA.
Figure A2-3: A population of LECs are present in VECs. (A) Flow cytometry analysis of lymphatic markers LYVE1 and Prox1 in AEC and VEC. There is a clear population of LYVE1+/Prox1+ cells in the VECs that is absent in the AECs. (B) Immunofluorescent stain of AECs and VECs on 1% gelatin. Cells stained for LYVE1 (green) and DAPI (blue). Scale bar=50µm.
Discussion

Although VECs demonstrate the capacity to transdifferentiate into a mesenchymal phenotype as well as differences in osteogenic markers under stress in comparison to vascular ECs [35], these valvular cells are often treated as a uniform population with general, pan-endothelial traits such as the expression of CD31 and von Willebrand factor and the production of basement membrane extracellular matrix [128]. Although that generalization is not without merit, this study evaluated the complexities and heterogeneity unique to this cell population and showed the existence of two distinct populations on the valve surface endothelium: non-angiogenic VECs and LECs. The understanding of such nuances will be fundamental to future study of normal and pathological heart valve function. In addition, as progress is made towards generating engineered tissues that are suitable for valve replacements, it becomes necessary to ensure that population of cells seeded within these engineered tissues represents a phenotype that resembles the in vivo characteristics as closely as possible. Valve cells are particularly challenging in this regard, since harvesting cells from a patient is considered to be extremely invasive if not impossible. Therefore, a suitable VEC facsimile would need to be derived from a different source, such as peripheral blood endothelial progenitor cells (EPCs), umbilical cord blood EPCs, or possibly induced pluripotent stem cells (IPSCs).

Pronounced expression of Flt-1, as observed here in VECs, has become a hallmark trait of non-angiogenic/phalanx EC, and may be a marker for the stability of the endothelial layer atop valves. Flt-1 appears to mitigate angiogenic signaling by serving as a sort of “trough” for excess VEGF [232]. VECs expressed not only high levels of Flt-
1, but also showed reduced migration towards angiogenic stimulus, both of which are traits observed in non-angiogenic ECs [227,228]. These aspects of non-angiogenic behavior by VECs was further supported by their low-to-moderate expression of angiogenic EC markers CXCR4 and DLL4, which are known to play an important role in angiogenesis and are found on tip ECs [233,234]. As expected, AECs expressed low levels of Flt-1 [235] and high expression of DLL4 and CXCR4. With respect to recapitulating these VEC characteristics in a tissue engineered valve, future investigators could perform directed differentiation towards a non-angiogenic EC phenotype; this approach has already been developed using embryonic stem cells as a source population [228] and the scheme could be modified to accommodate other EPC populations. Having a seeded EC population with the ability to remain stationery after implantation would be critical for the success of a tissue engineered or bioprosthetic valve. Not only will this EC population be subject to dynamic shear stresses from the unique blood flow environment surrounding heart valves, but the cells would also need the capacity to withstand potential migratory signals that could result from the implantation procedure itself. If the VEC stand-in population could be derived from autologous cells such as EPCs, the transplanted valve would have a barrier that prevents or minimizes immune response.

The question of whether or not lymphatic ECs exist on the outside of the leaflets in the VEC layer was motivated by the finding of lymphatic vessels inside of valve leaflets [229,230], although the function of lymphatics within heart valves has not been fully elucidated. As the lymphatic system transports immune system-related cells, it is possible that lymphatic vessels in the interior of the valves serve as a prophylactic
measure to prevent, or subsequently combat, valve disease. Valve thickening has been attributed in part to changes in lymph flow [236]. Calcified areas within aortic valves, especially in cases of stenosis, show an increase in the number of lymphatic vessels and blood vessels, likely in response to inflammation [230,237]. A common way to distinguish LECs from vascular ECs is by examining the expression of two or more known lymphatic markers co-expressed in a single population [238]. Markers include LYVE1, a hyaluronan receptor [239], and Prox1, a transcription factor that is considered to be the best functional marker for LECs [240]. A LYVE1+/Prox1+ population was found within the VECs, indicating the presence of LECs on the outside of the valves in the VEC layer, although further studies are necessary to determine what role they play in normal and abnormal valves. A possible function is that these cells serve as a “reserve” population of LECs that can migrate into the valve when needed, thereby giving a boost to an immune response to valve disease.

In summary, the endothelial cells comprising the outer layer of the aortic valve leaflets are a heterogeneous population that includes LECs and non-angiogenic/phalanx phenotypes. In the future, it will be important to assess how these cells promote overall valve health or are affected by valve disease. Furthermore, this VEC heterogeneity has implications for heart valve tissue engineering. Although this study is preliminary in nature, it highlights how the in vivo valve cell populations should be examined just as carefully as the mechanical properties of the valve leaflets in which they reside. The consideration of both factors is necessary for maximizing the success of bioengineered constructs and therapies. In heart valve therapy specifically, understanding the changes in composition of the valve cell populations and leaflet matrix that occur as a patient ages
or as a result of pathological causes can shed light on how the constituents work together to form a functional tissue. This information would not only aid in the development of suitable engineered replacements, but also in how to prevent or minimize complications post-implantation.
Appendix 3: References


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