Endothelial Regulation of Extracellular Matrix in the Aortic Valve

A thesis submitted to Imperial College London for the degree of Doctor of Philosophy

by

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Abstract

It has been well known that the extracellular matrix (ECM) is important to the aortic valve function and that mechanical forces and cell-cell communication can regulate ECM remodeling. However, it has not been determined which cells regulate ECM production by valve interstitial cells (VICs). Thus, this study aimed to investigate if the valve endothelial cells (VECs) can influence ECM production and whether there is a differential role of VECs from either surface of the valve. In addition, the study aimed to assess the suitability of adipose derived stem cells (ADSCs) to differentiate into VECs with side-specific characteristics, so that could be used to populate tissue engineered heart valves.

Firstly, a reliable method of side-specific isolation and culture of VECs was developed. Consequently, the phenotypic characteristics of aortic VECs (aVECs) and ventricular VECs (vVECs) were investigated *in vitro* by immunostaining, western blots and protein array. Endothelial markers vWF, CD-31, VE-cadherin and eNOS and the release of cytokines IL-1β, IL-8, and TGF-β were assessed in cultured VECs from either side of the valve. However the distinct phenotypes between aVECs and vVECs in culture were not established.

The differential ability of side-specific VECs to regulate the production of ECM components by VICs was observed when VICs were cultured with media containing molecules released from side-specific VECs, in static culture, and when aVECs or vVECs were co-cultured with VICs. The media collected from vVECs increased amount of collagen by VICs while the media from aVECs showed no significant changes in the content of ECM. Interestingly, in the co-culture system, vVECs-VIC co-culturing enhanced the amount of both collagen and glycosaminoglycans (GAGs) whereas aVECs did not affect the ECM proteins.

Moreover side-specific VECs were exposed to the oscillatory and laminar shear stresses (flow patterns experienced by aortic and ventricular surface of the valve, respectively). Their distinct responses on the production of ECM by VICs were investigated. Aortic VECs exposed to oscillatory flow had higher content of collagen and GAGs while vVECs did not share this effect. The laminar shear stress on both sides of the valve maintained elastin content (as compared to fresh tissue).

ADSCs were also exposed to side-specific patterns of flow to assess their ability to differentiate into side-specific VECs. With respect to these factors, the exposure of ADSCs to the oscillatory shear stress induced the differential expression of NOS III and SMαA, similar to reported differences found between porcine aVECs and vVECs. Both flow patterns also increased the endothelial function of ADSCs by up-taking low-density lipoprotein.

In conclusion, this study reveals the unique functional phenotypes between the aortic and ventricular VECs, and the unique communication between VECs and VICs that is mediated by shear stress to regulate the specific production of ECM components. ADSCs appear to have the capacity to express endothelial markers, but a full functional assessment of their communication with VICs remains to be investigated. This information is important to the development of a living tissue engineered heart valve.

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Declaration

I declare that this thesis is my own work except from the scanning electron microscopic picture in the Figure 2.5 that was kindly performed by Dr. Padmini Sarathchandra. All else of the pictures that were adapted from publications are appropriately referenced.

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Napachanok Mongkoldhumrongkul, February 2014

Abbreviations

2-D	2 -Dimension
3-D	3-Dimension
5-HT	Serotonin
AcLDL	Acetylated low density lipoprotein
ADSCs	Adipose-derived stem cells
AF	Aortic flow
AMC	Amino-4-methylcoumarin
AS	Aortic side
AV	Atrioventricular
aVECs	Aortic valve endothelial cells
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin
CAMs	Cell adhesion molecules
cDNA	Complimentary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CM	Conditioned media

COL1A1	Collagen1α1
COL3A1	Collagen3α1
COX-2	Cyclooxygenase-2
CRE	cAMP-reponsive element
CS	Chondroitin sulfate
CSP4	Chondroitin sulfate proteoglycan 4
Cx43	Connexin 43
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
dNTP	Deoxyribonucleotide triphosphate
DS	Dermatan sulfate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
eEF2	Eukaryotic elongation factor 2

EGM2	Endothelial growth media 2
ELISA	Enzyme-linked immunosorbent array
EndoMT	Endothelial mesenchymal transition
ET-1	Endothelin 1
FACS	Fluorescence-activated cell sorting
FBN1	Fribillin 1
FBN2	Fribillin 2
FBS	Fetal bovine serum
FLK-1	Fetal liver kinase
FSA	Fibroblast surface antigen
FT	Fresh tissue
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage-colony stimulating factor
HA	Hyaluronic acid
HAS2	Hyaluronan synthase 2
HRP	Horseradish peroxidase
HS	Heparan sulphate

HSP	Heat shock protein
ICC	Immunocytochemistry
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
IQR	Interquartile range
LDL	Low-density lipoproteins
M	Molar
miRNA	Micro ribonucleic acid
MMPs	Matrix metalloprotease
mRNA	Messenger ribonucleic acid
MTS	Tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]
MW	Molecular weight
NO	Nitric oxide
NOS III	Endothelial nitric oxide synthase
OD	Optical density

PASMCs	Porcine aortic smooth muscle cells
PAVICs	Porcine aortic VICs
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween 20
PGI2	Prostaglandin I2 or prostacyclin
PGs	Proteoglycans
PKI	Protease K inhibitor
PMS	Phenazine methosulfate
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT-PCR	Real time-polymerase chain reaction
SC	Static control(s)
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
siRNA	Small interfering RNA
SMαA	Smooth muscle alpha-actin
SMCs	Smooth muscle cells
SVF	Stromal vascular fraction

T25	Tissue culture flask 25 cm^2
T75	Tissue culture flask 75 cm^2
TCP	Tissue culture treated polystyrene plates
TGF-β	Transforming growth factor beta
TIMPs	Tissue inhibitors of matrix metalloproteases
TNFα	Tumor necrosis factors alpha
TPPS	5,10,15,20-tetraphenyl-21H,23H-porphine tetra-sulfonate
VEGF	Vascular endothelial growth factor
VF	Ventricular flow
VICs	Valve interstitial cells
VS	Ventricular side
vVECs	Ventricular valve endothelial cells
vWF	von Willebrand Factor

CHAPTER 1 Introduction

1.1 The Aortic Valve

The aortic valve is a tricuspid structure that ensures the uni-directional flow of blood out of the left ventricle into the ascending aorta and optimises coronary perfusion. It is calculated to open and close, approximately 3 billion times in an average lifetime.

The valve was previously believed to be a passive structure that functioned in response to differences in transvalvular pressures between left ventricle and aorta. The mechanism for valve function was thought to rely only on the changes in pressure that occur during the cardiac cycle, whereby, during systole, the left ventricle contracts to produce a rise in pressure in the chamber higher than that in the aorta, thus causing the aortic valve to open. When systole finishes, the left ventricle relaxes (turning to diastole) causing the pressure in it to drop. At this moment the pressure in the aorta is higher than left ventricle letting the aortic valve close thereby preventing flow of blood back into the left ventricle.

However, it is now widely accepted that the aortic valve is a highly complex structure that relies on its cellular components for the coordinated movements of its constituent parts (Yacoub *et al*., 1999). This is illustrated by the fact that the valve actually starts to open before there is outflow of blood from the left ventricle (Higashidate *et al*., 1995). Furthermore viable cells in the valve are needed to maintain the durability and functionality of the valve (Schneider and Deck, 1981).

Understanding the function of the cellular components of the valve during development, in the post-natal period and into adulthood, is to be able to fully comprehend how the valve maintains its function over the course of a lifetime as well as how and why the valves may become diseased in certain individuals.

1.2 Development of Aortic Valve

During embryonic development, the aortic valvulogenesis starts from the creation of endocardial cushions in the atrioventricular (AV) canal. It involves a series of delamination, migration and transdifferentiation of particular cells as well as remodeling of extracellular matrix (ECM). The endocardial cushion formation is induced by the myocardium that produces a hyaluronan-rich matrix. The hyaluronic matrix has hydrophilic properties; thus, it is termed "cardiac jelly". It is secreted and accumulated between the myocardium and the endocardium at the AV canal creating swelling regions, known as the endocardial cushions (Figure 1.1). Moreover the hydrophilic cardiac jelly serves as a physical scaffold for the endocardial cells to migrate into, populate, synthesise matrix proteins and subsequently lay down the ECM of the valve. Although the endocardial cushions are formed at the early stage of embryonic development, they function as a physical barrier to prevent the regurgitation of blood through the primitive heart tube prior to the individual cushions becoming the heart valve cusps.

Following endocardial cushion development, overlying endothelial cells are subsequently delaminated from the single layer, allowing them to migrate into the cardiac jelly. The cushion-resident cells laterally develop into mesenchymal cells or valve interstitial cells. The process of endothelial cells differentiating into mesenchymal cells, known as Endothelial-Mesenchymal-Transition (EndoMT),

requires complicated signaling pathways that transduce signals from myocardium and endocardium (Figure 1.1).

The key mediators involved in the EndoMT process are transforming growth factor (TGF), Notch, vascular endothelial growth factor (VEGF), wnt/β-catenin and the NFAT family of proteins. The TGF superfamily, including TGFβ and bone morphogenetic proteins (BMPs), is also important for inducing endocardial cushion formation. Although Notch signaling is not essential for initial endocardial cushion formation, it is indispensible for EndoMT. VEGF plays an important role in regulating the proliferation of endothelial cells which are profoundly associated with the initiation of the endocardial cushion, the deposition of cardiac jelly and EndoMT. In addition, the wnt/β-catenin and the NFAT family of proteins have important roles for the migration and EndoMT of endocardial cells (Combs and Yutzey, 2009; Armstrong and Bischoff, 2004).

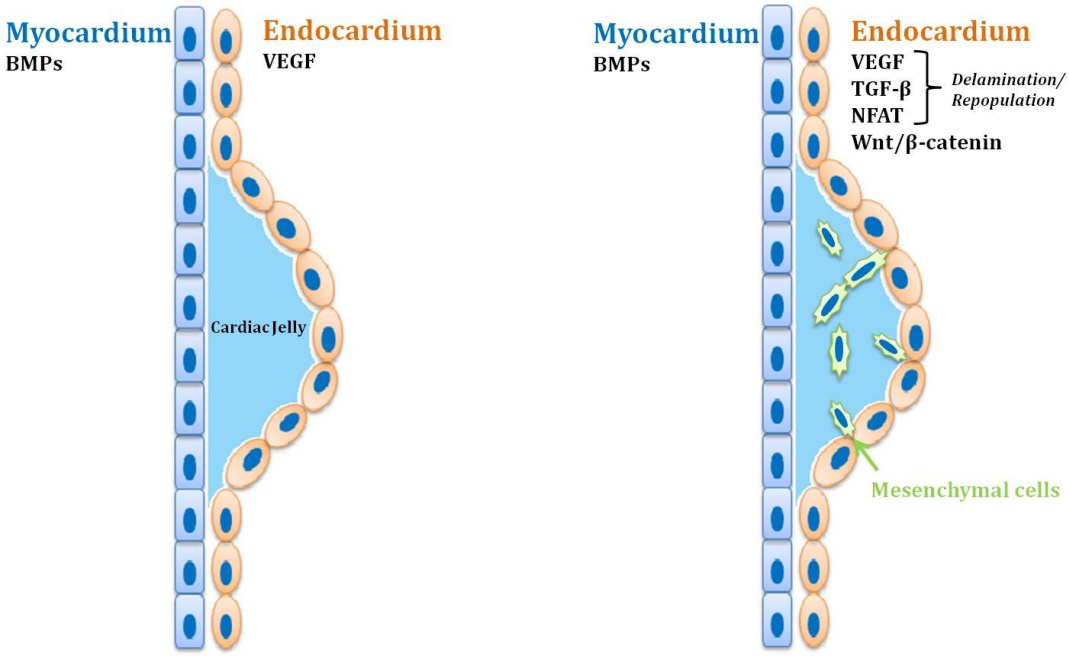


Figure 1.1 Model of heart valve development**.** Heart valve development occurs in the developing heart tubes containing the outer layer of myocardium and the inner layer of endocardium. BMP expression in myocardium results in the production and deposition of hyaluronic matrix, cardiac jelly, between myocardium and the endocardium. It generates the protruding area known as endocardial cushion. Endocardial VEGF expression increases the proliferation of endocardial cells during the cushion formation, a), and endothelial-to-mesenchymal transition (EndoMT), b). During EndoMT, the endocardium express VEGF, TGF-β and NFAT to increase the proliferation, delamination and migration of themselves into the cardiac jelly to become mesenchymal cells (represented in star-shaped green model). The expressions of Wnt/β-catenin by endocardium and BMPs by myocardium are important to EndoMT.

Once in the cardiac jelly, endocardium undergoing EndoMT digests the previously deposited ECM and produces new ECM components such as collagen type I, II and III, versican and other proteoglycans to create more rigid cusps (Liu *et al*., 2007). After birth, the aortic valve adapts its strength by modulating the ECM in response to the increased systemic pressures. There is also adaptive change in cell-loss of myofibroblasts and a gain of quiescent valve interstitial cells (VICs) over the period of a lifetime (Rabkin-Aikawa *et al*., 2004b). In fetal valves, myofibroblasts are

prominently found at about 62% of total amount of VICs as their function is required to remodel the ECM components during development. However, they are less abundant (presenting at 2.5% of total VICs) in adult healthy valves (Rabkin-Aikawa *et al*., 2004b). The fully developed elongated thin valve cusp that has stratified layers of ECM and specific cellular components are further remodeled after birth and are important for the structure-function relationship of the valve.

1.3 Structure of the Aortic Valve

The aortic valve is a complex structure consisting of many functional units, for example, annulus, (three) cusps, sinotubular junction and (three) sinuses. The whole valve machinery is termed the 'aortic root' and it sits between the left ventricle and the ascending aorta.

The valve cusps are attached to a crown-shaped annulus in a semilunar pattern. The highest point of the attachment, where adjacent cusps are closest together (known as commissures), marks the boundary of the valve and the ascending aorta, which is identified as small ridge, termed the sinotubular junction.

At the basal point of attachment of each cusp, the annulus is bulged creating three "pockets"-called the sinuses of Valsalva. Two of the sinuses of Valsalva have ostia that give rise to left and right coronary arteries. Thus, the sinuses and cusps are named according to the coronary arteries arising from; namely the left, right or non-coronary sinus or cusp (Figure 1.2).

The competent seal of the aortic valve prevents the regurgitation of blood flow back into the left ventricle and involves the coapting areas on individual cusps. In the middle of the free edge of each cusp, nodules of Arantius are created from bundles

of fibrous tissue. The co-aptation of 3 nodules at the centre of the aorta ensures a tight seal during valve closure. Two crescentic areas of each cusp known as lunulae are located along the free edge between the commissures and the nodule of Arantius. The lunulae affect the competent seal during diastole by contact with the corresponding region of the contiguous cusps. The remaining area of the cusp, apart from coapting area, is termed the "belly" region. The microstructure and cellular compositions of each of the structures within the valve dictate the durability and function of the valve.

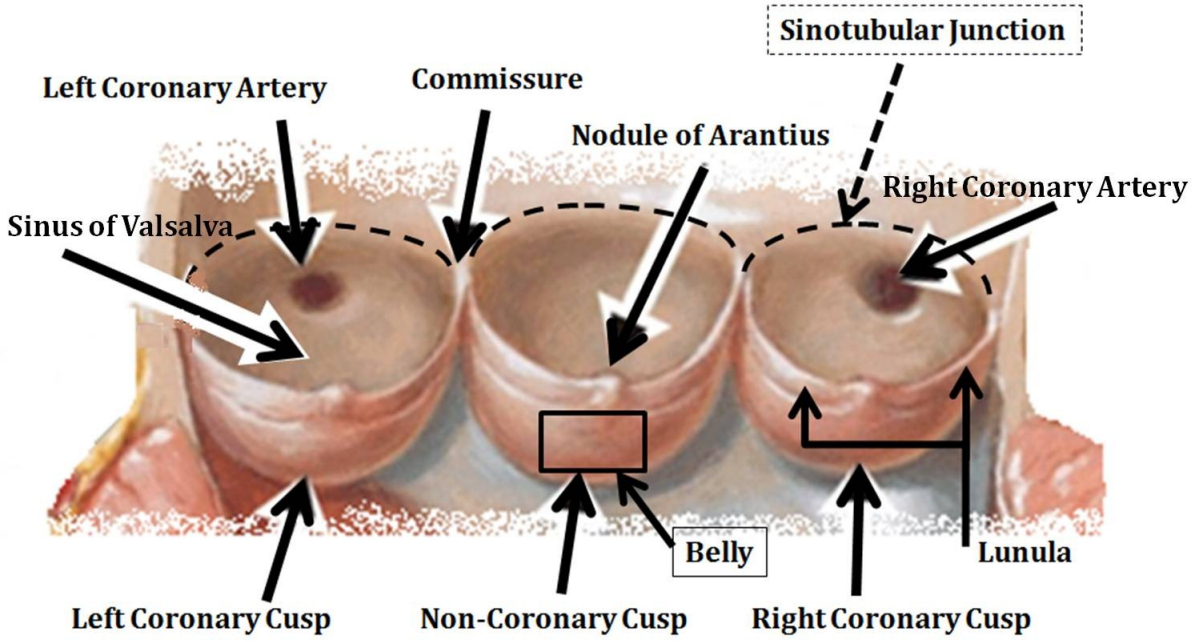


Figure 1.2 Anatomy of the aortic valve. Aortic root consists of 3 semilunar cusps sitting between the aorta and left ventricle. The bulged pockets in the aortic root are called Sinus of Valsava in which two of them have the ostria known as left and right coronary artery. The edge of fibrous ring on top of the aortic root is Sinotubular junction. The coronary cusps are attached on the aorta wall in a crown shape and join to each other at the commissures. Free edge of each cusps consists of Nodule of Arantius and Lunalae. During dystole, the free edges are tightly in contact to the adjacent leaflet and asssure the closing of the valve. Adapted from http://www.cicmd.com/images/cicmd/anatomy%20images/aortic-valve.gif

1.3.1 Histological profile of the aortic valve

Histological examination of aortic valve leaflets reveals 3 distinct layers, which are different in terms of extracellular matrix (ECM) components and cellular components.

The fibrosa layer, which is mainly composed of collagen, is on the aorta side of the valve. The middle layer, termed the spongiosa contains glycosaminoglycans (GAGs) as a major component. Finally the ventricularis layer is on the ventricular side of the valve and is composed of abundant amounts of elastin (Figure 1.3). The microstructure architecture of the aortic valve is preserved among different species. *Sus Scrofa* (pig) and *Ovis Aries* (sheep) have cellular and extracellular composition similar to human; thus, they are commonly employed as animal models for studying valve biology and disease.

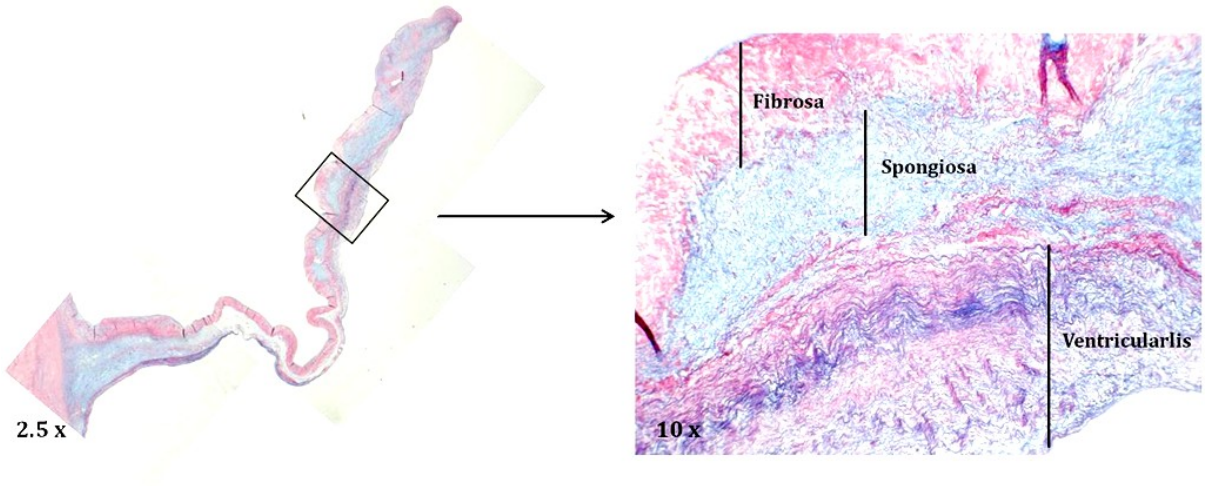


Figure 1.3 Histological structure of an aortic valve cusp. ECM component in a human aortic valve leaflet was stained with sirius red, alcian blue and Millaer's elastin. Collagen, GAGs and elastin are represented in pink, blue and purple, respectively. Provided by Dr. Padmini Sarathchandra.

1.3.2 Cellular components

The durability and functionality of the valve is maintained by its cellular population. Rabkin-Aikawa and colleagues showed that a pulmonary autograft (where the pulmonary valve is placed in the aortic position) could adapt its functionality to cope with the new heamodynamic environment on the left side of the heart (Rabkin-Aikawa *et al*., 2004a). The ability of the pulmonary valve to adapt in this way and the importance of living cells to the valve was recently illustrated by El-Hamamsy *et al*. who showed that the survival of patients following the implantation of a living valve substitute (such as a pulmonary autograft) was indistinguishable to the survival rate of an aged matched population and superior to the recipients who received a homograft valve (that contains no living cells) (El-Hamamsy *et al*., 2010).

There are 2 principal types of cells found in the aortic valve. Valve endothelial cells (VECs) are on the surface and VICs that are distributed throughout the tri-layered structure of the leaflet.

1.3.2.1 Valve Interstitial Cells

VICs is the collective name for cells that populate the ECM of the valve cusps. They have been observed to have distinct characteristics different to other fibroblast-like cells from pericardium and skin (Taylor *et al*., 2000). Moreover the unique phenotypes among subpopulations of VICs from different valves are also evident. In mature valves, VICs are comprised principally of 3 distinct phenotypes; fibroblasts, myofibroblasts and smooth muscle cells.

(i) Fibroblasts express fibroblast surface antigen (FSA) and vimentin, an intermediate filament of mesenchymal cells. The unique characteristic, which

differentiates fibroblastic VIC phenotype from other two phenotypes, is the absence of smooth muscle alpha-actin (SMαA). About 80% of cultured human VICs from all 4 heart valves are positive for FSA (Rabkin-Aikawa *et al*., 2004b; Taylor *et al*., 2000). Moreover, fibroblastic VICs possess ECM sectretory and remodeling properties. All cultures of VICs express prolyl 4-hydroxylase (the collagen triple helix cross-linking enzyme) (Taylor *et al*., 2000). The electron microscope pictures of VICs also show the secretory organelles associating with prolyl 4-hydroxylase expression (Messier, Jr. *et al*., 1994).

VICs are believed to remain quiescence in normal healthy valves but become activated under pathological circumstances. Chemokines and growth factors are produced by VECs and macrophages following injury or abnormal hemodynamic mechanical stress. They are considered as precursors of the trans-differentiation pathway that normal VICs enter in becoming the osteoblast-like cells and associating with calcification of the valve (Osman *et al*., 2006; Rajamannan *et al*., 2003).

(ii) VICs identified as myofibroblasts express intermediate filaments (vimentin and desmin), cytoskeletal filaments (SMαA) and non-muscle myosin (SMemb) which are markers for activated mesenchymal cells. Additionally, they are negative for myosin, which is expressed by smooth muscle cells (Rabkin *et al*., 2001). Myofibroblasts are considered as activated VICs. After become activated, they increase the production of one autocrine, TGF-β (Liu *et al*., 2007), which plays important role in the trans-differentiation of VICs into osteoblast-liked VICs (Osman *et al*., 2006). Primary VICs, which were isolated from normal human valves and treated

with osteogenic media, bone morphogenic proteins and TGF-β, increased expression and activity of alkaline phosphatase (Osman *et al*., 2006).

Activated VICs actively remodel ECM components during development, adaptation and pathology (Rabkin-Aikawa *et al*., 2004b). During fetal development, SMαA and SMemb possitive cells were found abundantly in about 62% of VICs. Interestingly, in normal adult valves, the amount of myofibroblasts are significantly decreased to 2.5% (while the majority phenotype of VICs are fibroblasts). Similarly, the amount of myofibroblasts was decreased in long-term pulmonary-autografted aortic valves, compared to newly transplanted valves which have not adapted to their new heamodynamic environment. Lastly, myofibroblastic phenotypes were found abundantly in myxomatous degenerative valves (Rabkin-Aikawa *et al*., 2004b). The myofibroblasts in all valves (fetal, newly autografted and diseased valves) also expressed ECM remodeling enzymes (Rabkin *et al*., 2001; Rabkin-Aikawa *et al*., 2004b). Matrix metalloprotease (MMPs), which are a family of collagen and elastin degrading enzymes, and their inhibitors, tissue inhibitors of matrix metalloproteases (TIMPs), were also observed in normal human valves around VICs positive for SMαA. These results demostrate that myofibroblastic phenotype and, thus, function are required for heart valves to modulate ECM components in reposnse to the microenvironment.

(iii) Smooth muscle cells (SMCs) are found less frequently in the valve than the other two cell types. They express vimentin, SMαA and smooth muscle myosin (SMM) heavy chain, but not desmin nor SMemb (Rabkin *et al*., 2001). SMCs also exhibit contractile properties; thus, they are believed to mediate the observed contractile properties in the valve (Chester *et al*., 2000).

Moreover, the comparison between porcine aortic VICs (PAVICs) and porcine aortic smooth muscle cells (PASMCs), from the same animal, substantiated the unique abilities of VICs to contract and synthesise matrix component (Butcher and Nerem, 2004). When PAVICs and PASMCs are encapsulated in collagen type I, they both showed the ability to compact the gel at similar extent. Additionally they express SMαA to a similar level. However PAVICs expressed different amounts of desmin which is a muscle cell marker and produced more protein and glycosaminosglycans (GAGs) (Butcher and Nerem, 2004).

Valve interstitial cells actively communicate with each other and the ECM as well as dynamically respond to the mechanical changes through particular (membrane) proteins. The presence of N-cadherens, desmogleins, connexins (Cx) 26 and 43 suggests that VICs have cell to cell communication through junctional proteins (Latif *et al*., 2006); although tight junctions were not found. They are thought to use cell-matrix communication to respond to the ever changing mechanical environment. Integrins, are abundant membrane bound proteins expressed by VICs, and play an importance role in the transmission of mechanical force experienced by the ECM into cells (Latif *et al*., 2005). The extracellular part of the integrin binds to collagen and senses the mechanical stress in ECM, while the intracellular components are linked to the cell's cyotoskeleton.

1.3.2.2 Valve Endothelial Cells (VECs)

VECs inhabit the surface of the valve on both the aortic and ventricular aspects of the valve and are named, aortic valve endothelial cells (aVECs) and ventricular valve

endothelial cells (vVECs). They populate the surfaces of the cusps and are, continuous with the endothelium of aorta and endocardium of the left ventricle. During the cardiac cycle, VECs on the opposite sides of the valve are exposed to different flow patterns. While VECs that lie on the aortic side encounter low oscillatory shear stress, VECs on the ventricular side experience high laminar shear stress. The different magnitude of the shear stress is therefore speculated to effect VEC morphology – with cubodial shape of aVECs and a more elongated and flat shape of vVECs (Hurle and Colvee, 1983; Maron and Hutchins, 1974).

VECs are considered to have a function similar to vascular endothelial cells (ECs) elsewhere in the cardiovascular system. For example, they regulate inflammatory reactions between blood and tissue (Muller *et al*., 2000), deliver nutrients to the underlying cells (Tompkins *et al*., 1989) and regulate the phenotype of cells within the valve (Butcher and Nerem, 2006).

Cell adhesion molecules (CAMs) expressed by activated vascular ECs play an important role in the interaction between vascular ECs and circulating blood cells. Likewise, adhesion molecules in VECs have been identified (Muller *et al*., 2000). Muller reported the expression of a range of CAMs including ICAM-1, CD34, PECAM-1 (CD31) and VCAM-1, all of which were constitutively expressed by VECs in calcified and inflamed valves.

Circulating macromolecules can be delivered to VICs within the valve through the function of VECs (Tompkins *et al*., 1989). *In vivo* autoradiographic studies by Tompkins and colleagues demonstrated that the radiolabeled low-density lipoproteins (LDL) and albumin were able to immediately permeate into the aortic valve through VECs. There was no difference in permeability between VECs on the aortic and ventricular sides in spite of the different magnitudes of shear stress between each side of the valve. However, the average LDL permeability of VECs was

found to be significantly higher than those of ECs on the aorta (Tompkins *et al*., 1989).

VECs have been shown to regulate VIC proliferation and trans-differentiation (Butcher and Nerem, 2006). In the presence of VECs in a 3-dimensional co-culture model with laminar flow, VICs remain quiescent by showing less proliferation and expressing less SMαA. When cultured with osteogenic differentiation media without the effect of shear stress, VECs prevent VICs differentiation into myofibroblasts.

Paracrine mediators produced by vascular ECs are well characterised and they function to regulate the contraction of the vessel wall (Vanhoutte and Tang, 2008). Likewise, valvular ECs have the potential capability to control the contraction of the aortic valve by releasing vasodilators such as prostacyclin and nitric oxide (NO) (Ku *et al*., 1990; Pompilio *et al*., 1998) and vasoconstrictor agents such as endothelin-1 (ET-1) (Peltonen *et al*., 2009).

Evidence of the regulation of valve stiffness by VECs was revealed in porcine aortic and pulmonary valves. The contraction of valves cusps was increased with increasing concentration of the exogenous ET-1 in both intact and VEC-denuded valves (Pompilio *et al*., 1998). However, the contraction of the denuded valves was higher than the intact valves at the same concentration of exogenous ET-1. Moreover, the contraction of the valves was profoundly increased when an NO synthase inhibitor was used to block the release of NO. It was noted that the NO syhthase inhibitor did not affect the contraction of the valves from which ECs had been intentionally removed (Pompilio *et al*., 1998). Recently El-Hamamsy *et al.* have shown that VECs regulate the valve mechanical properties through an endothelium-dependent mechanism that involves NO. They revealed the effect of serotonin (5-HT), which releases NO, decreases the stiffness of the valve. However,

the effect of 5-HT was reversed when VECs are intentionally removed from the valve or in the presence of a NO synthase inhibitor (El-Hamamsy *et al*., 2009).

The valve endothelial cells do not only maintain valve homeostasis at the tissue level, they also regulate the cellularity of the valve. VECs are thought to have the property of valve progenitor cells that act to replenish VICs through activation of EndoMT. Evidence of this process can be seen by the co-expression of endothelial and mesenchymal markers by endothelial cells in both on the mature valves and in culture. Co-expression of CD31 (an endothelial specific marker) and SMαA by VECs have been reported in mature ovine aortic valves (Paranya *et al*., 2001), mitral valves (Wylie-Sears *et al*., 2011) and human pulmonary valves (Paruchuri *et al*., 2006). The ability to express SMαA by VECs is enhanced by the exogenous treatment of TGF-β (Paranya *et al*., 2001; Paruchuri *et al*., 2006). Although the mechanisms were not pursued by these studies, it is now well known that TGF-β increases the expression of SMαA through Snail1 (Piera-Velazquez *et al*., 2011; Li and Jimenez, 2011). Moreover, the cultures of mitral VECs are able to be differentiated into osteogenic and chondrogenic lineage in a comparable way to VICs and bone marrow-derived mesenchymal stem cells (Wylie-Sears *et al*., 2011).

Although VECs exhibit functions similar to ECs elsewhere in the circulation, there is increasing evidence showing that VECs have distinct properties compared to the vascular ECs. Studies in the canine aortic valves revealed that VECs on both side of the valves lie circumferentially to the free edge (Deck, 1986) which is, in fact, parallel to the collagen fiber alignment but perpendicular to the flow experienced on the ventricular surface. Moreover the unique alignment of VECs is conserved in cultured cells that were maintained in a culture dish without an aligned substrate (Butcher *et al*., 2004). Porcine VECs and ECs from the aorta from the same animals were expanded *in vitro* and exposed to steady laminar shear stress for 48 hours. Aorta ECs responded to the flow by aligning parallel to the direction of flow, while

VECs maintained perpendicular alignment to the flow similar to the observation made *in vivo* (Butcher *et al*., 2004; Deck, 1986). The signaling pathway in response to the shear stress revealed differences between VECs and aorta ECs. While the shear stress induced only Rho-kinase signaling in VECs, both Rho-kinase and phosphatidylinositol 3-kinase pathways were involved in alignment of EC from the aorta (Butcher *et al*., 2004).

In addition, VECs from aortic valves also appear to have a different transcriptional profile and a higher proliferation rate than ECs from the aorta. Under static conditions, VECs are most proliferative when cultured on gelatin and collagen substrate, whereas ECs from the aorta grow most rapidly on lysine and laminin (Farivar *et al*., 2003). The differential proliferation rate could be due to an effect of different cell-matrix interactions between VECs and vascular ECs. Different materials coated on the culture plates may have unique interactions to membrane proteins which are differentially expressed by ECs and VECs. The unique cell-matrix interactions subsequently induce differential intracellular signaling pathways and may lead to differential cell phenotype and proliferation rate.

Microarray results also suggested a higher expression of mitogenic mRNA in VECs e.g. the fibroblast growth factor, protein kinase C and jun D. Furthermore, some endothelial markers are differentially expressed. For example, platelet-derived growth factor receptor (PDGF-R) and platelet endothelial cell adhesion molecule (PECAM) mRNA are found only in VECs. Whereas vascular endothelial cell growth factor (VEGF)-B and vascular cell adhesion molecule (VCAM)-1 mRNA are expressed only in vascular EC (Farivar *et al*., 2003).

Transcriptional profiles of VECs and aorta ECs exposed to laminar shear stress have also been compared (Butcher *et al*., 2006). Under laminar flow, VECs increased expression of chondrogenic factors, compared to un-stimulated VECs. Whereas

aorta ECs illustrated higher expression level of osteogenic factors, compared to static ECs. Additionally, VECs expressed higher amounts of anti-inflammatory mRNA but less pro-inflammatory mRNA when compared to vascular ECs. Phenotypic heterogeneity of ECs is not conserved to only cells from individual tissue or organs. VECs on the aortic and ventricular side of the aortic valve also have the capacity to regulate valve/VIC function in different ways.

1.3.3 Side-specific Heterogeneities of VECs

Valve endothelial cells from the opposite sides of the valve are exposed to different types of flow and exhibit divergent gene expression. A transcription profile of *in situ* porcine VECs revealed 584 genes differentially expressed by VECs on the aortic aspect of the valve and those on ventricular aspect (Simmons *et al*., 2005). The disease-prone aortic side of the valve expressed less genes of inhibitors of calcification such as osteoprotegerin, C-type natriuretic peptide, parathyroid hormone and chordin, but compensated by higher expression of genes for antioxidants such as glutathione S-transferase and arachidonate 12-lipoxygenase as well as the oxidation related gene, endothelial NO synthase (NOS III). aVECs and vVECs have also been shown to have a differential expression of some endothelial markers; expression ratio of aortic over ventricular side VECs of connexin 43 and von Willibrand factor (vWF) were -1.72 and 1.60, respectively (Simmons *et al*., 2005).

Subsequently, mRNA microarray of cultured human aVECs and vVECs which were exposed to their physiological shear patterns has been reported (Holliday *et al*., 2011). Although there are differences among experimental settings, *in situ* verses *in vitro*, and species of animal models, mechanosensitive gene expression is conserved

between the 2 studies. More importantly, Holliday and colleagues identified side-specific micro RNAs (miRNA). miRNA-370 which has been reported to be up-regulated during EndoMT (Castilla *et al*., 2011) has increased expression in vVECs exposed to laminar flow when it was compared to aVECs exposed to laminar flow. This result suggested that miRNA-370 expression was not flow-dependent but, in fact, differentially expressed in a side-specific manner (Holliday *et al*., 2011). However the comparison between laminar and oscillatory shear stress effect on miRNA-370 expression by vVECs was not validated.

In addition to the gene array studies, *in situ* staining of Cx, a component of the gap junctions, have shown differentially expression by VECs from opposite sides of the valve (Inai *et al*., 2004). Cx37, Cx40, and Cx43 are differentially expressed in vascular ECs depending on where the ECs are in the vascular tree. Inai *et al.* reported 70-200-fold greater expression of Cx43 on the ventricular surface of the valve, but no difference in the expression of Cx37 and Cx40 of VECs on both sides. They also showed size-differences in VECs, with those on the ventricular surface being 35% to 65% smaller than those on aortic side.

Endothelial nitric oxide production is stimulated by fluid shear stress. Recent studies in human aortic valves revealed that VECs expressed NOS III enzyme in a side-specific manner, which is regulated by shear stress (Richards *et al*., 2013). In non-calcified valves, VECs on the ventricular side expressed significantly higher levels of NOS III than the cells on the aortic side. The expression of NOS III on calcified valves was also observed to have the same trend as cells on non-calcified valves. However NOS III expression of VECs on both sides of the calcified valves was significantly reduced, compared to normal valves (Richards *et al*., 2013). The production of NO by porcine aVECs and vVECs, exposed to their respective shear stresses or, kept under static conditions, was further investigated *in situ*. By using cGMP as a surrogate marker for NO production, the authors also confirmed higher

production of NO by vVECs compared to aVECs. These results were observed both from static valves and valves exposed to side-specific patterns of flow (Richards *et al*., 2013).

The observed heterogeneities of VECs from each side of the valve are possibly due to the effect of the different mechanical forces exposed to the cells. While the ventricular surface experiences high shear stress, the aortic surface of the valve is exposed to low shear disturbed flow. Sucosky *et al.* exposed 2 types of flow to VECs from both sides of the valve and revealed a different response of aVECs and vVECs to each pattern of flow (Sucosky *et al*., 2009). The authors revealed that expression of mediators involved in pathological inflammatory responses, VCAM-1, ICAM-1, TGF-β1 and BMP-4, were not changed on vVECs exposed to both physiological force (laminar shear) and altered shear stress (oscillatory shear). Surprisingly, aVECs had an increased expression of the same meadiators when they were exposed to laminar shear stress (Sucosky *et al*., 2009). This work suggests different intrinsic properties of aVECs and vVECs are independent of the flow patterns to which they are exposed. This property could lead to differences in the regulation of cells within the valve in terms of the production of ECM components - which are spatially distinct in different regions of the valve.

1.3.4 The extracellular matrix of the aortic valve and their functions

The ECM represents the key structural elements of heart valves. Up to 80% of total human valve proteins are ECM components which mainly comprises of collagen, GAGs and elastin (Bashey *et al*., 1967).

Collagen: Collagen constitutes about 43-55% of the human valve (Bashey *et al*., 1967). Collagen fibres are present in all 3 layers of the mature valves; however, they are most abundant and more organised in the fibrosa layer than those in ventricularis layer (Scott and Vesely, 1995). The collagen fibres in the leaflet predominantly consist of type I collagen and some type III collagen. They form large bundles in a tubular shape which align in a circumferential direction in the valve leaflet (Clark and Finke, 1974). The fibres have high stiffness but limited extensibility so that they can bear high loading pressure, but not strain. During diastole, the circumferential-aligned collagen maintains co-aptation by providing strength and stiffness to the valve (Sacks *et al*., 1998). Additionally, they adapt their structures by crimping and corrugation to minimize the stretching in the radial direction during systole (Figure 1.4)(Scott and Vesely, 1995).

Glycosaminoglycans (GAGs): GAGs are poly-anionic carbohydrates which have hydrophilic properties. They can absorb water and act like a sponge; hence the middle layer of the valve where they are located is termed the spongiosa. The complex carbohydrate structures are biochemically classified e.g. heparan sulphate (HS) or heparin, dermatan sulfate (DS) and chondroitin sulfate (CS). While DS and CS are the main GAG components in valve, HS represents approximately 20% of the GAGs in the aortic valve (Rothenburger *et al*., 2002). When sulfated GAGs interact with the non-sulfated GAG, hyaluronic acid (HA), they produce long polysaccharide chains in which enable them to bind water in quantities that are 1000 times greater than their volume (J.Koolman and K.H.Roehm, 2006). Due to their properties and location between the fibrosa and ventricularis, GAGs are believed to act as lubricator and shock absorber by dampening compressive forces and stress between the different layers of the cusp.

Elastin: Elastin comprises of 13% of dried weight in the human aortic valve (Bashey *et al*., 1967). Its fibres are a major component of the ventricularis layer and are

arranged in sheets. They are also present in scarce amounts in the fibrosa layer by forming a network around collagen bundles (Vesely, 1998). Elastin fibres align in the radial direction in the ventricularis and fibrosa, perpendicular to the circumferentially arranged collagen fibres. In contrast to collagen, elastin has high elastic properties but low stiffness. As such, elastin maintains collagen fibre orientation through its network around the collagen bundles and contracts to minimize cusp area and facilitates valve opening during systole (Figure 1.4)(Vesely, 1998).

The unique composition and structure of the individual layers of ECM result in the anisotropic behavior of the aortic valve leaflets, which has less mechanical stiffness (more compliant) in the radial direction than in the circumferential direction. Thus, the valve is capable of responding to the high strain, low stress in the radial direction, with minimal strain but significant pressure in the circumferential direction during every cardiac cycle, without permanent changes to the valve's structure (Billiar and Sacks, 2000; Yap *et al*., 2010). In addition to its load bearing role, the ECM also plays a key role in communicating with the cellular components of the valve to maintain ECM production and respond to chronic changes in hemodynamic pressures.

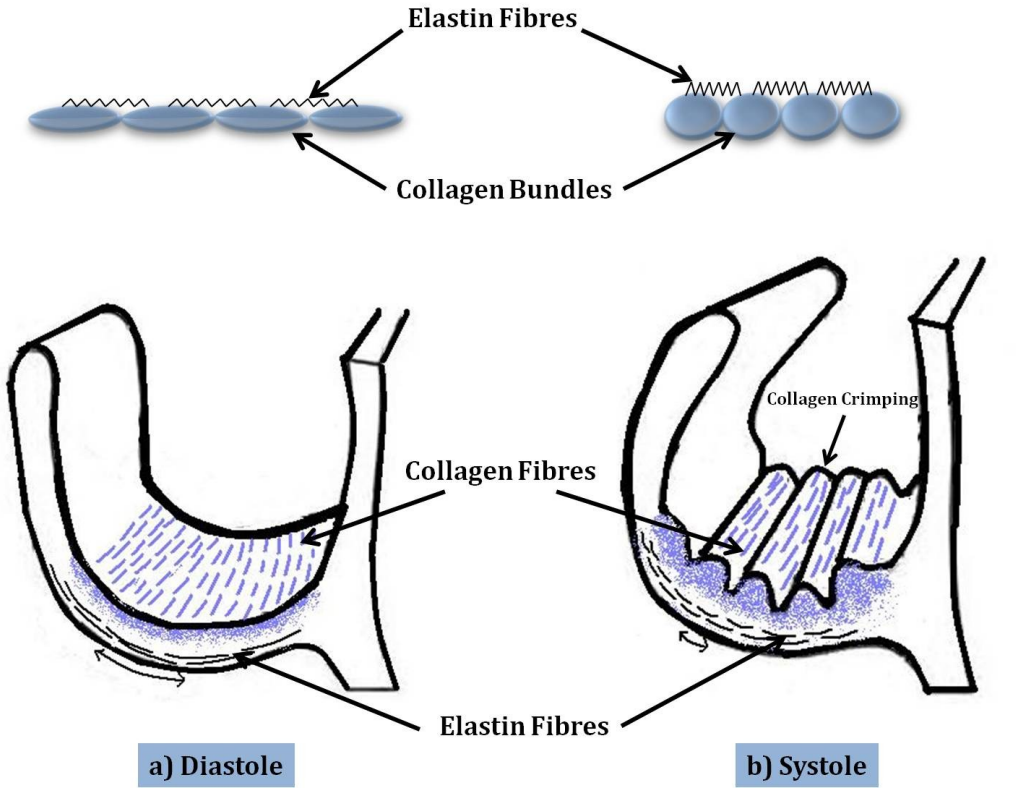


Figure 1.4 The intrefibre connection and configuration of collagen and elastin fibres inside an aortic valve cusp during a) diastole (on the left); and b) systole (on the right). Collagen bundles adapt their structures to create crimping and corrugation to reduce the stretchng in radial direction during systole whereas elastin is contracted to minimise to leaflet's area of the aortic valve . Adapted from Schoen F.J and Scott *et al*. (Schoen, 2008; Scott and Vesely, 1995)

Certain ECM components have been demonstrated to have the ability to maintain VIC phenotype in a quiescent fibroblastic state and modulate the production of TGF-β by VICs (Cushing *et al*., 2005). Porcine VICs cultured on the collagen-coated plates, significantly reduced the production of SMαA to 66% of that expressed by cells grown on tissue culture-treated polystyrene plates (TCP). Whereas VICs grown on a plate coated with heparin, a GAG component, increased the expression of SMαA to 258% of cells on untreated TCP. The addition of heparin, which can interact with several growth factors, also has the effect of enhancing the absorption of TGF-β on the culture plate and increases the production of TGF-β by VICs. Moreover, the

modulation of osteoblastic differentiation of VICs by ECM components has also been demonstrated (Rodriguez and Masters, 2009). Porcine VICs cultured on collagen and heparin coated plates have a significant reduction in cell contraction and form less calcified aggregates containing apoptotic cells. They also express less alkaline phosphatase (ALP), an osteogenic differentiation marker, when compared to the cells cultured on TCP plates (Rodriguez and Masters, 2009).

Interestingly, the elasticity of ECM can modulate the activation of VICs. In 2010, Kloxin *et al.* created a substrate with a gradient stiffness, by carrying out photodegradation at various time points. They illustrated that, on the same substrate, VICs on the higher stiffness area are activated and expressed higher amounts of SMαA than those on the lower stiffness area (Kloxin *et al*., 2010). The *in situ* modulation of substrate elasticity also confirmed that VICs are de-activated (expressed less SMαA) when the stiffness of the matrix was decreased locally.

Since the ECM serves to maintain valve function and regulate VIC phenotypes, continual regulation by synthesis and degradation is important to the valve's durability.

1.4 Regulation of the ECM

The ECM functions to allow the valve to withstand the cyclical nature of the changing loads of which it is exposed to during each cardiac cycle. The ECM is in continual contact with the cells within the valve and it therefore transduces the effect of these mechanical forces to the cells, which responding accordingly. The transduction of forces to the cells via the ECM is believed to result in further ECM production and expression of matrix remodeling enzymes by VICs (Mendelson and

Schoen, 2006). This allows the cells to constantly renew the ECM components in the aortic valve. In general, the homeostasis of valve ECM of the valve relies on a dynamic balance between synthesis and degradation.

1.4.1 Synthesis

There is evidence indicating that VICs have a secretory function. For example, they express soluble and insoluble matrix proteins, such as fibronectin and chondroitin sulphate, and the procollagen hydroxylating enzyme, prolyl 4-hydroxylase which is an essential enzyme for collagen synthesis (Messier, Jr. *et al*., 1994; Taylor *et al*., 2000).

Fibronectin is readily produced and localized adjacent to VICs in early passages of cultured cells. With time, the amount of fibronectin is dramatically increased in confluent cultures of VICs and is diffused throughout the culture. Likewise, chondroitin sulphate is also secreted in new cultures of VICs and continues in passaged cells. However, it appears to be less adherent to the VICs compared to fibronectin.

Prolyl 4-hydroxylase is also expressed by the VICs but restricted to spindle shaped cells, suggesting that it is produced by fibroblast-like VICs. Moreover, electron microscopic examination of VICs illustrated the presence of secretor organelles such as Golgi apparatus, rough endoplasmic recticulum and free polyribosome (Messier, Jr. *et al*., 1994). *In situ* detection of collagen synthesis is also observed in normal valves containing quiescent VICs and diseased valves containing activated VICs (Rabkin *et al*., 2001).

The elastic fibres, on the ventricular side of the valve, are composed of a variety of components including linking proteins, microfibrillar proteins (fibrillins) and the core protein elastin. The comprehensive study on the expression of fibrillin (FBN) genes which involve in elastogenesis was performed in developing and aging valve tissues (Votteler *et al*., 2013). FBN1 showed the different gene expression from the FBN2 and 3. The expression of the FBN1 gene and protein continued through all developmental stages, whereas FBN2 was mainly produced during fetal development and had a reduced production in postnatal life (Votteler *et al*., 2013).

1.4.2 Degradation

In the face of continued ECM production by VICs, the degradation of existing ECM is important to maintain the optimal ECM content of the valve and thereby valve durability. Proteolytic enzymes for ECM degradation are expressed by quiescent VICs and activated VICs, which were identified in normal and diseased mitral valves, respectively (Rabkin *et al*., 2001); for example, collagenases (MMP type 1 and 13) and gelatinases (MMP-2 and MMP-9). However, elastases (cathepsin S and K) were found only around activated VICs.

The expression and distribution of MMPs along with their inhibitors, TIMPs, are distinct among heart valves. While MMP-1, TIMP-1 and TIMP-2 are expressed by cells in all four heart valves, MMP-2 is only expressed in the aortic and pulmonary valve. However, MMP-3 and MMP-9 are not found in any heart valves (Dreger *et al*., 2002). Expression of MMP-1 is seen throughout the aortic valve while pulmonary valve positive cells are only observed in the belly and free edge areas. TIMP-1 is localized in the commissures regions of all valves except for the pulmonary valve,

where it is found only in the belly region. Unlike TIMP-1's specific distribution, TIMP-2 is found throughout the leaflets of all four valves (Dreger *et al*., 2002).

The expression of MMPs and TIMPs, in the study by Dreger *et al*., are predominantly localised around VICs. However, VICs were stained only with SMαA on separate tissue sections rather than true co-localisation with double-staining. MMPs and TIMPs are also found intermittently in endothelial cells in the valve (Dreger *et al*., 2002). Moreover, the living pulmonary autografts adapt to their new mechanical environment by increasing the production of MMP-13 (Rabkin-Aikawa *et al*., 2004a), indicating the potential role of changes in mechanical force mediating ECM remodeling by VICs.

1.4.3 Role of mechanical force

Aortic valves experience a myriad of complex mechanical forces during the cardiac cycle. For example, they experience stretch and transvalvular pressure during the diastole as well as the shear stress produced by blood flow during diastole and systole (Figure 1.5). Animal studies have shown a capability of valves to renew their ECM in response to these mechanical forces. Autoradiography has shown that the site of GAG and protein synthesis correlates to the distribution of the stress within the aortic valve (Schneider and Deck, 1981).

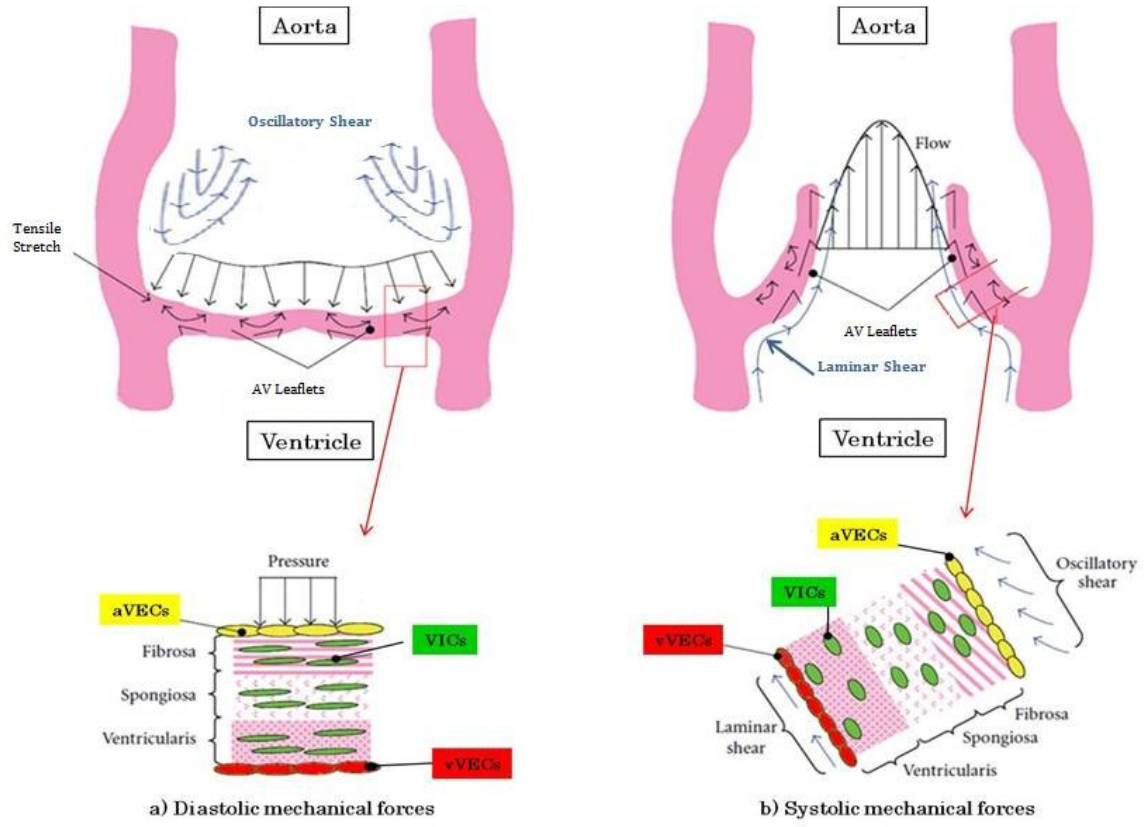


Figure 1.5 Mechanical forces during diastole and systole of an aortic valve. Heamodynamic generates various mechanical forces. Whereas the aortic valves experince stretch and transvalvular pressure during the diastole, oscillatory and laminar shear stress are produced by blood flow during diastole and systole Adapted from Balachandran *et al*., (Balachandran *et al*., 2011)

1.4.3.1 Stretch

The aortic valve is stretched both circumferentially and radially. During the diastole, the transvalvular pressure causes the valve cusps to extend and allow co-aptation to occur between the adjacent cusps. The cusps have a diastolic stretch ratio that is significantly higher in the radial than that in the circumferential direction (Yap *et al*., 2010). *Ex vivo* aortic valve leaflets that are stretched in circumferential a direction (by 15% elongation) induce ECM remodeling and the expression of contractile VIC phenotypes (Balachandran *et al*., 2006).

Colourimetric-quantitative methods revealed that cyclically stretched leaflets have increased collagen content, reduced levels of GAGs, but no change in elastin levels when compared to static valves. While the structure of ECM layers is preserved during stretch, immunohistochemistry (IHC) showed an increasing expression of SMαA in VICs on the ventricular side (Balachandran *et al*., 2006). Likewise, *in vitro* porcine VICs increase their total collagen production when subjected to a cyclic stretch using the Flexercell machine. VICs significantly enhance the production of both membrane-bound and secreted collagen when they are stretched by 14% for 2, 3, 4 and 5 days. This is associated with an increase in collagen III gene expression in the stretched samples (Ku *et al*., 2006).

1.4.3.2 Pressure

During normal diastole, transvalvular pressure, which is applied perpendicular to the leaflet area, varies from 80 mmHg to 120 mmHg. Collagen fibres in the fibrosa layer mainly take up this load as previously described. This cyclical pressure is considered to be one of the important mechanical forces that the cells respond to in secreting new matrix to maintaining the durability of the valve.

Ex vivo porcine aortic valves cultured in a pulsatile culture system, at physiological pressures (at 80-120 mmHg) and cardiac output (4.2 l/min), maintain their expression of ECM components (Konduri *et al*., 2005). The content of collagen, sulphated-GAGs and elastin are not significantly changed from those within the fresh valve leaflets. However, Konduri and colleagues also observed that GAGs and elastin content are significantly reduced in the samples kept under the static culture conditions.

The production of ECM in the aortic valve can be regulated by the magnitude of the pressure that the valve is exposed to, rather than frequency with which it changes (Xing *et al*., 2004). When the frequency was fixed at 70 cycles per minute (analogous to the average heart rate), increasing magnitudes of pressure induced increases in both collagen and sulphate-GAG synthesis. However, there is no proportional relationship between the magnitude of compression and the amount of ECM synthesis. As per the finding by Konduri *et al*, the tissues subjected to the physiological pressure (80-120 mmHg) maintain their collagen and GAGs production. Compared to constant pressure, cyclic pressure significantly enhances the production of sulfated GAGs in porcine valve leaflets; suggesting the physiological mechanical environment is important to the control ECM synthesis (Konduri *et al*., 2005).

Indeed, Merryman and colleagues have shown evidence of cellular responses to differing physiological pressure. The function of VICs to synthesise collagen and thereby the stiffness of the valve was compared between valvular cells which were isolated from the higher (left) and lower pressure (right) sides of the heart. A higher expression of HSP47 and SMαA is observed in VICs isolated from the higher transvalvular pressure side of the heart (Merryman *et al*., 2006). There was a correlation between collagen synthesis, HSP47, and SMαA expression. This relationship is maintained when VICs were either *in vitro* or *in situ*. Moreover, cellular stiffness of cultured VICs that are isolated from the left side of the heart were found to be significantly greater than those from the right sided heart valves (Merryman *et al*., 2006).

1.4.3.3 Shear Stress (Flow)

The aortic valve cusps have their surfaces exposed to two patterns of shear stress during the cardiac cycle. On the ventricular surface of the valve, the unidirectional pulsatile laminar shear stress occurs with high magnitude, ranging from 0 to 80 dyn/cm^2 (Weston *et al*., 1999). In contrast, the low magnitude bidirectional oscillatory shear stress generated on the aortic surface of the valve, ranges between -8 to +10 dyn/cm^2 (Figure 1.6)(Kilner *et al*., 2000; Sucosky *et al*., 2009) .

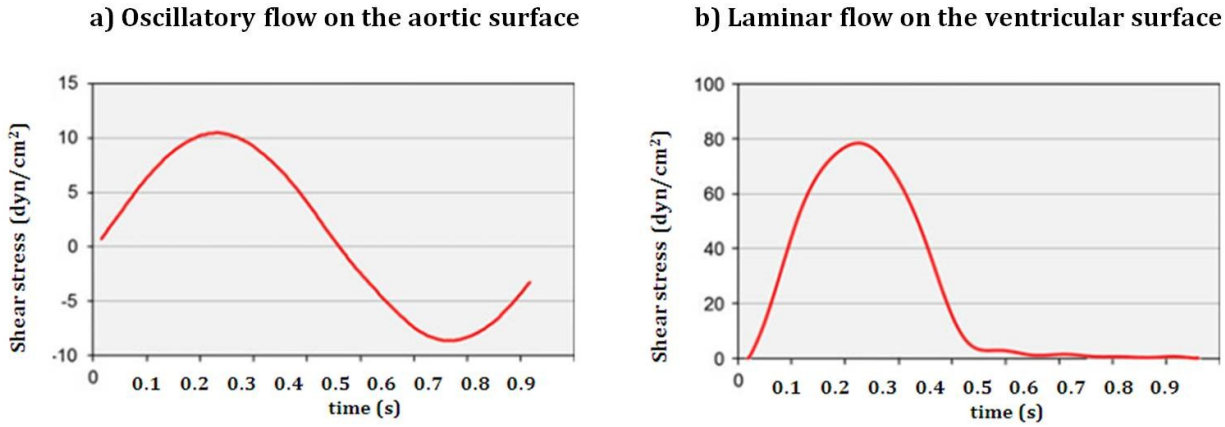


Figure 1.6 Native shear stress velocities experienced by the aortic valve. a) Oscillotory shear stress velocity experienced by aVECs on the aortic surface. b) Laminar shear stress velocity experienced by vVECs on the ventricular surface. Adapted from Sun *et al*. (Sun *et al*., 2011).

Ex vivo porcine valves were used to investigate the effect of shear stress on cell function. DNA synthesis, protein and GAGs production were increased during static incubation. Moreover, total protein synthesis by tissues exposed to steady flows at different magnitudes was significantly higher than seen in fresh tissue. Regardless of the magnitudes and dynamics of the applied shear stresses, tissue under flow conditions sustained its cellular proliferation and GAGs synthesis at a similar extent to fresh tissue (Weston and Yoganathan, 2001). These results illustrate that valve

cells responded to the flow by synthesising new proteins and retaining GAGs content similar to the native valve.

Further *ex vivo* experiments were recently reported in a review article (Balachandran *et al*., 2011). They investigated the effect of the valve endothelial cells and the shear stress on the production of collagen and GAGs. The endothelial cells were either intentionally removed from the valve (VEC-denuded valves) or kept intact. Both the VEC-denuded and intact valves were then exposed to laminar shear stress at ranges of magnitudes. In the endothelial-denuded valves, both collagen and GAGs were increased by the effect of shear stress. However, in the intact valve leaflets, collagen content and GAGs were not affected by shear stress (Balachandran *et al*., 2011).

These findings suggest that a "shielding effect" is provided by the VECs and the communication between VECs and VICs is important to maintain the balance of ECM production and degradation in the active valve.

1.5 Evidence of ECs Regulation on the ECM Production by Underlying Cells

Endothelial cells have been proven to sense mechanical forces and communicate with the underlying cells. While well characterised for their regulation of vascular tone and inflammation, endothelial cells have also been shown to be able to affect the composition of the ECM.

In 1981, Merrilees *et al.* seeded endothelial cells from the aorta on top of SMCs. The production of HA and sulphated GAGs is significantly increased when compared to

the culture of SMC only (Merrilees and Scott, 1981). When ECs are seeded on a porous membrane (Transwell®) and co-cultured with cardiac fibroblasts, there is increase in collagen synthesis by the fibroblasts by almost 2 fold compared to fibroblasts alone (Guarda *et al*., 1993). In contrast to Guarda's study, vascular ECs reduce the production of collagen by SMCs when they are seeded onto collagen type I or matrigel. The SMCs experience a 40% reduction in collagen synthesis and a 60% reduction of collagen type I gene expression when co-cultured with ECs (Powell *et al*., 1997).

The expression of collagen type VIII, MMP-13, TIMP-3 mRNA by human hepatoma cell lines is increased when they are co-cultured with bovine pulmonary ECs, as compared to hepatoma cells alone (Ohno *et al*., 2009). More recently, the synthesis of collagen is reduced when venous ECs were grown onto 3-D fibrin gels containing myofibroblasts (Pullens *et al*., 2009).

Molecules released by ECs also show an effect on the production of ECM. ET-1, an endothelium-derived peptide, affects vascular SMC function by increasing collagen type I production (Rizvi *et al*., 1996). Similarly co-culture of vascular SMC and coronary artery endothelial cells increases the production of collagen type I but decreases synthesis of collagen type III. However, when NO production by ECs was inhibited, both collagen type I and III are increased (Myers and Tanner, 1998).

The function of ECs in the regulation of ECM production has been analysed in porcine VICs that are encapsulated in a collagen hydrogel and seeded with porcine VECs on the surface of the hydrogel. The 3-D construct was then either exposed to a 20 dyn/cm^2 laminar shear stress or kept under static conditions. The presence of VECs decreases the loss of GAGs in the hydrogel under both static and conditions of flow (Butcher and Nerem, 2006). VICs also become less activated when they were co-cultured with VECs.

VECs have been demonstrated to have a role in the regulation of the mechanical and biological integrity of the valve (Butcher and Nerem, 2006; El-Hamamsy *et al*., 2009; Richards *et al*., 2013), as previously discussed. Changes in the function of these cells may contribute to the disease process that results in the valve becoming calcified.

1.6 Pathogenesis of the aortic valve disease and Therapy

Aortic valve disease was originally believed to develop over time as a consequence of continuous exposure to hemodynamic and mechanical forces. However recent evidences imply an active progression involving the mechanisms of inflammation, apoptotic and osteogenic calcification (Rajamannan *et al*., 2003; Lee and Chou, 1998; Tanaka *et al*., 2005). These mechanisms compose of responses by valvular cells at molecular levels.

1.6.1 Cellular and molecular mechanism

Activated VICs which express SMαA are believed to differentiate into osteoblast-like cells and responsible for the accumulation of calcium and formation of calcified nodules in diseased valves (Osman *et al*., 2006; Mohler, III *et al*., 2001; Aikawa *et al*., 2007), where as VECs may contribute to valve disease by expressing molecules that can both promote and protect calcification.

Analysis of gene expression by side-specific VECs revealed that aVECs express genes associated with valve calcification at greater amount than vVECs (Simmons *et al*., 2005). In contrast, Kruppel-like factor2, which is expected to down regulate calcification, was increased by VECs subjected ventricular flow or the surrogate of

vVECs (Weinberg *et al*.,). Similarly, atheroprotective mediator like NOS III is greater increased by vVECs (Richards *et al*., 2013). Dysfunction or imbalance of the function of VECs is believed to be associated with aortic valve calcification.

Inflammatory

The theory that valve calcification is mediated through an inflammatory condition is supported by the presence of inflammatory cells, macrophages and T-cells within the cusps (Muller *et al*., 2000; Aikawa *et al*., 2007; Otto *et al*., 1994). Eventually, VICs adopt osteoblastic phenotypes by the activation of cytokines produced by the inflammatory cells e.g. TGF-β1 and osteopontin (Rajamannan *et al*., 2003; O'Brien *et al*., 1995).

Apoptosis

Apoptotic bodies in the aortic valve are considered as a source of calcium and lead to the formation of calcium nodules in calcified valves (Jian *et al*., 2003; Clark-Greuel *et al*., 2007). The apoptosis-dependent calcification is also believed to be stimulated through TGF-β1, under SMAD-dependent pathway (Jian *et al*., 2003; Clark-Greuel *et al*., 2007).

Osteogenic calcification

The expression of osteoblast markers and a mineralisation of the matrix is stimulated by potential mediators including TGF-β1, BMP 2, Wnt, β-catenin and RANK. The mechanism of these mediators is focused on the regulation of an osteoblast transcription factor, RUNX2 (Chester, 2014).

Whereas TGF-β1 may contribute to osteogenic calcification by directly transdifferentiating VICs into osteoblast-like cells (Osman *et al*., 2006; Clark-Greuel *et al*., 2007; Kennedy *et al*., 2009), BMP2 implicates bone formation and

calcification in aortic valves through Wnt pathway (Hruska *et al*., 2005). The binding of Wnt to its receptor and co-receptor increases level of β-catenin. This eventually activates the translocation of β-catenin into nucleus to form an active enhancer of RUNX2 (Pandur *et al*., 2002).

The binding of RANK (receptor activator of NF- ϰB) and its ligand directly increases DNA binding of RUNX2 (Kaden *et al*., 2004). Subsequently, osteoclastogenesis is stimulated and extracellular calcium concentration is increased.

1.6.2 Therapy

At the present, the treatment for valvular calcification remains the surgical replacement with mechanical or bioprosthetic implants (Baxley, 1994). The procedures taken for valve replacements account for 22% of all cardiac surgeries (Iung and Vahanian, 2011) in which aortic valve replacements are by far the most among those valve procedures.

Although surgical valve replacement is the only promising treatment, all valve substitutes have limitations; for example, calcification of the bioprosthetic and requirement of anti-coagulants for the patients who have mechanical implants. More importantly, the importance of living cells in the valve substitutes has been highlighted (El-Hamamsy *et al*., 2010) which has stimulated the need for a valve that contains living cells. Tissue engineering of heart valves (TEHV) has the capacity to produce such a valve in the laboratory and is considered as an alternative solution for valve replacement. Suitable cells are required to regenerate a living functional heart valve and maintain the durability of engineered valve, similar to native aortic valves.

1.7 Hypothesis

It is well known that collagen, GAGs and elastin, are stratified into 3 layers inside the aortic valve, and that specific distribution is important to valve structure and function. Moreover ECM remodeling can be regulated by mechanical forces and cell to cell communication. However, there have been no studies that have investigated which factors regulate ECM production by VICs. In order to address this question, VECs phenotypes from the opposite sides of the aortic valve and the effect of side-specific patterns of flow are necessary to be understood. The informative data could help to choose an appropriate cell type to re-create the valve endothelial function on a tissue-engineered valve, in order to ensure that the engineered valve can maintain a similar ECM content, and in turn the durability associated with native valves.

I hypothesise that, VECs are able to modulate the ECM components produced by VICs in the aortic valves. Passive release of mediators and release in response to the specific patterns of flow that are experienced by the aortic and ventricular surfaces of the aortic valve may both contribute to this effect. Moreover, the information of the response to particular patterns of shear stress could be used to derive stem cells into side-specific VECs. The differentiated stem cells can serve as a potential source to endothelialise a tissue engineered heart valve.

1.8 Aims

The specific aims of this works were:

1. To establish a robust and repeatable method for isolation and culture of VECs from each side of the valve and compare the phenotypic characteristics of EC from the aortic and ventricular surfaces

2. To investigate the effect of stable mediators released by aVECs and vVEC on VIC function with respect to:

   Cell proliferation

   Collagen synthesis

   Collagen Cross linking

   GAGs synthesis

   Elastin synthesis and cross-linking

3. To examine the effect of labile mediators produced by aVECs and vVECs in a non-contact co-culture with VICs on their ability to proliferate and produce ECM components

4. To determine the effect of specific flow patterns on the functional effects of VECs on the ECM homeostasis in the aortic valve cusps

5. To evaluate the suitability of adipose derived stem cells for use as a valve endothelial cells in tissue engineered valves.

CHAPTER 2 Side-Specific Valve Endothelial Cells

2.1 Background

While endothelial cells share basic functions in the control of vasomotor tone, permeability, innate and adaptive immunity, there is considerable heterogeneity among endothelial cells from different anatomical locations and organs throughout the body (Aird, 2007). The EC heterogeneity results from the interaction of ECs with their microenvironment including cytokines, paracrine factors, growth factors, cells, ECM and mechanical forces. Moreover, certain "site" specific phenotypes of ECs are epigenetically programmed and transmitted during mitosis which is independent of the extracellular factors (Aird, 2007).

For example, the distinct transcription profiles of multiply passaged ECs were established from 14 different sites of the human vasculature (Chi *et al*., 2003). ECs from large vessels expressed genes involving in the synthesis of ECM e.g. fibronectin and collagen type V. In contrast, ECs from micro-vessels transcribed genes for basement membranes and the ECM binding proteins such as laminin and integrins. ECs which were isolated from skin expressed fibroblast growth factor and cholesterol-synthesis genes, whereas ECs from lung tissue expressed the gene encoding an enzyme for surfactant secretion. These results suggest that the distinct expression patterns of ECs, referring to their specialised function of the respective tissue, are preserved thorough the generations in cell culture.

In addition to gene expression, the distinct barrier function of ECs from different sites persisted in several passaged cells (Kelly *et al*., 1998). *In vitro* pulmonary microvascular ECs form smaller intercellular gaps than pulmonary artery ECs

(similar to *in situ* observation) and are correspondingly less permeable to large molecules (72 kMW). A conclusive study for the dual roles of microenvironments and epigenetics was investigated by comparing gene expression profiles of *in situ* ECs to those of *in vitro* ECs. The functional expression profiles of ECs isolated from tonsils showed that 37 genes were maintained *in vitro* after culturing, but 23 genes were down regulated.

The heterogeneous phenotypes of VECs from the aortic and ventricular surface have also been established. As mentioned earlier, DNA microarray of *in situ* aVECs and vVECs revealed differential expression of 584 genes (Simmons *et al*., 2005). Moreover, the differential production of Cx43 (Inai *et al*., 2004) and NOS III (Richards *et al*., 2013) was exhibited on the aortic valve surfaces by immunostaining. It is well established that, during the cardiac cycle, aVECs experience different mechanical shear stress from the vVECs and, in turn, the different microenvironment are considered to be the factor that determines the unique characteristics of EC on each side of the valve. However, whether VECs from the opposite surfaces maintain their distinct phenotypes in cultures (in the same environment) has not been investigated yet.

In order to pursue the heterogeneity of side-specific VEC phenotypes *in vitro*, a technique to isolate pure populations of VECs from the opposite surfaces independently is required. Additionally, VEC phenotypes *in situ* are investigated in comparison to the VEC phenotypes *in vitro* by immunostaining and western blotting. Lastly, the secretory function of side-specific VECs to release cytokines will be examined.

2.2 Materials and Methods

2.2.1 Porcine hearts

Porcine hearts (24-36 months old) were obtained from a commercial slaughterhouse (Cheale Meats, Essex, United Kingdom). The Hearts were immediately placed in fresh ice-cold Hanks solution after their removal from the sacrificed animals and transported to the laboratory within 4 hours; 4-6 hearts were processed at a time Aortic valve leaflets were dissected under sterile conditions in a laminar flow hood. During the process, the cusps were handled with fine forceps to avoid damaging the endothelial cells on the valve surfaces. The dissected cusps were then washed with phosphate-buffered saline (PBS) twice. Up until being used, all 3 aortic cusps from each pig were kept in PBS separately to avoid mixing between individual animals. Specific sides, aortic and ventricular, of the excised valves were identified by the fact that the aortic surface appears rough while the ventricular surface is smoother. In order to obtain 3 types of cells (VICs, aVECs and vVECs), from the same pig, one leaflet was used to isolate each cell type from each valve used.

2.2.2 Aortic and Ventricular VECs isolation and culture

Since aVECs and vVECs are populated as monolayers on the opposite sides of the valve, a specific protocol was developed to selectively isolate each cell type. In order to obtain and grow aVECs and vVECs from the same animal, a number of isolation protocols were tested, for example, enzyme absorbed filter papers, scraping and varying times of enzyme digestion. A successful method was devised by adapting an enzymatic digestion and clonal expansion protocols. Briefly, aVECs and vVECs were

isolated from a separate leaflet (of the same animal) by exposing one surface of the leaflet to the enzyme, as depicted below (Figure 2.1).

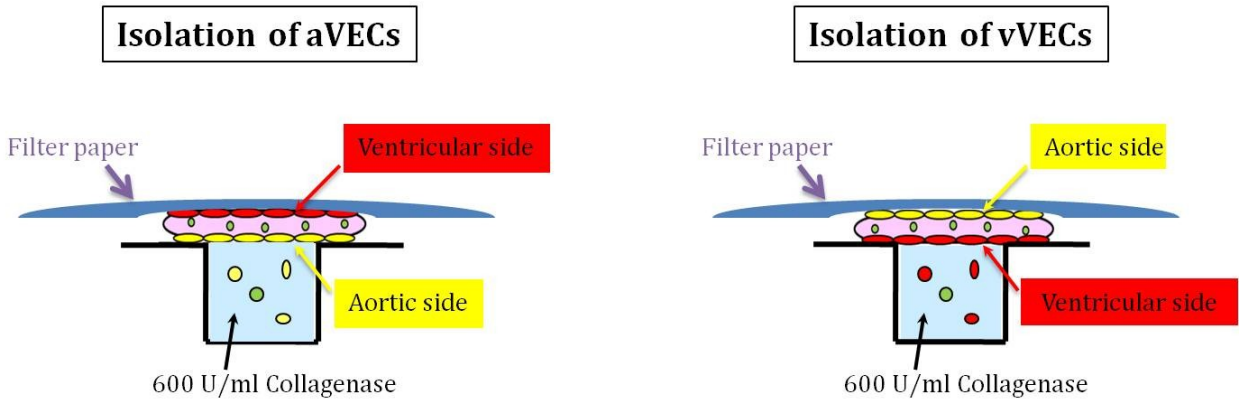


Figure 2.1 Schematic picture of cell isolation mehthod. Either aortic or ventricular surface of the cusps was exposed to the collagenase enzyme to isolate either aVECs or vVECs, whereas another cusp was removed VECs and isolated VICs.

2.2.2.1 Enzyme digestion

In order to isolate a particular type of VECs, the valve cusps were stretched on filter paper making sure that the side from which cells were to be isolated was facing upwards. These tissues were then exposed to 600 unit/ml of Collagenase II (Sigma-Aldrich, UK) contained in separate wells of a custom-made plate (used for the Cone-and-Plate machine, see Chapter 5.3). The cusps were subsequently fixed in position by screwing a lid (Figure 2.2) over the discs and incubating at various conditions e.g. incubation times (at 10, 20, 30 and 40 minutes) and rotating speeds (at 150 and 170 revolutions per minute (RPM)) to optimize the isolation conditions.

Side-specific VECs were successfully isolated at 37° C incubation for 30 minutes and by 170 RPM of shaking by an orbital shaker. After enzyme incubation, VECs were

physically dispatched from monolayer by vortexing the plate at moderate vigor for 1 minute. In order to stop digestion process, aVECs and vVECs solution in the wells was transfered to plates containing Endothelial Growth Media 2 (EGM2) (Promocell, UK) supplemented with 20% fetal bovine serum (FBS).

2.2.2.2 VECs purification (colony expansion) and culture

Primary VECs were cultured for 5-7 days as to achieve enough cells to be able to differentiate them from contaminating VICs by morphology. Cobblestone-like colonies were selected under microscope and covered with cloning cylinders (Sigma-Aldrich, UK; Figure 2.2) while the colonies that had spindle and elongated shape cells growing nearby were discarded since these cells were most likely VICs. To prevent a subsequent leaking of solution from the cylinders, sterile grease (Sigma-Aldrich, UK) was applied at the bottom and pressed tightly on the culture plates. VECs were isolated inside the cylinders by 50 µl of Trypsin (SAFC Bioscience, UK) and incubated at 37°C for 5 minutes. To facilitate the growth of VECs, it is important to expand cells to the right degree of confluence, such that each colony of primary VECs was initially passaged into a single well of 24-well plate, without mixing between colonies. Once reaching confluence, the VECs was serially passaged to a well of 12-well plate, a well of 6-well plate and so on.

VECs were cultured with EGM2 media (which contained 0.02 ml/ml Fetal calf serum, 5 ng/ml Epidermal growth factor, 10 ng/ml Basic fibroblast growth factor, 20 ng/ml Insulin-like growth factor, 0.5 ng/ml Vascular endothelial growth factor, 1 µg/ml Ascorbic acid, 22.5 µg/ml Heparin and 0.2 µg/ml Hydrocortisone) (Promocell, UK) and supplemented with 20% FBS (GE Healthcare, UK), 100 U/mL (1%) penicillin (Life Technologies, UK), 100 µmol/L (1%) streptomycin (Life

Technologies, UK) and 4 mmol/L (1%) L-glutamine (Invitrogen, UK). Before culturing, all VEC plates were coated with attachment factor containing 0.1% gelatin (Invitrogen, UK) at 37° C for 30 minutes and then maintained at a 37° C in an incubator gassed with (95% air/5% CO_2). VECs were expanded and used up to the 4^{th} or 5^{th} passage.

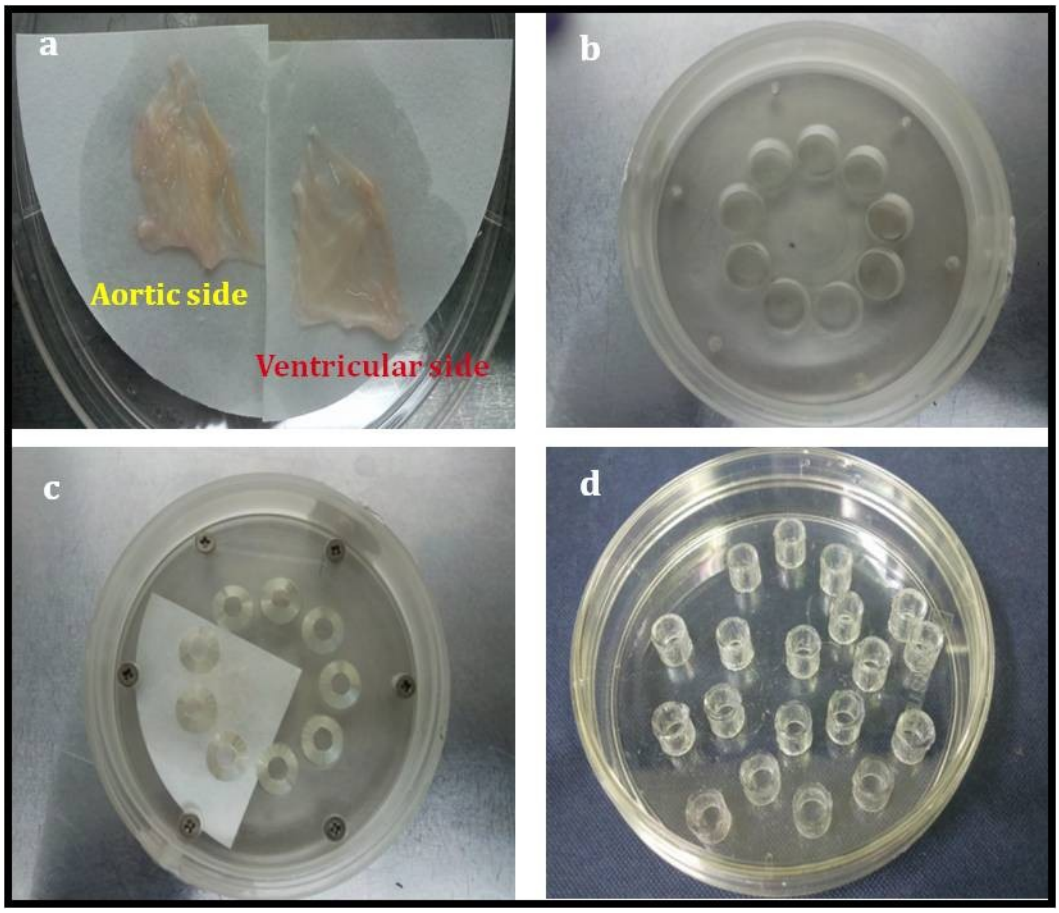


Figure 2.2 Materials for VECs isolation. a) valve leaflets on filter papers for aVECs and vVECs isolation on the left and right hand respectively, b) the plate composed of wells containing collagenease enzyme, c) particular side of valve put over the well, fixed by the screwed lid and incubated with enzyme in the well, d) cylindrical disk to cover cobblestone-liked VEC colony and contain Trypsin solution.

2.2.2.3 Scanning Electron Microscopy

VEC isolation method was validated using a scanning electron microscopy (SEM). After VEC isolation was finished, the valve cusps were pinned on a waxed plate containing 2.5% glutaraldehyde (Sigma-Aldrich, UK) to preserve structure of the valve and the remaining cellular components. The fixed valves were washed with 0.1M phosphate buffer two times. The specimens were then post fixed with 1% osmium tetroxide (Agar Scientific ltd., UK) in 0.1M phosphate buffer for 1 hour followed by 2 times washing with the buffer. The specimens were then incubated with 1% Tannic acid (Sigma-Aldrich, UK) in 0.1M phosphate buffer for 1 hour and washed again with the buffer twice. They were dehydrated by immersing the specimens in an ascending series of ethanol solutions starting from 25% up to 100%. They were then dried in hexamethyldisilazane (Agar Scientific ltd., UK) and then air-dried. Lastly the specimens were mounted on Stubbs with Acheson Silver Dag (Agar Scientific ltd., UK) and viewed in JEOL 6010 SEM microscope (Jeol, Japan). The cusps were examined by with the SEM, which was kindly completed by Dr. Padmini Sarathchandra.

2.2.3 VIC isolation and culture

In order to obtain pure VICs from aortic valve cusps, VECs from both sides of the valve leaflet were removed by incubation with 600 unit/ml collagenase II at 37° C in a water bath while being vigorously shaken for 15 minutes. The leaflet was then removed from the enzyme solution and washed with PBS twice under sterile conditions. It was then cut into small pieces by a sterile blade to increase the accessibility of the enzyme to digest the tissue and facilitate releasing of the VICs from the ECM of the valves. To obtain VICs, the pieces of the leaflet were transferred

to a new falcon tube containing fresh 600 unit/ml collagenase II in PBS and digested at 37° C in a water bath for further 30-45 minutes. After enzymatic digestion, 1 volume of DMEM (Sigma-Aldrich, UK) containing 10% serum was added to stop the reaction and the residual tissue was removed by filtering through a cell strainer with a mesh size of 100 µm (vWR, UK). Cells were collected by centrifugation at 700 g for 5 minutes. VIC pellet was resuspended and grown in DMEM with 10% FBS in a T25 flask (Sarstedt, USA). After 2 hours of incubation, floating cells were removed by changing the media. Once VICs were confluent, they were dislodged by incubating cells with 1% Trypsin solution for 3 minutes. VICs were cultured in a bigger, T75 flask, and used up until passage 4-6.

2.2.4 Cell phenotyping by immunocytochemistry

VECs were not only identified by morphology but also immunocytochemistry (ICC) at passage 2, 3, and 4 or the passage before used, for further experiments. Only cells that showed positive staining for endothelial cell markers such as CD31, vWF and VE-Cadherin, but were completely negative for SMαA were considered as pure endothelial cell colonies. To perform ICH, VECs were seeded on gelatin coated 13 mm coverslips (Thermo Scientific, UK) and cultured overnight in an incubator to allow the cells to attach on the coverslips. Before the staining, cells on coverslips were washed with PBS 3 times for 3-5 minutes each and fixed with cold acetone for 10 minutes. Cells were then blocked with 3% bovine serum albumin (BSA) in PBS (known as blocking solution) for 30 minutes at room temperature to prevent non-specific staining. Primary antibodies were applied at appropriate concentration in 1.5% BSA in PBS (1:100 dilution of CD31-FITC (Mouse Anti-Porcine, Serotec, UK), 1:400 dilution of vWF (Rabbit Anti-Human, Dako, Denmark), 1:200 dilution of VE-Cadherin (Rabbit Anti-Human, Cell Signaling, UK) and 1:200 dilution of SMα-A

(Mouse Anti-Human, Dako, Denmark) and incubated at room temperature for 1 hour. A negative control was incubated with blocking solution instead of the primary antibody. Excess primary antibodies were removed by washing with PBS 3 times for 3-5 minutes each, before applying the secondary antibody. AlexaFluor 488 Goat Anti-Mouse (Invitrogen, UK) and/or AlexaFluor 594 Goat Anti-Rabbit (Molecular Probes, UK) were incubated with cells at 1:1000 dilution in 2% normal goat serum at room temperature for 1 hour. Non-bound secondary antibodies were washed twice with PBS containing 1:20000 dilution of DAPI (Sigma-Aldrich, UK) for 10 minutes. Concurrently, nuclei were stained with DAPI during the washing. Coverslips were blotted to dry with tissue papers and mounted with Permaflour (Beckman Coulter, UK) on glass slides (Thermo-Scientific, UK). The cells were observed using a fluorescent microscope (Nikon, Japan).

2.2.5 Cell phenotyping of *in situ* side-specific VECs by immunohistochemistry

In order to understand phenotypes of valve endothelial cells, intact VECs on the fresh valves were also observed for EC markers by IHC technique and compared to the cells isolated by side-specific method described above.

After aortic valves were dissected from sacrificed pigs, each leaflet of the valves was cut into 2 pieces; 1 was stained on the aortic side and another on the ventricular side. Tissues were then held in place by pins on a wax plate, with the side to be studied facing upward. Briefly, every tissue section was fixed with 4% PFA, permeabilized by 0.5% Tritron X in PBS for 3 minutes, non-specific staining was blocked with 3% BSA in PBS for 30 minutes and immunostaining performed using the same methodology as section 2.2.4. Following staining, tissues were moved to

cavity slides, with the side to be observed facing upwards to create a flat surface. Fluorescent staining was fixed by Permaflour and covered with coverslips. The IHC was observed and captured pictures by the confocal microscope (Zeiss, Germany).

2.2.6 Proliferation of side-specific VECs by MTS assay

The proliferation rate of aVECs and vVECs after certain incubation times and media were investigated. It was important to seed a sub-confluent number of cells to avoid contact inhibition of cell growth. Thus aVECs and vVECs isolated from 4 animals were seeded on a gelatin pre-coated 96 well plate at 2000 cells per well. VECs were maintained in complete EGM2 media in the CO_2 incubator overnight to allow attachment to the wells. Triplicates of each cell isolate were changed to 0.4% FBS EGM2 media and used as a control. Another set of triplicates was refreshed with EGM2 with 20% FBS. VECs were cultured in the CO_2 incubator for 48 hours before the proliferation was observed by using CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS assay; Promega, UK).

The proliferation assay is composed of tetrazolium compound (MTS) and an electron coupling reagent (PMS). The complex solution of MTS and PMS was prepared freshly under sterile condition before being used. By gently mixing 2.0 ml of MTS solution with 100 µl of PMS, MTS/PMS solution was ready to be used at 20 µl per well of 96-well plate. During the reaction, MTS is converted to UV-absorbable formaman product (absorption at 490 nm) by dehydrogenase enzymes found in metabolically active cells. Consequently, it is important to keep cells alive while the reaction is performed by preparing 20 µl of MTS/PMS solution in 100 µl of culture media for each well.

At the start of the MTS assay, media over aVECs and vVECs were aspirated. It is unnecessary to wash cells with PBS in order to prevent cell removal during washing. 120 µl of MTS/PMS complex in EGM2 was added to every well. To avoid light exposure, the plate was wrapped with foil and the reaction was carried out at 37°C, in a CO_2 incubator for 45 minutes. The amount of living cells was calculated from the relative amount of formaman products by measuring the absorption at 490 nm, using a microplate reader (Bio-Tek Instruments INC, USA).

2.2.7 Isolation and protein extraction of side-specific VECs from fresh tissue

Immediately after the porcine aortic valves were dissected and washed with PBS, VECs from the aortic and ventricular side were obtained on separate slides by snap frozen method. Protein extraction was then performed to obtain protein for western blot analysis.

To prepare for the snap frozen isolation, slides (Superfrost® Plus, Thermo Scientific, UK) were kept on dry-ice, for at least 15 minutes. Two frozen slides were used per valve leaflet. While one frozen slide was placed in contact with VECs on the aortic side, another slide was laid on the ventricular side, like a sandwich. It was made sure that the leaflet was not folded when placed between the 2 slides. Both frozen slides were then pressed, resulting in VECs attachment on them. They were then moved to the area that had not been contacted with the valve, and the extraction of VECs repeated for minimum 3 times per a valve leaflet.

VECs on the slides were then collected into an eppendorf for protein extraction. 5 µl of protein extraction buffer (RIPA buffer) which was prepared before used was

added on the slide. The solution containing VECs were swept and pooled, in the middle of the slide, using a cell scraper (Sarstedt, USA). The solution was then transferred by pipetting into a 1.5 ml microtube (VWR, UK) which contained another 5 µl of RIPA buffer. Protein extraction by the reaction of RIPA buffer was performed on ice for 30 minutes time of incubation. The samples were then vigorously vortexed at regular intervals. The amount of protein of each sample was measured by BSA based assay as described below.

Protein extraction buffer was prepared before used by mixing 10 ml of RIPA buffer or Radio Immuno Precipitation Assay buffer (Sigma-Aldrich, UK) with 1 tablet of protease K inhibitor (PKI, cOmplete Mini, EDTE-Free, Roche, USA).

2.2.8 Western blot

To investigate the heterogeneity between aVEC and vVECs, endothelial markers and other proteins were quantitatively compared with Western blotting technique which has been developed by members of the lab, Dr Paul Riem Vis and Dr. Najma Latif. Proteins extracts from VECs isolated and/or cultured (passage 4-5) from the aortic and ventricular sides of fresh aortic valves were separated by size in polyacrylamide gel. They were then transferred to a nitrocellulose membrane to be stained with specific antibodies. The proteins of interest were detected by chemiluminescence and the bands developed on a film; the bands intensity which equates to the level of expression was assessed and normalised with the level of expression of a house-keeping protein, either GAPDH (38 kDa) or eEF2 (95 kDa), depending on their sizes which should not be similar to a size of any interested proteins. The expression of interested proteins between aortic and ventricular cells was then compared.

2.2.8.1 Protein extraction and concentration

Total protein from aVECs and vVECs isolated from fresh tissue were extracted by RIPA buffer as explained in the section 2.2.7. For proteins extracted from cultured VECs *in vitro*, cells at passage 5 in a T25 flask were washed with PBS twice before 30 µl of RIPA + PKI was added. Cells were then scraped off the flask and collected into 1.5 ml microtubes. The extraction incubation was carried on ice for 30 minutes with vortex at intervals. The debris was then removed by spinning the microtubes at for 16060 g 5 minutes. The supernatant which contained total proteins was stored at -20°C if the quantification was not performed immediately.

All samples were measured for the concentration of protein by Bicinchoninic acid (BCA) assay using bovine serum albumin (BSA) as a standard. To make a calibration curve, BSA was prepared in triplicates at different concentrations, 0.50, 0.40, 0.30, 0.20, 0.15, 0.10 and 0.05 µg/µl, at a total volume at 50 µl, in a 96-well plate. Samples were mixed with distilled water at the appropriate dilution in duplicate, to ensure that the samples to be tested would fall within the range of the standard curve. 50 µl of water was used as blank. 50 µl of standards, samples and the blank were then mixed with 200 µl of the mixture of $CuSO_4$ (Sigma-Aldrich, UK) and BCA (Sigma-Aldrich, UK) at 1:50 ratio. Protein in the reaction, which contained peptide bonds as well as certain amino acids e.g. tryptophan, tyrosine and cysteine, reduced Cu^{2+} into Cu^{+}. The reduced copper was then able to chelate with BCA and formed a purple complex. To allow such reaction to occur, the 96-well plate was incubated at 37 °C for 30 minutes and wrapped with light protection foil. The absorbance of the purple complex at 562 nm was measured in each sample after finishing the incubation using a microplate reader (Bio-Tek Instruments INC, USA). The actual

concentration of protein samples was later calculated from the standard curve and their dilution factor.

2.2.8.2 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins at different molecular weights, i.e. different length of polypeptides, were separated by their abilities to move within a polyacrylamide gel when an electrical current was applied. The positioning of the protein in the gel is dependent upon its size. To achieve this, proteins were mixed with SDS which is an anion detergent to denature secondary and non-disulfide-linked tertiary structure of proteins as well as to apply negative charge to the proteins proportionally to their polypeptide length/size. SDS-coated proteins were then able to move towards the anode when the electrical current was supplied. In a certain percentage of polyacrylamide gel, the longer polypeptide proteins migrated slower than the smaller proteins.

To denature tertiary structure of proteins, they were additionally mixed with dithiothourseitol (DTT) (Sigma-Aldrich, UK) and NuPAGE LDS Running Buffer (Life Technologies, UK), containing not only SDS but also lithium dodecyl sulfate at a pH of 8.4, to reduce the disulfide bonds. Loading volume was prepared at 20 µl for either 10 or 20 µg of total protein from each sample. 4 µL of Rainbow Marker (GE Health, UK) was also prepared in the loading buffer to be used as a molecular weight reference. All samples and markers were heated at 70^0C for 10 minutes before being quickly spun down and loaded on to polyacrylamide gels.

Most of proteins of interest had a size in the range of 14-200 kDa and were separated in 10% NuPAGE Bis-Tris gels (Life Technologies, UK) and NuPAGE MOS SDS Running Buffer (Life Technologies, UK). When vWF(250 kDa for monomer)

was investigated, 3-8% NuPAGE Tris-Acetate gel (Life Technologies, UK) and NuPAGE Tris-Acetate SDS Rnning Buffer (Life Technologies, UK) were used. The gels were prepared with the proper buffer in the XCell Surelock™ (Invitrogen, UK). Denatured samples were loaded into each well and run at 200 V using a Power PAC 300 (Bio-Rad, UK) power supply. The current was stopped at the appropriate time to obtain protein separation referring to the separation of the Rainbow markers.

2.2.8.3 Blotting

Following SDS-PAGE, proteins within the gels were then transferred by an electroblotting method onto a nitrocellulose membrane (GE Health, UK) to allow the detection of specific proteins by immunostaining.

Transfer buffer was prepared by mixing NuPAGE Transfer Buffer (Life Technologies, UK) with 20% Methanol (10% methanol was prepared for large protein blotting) and distilled water. Nitrocellulose membranes and filter papers were cut into appropriate size and soaked in transfer buffer along with sponges which were squashed firmly in the buffer to remove all bubbles. The blotting stack was then assembled on the XCell II™ Blot Module (Invitrogen, UK). On the cathode core, 2 sponges were placed, followed by 2 filter papers, gel, nitrocellulose membrane, 2 filter papers and finally 4 sponges. In cases of transferring 2 gels, 1 sponge was placed to separate from the first gel stack, followed by 2 filter papers, another polyacrylamide gel, nitrocellulose membrane, 2 filter papers and finally 2 sponges. The anode core was put on top and the assembled transfer stack was loaded into the blot module. It was necessary to ensure that nitrocellulose membrane was placed between gel and the anode to allow proteins driven by the buffer to move from cathode to anode (the same direction as the current). Transfer

Buffer was then added to soak the stack between the cathode and anode cores while distilled water was filled up outside the stack to prevent overheating. Gels were blotted at 30 V for 80 minutes.

To monitor the transfer efficiency, the membrane was stained with water soluble dye, Ponceau S (Sigma, UK) after electroblotting. The membranes were removed from the transfer system and handled carefully by placing the surface exposed to the gel upward all the time. Total proteins on the membranes were then stained with Ponceau S prepared at 0.1% (w/v) in 5% acetic acid. The staining should show continuous bands, representing no air bubble interference which could prevent protein transferring and binding on the membranes. The positions of markers were marked and the membranes were cut into small pieces to separate interested proteins according to their size. Ponceau S was washed off the membrane by rinsing with distilled water and 1% Tween20-PBS (PBST) for a few times to provide the membranes for the next step.

2.2.8.4 Protein Detection

Before the expression of proteins was detected by immunostaining, non-specific binding was prevented by incubating membranes with the blocking solution (3% skimmed milk in PBST) for 30 minutes. Primary antibodies were prepared in the blocking solution at the appropriate concentrations (Table 2.1) and applied to the membranes carrying interested proteins.

Table 2.1 Primary antibody concentration used for Western blot

Antibodies	Manufacturing	Host	Dilution	Expected Size
vWF	AbCam	Rabbit	1/500	250 kDa
NOS III	Santa Cruz	Rabbit	1/50	140 kDa
eEF2	Cell Signaling	Rabbit	1/1000	95 kDa
Cox2	Santa Cruz	Rabbit	1/200	70-72 kDa
CD43	AbCam	Mouse	1/1000	43 kDa
GAPDH	Chemicon	Mouse	1/2000	38 kDa

Primary antibody staining was performed at room temperature for 1 hour or at 4^0 C for overnight. The unbound primary antibodies were then washed off by incubating the membranes with PBST twice, for 15 minute each. The secondary antibodies linked with the reporter enzyme i.e. Horseradish peroxidase or HRP-linked enzymes (Dako, Denmark) were prepared at 1:1000 dilution in the blocking solution. They were applied to the appropriate membranes and incubated at room temperature for 1 hour. The membranes were then washed 4 times with PBST for 15 minutes to remove the unbound HRP-linked secondary antibodies before they were pooled on a clean plate to be stained with enhanced chemiluminescence or ECL solution. The ECL solution (GE Healthcare, UK) was freshly prepared by mixing solution A (Luminol Enhancer solution) and solution B (Peroxide solution) at 1:1 ratio and used to incubate all membranes for 5 minutes at room temperature to allow chemiluminescence reaction to happen. The substrates in the ECL solution were catalyzed by peroxidase enzyme in the vicinity of secondary antibody, in other words at targeted proteins, and produced a complex which emitted light. The excess ECL solution was subsequently drained off the membranes and the light referring to the targeted proteins was detected on an X-ray film.

2.2.8.5 Developing films

A black-and-white photographic film (Amersham Hyperfilm™ ECL, GE Healthcare, UK) was used to detect the light produced by HRP-antibody protein complex. The film is coated with silver halide crystals which are sensitive to not only to X-rays or gamma rays but also light. When the film is exposed to light, the crystals are divided into halide ions and Ag^+ producing a "latent" image or a sensitive area. The film can eventually turn into a virtually detectable image by light or the developer solution. At the optimal time in the developer solution, the light-exposed film can convert Ag^+

into pure metallic silver readily while maintain the silver halide crystal at the area of non-light exposure. The silver halide crystals can be then washed off from the film by the fixer reagent.

The ECL stained membranes from the previous step were mounted on a glass mirror and covered with cling film to prevent the dripping of ECL and the movement of the membranes during the developing step. This developing step was then continued in a dark room to prevent the unnecessary light exposure. The hyperfilms were placed over the membranes at a marked position in a cassette, for approximately 1 minute. They were then moved to be immersed under the developer (Agfa, UK) until the dark bands appeared. All chemical was washed off by tap water and the reaction was stopped by removing non-reacted crystals from the films with the fixer (Agfa, UK). The films were then washed with tap water, dried and the density of bands analysed.

2.2.8.6 Quantification of band density

The amount of protein present is proportional to the density of the bands on the film. The films were scanned by the densitometer (Biorad GS710, AGFA ArcusII). The images were acquired and analysed the bands density by QuantityOne Software (Biorad). To correct the amount of proteins loaded and proteins transferred on the membrane, the density of target proteins was normalized with the density of housekeeping protein(s) e.g. GAPDH and eEF2.

2.2.9 Protein array of side-specific VECs

Molecules produced by VECs and secreted outside the cells were collected to investigate the heterogeneity between aVECs and vVECs in terms of cytokine production. Cytokines which include the Interleukin (IL) family [IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12], granulocyte macrophage-colony stimulating factor (GM-CSF), interferon gamma (IFNγ), transforming growth factor beta (TGFβ1), tumor necrosis factors alpha (TNFα) were observed by using Quantibody® Porcine Cytokine Array Kit (Raybiotech Inc., USA). The Quantibody Array uses enzyme-linked immunosorbent array (ELISA) based technology. Briefly, the antibodies against 10 cytokines were pre-immobilised on a glass chip. The cytokines in sample media then bind to the antibodies on the glass chip and "sandwiched" with the biotin-tagged cytokine specific antibodies that bind to different targets of the cytokines. Subsequently, streptavidin-labeled Cy3 dye is applied to bind the cytokine-antibody-biotin complex trapped on the chip and visualised by a fluorescent laser scanner with Cy3 equivalent dye detector.

10^6 cells of aVECs or vVECs were seeded on the culture plates and maintained with EGM2 media overnight. The cultures were changed into low serum DMEM for 24 hours to synchronise cell cycle before protein array was performed by adding 3 ml of fresh low-serum media. Media collected from aVEC and vVEC cultures at 48 hours incubation was concentrated by centrifugation through a polyethersulfone membrane (Vivaspin2, SartoriusStedium Biotech, Germany). 3 ml of fresh low serum media without VEC contact (used as a negative control) was concentrated in the similar method as media from VEC cultures. The concentrated media from every sample was adjusted to the total volume of 300 µl, in order to be able to calculate the total production of the cytokines.

100 µl Sample Diluent was used to block non-specific binding of the pre-coated antibodies by incubating the solution at room temperature for 30 minutes. In the meantime, standard cocktails were prepared at different concentrations by serial dilution. Sample Diluent was decanted after finishing the blocking incubation. 100 µl of media control, sample media and standard cocktails were then incubated with the protein array chips for 90 minutes at room temperature. Unbound molecules in each array were washed off with 150 µl of Washing Buffer I at room temperature for 5 minutes, 5 times with gently shaking. Afterwards, 150 µl of Washing Buffer II was added to each array to repeat the washing at room temperature for 5 minutes, twice. The Washing Buffer was removed completely from the array before 80 µl of the (biotin labelled) detection antibody cocktail was added. The antibody-cytokine-detection antibody complex was incubated at room temperature for 90 minutes. The unbound detection antibodies were then washed with 150 µl of Washing Buffer I for 5 minutes and 150 µl of Washing Buffer II for 5 minutes, 2 times at room temperature. The Biotin on the cytokine complex was then detected by 80 µl of Cy3 equivalent dye-conjugated streptavidin. The binding reaction between streptavidin and biotin complex was incubated at room temperature for 60 minutes with light protection. The solution was decanted and the unbound Cy3- Streptovidin was washed off with 150 µl of the Buffer I for 5 minutes at room temperature 5 times, followed by 15 minutes washing with the Washing Buffer I on a shaker and Buffer II for 5 minutes at room temperature. The solution was then completely removed by centrifugation of the glass slide at 216 g for 3 minutes. The amount and position of Cy3 on the chips represents the amount and types of the cytokines detected by a laser scanner equipped with a Cy3 wavelength (RayBiotech Service Department, USA). The dilution factor was used to calculate the total amount of the cytokines which were produced by 10^6 cells of VECs in a volume of 3 ml media.

2.2.10 Statistic analysis

The proliferation of VECs in the complete media was normalised to that in serum starved cells, grown with 0.4% FBS EGM2, and reported as percentage over the control. The proliferation rate of aVECs and vVECs, was compared using a Mann-Whitney test and the data plotted using by GrapPad Prism 5.

The data of protein expression was also scatter-dot plotted by GraphPad Prism5 and represented as optical density (O.D.) units over the housekeeping protein. Mann-Whitney Test was used to statistically compare the expression of specific proteins between aVEC and vVEC. P value less than 0.05, 0.01 and 0.001 are represented by *, ** and ***.

2.3 Results

2.3.1 Optimisation of Side-specific Valve Endothelial Cell Isolation Condition

The reliable and reproducible method to isolate aortic and ventricular VECs, and VICs from the same animal was established. The method was developed from previous protocols of using enzyme digestion and adapted from a clonal expansion method.

When placing the valve on the isolation wells containing collagenase, there was spilling of the enzyme out of the wells. This resulted in the lower level of the enzyme present after fixing the cusps in place with the lid of the chamber, and no contact of the valve surface to the enzyme. Thus, the orbital shaker was implemented during the incubation to ensure surface contact of the enzyme with the valve surface.

The isolation incubation time was evaluated at various time points, 10, 20, 30 and 40 minutes (from 6 pigs for each time variations), by the appearance of VEC colonies on a culture plate (at day 2) and the ability to proliferate after the isolation process (Figure 2.3 and Figure 2.4).

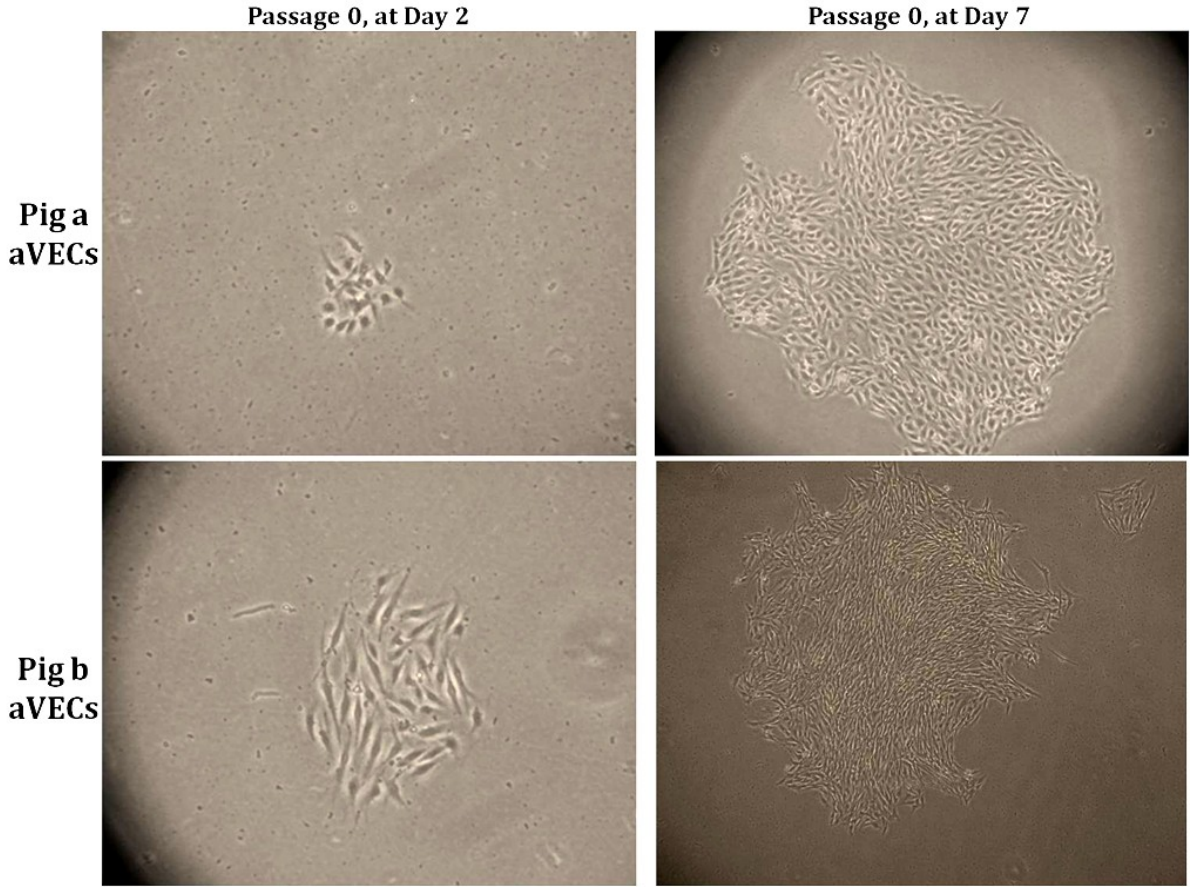


Figure 2.3 Light Microscopic photographs of aVECs isolated by enzymatic digestion at 30 minutes and 170 RPM incubation. VECs freshly isolated from the aortic surface of the porcine aortic valves demonstrated separate clusters of cells after the second day (left panel). As the colony expanded, cells displayed more cuboidal shape of cells at day 7 (right panel) without contamination of elongated and spindle shape cells. Pictures were taken from 2 out of 6 pigs at 100x magnifiction.

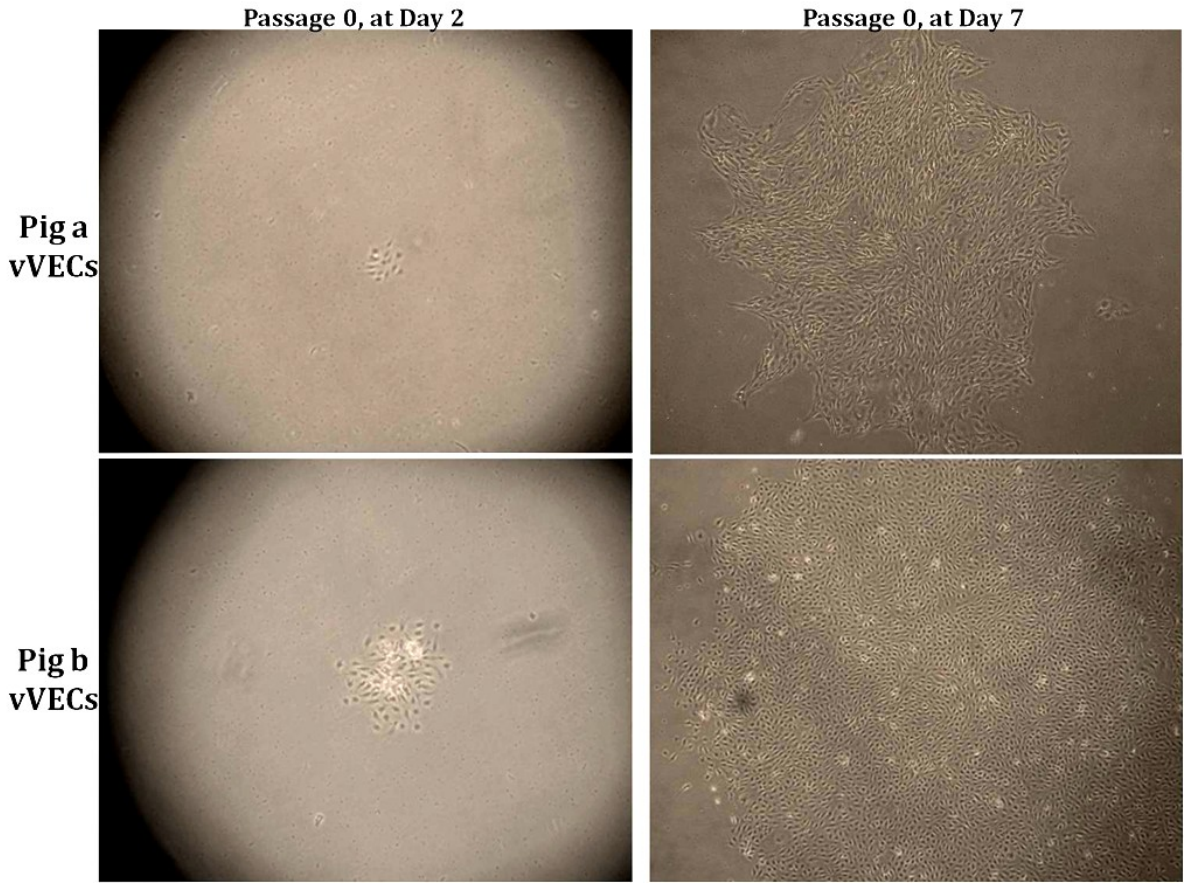


Figure 2.4 Light microscopy photographs of vVECs isolated by enzymatic digestion at 30 minutes and 170 RPM incubation. VECs freshly isolated from the ventricular surface of the porcine aortic valves demonstrated separate clusters of cells after the second day (left panel). As the colony expanded, cells displayed more cuboidal shape of cells at day 7 (right panel) without contamination of elongated and spindle shape cells. Pictures were taken from 2 out of 6 pigs at 100x magnification.

The results validated the optimal incubation time and optimal speed for orbital shaking at 30 minutes and 170 RPM. aVECs and vVECs were removed from the valve surface by the collagenase following 30 minutes of incubation and appeared to adhere on the culture plates at day 2 as clusters of cells. There were no VECs attached on the culture plates when they were isolated at the shorter periods of incubation (10 and 20 minutes).

Moreover, at the optimal isolation-condition (30 minutes – 170 RPM incubation), aVECs and vVECs proliferated and maintained their clusters. VECs incubated at 40 minutes were isolated and also attached on the plate; however, they proliferated at significantly slower rate than those isolated at 30 minutes. More importantly, VECs isolated using the optimal conditions showed cuboidal morphology without the contamination of VICs, which have a distinctive spindle-elongated shape. After the isolation conditions were optimised, cellular components of the valve, both on the area where it was exposed to the enzyme and the outside area, were investigated by scanning electron microscope (Figure 2.5).

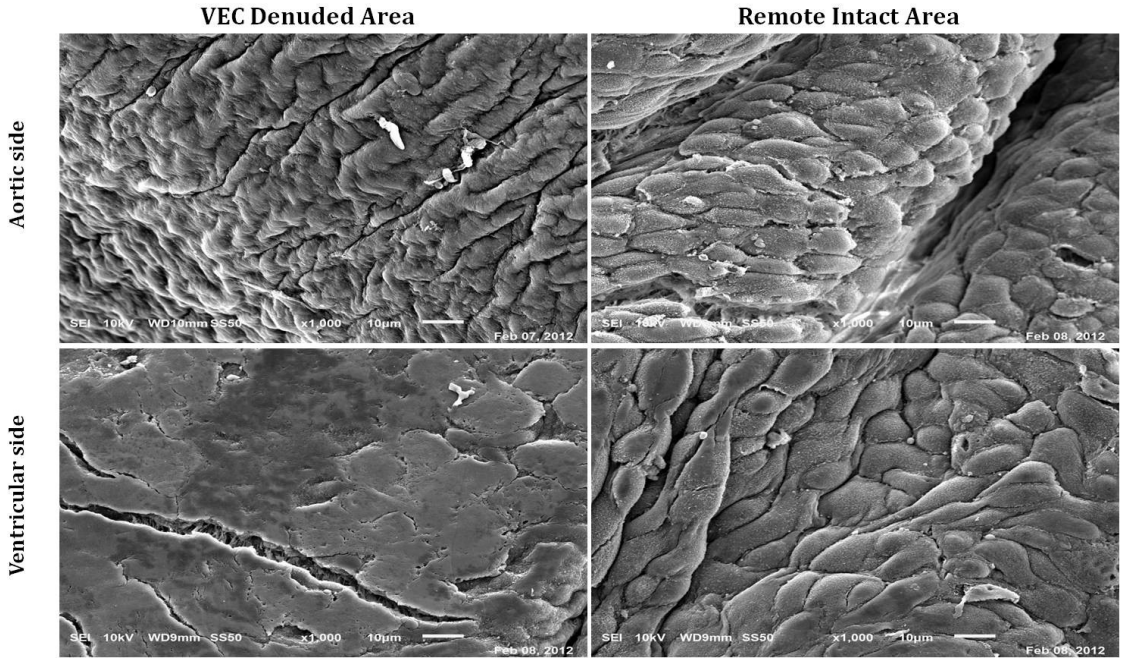


Figure 2.5 Scanning Electron photographs of the aortic valves after side-specific isolation. The area of the aortic surface (top) and the ventricular surface (bottom row) was investigated for the VEC components. VECs were absent from the area where exposed to collagenase (left panel). However, the remote area of the valves where VECs were not exposed to the enzyme (right panel) displayed the preserved VECs on both aortic and ventricular surface.

The valve surface of the isolating area (exposed to the enzyme in the wells) contained no VECs, suggesting the success of the isolation at 30 minutes of the incubation time. The left panel of Figure 2.5, where VECs were removed by

collagenase revealed the structure of crimping collagen and elastin on the aortic and ventricular side, respectively. Moreover, VECs were maintained on the area outside the enzyme contact.

2.3.2 Phenotypes of side-specific VECs

Immunostaining for endothelial markers vWF, CD31 and VE-Cadherin and VIC, myofibroblast marker SMαA, was performed on the aortic and ventricular sides of the fresh tissue as well as cultured aVECs and vVECs at passage 2, 3 and 4. VICs at passage 5-6 were also stained for those antibodies and used as a negative control for endothelial markers but as a positive control for SMαA.

As depicted in the Figure 2.6 and Figure 2.7, all aVEC and vVEC cultures were shown to have positive staining for CD31 and VE-Cadherin on their cell membranes; however, only certain cells were positive for vWF (staining in secretory vesicles and cytoplasmic organelles known as Weible-Palade body). These characteristics were also observed in VECs on the fresh valve. The reduction of EC markers (Figure 2.6 and Figure 2.7) was observed in VECs at passage 3 for every isolation, n=4. Moreover, VICs showed negative staining for all endothelial markers (vWF, CD31 and VE-Cadherin) but positive staining for SMαA.

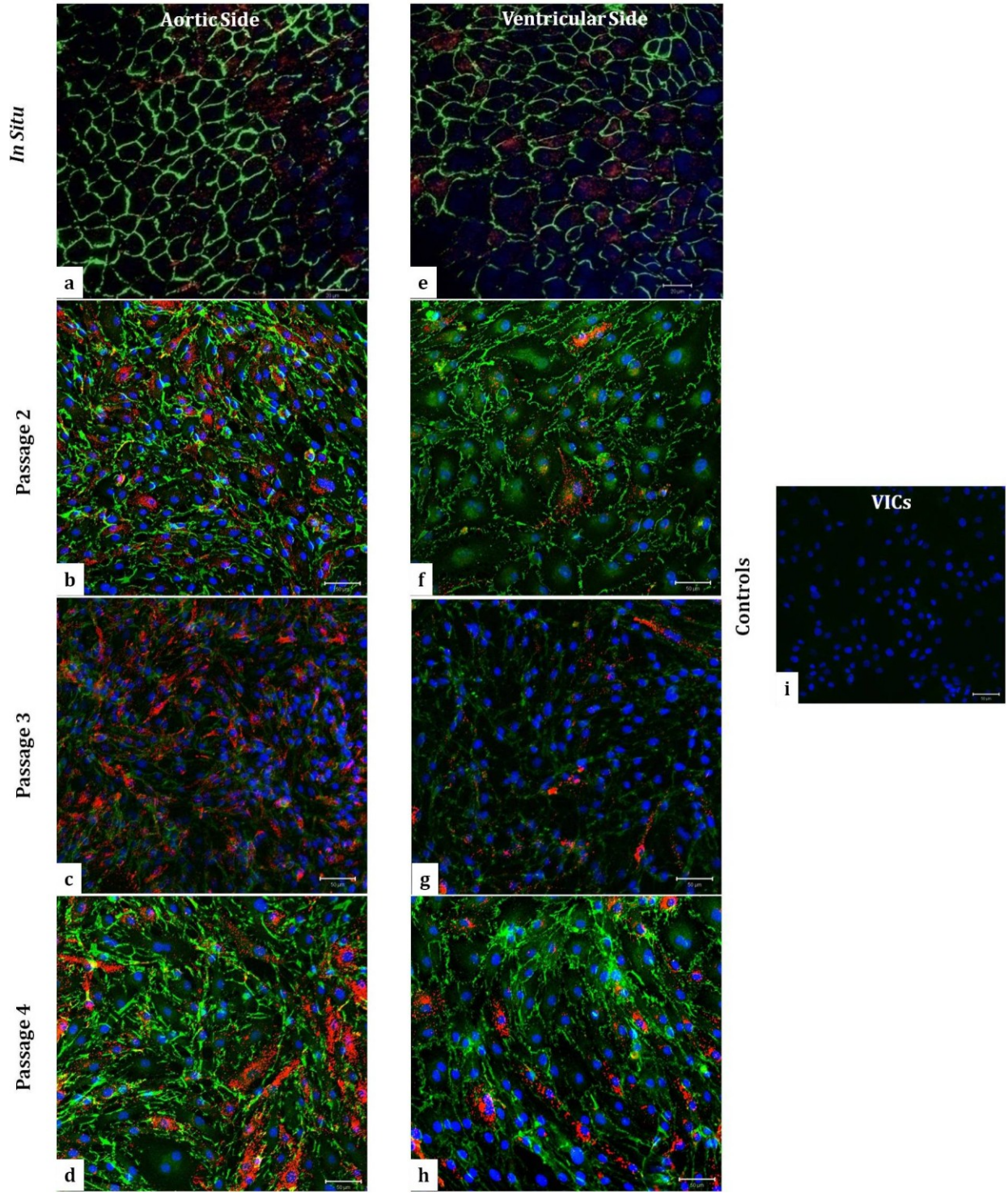


Figure 2.6 Immuno-staining of endothelial markers expressed by aVECs and vVECs from *in situ* and *in vitro*. Co-staining of endothelial markers (vWF in red, CD31 in green and nuclei in blue) was maintained in aVECs (left) and vVECs (right panel) on fresh tissue and over passage 2, 3 and 4 (a-h), n=4. VICs were negative for all statining of the EC markers (i). Scale bars equal to 50 μm.

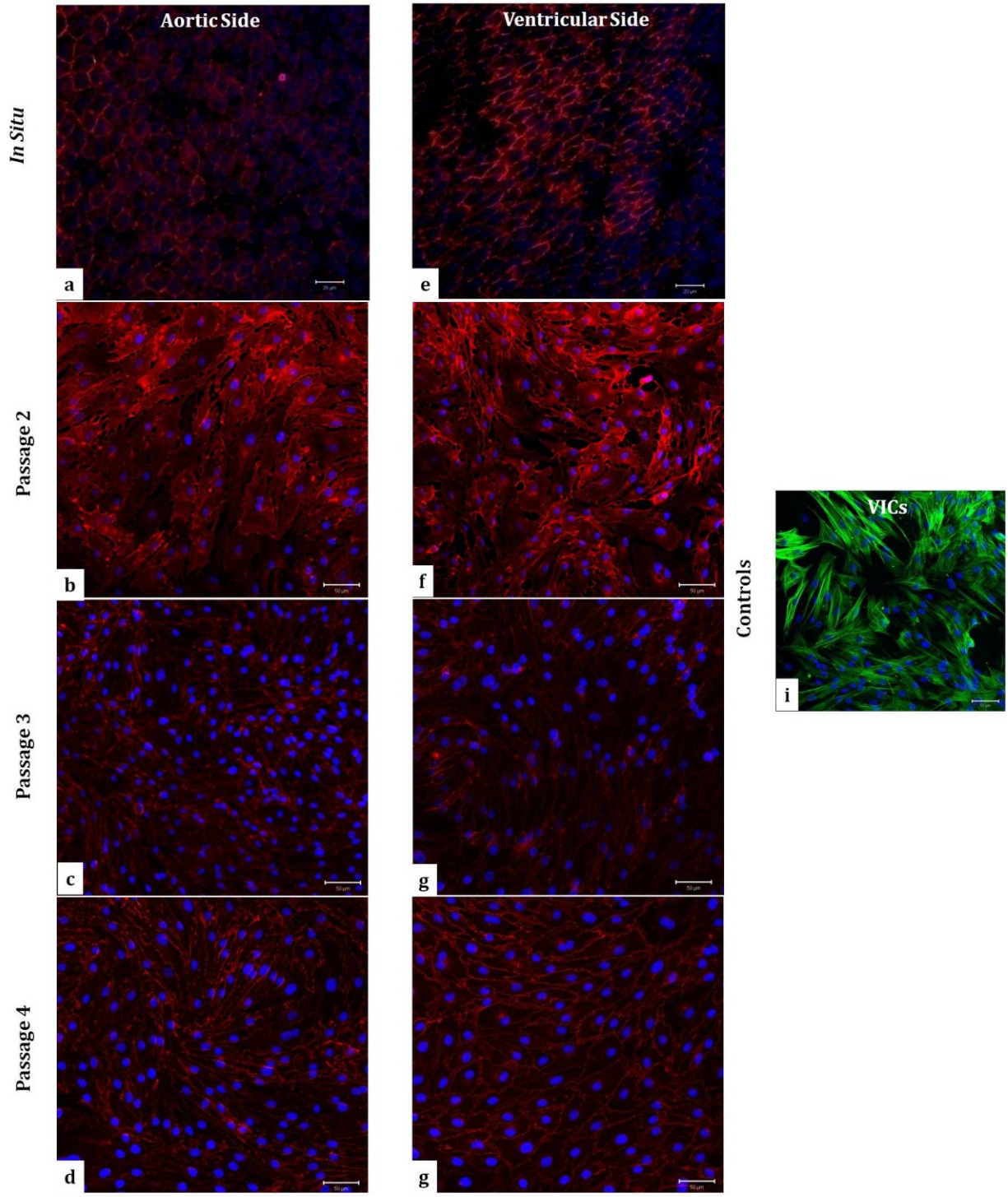


Figure 2.7 Immuno-staining of endothelial and VIC markers expressed by aVECs and vVECs from *in situ* and *in vitro*. Nucleus was stained in blue. Endothelial marker (VE-Cadherin, in red) and VIC marker (SMαA, in green) were co-stained in aVECs (left) and vVECs (right panel), (a-h). Both aVECs and vVECs, n=4, from *in situ* and *in vitro* expressed VE-Cadherin but not SMαA. In contrast, VICs were negative for VE-Cadherin (red) but positive for SMαA (green) in figure i. Scale bars equal to 50 μm.

2.3.3 Proliferation of side-specific VECs

The ability of aVECs and vVECs to proliferate in the EGM2 media for 48 hours of incubation was compared (Figure 2.8). The amount of cells cultured with 20% FBS in EGM2 was normalised to cells cultured with 0.4% FBS in EGM2 for 48 hours and reported as proliferation percentage over the control.

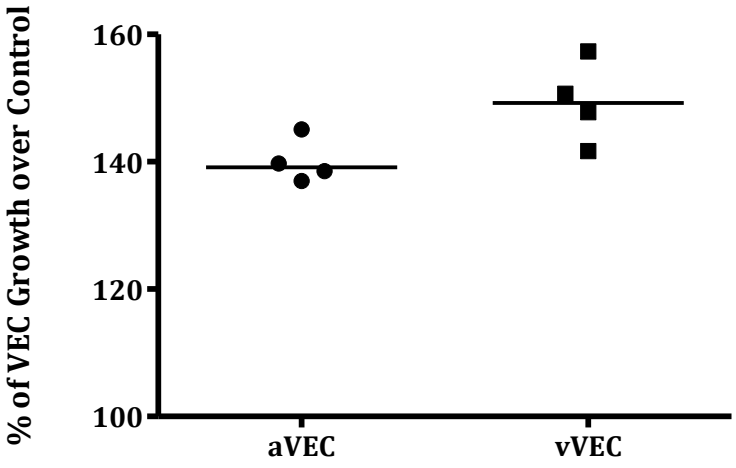


Figure 2.8 Growth rate of aVECs and vVECs. The proliferation of *in vitro* aVECs and vVECs during 48 hours incubation in complete EGM2 media was normalised to that of aVECs and vVECs under the starved condition. aVECs and vVECs from 4 animal models at passaged 4-5 were used for the investigation.

Both aVECs and vVECs proliferated in EGM2 media. The median of proliferation percentage, over control by aVECs was 139.1% while vVECs had a median of 149.2%, of the control. However, there was no significant difference of the proliferation between aVECs and vVECs (n=4, p=0.0571).

2.3.4 Quantitation of potentially difffential markers for side-specific VECs

The expression of NOS III, vWF, Cx43 and Cox2 by aVECs and vVECs, was examined by western blot analysis and normalised to the production of house-keeping protein i.e. either GAPDH or eEF2 (depending on size of the target proteins). The differential expression by VECs was investigated from both fresh cusps and the cultures (at passage 4-5), Figure 2.9.

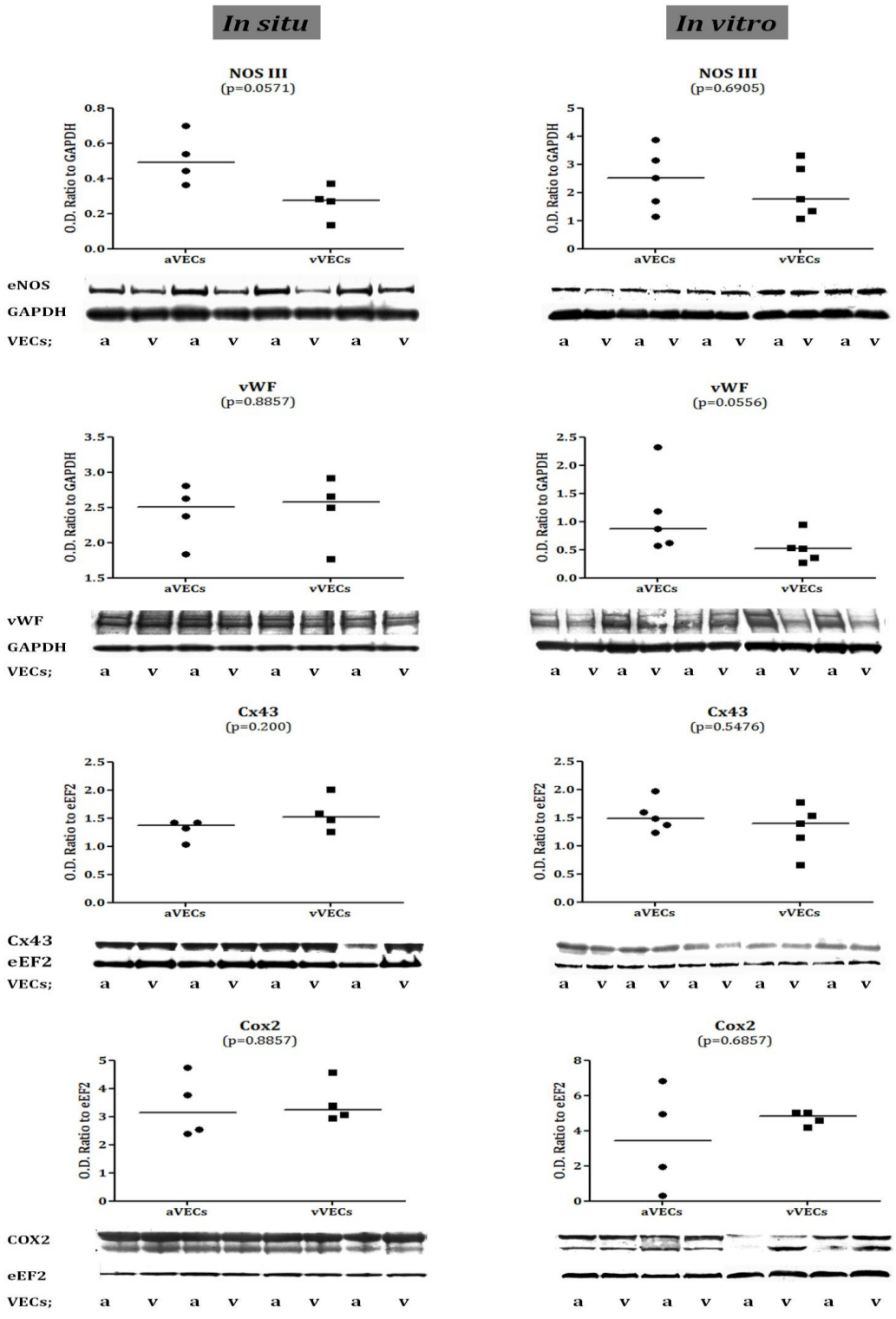


Figure 2.9 Quantitative analysis of potential markers for side-specific VECs. Expression of NOS III, vWF, CX43 and Cox2 by side-specific VECs from *in situ* (left) and *in vitro* (right panel) was quantified by Western Blot and normalized with housekeeping protein, as reported in O.D. ratios to the housekeeping genes. n number for each analysis eqauls to data plots.

There was a trend towards significant differences between side-specific VECs, for expression of NOS III (*in situ*), p=0.0571 and vWF (*in vitro*), p=0.0556; however, the difference was not statistically different (n=4-6). The median values of the O.D. Units are summarised in Table 2.2. It was noted that there was no significant difference in all observations comparing the expression of endothelial associated proteins on each side of the valve.

Table 2.2 Median value of O.D. Units by western blot analysis of *in situ* and *in vitro* VECs

Proteins	***In situ***		***In Vitro***	
	aVECs	**vVECs**	**aVECs**	**vVECs**
NOS III	0.491	0.276	2.521	1.763
vWF	2.503	2.574	0.865	0.518
CD43	1.370	1.524	1.481	1.396
Cox2	3.152	3.231	3.446	4.814

Note: All comparison beweet aVECs and vVECs within either *in situ* or *in vitro* group has p-value > 0.05

2.3.5 Cytokine Productions by side-specific VECs at resting state.

Low serum media which was used to culture either aVECs or vVECs (at passage 4), was collected after 48 hours in contact with the cells. The production of 10 cytokines was investigated by protein array and compared to the basal level present in the low serum media. 5 out of 10 cytokines were detected by the array. Those were IL-1β, IL-8, TGF-β1, IFNγ and TNFα (Figure 2.10).

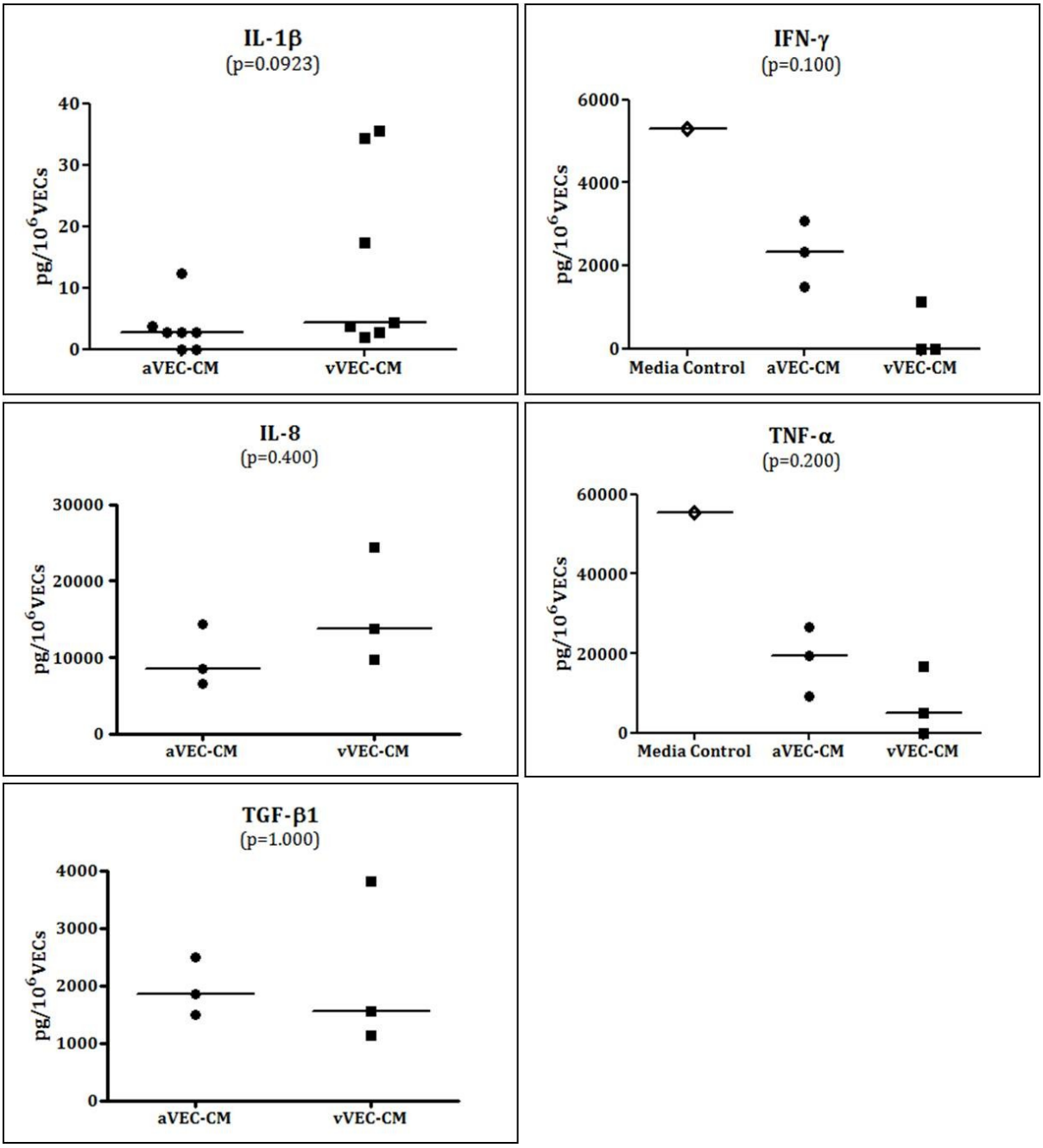


Figure 2.10 Cytokine production by *in vitro* side-specific VECs**.** aVECs and vVECs at passage 4-5 were cultured with low-serum media for 48 hours and investigated for the production of cytokines released in the media by protein array. There is no significant difference of cytokine modulationbetween aVECs and vVECs. Whereas IL-1β, IL-8 and TGF-β1 were not present in the media control, the amount of IFNγ and TNFα in the media control were higher than media over VEC cultures.

Both aVECs and vVECs released IL-1β, IL-8 and TGF-β1 into the culture media at 48 hours incubation of static culture. These cytokines were not detected in the low serum media without the contact with VECs. Moreover both aVECs and vVECs reduced the amount of IFNγ and TNFα present in the media. The median value of the production and degradation of the cytokines by aVECs and vVECs are summarised in Table 2.3.

Table 2.3 Median value (pg/10^6 cells of VECs) of the increased (+) and reduced (-) amount of cytokines in the media cultured with aVECs and vVECs *in vitro* as compared to the media without the contact with VECs.

Cytokines	**aVECs (pg/10^6 cells)**	**vVECs (pg/10^6 cells)**
IL-1β	+2.670	+4.410
IL-8	+8558	+13790
TGF-β1	+1859	+1551
IFNγ	-3387	-5302
TNFα	-36485	-50601

There were no significant differences in the production and degradation of the cytokines by VECs from the different sides.

2.4 Discussion

There is increasing interests in the function of VECs from the opposite sides of aortic valve due to the preferential formation of calcific disease on the aortic surface of the valve. However most of the investigations are *in situ* studies. In order to examine the function of these cells in greater detail, the isolation and culture side-specific valve endothelial cells is essential. In this work, a successful method to isolate and culture aVECs and vVECs from the same animal was developed by adapting existing enzymatic digestion methods and using a purpose built chamber. Consequently, the potential phenotypic and functional differences between the two cell types were then investigated.

One of the key steps in side-specific VEC isolation is to exclude VICs from the initial isolated cell population. Magnetic cell sorting and fluorescence-activated cell sorting (FACS) have been widely used; however, these techniques depend on specific antibody labeling that was not available for porcine tissue. An alternative prospective method is clonal expansion of cells which has been employed by others (Gould and Butcher, 2010) and our laboratory previously. With this technique, VECs are required to be diluted and expanded from a single cell in a well. As a result of the lack of cell-cell adhesion to promote cell growth, single VECs initially proliferated slowly. It is noted that this condition contradicts the physiological conditions in which ECs line the vasculature as a continuous monolayer. Thus, the colony expansion technique was adapted whereby groups of VECs, detached from valve cusps during the enzyme digestion, were grown on the plate and expand as clusters of the cells.

SEM was used to validate the isolation of VECs from the valve surfaces that were exposed to collagenase. VECs were successfully removed from the aortic and ventricular surfaces at the optimum condition of 170 orbital shaking for 30 minutes

at 37^0C. The scanned surface at the denuded area was absent of VECs and revealed the crimped collagen and elastin sheaths on the aortic and ventricular surfaces, respectively. After detaching from the valve surface, the primary culture of VECs maintained a cobblestone-like morphology. However not every VEC had cobblestone structure at later passages. This characteristic of cultured VECs has also observed in cells cultured from human pulmonary and calf atrioventricular valves (Paruchuri *et al*., 2006; Manduteanu *et al*., 1988).

Phenotypes of aVECs and vVECs in cultures were investigated and compared to the phenotypes of those on the fresh valve (*in situ*). Both aVECs and vVECs *in vitro* preserved endothelial phenotypes (by expressing CD31, vWF and VE-Cadherin) throughout several passages even when they were maintained in the culture conditions, without exposure to a physiological heamodynamic environment.

Although vWF is widely considered as a specific endothelial marker, there is evidence showing a differential capacity of ECs from different sites and species to produce vWF (Giddings *et al*., 1983). Porcine aorta ECs were weakly positive when stained with the vWF antibody, whereas the porcine umbilical vein ECs were consistently positive for vWF. In this study, VECs *in situ* showed an inconsistent positive staining against vWF. The granular fluorescent staining was localised in cytoplasm of certain VECs on each side of the valve. Additionally, this characteristic was maintained for several passages in cultured aVECs and vVECs. The possibility of the contamination by VICs was also examined by staining for SMαA. The negative staining of SMαA in VECs assured the reliability of the isolation method to exclude the contamination by VICs, which express SMαA in culture.

The ability of aVECs and vVECs to grow in complete EGM2 media, relative to those maintained in starved serum media over 48 hours, was not statistically different, although the p-value was near to significance. Moreover, the doubling time of mixed

VECs from both sides of bovine heart valves has been reported at 36 to 42 hours (Manduteanu *et al*., 1988). Consequently, side-specific VECs were not maintained in EGM2 media longer than 36 hours in subsequent experiments in order to reduce the differential effect of varied numbers of aVECs and vVECs in each experimental group (Manduteanu *et al*., 1988).

There have been attempts to identify the phenotypic difference of *in situ* side-specific VECs in terms of gene expression (Simmons *et al*., 2005; Holliday *et al*., 2011) and protein expression (Richards *et al*., 2013; Inai *et al*., 2004). The transcription profiles of VECs isolated from the aortic and ventricular surface of porcine aortic valves revealed different gene expression patterns such as actin cytoskeletal organisation, transforming growth factor-β signaling, cell adhesion proteins (CD31), gap junctions (Cx43), specific endothelial proteins (NOS III and vWF), mediator of vascular calcification (BMP4) and skeleton development (fibrillin 1) (Simmons *et al*., 2005). However, it is noted that the porcine gene profile generated from the human microarray may provide some inaccuracy due to the species differences. Recently, human aVECs and vVECs were exposed to oscillatory and laminar shear stress *in vitro* to mimic physiological conditions, as well as to the flow pattern experienced on the opposite-side of the valve to investigate the dependence of the type of flow on EC phenotype (Holliday *et al*., 2011). In comparison to the porcine transcription profile, human mRNA expression of aVECs and vVECs shared the same trend of differential expression. Similarities existed in the differential expression of BMP4, cathepsin K, Asparagine synthetase, Histone deacetylase 1 and prostagladin endoperoxidase synthase 1. However, the semi-quantitative analysis of mRNA expression of CD31, vWF and VE-Cadherin did not identify any significant differences between human aVECs and vVECs. Additionally, there were no side-dependent differences of any mRNA expression detected in the study. It was demonstrated that aVECs and vVECs did not show the same response

to the same pattern of flow (Holliday *et al*., 2011). These results suggested that the microenvironment i.e. shear stress could play an important role to those phenotypic difference observed *in vivo*.

This study attempted to quantify the reported differential production of proteins by side-specific VECs and investigate whether the differential phenotypes were maintained in cultured cells, under the static condition. Protein candidates such as NOS III, vWF, Cx43 and Cox2 were thus quantified by western blot analysis of *in situ* and *in vitro* VECs.

NOS III stimulates the production of NO from L-arginine. NO is postulated the function to protect the valve against calcification via decreasing the myofibroblastic phenotype of VICs in the ventricularis (Richards *et al*., 2013). Human aortic valves have also been shown to have the higher production of NOS III in vVECs compared to aVECs, suggesting that this may contribute to the greater resistance of the ventricularis to the formation of calcific lesions compared to the disease prone fibrosa side. However, these observations are different to those reported in porcine valves where higher mRNA expression of NOS III was seen on the aortic side (Simmons *et al*., 2005). It is noted that NOS III was investigated at different level (protein vs mRNA production) as well as in the different species (human vs porcine) which may cause the discrepancy of data observed by studies by Richards *et al.* and Simmons *et al*. In this study, protein quantification of NOS III of porcine aortic valves showed a tendency of higher production on aVECs than vVECs, however there was no statistically significant difference in these data. Moreover the side-specific VECs cultured under static conditions for several passages also exhibited no difference of NOS III production between ECs isolated from the different aspects of the porcine aortic valve.

The pattern of shear stress experienced by ECs is an important factor in the regulation the expression of NOS III by bovine aortic and venous ECs. The oscillatory shear stress (-3 to +3 dyn/cm2) dramatically increased the function of regulatory element (promoter) of the NOS III gene, compared to the unidirectional shear stress and static culture (Ziegler *et al*., 1998b). In contrast, mRNA expression of NOS III was significantly increased in proportional to increases in the magnitude of unidirectional flow between 3 to 6 dyn/cm^2. This led to the conclusion that the oscillatory and unidirectional shear stress than exert differential regulation of NOS III expression (Ziegler *et al*., 1998b). Although these experiments were performed with vascular ECs, that experience a lower magnitude of the shear stress that occur on the aortic valve, it indicates how complex of the regulation of NOS III transcription can be. In addition, the regulation on the protein production and activity of NOS III by different flow patterns has not been studied.

Von Willebrand factor (vWF) is a glycoprotein component of factor VIII that is produced by endothelial cells to help maintain the haemostasis. vWF mRNA expression observed in porcine aortic valves was higher in aVECs than those in vVECs (Simmons *et al*., 2005). Intracellular vWF can be detected, by immunostaining, as granules in cytoplasm. Although vWF is widely considered as an endothelial marker as discussed earlier, the production of it was decreased in porcine ECs maintained in cultured for only 48 hours (Jaffe, 1982). The IHC and ICC revealed that both aVECs and vVECs *in situ* and *in vitro* produced vWF. The quantitative analysis confirmed the production of vWF by VECs from *in situ* and *in vitro* of which there was no significant difference among side aspects. Although there was a trend that in vitro aVECs had slightly higher production of vWF than those of vVECs, this difference was also not statistically significant.

Cx43 is a component protein of gap junctions facilitating the propagation of electrical and chemical signals between cells. mRNA of Cx43 has been demonstrated

to be up-regulated by the turbulent shear stress in certain areas of arteries (Gabriels and Paul, 1998). In addition, the expression of Cx43 mRNA on porcine aortic valves exhibited differential expression in which the higher expression of Cx43 was found on the ventricular surface (Simmons *et al*., 2005). Likewise, the staining of the gap junctions on the mouse aortic valve also showed higher expression of Cx43 on the surface exposed to laminar flow. The semi-quantitative analysis showed a broad range of the expression of 70-200 folds greater on the ventricular surface (Inai *et al*., 2004). In this study, however, there was no significant difference of the Cx43 protein between aVECs and vVECs, both from *in situ* and *in vitro* analysis. The difference between this result to the semi-quantitative analysis of gene expression of porcine valves and of protein production of murine valves may relate to the numbers of valves studies and requires further investigation.

Cyclooxygenase-2 (COX-2) is also referred to prostaglandin H synthase-2. Its function is to synthesise Prostaglandin I_2 (PGI2) or prostacyclin, a potent inhibitor of platelet aggregation and a powerful vasodilator (Morita, 2002). There are several factors that could regulate the COX-2 expression by endothelial cells; for example, the down-regulation by glucocorticoids and anti-inflammatory cytokines (IL-4 and IL-10) and the up-regulation by growth factors, cytokines and mechanical stress. One of the COX-2 promoters that responds to shear stress is the cAMP-responsive element (CRE) (Inoue *et al*., 2002). Asynchrony of shear stress and strain reduce the expression of COX-2 mRNA and the secretion of prostacyclin by bovine aortic ECs (Dancu *et al*., 2004). Although the investigation of *in situ* VECs can be a surrogate marker for *in vivo* mechanical environment, there was no significantly difference of COX-2 production by the VECs freshly isolated from the tissue and cultured cells from the aortic and ventricular side. This preliminary observation was the first investigation of the expression of COX-2 in porcine aortic valve.

Cytokines are a group of short–life soluble molecules (peptides, proteins and glycoproteins) which are produced by various immune cells and vascular cells (Sprague and Khalil, 2009). They act in picomolar to nanomolar concentration to modulate functions of ECs and vascular SMC with a subsequent effect on remodeling of ECM. Cytokines can be generally categorized as: Interleukins (ILs), Interferons (IFNs), Transforming growth factors (TGFs), Tumor necrosis factors (TNFs), Colony stimulating factors (CSFs), Lymphokines, Monokines and Chemokines.

The ability of vascular ECs to produce cytokines in response to external chemical or mechanical (both physiological and pathophysiological) stimuli has been established (Sprague and Khalil, 2009). However the differential function to release cytokines by side-specific VECs has not been studied yet. In this study, the production of cytokines by aVECs and vVECs was performed using a protein array that detected interleukin (IL) family [IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12], granulocyte macrophage-colony stimulating factor (GM-CSF), interferon gamma (IFNγ), transforming growth factor beta (TGFβ1) and tumor necrosis factors alpha (TNFα).

At resting state (without any immune activation and heamodynamic stimuli), both aVECs and vVECs released detectable levels of IL-1β, IL-8 and TGFβ1 (pro-inflammatory and atherogenic molecules) which are known to affect the migration, proliferation and activation of ECs and SMCs (Sprague and Khalil, 2009). In contrast, the media from VEC cultures had a reduced amount of some pro-inflammatory molecules such as IFNγ (inhibits ECM synthesis by SMC) and TNFα (suppresses adhesion molecule expression and increases permeability of ECs). This decreased amount of the cytokines was relative to those the levels of the low serum media without the contact with VECs. This effect may be related to the sensitivity/variability of the assay, or suggest that VEC degrade these cytokines. The potential mechanisms involved in this effect were not pursued further.

GM-CSF that affects the growth and proliferation of monocytes, macrophages, and granulocytes (Sprague and Khalil, 2009) was not detected in this study. This could be explained by the non immunity-activation condition with which VECs were maintained for the study. IL-4, IL-6, IL-10 and IL-12 were also not detectable in this assay and have not been reported to be produced by any ECs before (Sprague and Khalil, 2009).

This study demonstrates that VECs can modulate the cytokine production of the inflammatory factors (IL-1β, IL-8, TGFβ1, IFNγ and TNFα); however there is no significant difference of those productions between VECs from the different aspects of the valve. Similarly, aVECs and vVECs, on the valve, that were exposed to their physiological flows also produced inflammatory markers at the non-different level (Sucosky *et al*., 2009). However, the expression of imflammatory molecules was increased when aVECs were exposed to the reverse flow from the normal condition. The lack of difference of cytokine production, seen in these studies, might be due to an inherent ability of the cells to produce the inflammatory molecules when they are maintained under non-activated conditions.

2.4.1 Limitations of the study

The differential cellular isolation and the attempt to identify the side-specific VECs markers in these studies have some limitations.

The isolation technique developed by this study allows aVECs, vVECs and VICs to be obtained from different cusps of an aortic valve. This study is based on the assumption that there is no phenotypic difference of cells from the same aortic valve/animal. Thus, the left-, right- and non-coronary leaflets were randomly selected for cellular isolation.

The amount of cytokines in the media, without contact with VECs, should be compared between 0 and 48 hours incubation, in order to include the possibility that TNFα and IFNγ are degraded independent of cellular function.

The experiments in this study were carried out under static, non-activated conditions. Further experiments are required to investigate the influence of flow and immune stimulation on the ability of VECs to release cytokines and chemokines in order to obtain a clear picture of the physiological and pathophysiological role of cytokine production by VECs. However, the cost of the Porcine Protein Array kit limited the number of experiments that could be conducted.

Finally, the quantitative analysis of the potential markers and the cytokine productions were performed at low sample number, due to cost and time limitation. Consequently, it is impossible to achieve conclusive results. However, the result data from low n-number can be useful for Power Analysis and predict the sample size for later experimental design. For example, means and standard deviation of the ratio value of NOS III expression by *in situ* VECs (Figure 2.9) were used to calculate sample size and the effect size (the difference between the null hypothesis the alternative hypothesis) by G*Power programme (http://www.gpower.hhu.de/en.html). The significant difference is predicted to be achieved if the sample size of aVEC and vVEC is bigger than 12 pigs for each group (at effect size=1.64, p-value=0.05 and the probability of accepting the null hypothesis=0.05).

2.5 Conclusions and Future Directions

In conclusion, the efficient and repeatable method to isolate side-specific VECs and VICs from the same aortic valve is established. This enables the study of aVEC and vVEC function in separated conditions *in vitro*. Both aVECs and vVECs maintain their endothelial phenotypes (expressing vWF, CD31 and VE-Cadherin) between passages. Moreover the quantitative analysis of the potential markers for side-specific aVECs and vVECs was investigated. The conclusive method to "screen" for the unique phenotypes among aVECs and vVECs could be achieved using proteomics. Protein patterns (2-D gel electrophoresis) of aVECs and vVECs from both *in situ* and *in vitro* could be compared to each other in order to identify the protein profile produced by VECs under different conditions. Mass spectrometry could also be used to identify the unique proteins found under physiological conditions and to assess if inherent differences in cell phenotype are maintained independently of the mechanical forces exposed to the cells. In addition, these experiments would demonstrate the function of VECs (in cultures) to secrete cytokines that can affect function of VECs themselves, nearby interstitial cells and the surrounding ECM. In these studies, nevertheless, markers showing differential expression and the release of cytokines by aVECs and vVECs were not reliable identified. The differential characteristics of aVECs and vVECs to regulate the function of VICs require further investigation. This thesis will attempt to identify functional differences in the functional effects of aVECs and vVECs on the ability of VICs to secrete ECM proteins.

CHAPTER 3 Effect of Conditioned Media by Side-Specific VECs

3.1 Background

It is well acknowledged that vascular ECs secrete a myriad paracrine factors that have an influence on the proliferation and function of cells such as smooth muscle cells and fibroblasts (Sprague and Khalil, 2009). The endothelial cells produce a source of molecules that can stimulate and inhibit the proliferation of underlying cells. For example, concentrated serum-free media which was pre-cultured with bovine aorta ECs (at later passages) increases the proliferation of bovine SMCs (Gajdusek *et al*., 1980). In contrast, the 20% serum media used to maintain the primary culture of bovine aorta ECs has reduced the replication of growth-arrested bovine SMC (Castellot, Jr. *et al*., 1981).

These contradictory observations from those different experimental settings could be explained by the different cellular conditions of ECs. The density of ECs was recently reported to determine the stimulatory or inhibitory effect on cell SMC proliferation (Dodge *et al*., 1993). Media, with 10% serum, containing EC-derived factors that were collected from the sparsely cultured aorta ECs stimulated the proliferation of SMCs. In contrast, when the same ECs grew until they reach confluence, the media collected from the dense culture of ECs inhibited the proliferation of SMCs (although it was prepared with a fresh 10% serum media at the same conduct as the sparsely cultured ECs before being used to culture SMCs) (Dodge *et al*., 1993).

In addition to the effect on proliferation, the media containing EC-derived factors also affects the regulation of the ECM production by SMCs and fibroblasts.

The production of collagen by the cardiac fibroblasts was increased when they were cultured with the conditioned-media (CM) obtained from the pre-incubation with endocardial ECs (Kuruvilla *et al*., 2007). Regardless of the species difference, the CM achieved from bovine ECs increased the production of collagen by human fibroblasts in a concentration-dependent manner (Villanueva *et al*., 1991). Moreover, bovine aorta EC-CM increased the *de novo* synthesis of GAGs by SMCs in a concentration and TGF-β dependent manner (Ciolino *et al*., 1992). The production of GAGs was diminished when the TGF-β antibody was added into the EC-CM. The evidence that TGF-β is the active factor in CM and responsible for the increasing production of GAGs was supported by the culturing of porcine SMCs with bovine EC-CM (Merrilees and Scott, 1990).

In the previous chapter, VECs were demonstrated to be able to secrete and/or modulate the concentrations of a range of different cytokines. It is hypothesised that, under non-stimulated (non-activated) conditions, VECs can also produce molecules that have effects on the VICs, similar to the function of ECs found elsewhere in the vascular system. In this study, investigations were carried out into the ability of side-specific VEC-derived molecules to regulate the proliferation and production of ECM components of VICs. Low serum medium is pre-incubated with either aVECs or vVECs to make aVEC-CM or vVEC-CM. These CM were examined for their ability to affect the proliferation of VICs. Additionally the effect of VEC-CM on the production of ECM components by VICs was also quantitatively assessed.

3.2 Materials and methods

3.2.1 Valvular Endothelial Cell Conditioned Media (VEC-CM)

To investigate the effect of factors released by VECs under resting conditions on VICs, aVECs and vVECs were isolated (as previously described in the section 2.2.2) and grown in 25 cm^2 flasks until reaching confluence (at about 1.5 x 10^6 cells). Freshly prepared DMEM with 0.4% FBS was then added and left in contact with the cells for 6, 12, 24 or 48 hours. Thereafter, the VEC-CM was collected and prepared at different dilutions (1:50, 1: 20, 1:10, 1:5, 1:2 and undiluted) and added to cultures of VICs to investigate whether VEC-CM has a concentration-dependent effect. The effect of VEC-CM on VIC proliferation and ECM production was also investigated.

A low serum DMEM media was used to generate the VEC-CM, so as the basal growth factors necessary for culturing VICs could be maintained but with little or no mitogenic factors present that might mask effects of mediators produced by the ECs. Consideration was also given to the endothelial phenotype of VECs that were maintained with low serum DMEM. Thus, endothelial markers such as VE-Cadherin and vWF were examined in VECs cultured low serum DMEM for the longest culture period of, 48 hours. The expression of the markers was investigated by the immunostaining technique and compared to those of the same VEC isolations, which were cultured with the endothelial media.

3.2.2 VIC proliferation under the effect of VEC-CM by MTS assay

VEC-CM at 6, 12, 24 and 48 hours time points were diluted with DMEM containing 0.4% serum at 1:50, 1:20, 1:10, 1:5 and 1:2 to investigate its effect on VIC growth. VEC-CM at each time points and concentration were incubated with VICs seeded at

3.0 x 10^3 cells/well in 96-well plates for 48 hours. The VICs were cultured from the same animal as the VEC used to make the CM. Fresh 0.4% FBS DMEM was used to culture VICs to serve as a negative control, whereas complete DMEM with 10% FBS was used a positive control for the proliferation.

The proliferation of the VICs in response to the incubation with VEC-CM was assessed by MTS assay (section 2.2.6).

3.2.3 The effect of VEC-CM on the content of ECM by VICs

aVEC-CM and vVEC-CM generated over 48 hours at 1:5 dilution was prepared by diluting the media with fresh low-serum media. It was then used to culture VICs, for 48 hours, to investigate the effect of VEC-CM on the ability of VIC to produce ECM proteins.

The VICs were seeded at 5.2 x 10^5 cells per well of 6-well plate and maintained in complete DMEM. After reaching confluence, the culture media was removed and VICs were washed twice with PBS before being staved with 0.4% FBS DMEM overnight. The media was aspirated and 3 ml of VEC-CM at the dilution of 1:5 was subsequently added to culture VICs. A newly prepared low-serum media was for the controls. After appropriate media were applied, the VICs were cultured for 48 hours in the 95% air/5% CO_2 incubator. The media over VICs was then collected for assessment of the amount of collagen and GAGs, while elastin levels were measured in the VICs.

3.2.4 Collagen content by VICs in vitro

Soluble collagen can be measured by Sircol assay (Biocolor, UK), which is a dye-binding method. The quantity of collagen is proportional to the amount of the Sirius Red dye that binds specifically to [Gly-X-Y] repeating units in a helical structure of collagen type I, II, III, IV and V. In the presence of alkali reagent (0.5 M sodium hydroxide), Sirius Red dye is released from collagen and has a peak of absorption between 550-555 nm. By measuring the absorbance at 550 nm using a BioPhotometer (Eppendorf, UK), the amount of Sirius Red can be quantified and correlated to the amount of collagen using the a standard curve of known collagen concentrations.

Collagen concentrations in the media were measured after 48 hours incubation with VEC-CM. The amount of collagen production stimulated by aVEC-CM and vVEC-CM was compared to the control (VICs isolated from the same animal but incubated with just low-serum DMEM). To exclude the possible amounts of collagen in the VEC-CM, the collagen concentration in the 1:5 dilution of VEC-CM was also quantified.

To avoid the variations in the concentration of collagen due to the evaporation of media from the culture wells during the experiment, all each culture well was topped up to 3 mlof distilled H_2O. Duplicates of each sample (1 ml) were transferred to clean 1.5 ml micotubes and then mixed with 200 µl of Isolation and Concentration reagent containing polyethalene glycol in a Tris-HCl buffer at pH7.6 at 4° C for overnight. Collagen was then precipitated by centrifugation at room temperature at 13684 g for 10 minutes and the supernatant was removed. 1 ml of Sircol dye (Sirius Red) was added to the blank (100 µl of distilled water) and all experimental samples including collagen reference standards prepared at 5, 10 and 15 µg, at total volume of 100 µl. Samples were gently shaken at room temperature

for 30 minutes to allow Sirius Red bind to the collagen. The collagen-dye complex was separated from unbound dye by centrifugation at 13684 g for a further 10 minutes. The supernatant was carefully removed by pipetting. Collagen-dye pellet was then washed with 750 µl of ice-cold Acid-Salt Wash Reagent containing acetic acid, sodium chloride and surfactants. All samples were spun down at 13684 g for 10 minutes to precipitate the complex again. The wash reagent was carefully aspirated with a fine needle syringe. The collagen-dye complex was released by 250 µl of Alkali Reagent and the measured absorbance at 550 nm. The collagen concentration present in the sample was then calculated by using the standard curve and expressed as the total amount of collagen produced by ~5.2 x 10^5 VICs.

3.2.5 GAGs content by VICs *in vitro*

Blyscan kit (Biocolor, UK), a colorimetric method, was used to quantify the amount of sulphated proteoglycan and glycosaminoglycan. At acidic pH, Blyscan Dye or 1,9-dimethyl-methalene blue proportionally binds to sulphate-GAGs. Subsequently the dye is released to a free form by dissociation reagent containing chaotropic salt in an aqueous propanol and it has a peak absorption at 656 nm. The amount of GAGs in the samples is calculated from the amount of dye that is recovered from sulphated-GAGs.

In order to concentrate GAGs secreted by VICs, the media from VIC cultures was freeze-dried (HETO Dry Winner, Denmark) overnight. VEC-CM (without contact with VICs) was also concentrated to investigate the amount of GAGs produced by VECs. Freeze dried samples were subsequently resuspended with 500 µl of distilled water prior to being used in the Blyscan assay.

75 µl of samples were taken to carry out GAGs quantification. GAGs standards at 1, 2, 3 and 5 µg, and the experimental samples were prepared up to 100 µl with Millipore distilled H_2O. 100 µl of dH_2O was used as a blank of the assay. 1 ml of the dye was added to all the samples and gently shaken for 30 minutes at room temperature to allow the dye bind to the sulphated-GAGs present. Insoluble GAGs-dye complex was centrifuged at 16060 g for 12 minutes. An unbound dye in supernatant was carefully aspirated using a fine-needle syringe. The dye was dissociated from a GAGs-dye pellet by resuspending with 1ml Dissociation Reagent. The amount of the dye corresponding to the amount of GAGs and was measured the absorbance at 650 nm by BioPhotometer plus (Eppendorf, UK). The total amount of GAGs was calculated from the standard curve and, following correction for the dilution factor used for sample preparation, was calculated as µg.

3.2.6 Elastin content by VICs *in vitro*

Tropoelastin is a monomer of elastin and can be quantified by a dye-binding assay, Fastin kit (Biocolor, UK). 5,10,15,20-tetraphenyl-21H,23H-porphine tetra-sulfonate (TPPS) in the Fastin Dye binds to the basic and non-polar amino acid sequences of α-elastin in a soluble form. The amount of TPPS bound is proportional to the amount of elastin in the sample and is measured by the absorption of light at 513 nm.

Tropoelastin is exported by microfibril glycoprotein and bound on the outer surface of the cell, hence measurements were made on the VICs, rather that the media. α-elastin matrix on the cell membrane of VICs was subsequently extracted into water-soluble form by oxalic acid.

After 48 hours of incubation either with aVEC-CM or vVEC-CM or fresh low-serum media, VICs were washed gently with PBS twice and scraped out of the plate with 200 µl of 0.25 M Oxalic acid added. Cell suspension was then transferred to a 1.5 ml microtube and another 800 µl of 0.25 M oxalic acid was added into the culture wells to collect all VICs that may have been left on the plate. The extraction of elastin was performed at 100°C by placing the microtubes on the heat box. After 1 hour of heating, the microtubes were centrifuged at 9503 g for 10 minutes. Soluble elastin in the supernatant was then transferred into new microtubes and ready to be assayed. The extraction was then repeated twice by adding another 1 ml of 0.25 M oxalic acid into the pellet and re-heating the microtubes for 1 hour.

Triplicates of the heat extraction from each sample were added with 1 equal volume of precipitating reagent containing trichoroacetic and hydrochloric acids. Elastin in samples and standards, which were prepared at 2.5, 5, 10, 20, 40, 80 and 100 µg, were precipitated at room temperature for 15 minutes and centrifuged at 9503 g for 10 minutes. Distilled water was used as blank for the assay. After centrifugation the supernatant was then removed carefully by inverting microtubes and tapping the tubes on a clean paper towel.

1 ml of Dye reagent was added to the α-elastin that remained at the bottom of microtubes and dispersed by vortexing. The binding reaction between elastin and TPPS dye was performed at room temperature for 90 minutes with gentle shaking. Unbound TPPS was separated from the elastin-dye complex by centrifugation at 9503 g for 10 minutes until the complete removal of the supernatant was possible. Thereafter, 250 µl of Dye Dissociation Reagent containing guanidine HCl and isopropanol was added into every sample to release TPPS from α-elastin. 100 µl of each reaction was then transferred in duplicate into wells of 96-well plates to enable the absorbance of TPPS at 513 nm to be measured by the microplate reader. The concentration of elastin in each sample was calculated using the standard curve.

3.2.7 Data and statistic analysis

Proliferation, collagen, GAGs and elastin production of VICs under VEC-CM were represented as a percentage change by comparing the test sample to its individual control. Data was expressed showing mean±SEM and plotted using Excel for the proliferation studies. For collagen, GAGs and elastin experiments, the median was presented by the bar in the Box and Whiskers Plot, using GraphPad Prism 5. The top of the box represents the third interquartile (IQ3) whereas the bottom of the box shows the first interquartile (IQ1). The top and bottom whiskers illustrate the maximum and minimum value, respectively. Data was statistically analysed using Kruskal-Wallis Test which is a non-parametric one-way ANOVA. The data were then compared in a pair-wise manner using Dunn's post-hoc Test. P value less than 0.05, 0.01 and 0.001 are represented by *, ** and ***.

Non-parametric Spearman test was used to analyse the correlation between the dilution of each CM and VIC growth. Spearman's correlation coefficient (r) indicates the strength of the relationship between the two variables using a monotonic function. "r" equals to -1 or +1 when there are no repeated data and the variables are a perfect monotone function. P value less than 0.05 suggests the real correlation which is not due to random sampling.

3.3 Results

3.3.1 Phenotypic characterisation of VECs in low serum DMEM

The expression of endothelial markers such as VE-Cadherin and vWR by VEC isolates grown in EGM2 media and, later, in the low serum DMEM for approximately 72 hours were examined.

Both aVECs and vVECs that were cultured in the endothelial media were positively stained for VE-Cadherin and vWF (Figure 3.1 and Figure 3.2). Moreover, the expression of those endothelial markers was maintained in both aVECs and vVECs when the media was changed to with low serum DMEM, from which the conditioned media was. The morphology of VECs appeared to retain the cobblestone shape in the low serum DMEM.

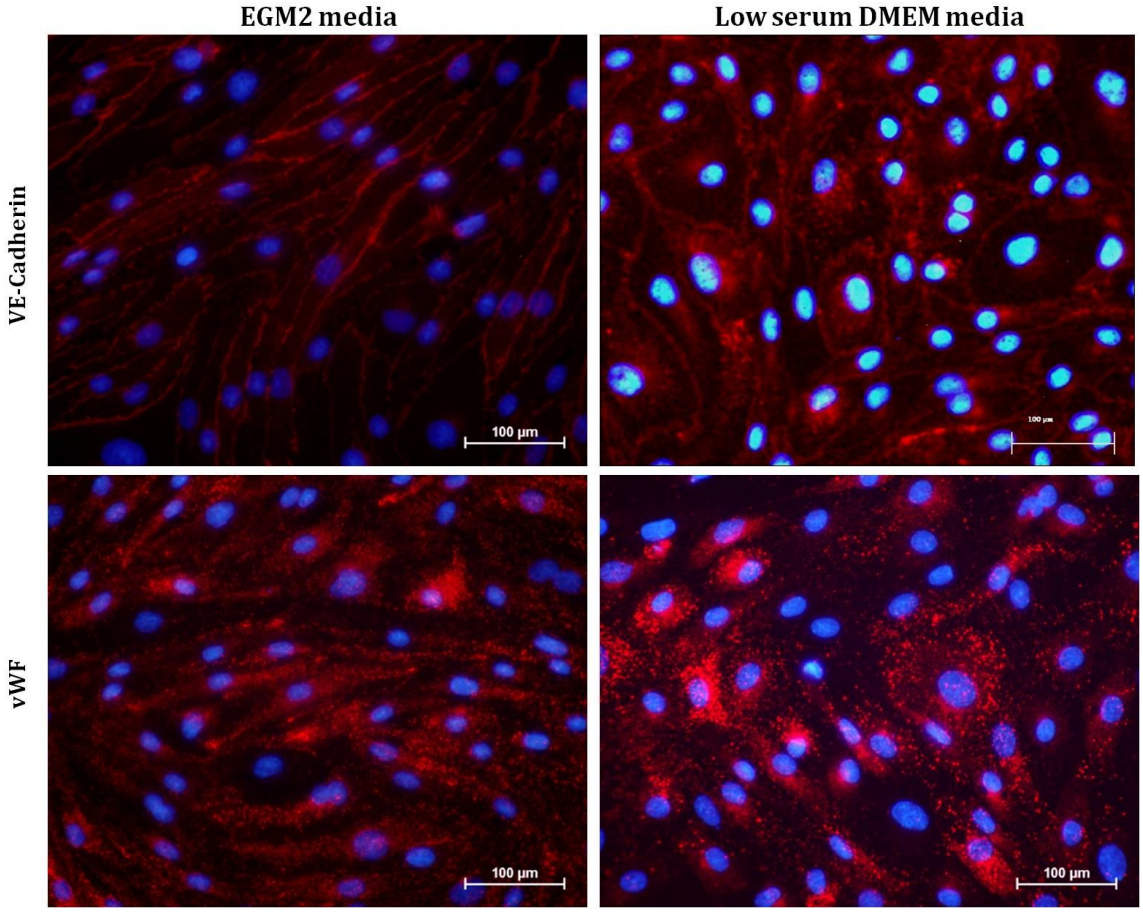


Figure 3.1 Endothelial phenotypes of aVECs with EGM2 media and low serum DMEM media. The expression of VE-Cadherin (top row) and vWF (bottom row) was positively stained in red colour by aVECs, at passage 4-5, which were cultured in EGM2 and subsequently maintained in low serum DMEM.

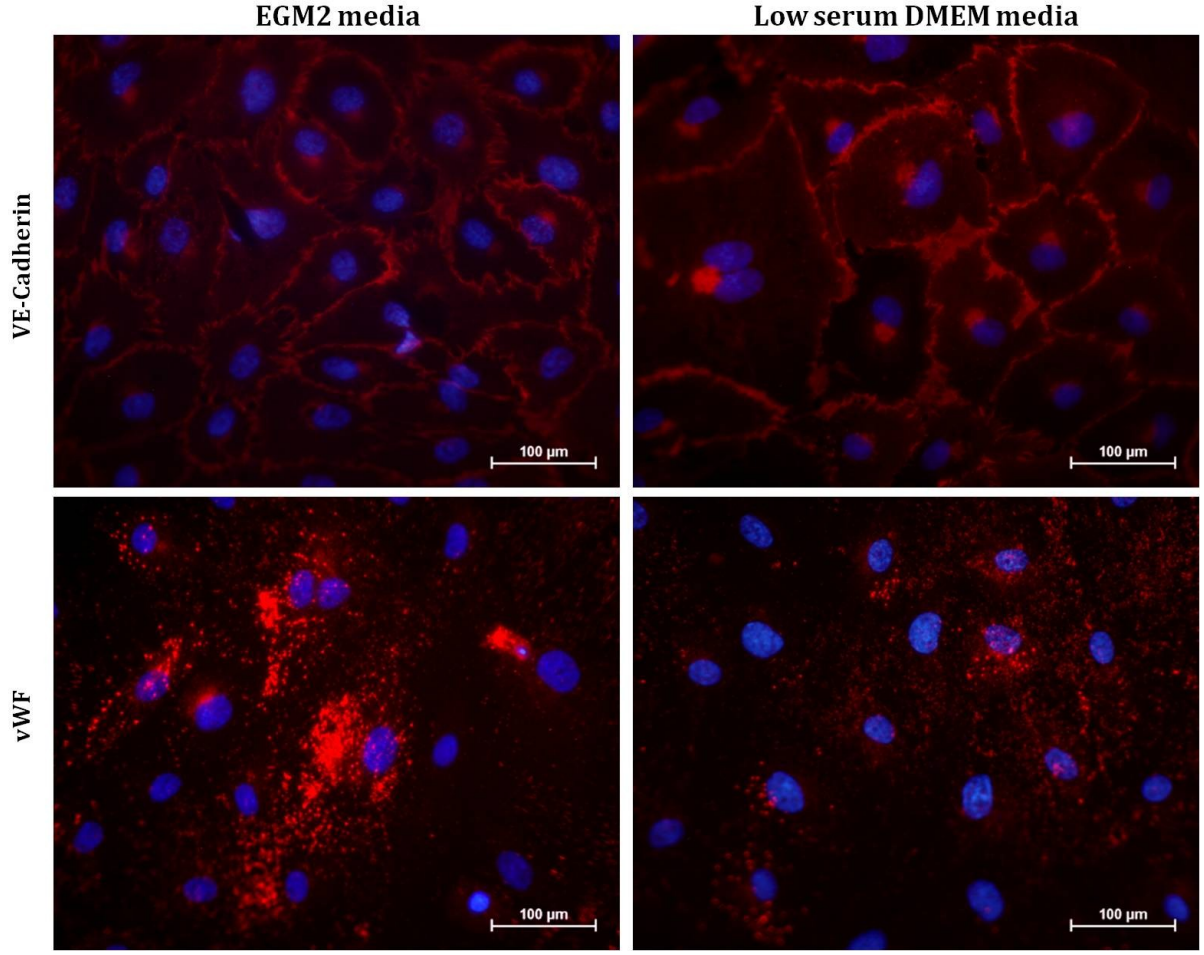


Figure 3.2 Endothelial phenotypes of vVECs with EGM2 media and low serum DMEM media. The expression of VE-Cadherin (top row) and vWF (bottom row) was positively stained in red colour by vVECs, passage 4-5, which were cultured in EGM2 and subsequently maintained in low serum DMEM.

3.3.2 Effect of VEC conditioned media on VIC proliferation

The effect of aVEC-CM and vVEC-CM that was collected at after 6, 12, 24 and 48 hours in contact with the respective VEC cultures was diluted to give a concentration range of 1:50 to 1:2. The effect of these dilutions was investigated on VIC proliferation (at 48 hours) and compared to the response of VICs in low serum media without VEC contacts (Figure 3.3).

The VEC-CM generated over 48 hours shown evidence of a concentration-dependent increase in the VIC cell number. Both aVEC-CM and vVEC-CM at 48 Hours had an increased effect on VIC proliferation, Spearman r = 1 and p-value = 0.02. The maximum effect was seen at a dilution of 1:2 by aVEC-CM and vVEC-CM at 184.74±5.47% and 199.25±2.60% of control, respectively. However, there was no difference between the effect of aVEC-CM and vVEC-CM ($p>0.05$, n=3). In addition, the sub-maximum effect was observed at a dilution of 1:5 by aVEC-CM and vVEC-CM at 169.05±4.26% and 179.28±7.23%, respectively. However there was still no difference among VIC controls and those cultured with VEC-CM ($p>0.05$, n=3).

There appeared to be an effect with time taken to generate the CM but the effect seen was not consistent. For example, the CM from the contact with VECs for 6 hours seemed to increas the proliferation of VICs but the correlation is not real as demonstrated by p=0.90. The VEC-CM at 12 hours appeared to decrease the proliferation of VICs when the concentration of VEC-CM was increased (inverse correlation at p=0.02). Moreover the effect of VEC-CM at 24 hours was variable, without the concentration dependence (p=0.95). The maximum effect on the proliferation of VEC-CM at 24 hours was found at the dilution of 1:10.

These results suggested that 48 hours was the optimal amount of time that VECs could generate growth factor in the CM to have a consistent effect on the proliferation of VICs. Moreover the sub-maximum effect by VEC-CM at the dilution of 1:5 would allow the increasing or decreasing effects on VIC response possible to be observed. Thus the VEC-CM was prepared at after 48 hours in contact with side-specific VECs at a 1:5 dilution to use in further investigations relating to the modulation of ECM proteins in VICs.

Figure 3a: VIC Proliferation under 6Hr VEC-CM Effect

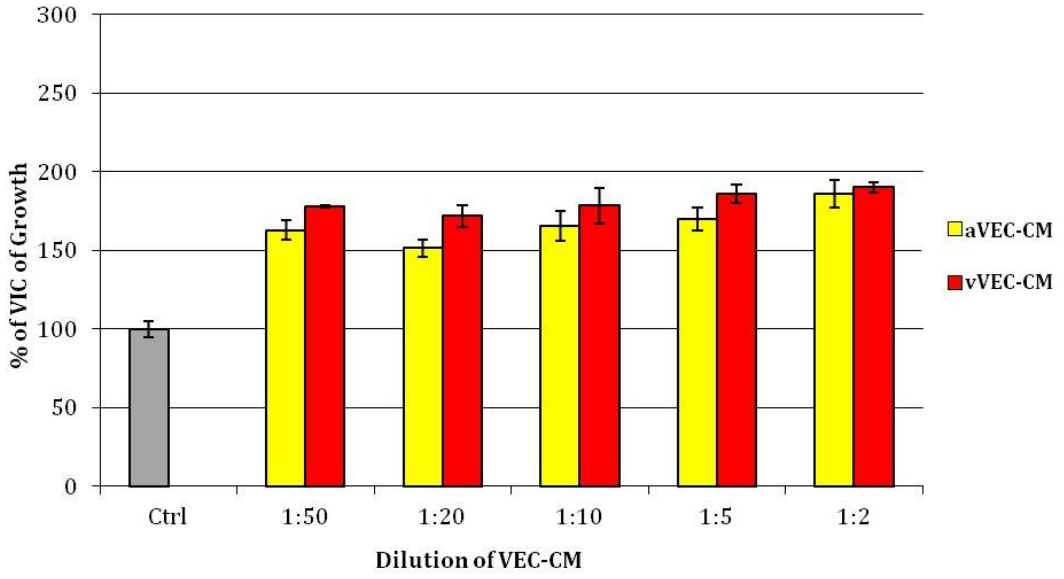
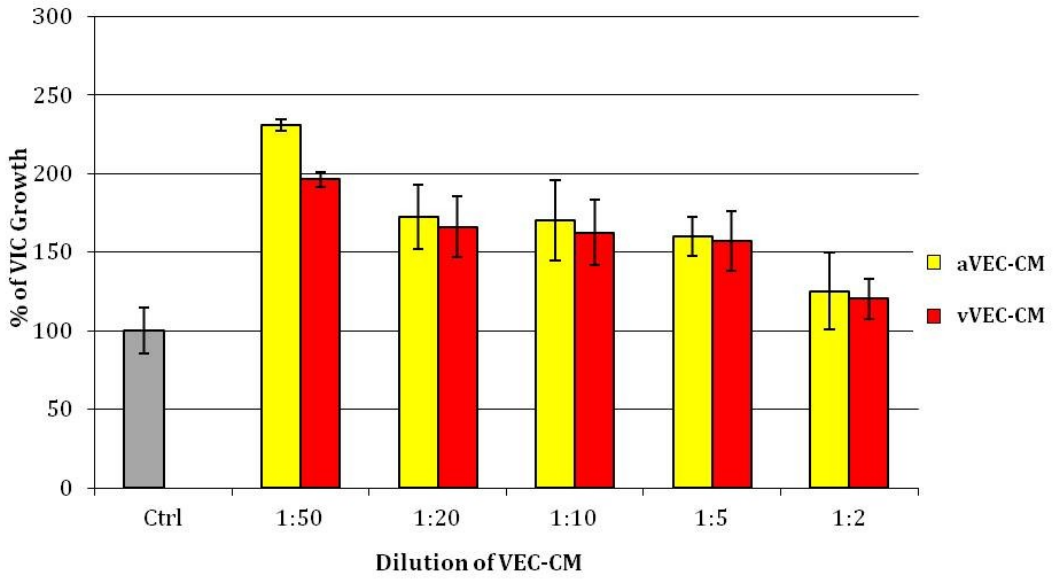
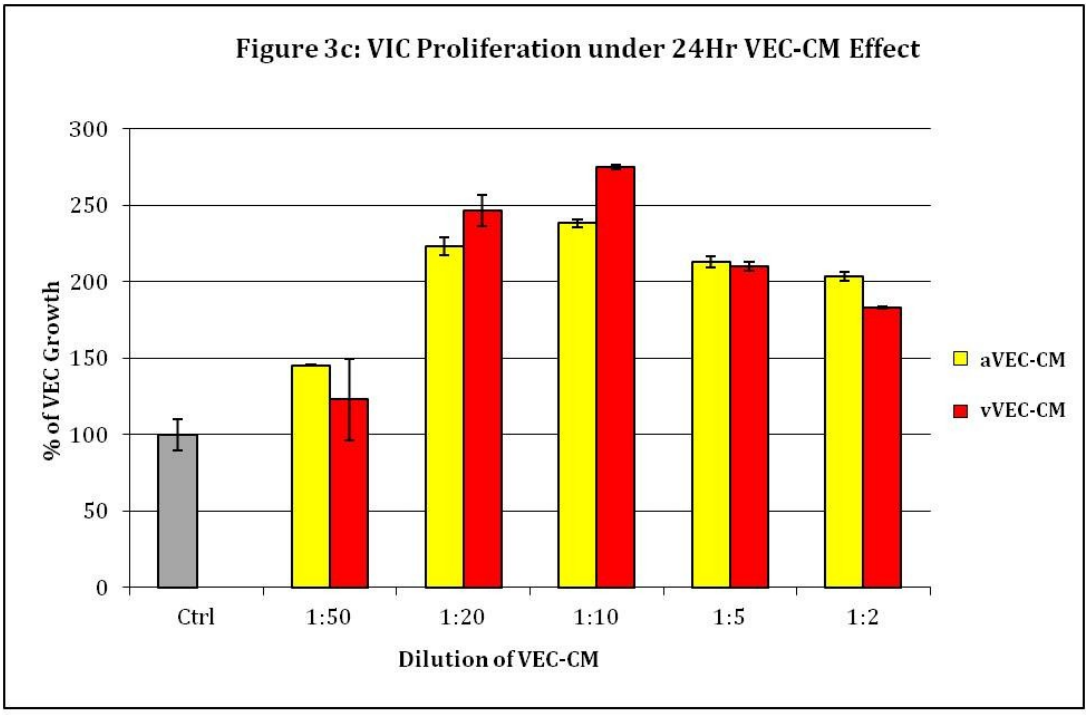


Figure 3b: VIC Proliferation under 12Hr VEC-CM Effect





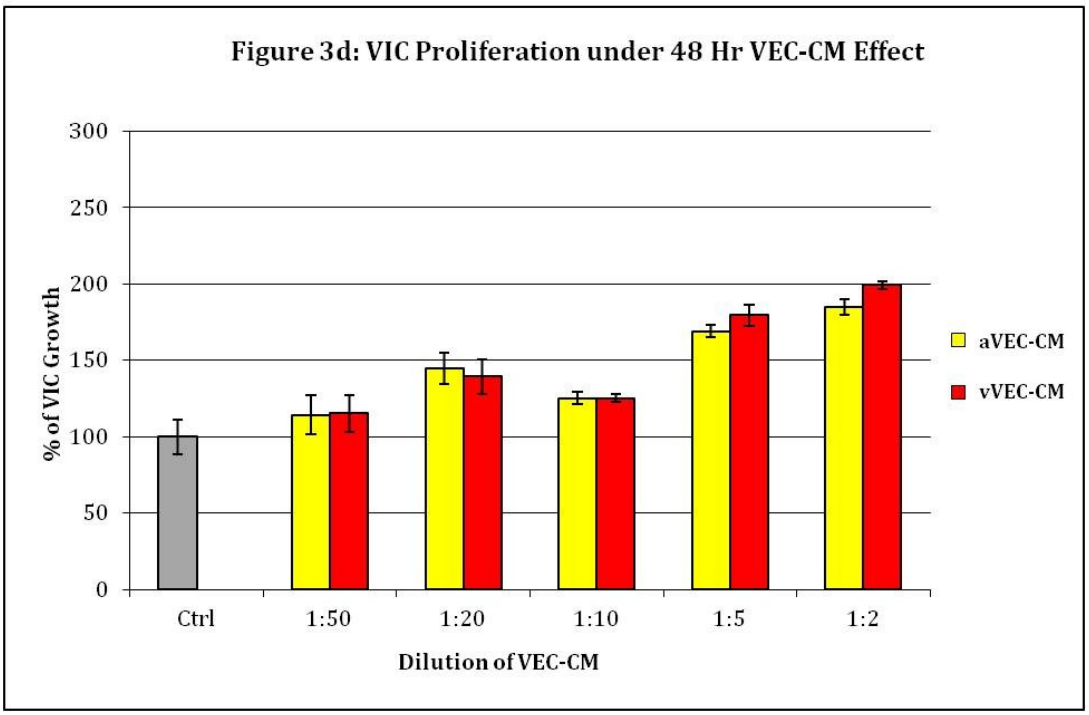


Figure 3.3 The effect of VEC conditioned media on VIC proliferation. VICs were cultured with aVEC-CM and vVEC-CM, n=2-3, generated at 6, 12, 24 and 48 hours and diluted with fresh 0.4% FBS DMEM at various concentration, 1:50, 1:20, 1:10, 1:5 and 1:2. All proliferation was examinated by MTS and compared to the effect of 0.4% serum DMEM. Graps were plotted by mean ± SEM using Excel and analysed the concentration-dependent effect by Spearman test. Whereas the VEC-CM at 12 and 48 hours showed the decreasing and increasing correlation, respectively, the VEC-CM at 6 and 24 hours had no concentration-depedent effect to the proliferation of VICs.

3.3.3 The effect of VEC-CM on the content of collagen

The production of collagen by VICs cultured with 0.4% FBS DMEM and VEC-CM, obtained at 48 hours, at a 1:5 dilution, were quantified after 2 days incubation with the CM (n=6). As demonstrated in the Figure 3.4, vVEC-CM significantly increased the production of collagen by VICs (median = 137.1%) as compared to the effect of VICs in 0.4% FBS DMEM, p=0.0240. Although media collected from the aVECs also increased the production of collagen by VICs (116.5%), this effect was not statistically significant.

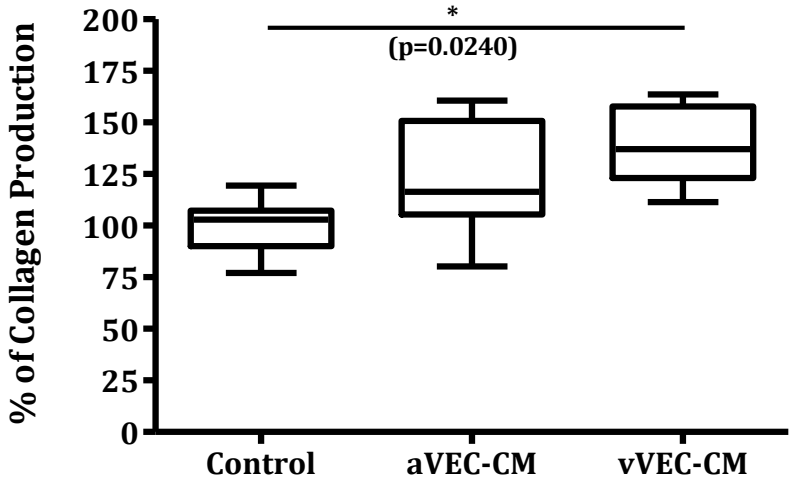


Figure 3.4 Collagen production by VICs incubated with conditioned from aVECs and vVECs. VICs were cultured with 0.4% FBS DMEM (the control) or conditioned media collected from aVECs and vVECs (n=6). Percentage of the amount of collagen produced by VICs cultured with VEC-CM was calculated relative to the 100% production by the control, obtaining from the same valve. vVEC-CM significantly increased the amount of collagen as compared to the control.

3.3.4 The effect of VEC-CM on the content of GAGs

The effect of VEC-CM on the production of secreted GAGs by VICs was investigated by comparing to the effect to low serum DMEM. The median values of GAGs

production by VICs in aVEC-CM and vVEC-CM culture were 114.5% and 121.3%, respectively, as shown in the Figure 3.5. The effect of the conditioned media collected from aVECs was not statistically different from those collected from vVECs.

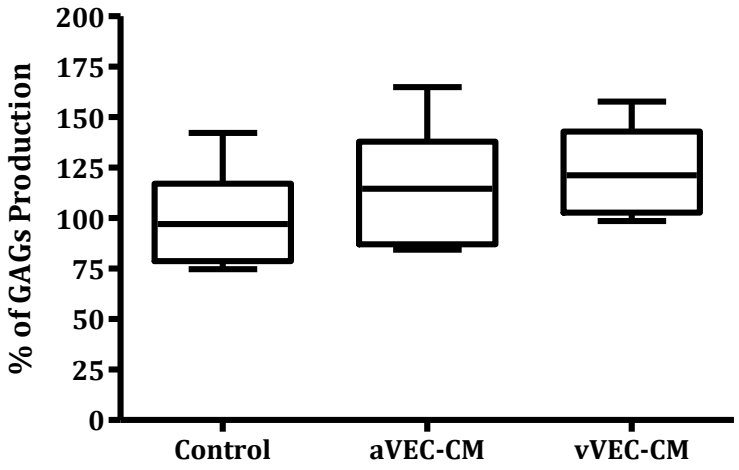


Figure 3.5 GAG production by VICs incubated with conditioned from aVECs and vVECs. VICs were cultured with 0.4% FBS DMEM (the control) or conditioned media collected from aVECs and vVECs (n=6). Percentage of the amount of GAGs produced by VICs cultured with VEC-CM was calculated relative to the 100% production by the control, obtaining from the same valve. There was no significant difference among VEC-CM treatments and control.

3.3.5 The effect of VEC-CM on the content of elastin

VICs were cultured with DMEM with 0.4% FBS, aVEC-CM and vVEC-CM for 48 hours and the production of elastin bound on the outer side of cell membrane was investigated. The percentage of elastin production by the CM was compared to the effect of low serum DMEM. Median values of the elastin for aVEC-CM and vVEC-CM were 148.6% and 152.1%, respectively. Although aVEC-CM and vVEC-CM showed an increase in elastin production compared to the control, there was no statistically significant difference observed between them (Figure 3.6).

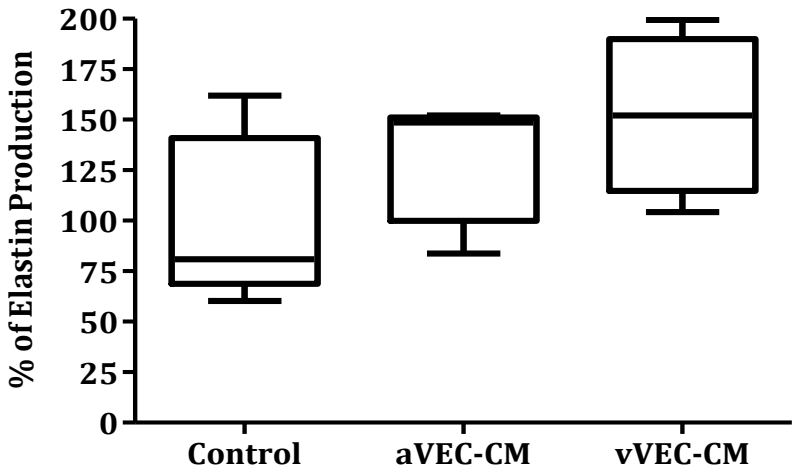


Figure 3.6 Elastin content of VICs incubated with conditioned from aVECs and vVECs. VICs were cultured with 0.4% FBS DMEM (the control) or conditioned media collected from aVECs and vVECs (n=6). Percentage of the amount of elastin produced by VICs cultured with VEC-CM was calculated relative to the 100% production by the control, obtaining from the same valve. There was no significant difference among VEC-CM treatments and control.

3.4 Discussion

The durability and function of valve tissue mainly depends on the ability of VICs to maintain the integrity of the ECM. Using porcine VICs and VECs, communication between these two cell types has been previously investigated (Butcher and Nerem, 2006). It was found that VECs decrease the proliferation of VICs and the expression of SMαA by VICs as well as the reduction of GAGs. Although it is well known that VECs on the aortic and ventricular side of the aortic valve are exposed to different patterns of blood flow, the differential ability of aVECs and vVECs to communicate with VICs has not yet been studied. In this study, accumulation of molecules released into the culture media by VECs at different time points were investigated to establish they have an effect on the proliferation of VICs. In addition, the effect of vVECs on the modulation of collagen content produced by VICs was studied.

During the generation of the CM, side-specific VECs were cultured with DMEM containing 0.4% FBS, in order to exclude the effect of factors within serum which may directly affect the response of VICs. Incubation in this low serum media had no appreciable effect of the phenotypic markers associated with ECs. After 72 hours incubation in the media, aVECs and vVECs, were seen to preserve the expression of the endothelial markers VE-Cadherin and vWF.

VEC-CM had some effect on the proliferation of VICs, but the pattern of this stimulation was inconsistent with different effects at different time points and dilutions of the CM. However, the concentration-dependent effect on the increasing proliferation of VICs was observed in the VEC-CM collected over 48 hours. In addition, 48-hour incubation was sufficient to investigate the modulation of ECM in the valves (Balachandran *et al*., 2006). The maximum proliferation of 48-hour VEC-CM was seen with a 1:2 dilution of the VEC-CM. In order to be able to observe any increasing and decreasing effect by CM, it is better to use a sub-maximum level

stimulation. Thus, a 1:5 dilution of VEC-CM was selected to be used in further experiments. It is noted that when the proliferation of VICs by the aVEC-CM and vVEC-CM at 1:5 dilution are compared to the negative control (VICs at growth arrested state), there is no statistical difference among this group (data not shown). This highlights the fact that the effect of VEC-CM on the modulation the ECM proteins will not be affected by changed number of VIC in these experiments.

In contrast to the effect seen at 48 hours of VEC-CM, the effects mediated by CM generated at earlier time point was inconsistent. The contradicting effect of the VEC-CM collected at 6 and 12 hours may be a result of the different rates of release by individual cultures of VECs. Similarly, vascular EC-CM collected from sparse EC culture stimulated the SMC growth, while dense ECs from the same culture inhibited the proliferation of SMCs (Dodge *et al*., 1993). Moreover, the concentration "independent" effect of any VEC-CM accumulated for 24 hours was also observed in vascular EC-CM (Dodge *et al*., 1993).

These data could also be explained by the fact that VECs may produce the combination of mitogenic and inhibitory factors at certain time points. The net combination among those counter-acting factors, at various dilutions may give rise to the variable results. It may take the longer time period in order to accumulate enough growth factors in the media to produce consistent effect.

In addition to the proliferation, the modulation of VEC-derived factors on the amount of collagen was also investigated by using VEC-CM that had been in contact with VECs for 48 hours and prepared at 1:5 dilution. vVEC-CM significantly increases the amount of secreted collagen by VIC culture. However, the amount of collagen remains the same as VIC control when VICs were cultured with aVEC-CM. The differing response of collagen production by VICs to the aVEC-CM and vVEC-CM suggests the distinct regulation by aVEC and vVEC derived factors in the CM.

While there is an observable effect of vVEC-CM on collagen content in the media additional experiments are needed to clarify what steps in the collagen synthesis process or the inhibition of collagen degradation that are modulated by vVEC-derived factors that ultimately result in this increase.

The transcription of collagen genes by vVEC-CM could be investigated by measuring mRNA of collagen type I and III genes. Additionally, the regulation at either transcriptional or translational levels could be further investigated by applying inhibitors of those processes i.e. Actinomycin D and cycloheximide, respectively. An alternative method would be to look only at newly synthesised collagens by using radiolabeled proline, which is incorporated preferentially into newly synthesised collagen in the form of hydroxyproline. However while the use of radiolabel ^{3}H-proline is a more sensitive assay for collagen synthesis, it is expensive, has a number of safety issues and not all the facilities were available at our laboratory at the time these experiments were performed. Moreover, the regulation of collagen degradation should be further studied to understand whether vVEC-CM inhibits the degradation of collagen and, in turn, increases the amount of collagen as compared to the VICs alone. Thus the production of MMPs e.g. MMP-1, 2 and 13 and their inhibitors, TIMPs, should be investigated using zymography to compare the activities of MMPs in to aVEC-CM and control media.

In this study, the effect of VEC-CM on the production of secreted collagen was quantified using a dye-binding assay which binds to all the collagen isoforms found in the valves i.e. collagen type I and III. Although the dye is claimed to bind specifically to [Gly-X-Y]n, the helical structure of the collagens, preliminary data demonstrated that 10% FBS in fresh DMEM can non-specifically precipitate the dye. Thus all the ECM quantification was carried on by VICs cultured either with 0.4% FBS DMEM or by VEC-CM made from the low serum media.

Additionally, this study only measured the collagen released into the media which had previously been suggested to represent the majority of collagens released in these types of *in vitro* experiments (Myllyharju and Kivirikko, 2004; Villanueva *et al*., 1991). However, valve cells have been shown to be as efficient at incorporating the soluble collagen into the cells layer as releasing them into the media over 2 day period (Ku *et al*., 2006).

It is recommended to measure surface attached collagen by using acid-pepsin to release collagen into soluble form. An alternative method would be to analyse aged covalent crossed-link collagen using an acid hydrolysis method (6 N HCl, 110° C, 18 Hours). Under this harsh condition, protein would be broken down into amino acid and passed through ion-exchange chromatography to quantify the amount of hydroxyproline, a collagen marker, and then referred to the amount in the standards. However, there is likely to have some degree in inefficiency in this extraction process and the low levels measured in the current experiments may not have been suitable for this technique. Although the current experiments may not give an accurate measure of all the collagen produced by the cells, the methodology used does provide an index of collagen production and shows a clear relative difference in the collagen production secreted by VICs in response to aVEC-CM and vVEC-CM.

The regulation of collagen production by EC in vascular system has been well established. The effect of aorta and endocardial EC-CM on increasing the production of collagen by SMCs and cardiac fibroblast has been investigated (Villanueva *et al*., 1991; Kuruvilla *et al*., 2007). Villanueva *et al*. demonstrated that the amount of secreted collagen was significantly increased when the fibroblast were cultured with EC-CM for 24 hours, whereas the amount of membrane-bound collagen was minuscule and slightly higher than those of the fibroblast control. The authors also predicted the responsible molecule for the induction of collagen production would

be heat-stable molecule and have molecular weight of 8 -10 kDa. Such a molecule could be TGF-β, which has been shown to be present in EC-CM by Kuruvilla and colleagues (Kuruvilla *et al*., 2007). Moreover TGF-β in the CM was also demonstrated to reduce the proliferation of cardiac fibroblasts. Correspondingly in the previous chapter, TGF-β was detected in the media collected from the incubation with both aVECs and vVECs for 48 hours. However the question of whether TGF-β contributes to the increasing collagen production by vVEC-CM warrants further investigation by using neutralising antibodies against TBF-β.

GAGs are one of the ECM components important to the heart valve and vessel structure. The investigations of EC-derived factors revealed that EC-CM increased the production of GAGs by vascular SMCs (Ciolino *et al*., 1992; Merrilees and Scott, 1990). TGF-β was also identified as the inducer in EC-CM that activates the *de novo* synthesis of GAGs by SMCs (Ciolino *et al*., 1992; Merrilees and Scott, 1990). Moreover, the pulse-chase analysis of the secretion and degradation of radiolabeled ^{35}[S]-labeled GAGs confirmed that the increasing amounts of GAGs seen were not due to an effect of EC-CM on the secretion and degradation of existing GAGs but a synthesis of new proteins present in the media (Ciolino *et al*., 1992). In addition, 80% of the incorporated radioactivity was associated with the medium fraction, after 24 hours incubation.

As established in studies with valve cells, VECs have been shown to reduce the loss of GAGs by VICs, which were encapsulated in a collagen gel and exposed to laminar flow (Butcher and Nerem, 2006; Ciolino *et al*., 1992). In the investigation of the effect of VEC-CM, all sulfated GAGs in the media were collected and quantified using the dye binding method. The dye binds to sulphated polysaccharide component of proteoglycans and glycosaminoglycans e.g. chondroitin (4-, 6-), keratin, dermatan and heparin sulfates, which are the main GAGs component in the aortic valve (Rothenburger *et al*., 2002). The results reveal that VEC-CM collected after 48 hours

in contact with aVECs and vVECs, maintains the amount of secreted GAGs equal to the amount found in growth-arrested VICs. Even so, it is unclear, in this experiment, whether both VEC-CMs are unable to stimulate the synthesis of GAGs by VICs or they have an effect to protection against the degradation of GAGs that are produced by VICs under resting conditions. This effect may be influenced by the fact that in these experiments the VICs are maintained in a quiescent, growth arrested state.

Similar to the effect of VEC-CM on GAG production, VEC-CM does not change the amount of tropoelastin produced by VICs when compared to the VICs cultured with low serum media, without VECs contact. The tropoelastin, in this study, was extracted from α-elastin bound on the VIC membranes and quantified by the dye binding method. The amount of elastin secreted in the media was also investigated by using an ELISA technique, which has higher sensitivity (able to detect the minimum amount of elastin at 0.62 μg/ml) than the Fastin kit (able to detect a minimum amount of 2.5 μg/ml). However, elastin could not be detected in the media of any experimental samples using ELISA (data not shown). It has been well established that the entire intracellular synthesis of elastin is completed within 20 minutes; however, once the fibre of elastin is formed, the remodeling process is much slower and can take time up to a year (Starcher, 1986). Thus the conclusion from this study is any elastin which is produced by VICs in a growth-arrested state, is in the membrane bound form and its content is not modified by the effect of VEC-CM.

These results show an interaction between VECs and the VICs that reside within the valve. In addition, the results suggest the differing capability between aVEC-CM and vVEC-CM in regulating collagen content. Further experiments to characterise molecules in the CM, which are responsible for such function are required. For example, the VEC-CM can be treated with trypsin, proteinase K, heated up or blocked using antibody against potential mediators such as TGF-β and ET-1

(Kuruvilla *et al*., 2007; Dodge *et al*., 1993). The treated VEC-CM will then be used to culture VICs and any changes in the modulation of ECM by VICs will be determined.

Although this experiment reveals the effect of VEC-derived factors on the regulation of VIC proliferation and collagen content, it is not suitable to allow short-lived molecules, which are degraded over the 48 hours collection period to have an effect on VIC response. Therefore, further experiments to culture VICs in the vicinity of VECs are required to include the additional potential effects of labile mediator released by VECs.

3.4.1 Limitations of the study

There are few limitations in these experiments. The CM is usually limited by certain time points of the collection. This then means the method cannot include the extensive production of cytokines, which may be unstable at such collecting time or may require a longer period of the production time than the time of maintaining cells on low serum media may allow. Moreover the effect of low serum in the media might alter cellular activity and influence the responsiveness of both VEC and VIC.

3.5 Conclusions and Future Directions

The mitogenic effect of VEC-derived factors on the proliferation of VICs is demonstrated in these experiments. The increasing of cell numbers in VICs cultured with either aVEC-CM or vVEC-CM is established in the CM collected at different time points i.e. at 6, 12, 24 and 48 hours. Both aVEC-CM and vVEC-CM share a similar effect on the regulation of VIC proliferation without statistical differences between the different dilutions or time points at which the CM was obtained.

Whether side-specific VECs differentially regulate the production of ECM by VICs is also elucidated. The vVEC-derived factors significantly increases the amount of collagen secreted into the media by VICs when compared to VICs cultured with low serum media. Both aVEC-CM and vVEC-CM maintain the production of GAGs and elastin by VICs to the same extent as control.

In these experiments, the side-specific VECs reveal the function to regulate the proliferation and ECM production of VICs by the molecules that are stable for 48 hours. However the role of short-life molecules, which may affect the response of VICs, is neglected by this experimental setting. Thus VECs will be co-cultured with VICs in order to allow the continuous communication between VECs and VICs and thereby be able to assess any functional changes of VICs induced by VEC.

CHAPTER 4 Paracrine Effect of VECs on VIC Function by Co-Culture

4.1 Introduction

Previously in Chapter 3, soluble mediators secreted by VECs and accumulated in the media over 48 hours were seen to regulate the proliferation and production of ECM proteins by VICs. However the investigation of the effects mediated by VEC-CM may exclude some VEC-derived factors that are degraded in the media during the collection period or during storage prior to use. There are numerous methods for culturing ECs adjacent to other cells such as fibroblasts, SMCs or VICs. These methods have the advantage over CM in that the communication between the cells occurs in real time.

Direct contact between ECs, from the aorta, and SMCs revealed an increasing synthesis of sulfate-GAGs, when compared to the ECs and SMCs alone (Merrilees and Scott, 1981). Although the cells responsible for the greater production could not be identified due to mixing of the cells together, the direct contact of this 2-D co-culture had the benefit of direct cell-cell communication through membrane junction proteins (Merrilees and Scott, 1981).

The paracrine effect of ET-1, NO and TGF-β which were produced by ECs on co-cultured adjacent cells can also be investigated by growing ECs on a permeable membrane inserted into a culture well and culturing other cells underneath the membrane (Powell *et al*., 1997; Rizvi *et al*., 1996; Merrilees and Scott, 1990; Ciolino *et al*., 1992; Kolpakov *et al*., 1995). Aortic ECs have different effects on the regulation of collagen production by different types of cells such as SMCs and fibroblasts. While the ECs increase the production of collagen when co-cultured

with fibroblasts (Guarda *et al*., 1993), they have also been reported to reduced the production of collagen by adjacent SMCs (Powell *et al*., 1997).

Venous ECs were examined for their effect on the regulation of ECM production by myofibroblasts, which were encapsulated in the 3-D scaffold. The scaffold that was seeded with the ECs on the surface had a reduced amount of collagen but maintained the amount of GAGs when compared only to the scaffold containing myofibroblasts (Pullens *et al*., 2009). Recently, when the effect of laminar shear stress was studied in heart valve cells, mixed VECs from both sides of the valve protected the lost of GAGs in the scaffold containing VICs under both static and flow conditions (Butcher and Nerem, 2006). However, the regulation of side-specific VECs on the VIC function has not been examined.

Thus, in the present study, VICs will be co-cultured either with aVECs or vVECs grown on a permeable membrane, above a population of VICs. The effect of VECs on the proliferation and production of ECM components by VICs will be investigated and compared to the VIC cultures without VECs on the insert.

4.2 Materials and Methods

4.2.1 Co-culture of VICs and VECs by Transwell®

The effect of aVECs and vVECs on the VICs' functions was further observed by using Transwell® Permeable Supports (Corning, UK). VECs were grown on the Transwell® insert made from polyester microporous membrane (0.4 μm pore size at $1x10^8$pores/cm^2).

The insert was later hung over a culture plate containing VICs grown onto the base of the well. By using these supports it is practical to have a co-culture of VICs and VECs without direct cell contact. Thus the continuous effect of released factors by VECs on VIC function e.g. proliferation, ECM gene expression and ECM production can be examined.

4.2.2 Cell phenotyping of VECs on the insert membrane

VECs on the insert both with and without VICs (at the bottom wells) were characterised EC phenotypes after 48 hours of incubation. The polyester membrane was cut out off the insert of the co-culture system and held with a forceps to avoid the damage of VECs. The immunostaining against vWF, CD31, VE-Cadherin and SMαA were conducted in the similar way as section 2.2.4.

4.2.3 VIC proliferation under the effect of co-culture condition by MTS assay

The VICs were seeded at 3.0 x 10^3 cells per well of a 96-well plate, while 8.5 x 10^3 cells of aVECs and vVECs were separately seeded on the insert of Transwell® with a surface area of 0.143 cm^2. After the cells had become confluent, the insert containing VECs was placed into the culture plates over VIC culture. DMEM with 0.4% FBS was used to culture both VECs on the insert and VICs on the plates. At the same time, VICs were cultured at the identical condition without VECs being present on the insert and served as the control, as depicted in the Figure 4.1.

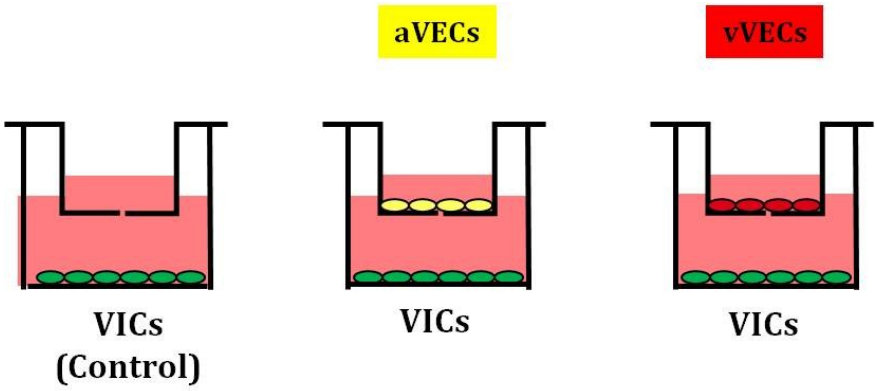


Figure 4.1 Co-culture model. aVECs (middle) and vVECs (right) are cultured on the permeable membrane insert without the direct contact with VICs. VIC culture which have not VECs on the inserts serves as the control. All cells on the inserts and the bottom wells are maintained with 0.4% FBS in DMEM and co-cultured for 48 hours before the amount of ECM compernents will be quantified.

Complete DMEM was used to culture VICs (without VECs on the insert) to ensure that they could proliferate under the experimental conditions, these cells served as a positive control. VICs and VECs were co-cultured for 48 hours in a 95% air/5% CO_2 incubator. At the end of the incubation period, both inserts containing VECs and

those with no-cells were removed from the culture plates. The proliferation of VICs was consequently measured by using MTS assay as already described in the section 2.2.6.

4.2.4 The effect of VEC co-culture on the production of ECM by VICs

aVECs and vVECs were co-cultured with VICs that were isolated from the same animal/aortic valve. aVECs and vVECs were cultured separately on larger permeable membrane with a surface area of 4.67 cm^2 in area at a density of 2.8 x 10^5 cells per insert.

VICs were seeded at a density of 5.2 x 10^5 cells per well of a 6-well plate. 3 ml of DMEM with 0.4% FBS was added to VIC culture and while 1.5 ml of the media was added on the insert containing VECs. The cells were maintained in co-culture for 48 hours. Upon completion, the media over VIC culture was collected to be analysed for the amount of collagen and GAGs while VICs were collected for the investigation of elastin production and ECM gene expression. Moreover, the wells that had only VECs on the insert, with no VIC on the base of the well, were also used so an assessment of ECM proteins release directly from VEC could be made.

Due to the possibility of media evaporation and changing the concentration of ECM in the media, media over VIC culture (including the media in 6-well plate of negative control) was topped-up with distilled water to 3 ml (equal to the starting volume). Subsequently, a volume of media was taken for collagen and GAG assay and calculated back for the total ECM production in 3 ml of media. From the same co-

culture settings, VICs were washed with PBS twice before the extraction of mRNA and elastin were begun.

4.2.4.1 Collagen production by VICs in co-culture model

The quantity of collagen was examined using a Sircol kit after 48 hours of the incubation as described in section 3.2.4. 1 ml of each sample was taken to perform the assay. The amount of collagen in samples was calculated relative to the amount of absorption of standard curve. The total production from 5.2×10^5 VIC cells was then calculated by allowing for the dilution factor.

4.2.4.2 GAGs production by VICs in co-culture model

1 ml from a total of 3 ml of media collected from the different cultures was concentrated by being freeze dried overnight. It was then resuspended in a volume of 500 µl of distilled water to quantify the amount of secreted GAGs by Blyscan kits as described in section 3.2.5.

Triplicates of 75 µl of every sample were made up to a volume of 100 µl with distilled water before starting the assay and then compared to the amount of GAGs relative to the standard curve. The total amount of GAGs produced by VICs or VECs on the insert was eventually calculated back by allowing for the dilution factor used to dilute sample media.

4.2.4.3 Elastin production by VICs in co-culture model

Membrane bound tropoelastin was extracted from the VICs' membrane by using hot oxalic acid, quantified by the Fastin kit as described in section 3.2.6. The total production of elastin from 5.2 x 10^5 VICs at different co-culture conditions was quantified by comparing the absorption of the dye to the standard curve and then multiplying the value to allow for the dilution factor.

4.2.5 Reverse Transcription and Real Time - Polymerase Chain Reaction (RT-PCR) of ECM genes

The ability of VIC to produce ECM proteins in response of co-culturing with VECs was also investigated at a transcriptional level. Gene expression of selected ECM components was observed after 48 hours of co-culturing with either aVECs or vVECs and then compared to VICs culturing in the absence of VECs.

4.2.5.1 RNA isolation

The co-culture system was stopped after a period of 48 hours by separating the inserts and the 6-well plates which contained VICs from each other. The VICs were washed twice with PBS and removed from the plates with Trypsin. After 5 minutes of incubation with Trypsin at 37°C, DMEM media with 10% FBS was added to stop the reaction. VICs were collected by centrifugation at 700 g for 5 minutes and the supernatant was removed carefully by aspiration. The RNA of VICs was subsequently obtained by using RNeasy Mini Kit (Quigen, Uk), otherwise cell pellets were kept at -80°C and the extraction performed at a later date. .

To break cells and nuclear membrane, 350µl of fresh RLT buffer containing Guanidine Thiocyanate and β-mercaptoethanol was added and mixed by pipetting up and down. 350 µl of 70% ethanol, which was prepared by mixing molecular grade ethanol with DEPC treated water (Sigma-Aldrich, UK), was then added to the lysate. This 700 µl solution was then transferred to an RNeasy spin column placed in a 2 ml collection tube. Released RNA was selectively promoted to bind to the RNeasy column's membrane in the presence of ethanol. The contaminants were flushed through the membrane by centrifugation at 8000 g for 15 seconds and the flow-through in the collection tube was then discarded.

The column membrane was then washed with 350 µl of RW1, centrifuged at 8000 g for 15 seconds and the flow-though was discarded. DNA bound on the membrane was specifically digested by DNaseI (Qiagen, UK) to ensure that the DNA did not interfere with absorbance at 260 nm during the RNA quantification step. As recommended by the manufacturer, 10 µl DNaseI stock solution was mixed with 70 µl Buffer RDD by pipetting.

The prepared enzyme was then added to the column and incubated at room temperature for 15 minutes for the DNA digestion reaction to take place. DNaseI was then washed from the membrane with 350 µl of RW1 buffer. Centrifugation at 8000 g for 15 seconds was performed again to remove unbound remnants and the flow-through was discarded. The column was subsequently was washed twice with RPE containing ethanol.

The second washing was extended, the spinning time being increased to 2 minutes to ensure that the ethanol was removed completely and did not interfere subsequent steps. The flow-through was discarded with the collection tube and the membrane column was transferred to a new collection tube. To eliminate all

remnants of RPE, the column was then spun at full speed for 1 minute, after which the membrane column was removed to a clean and labeled 1.5 ml microtube.

Finally RNA was eluted from the membrane by adding 30 µl of RNase free water directly to the membrane and centrifuged at 8000 g for 1 minute. The eluted RNA was handled on ice during quantification and cDNA synthesis or stored at - 80°C to be used later.

4.2.5.2 cDNA synthesis

RNA was quantified and the purity was assessed by its absorbance at 260/280 nm, before complimentary DNA (cDNA) was synthesised. A 2 µl sample of RNA was diluted with 58 µl of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and its absorbance at 260 and 280 nm was measured by a double beam spectrophotometer, GeneQuant II (Pharmacia Biotech, UK).

The RNA concentration was calculated from the rule that 1 unit of absorbance at 260 corresponds to 44 µg of RNA per ml. It is important to note that this equation (1 unit of A_{260} = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH. In addition, the ratio of A_{260}/A_{280} used to assess the purity of RNA is influenced significantly by pH; A_{260}/A_{280} is higher when pH increases from 5.4 to 7.5 as A_{280} decreases when A_{260} is unchanged. Thus TE buffer at pH 8.0 was used as a diluent when measuring its absorbance to ensure an accurate reading. Pure RNA should have a ratio of A_{260}/A_{280} in the range of 1.9-2.1. Since protein absorbs at 280 nm, a value lower than 1.9 indicates there is protein contamination. It is recommended that soon after quantification, RNA is used for making cDNA; when this was not possible RNA was kept at -80°C.

100 ng of total RNA was used as the templates to make cDNA by MultiScribe™ Reverse Transcriptase (Applied Biosystems, UK) composing of 1x RT buffer, 5.5 mM $MgCl_2$, 500 µM each dNTP, 2.5 µM Random hexamers, 0.4 U/µl RNase inhibitor and 1.25 U/µl MultiScribe reverse transcriptase. The reaction was prepared at a total 10 µl and cDNA synthesis was performed by the Techne PHC-3 PCR machine (Techne, UK). The reaction was started with primer binding at 25°C for 10 minutes, and then the cDNA was extended at 48°C for 30 minutes.

The last step was denaturing reverse transcriptase at 95°C for 5 minutes. After 3 steps of synthesis were completed, cDNA was diluted with RNA graded water to 2 ng/µl and kept at -20°C.

4.2.5.3 Amplification efficiency and RT-PCR of 6 ECM genes

6 genes involving ECM production were selected to examine the VICs functions. Primers were used that were tagged with reporter and quencher from TaqMan® Gene Expression Assays (Applied Biosystems) for Collagen1α1 (COL1A1), collagen3α1 (COL3A1), Chondroitin sulfate proteoglycan 4 (CSP4), Hyaluronan synthase 2 (HAS2), Fribillin 1 (FBN1) and Fribillin 2(FBN2) genes. The manufacturer would not disclose the sequences of these primers.

Various amounts of cDNA (4, 6, 8 and 10 ng) were prepared for the RT-PCR reaction to evaluate the amplification efficiency of target genes compared to that of endogenous gene, 18s RNA. All samples for RT-PCR were set up in a total volume of 20 µl composing of 1x Taqman® Fast Universal PCR Master Mix (Applied Biosystems, UK), 1x of 18s primers, 1x of target gene's primers and cDNA. The amount of cDNA was prepared at various amounts for the amplification efficiency investigation or at 6 ng for co-culture samples. The reaction was performed by

Applied Biosystems 7500 Fast Real-Time PCR System for 40 cycles. Each cycle, cDNA was denatured at 95°C for 15 seconds then primer binding and cDNA extension were achieved at 60°C for 60 seconds.

4.2.6 Data and statistic analysis

The results of the proliferation and the production of ECM by VICs were represented as a percentage change from the control values. The data was represented by Box and Whiskers plot using GraphPad Prism 5. Median value from each condition was expressed by a bar in the middle of the box and statistically compared using Kruskal-Wallis and Dunn's test.

The amplification efficiency of the RT-PCR was analysed by plotting the ΔC_T value against increasing amounts of cDNA. The slope of validated genes should be lower than 0.1. The expression of ECM genes was analysed by $\Delta\Delta C_T$ using a control from each animal as a reference at 1 fold expression. The data was scatter-dot plotted by GraphPad Prism 5when. The level of expression was statistically compared by Kruskal-Wallis and Dunn's test. P value less than 0.05, 0.01 and 0.001 are represented by *, ** and ***.

4.3 Results

4.3.1 Phenotypic characterisation of VECs in co-culture with VICs

Phenotypes of VECs were characterised after co-cultured with VICs, with low serum DMEM for 48 hours, in comparison to the phenotype of VECs in the culture without VICs. aVECs and vVECs were stained with antibodies against EC proteins such as CD31 (represented in green colour) and vWF (in red colour), as shown in Figure 4.2. Both aVECs and vVECs maintained EC phenotypes by expressing both CD31 and vWF, regardless of the presence of adjacent VICs and the absence of EC growth factors.

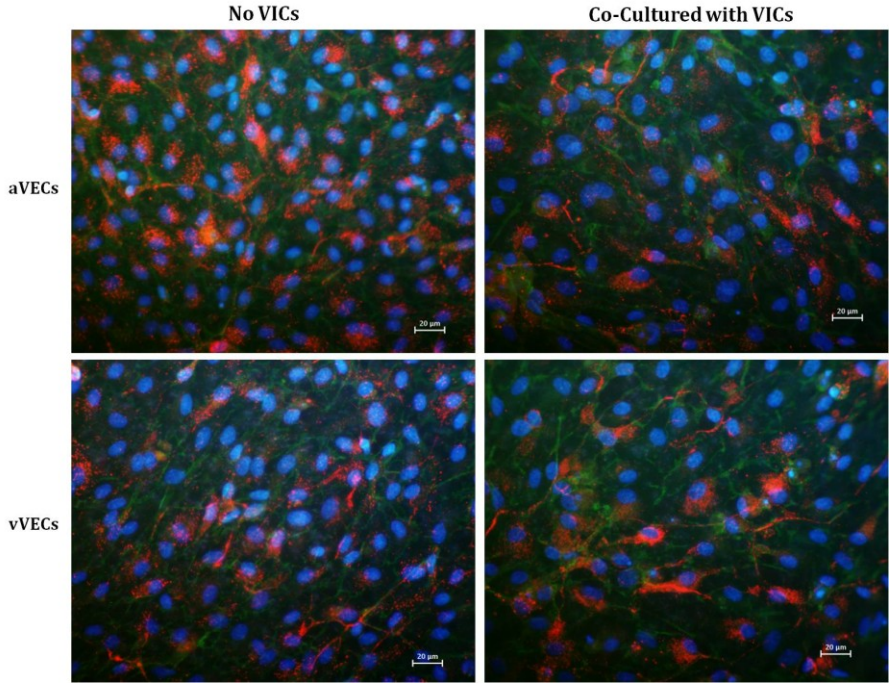


Figure 4.2 Expression of endothelial markers by VECs co-cultured with and without VICs. After 48 hours of incubation, both aVECs and vVECs were positvely stained against the antibodies of CD31 (represented in green staining) and vWF (in red staining), n=5. The left row displayed VECs cultured on the permeable membrane without VICs whereas VECs on the right row demonstrated the cultures which had adjacent VICs grown underneath, for 48 hours.

Moreover, the co-expression of VE-Cadherin and SMαA were also observed (Figure 4.3). Both aVECs and vVECs were negatively stained with the antibody against SMαA (green colour), despite of the presence of VICs in the co-culture. However VE-Cadherin staining (red colour) was faint but not relative to nuclei (in blue); suggesting non-specific staining and not related to the effect of co-culture with VICs.

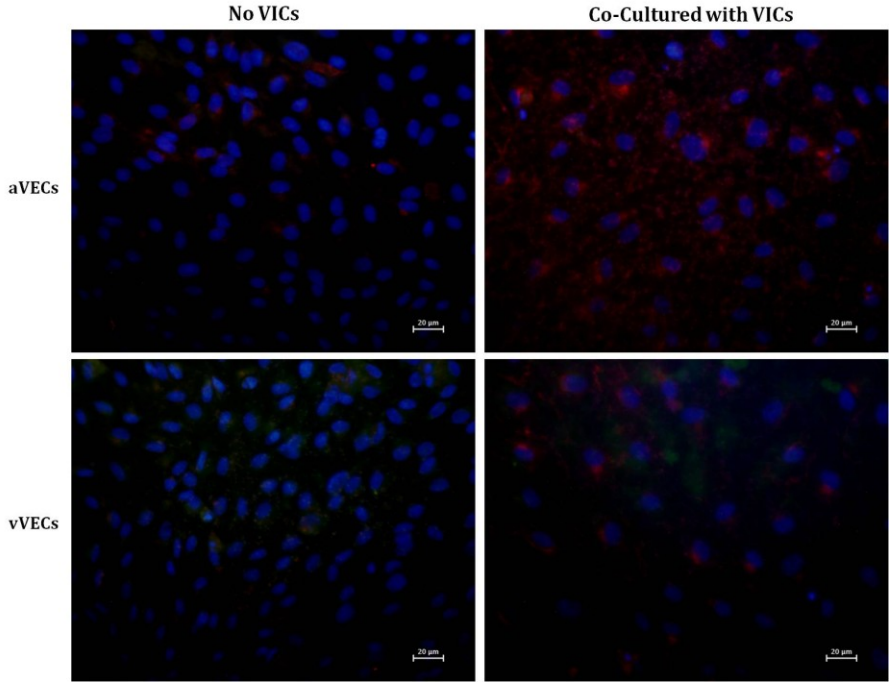


Figure 4.3 Expression of endothalial and interstitial marker by VECs co-cultured with and without VICs. VECs were weakly stained for VE-Cadherin (in red) and negative for SMαA (in green) after 48 hours of the incubation either with (the right panel) and without VICs (the left panel), n=5. All nuclei was stained in blue,

4.3.2 The effect of VEC co-culture on the proliferation of VICs

The regulation of VECs on the proliferation rate of VICs was investigated and compared to the growth rate of VICs cultured without VECs. All cells were maintained with low-serum DMEM and their proliferation was measured at 48 hours after the co-culturing or incubation. The results showed that both aVECs and

vVECs did not change the proliferation rate of VICs, at 48 hours of co-culturing (Figure 4.4).

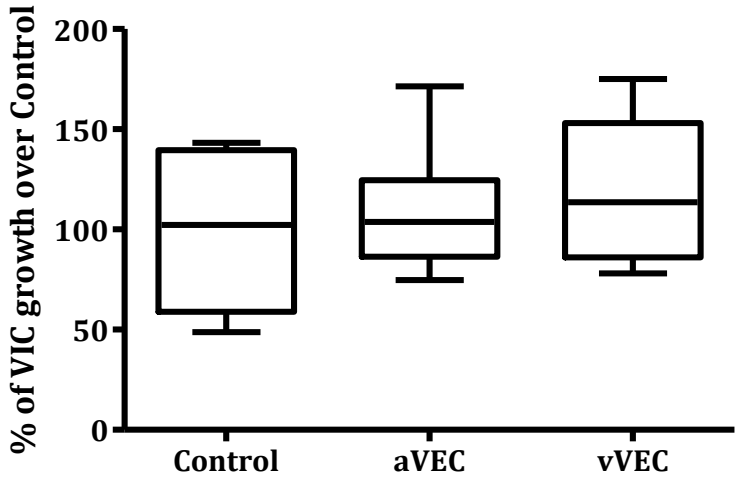


Figure 4.4 VIC proliferation in the presence of side-specific VEC co-culturing. VICs were co-cultured with either aVECs or vVECs (represented as aVEC and vVEC, respectively) for 48 hours. Their proliferations were normalised to the control (VICs alone) at 100% proliferation. The median values were presented by bars and the statistic comparison was conducted by Kruskal-Wallis and Dunn's Test, n=8.

4.3.3 The effect of VEC co-culture on the production of collagen by VICs

The amount of collagen produced by 5.2 x 10^5 VICs under different conditions was investigated. The collagen content of the control (VICs cultured with low-serum DMEM and in the absence of VECs), was normalised to 100%. The effect of VEC co-culturing for 48 hours was expressed as a percentage of the control (Figure 4.5).

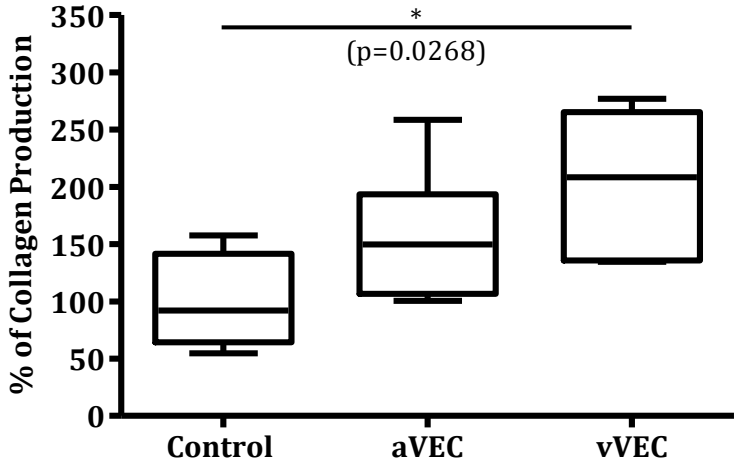


Figure 4.5 The effect of VEC co-culturing on the content of collagen by VICs. The amount of collagen in the media over VIC cultures was quantified at 48 hours of co-culturing. aVEC and vVEC represent the VIC cultures in the presence of aVECs and vVECs, respectively, on the permeable membrane. The control is VICs cultured with low serum DMEM alone. Data is represented in the percentage of collagen production over 100% production by individual controls. Median value among each group is represented by the bars and statistically compared by Kruskal-Wallis and Dunn's test (n=6).

VICs significantly increased the amount of secreted collagen when they were co-cultured with vVECs for 48 hours (p=0.0268), as compared to the VICs alone. The median value of percentage of collagen produced was increased to 149.7% and 208.5% by aVECs and vVECs co-culturing, respectively. However there was no significant difference between the effect of aVECs and vVECs.

4.3.4 The effect of VEC co-culture on the production of GAGs by VICs

Similarly, the amount of GAGs was quantified among VIC cultures which were either VICs cultured alone, that served as the control or in co-culture with either aVECs or

vVECs. The amount of GAGs secreted by 5.2 x 10^5 VICs in the co-cultures was normalised to the control from the same animal.

vVECs increased the amount of secreted GAGs (Figure 4.6) produced by VICs. The median value of the percentage of GAG production was significantly increased to 197.6% by the vVEC co-culture (p=0.0222). Although there was an increasing trend seen up to 150.8% by the aVEC co-culture, this was not a statistically significantly different from the control and the vVEC co-culture group.

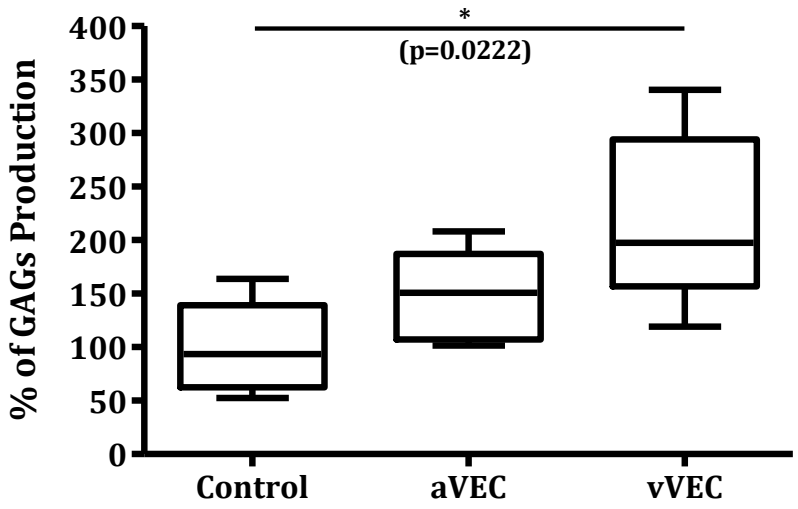


Figure 4.6 The effect of VEC co-culturing on the content of GAGs by VICs. The amount of GAGs in the media over VIC cultures was quantified at 48 hours of co-culturing. aVEC and vVEC represent the VIC cultures in the presence of aVECs and vVECs, respectively, on the permeable membrane. The control is VICs cultured with low serum DMEM alone. Data is represented in the percentage of GAG production over 100% production by individual controls. Median value among each group is represented by the bars and statistically compared by Kruskal-Wallis and Dunn's test (n=6).

4.3.5 The effect of VEC co-culture on the production of elastin by VICs

The tropoelastin that bound on to the VIC membrane was investigated. The amount of elastin produced by VICs under the VEC co-culture effect is depicted in the Figure 4.7.

Side-specific VECs had no effect on the amount of elastin which was produced by VICs over 48 hours of co-culturing. Neither were there any differences in the elastin content of aVEC (104.2%) or vVECs (110.1%) co-cultures.

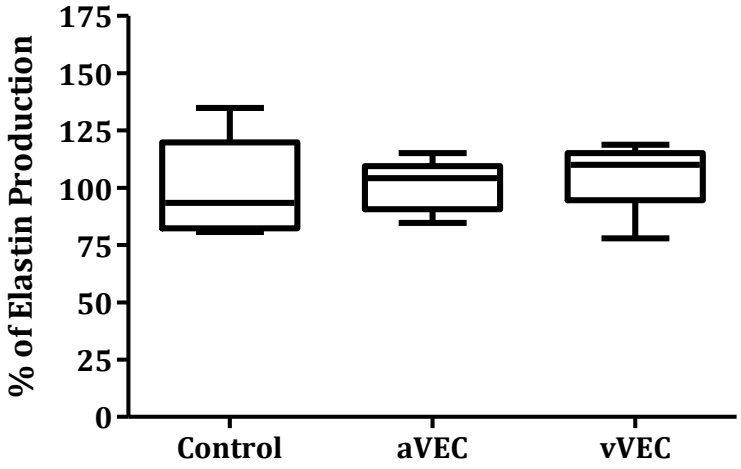


Figure 4.7 The effect of VEC co-culturing on the content of elastin by VICs. The amount of elastin in the media over VIC cultures was quantified at 48 hours of co-culturing. aVEC and vVEC represent the VIC cultures in the presence of aVECs and vVECs, respectively, on the permeable membrane. The control is VICs cultured with low serum DMEM alone. Data is represented in the percentage of elastin production over 100% production by individual controls. Median value among each group is represented by the bars and statistically compared by Kruskal-Wallis and Dunn's test (n=6).

The possibility that ECM components, produced by VECs, could "cross-contaminate" with the production by VICs was also examined. aVECs and vVECs were seeded on the permeable membrane inserts without the VIC culture. The media underneath

the inserts was collected at 48 hours so that the amount of ECM proteins could be measured. The quantification of collagen, GAGs and elastin was conducted in a similar way as the co-culture experiments. However, there were no detectable levels of the ECM components produced by VECs. The detection limit of collagen, GAGs and elastin assays were at 2.5, 1 and 0.625 μg/ml, respectively.

4.3.6 The effect of VEC co-culture on the expression of ECM components' genes by VICs

In order to complement the studies on ECM proteins release by VIC, expression of mRNA for each gene was also assessed. The expression of mRNA for ECM proteins by VICs was investigated after 48 hours of incubation (control) and co-culturing with either aVECs or vVECs.

Before the gene expression was investigated by comparative ΔC_T ($\Delta\Delta C_T$) method, the amplification efficiency of target genes was validated. The ΔC_T value between each target gene and 18sRNA were plotted on a graph against varied dilutions of the template. The slopes of the amplification efficiency of COL1A1, COL3A1, CSP4, HAS2, FBN1 and FBN2 were 0.0037, 0.0501, 0.0507, 0.0901, 0.0169 and 0.0107, respectively (Figure 4.8). All gradients that are less than 0.1 show that all target genes have approximately equal amplification efficiency to the reference gene and can be investigated expression by $\Delta\Delta C_T$.

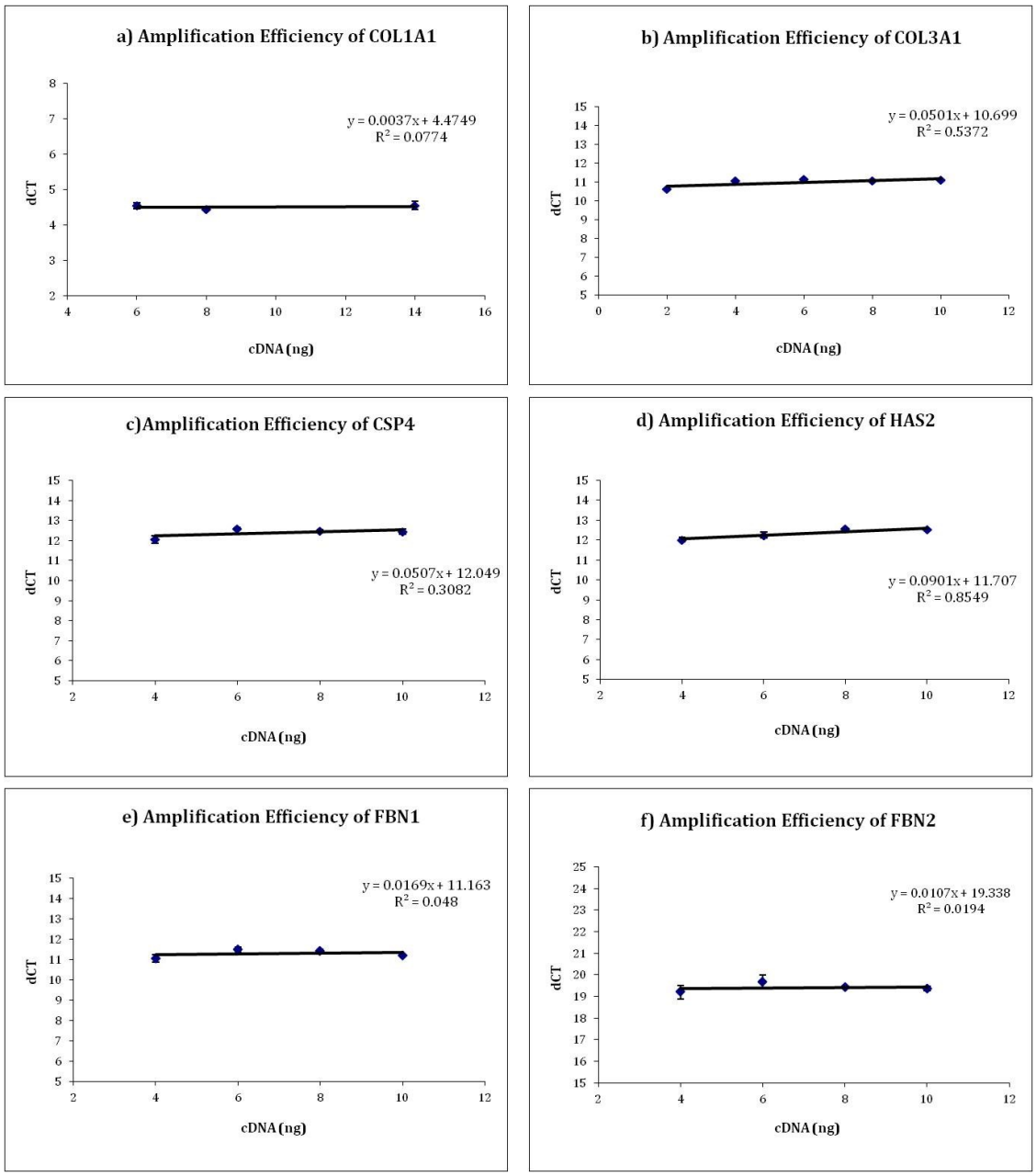


Figure 4.8 Amplification efficiency of 6 ECM genes. ΔC_T (C_T of target gene – C_T of 18s) was plotted graph against increasing amount of cDNA. Slope of each gene expression less than 0.1 represents equal amplification efficiency between endogenous control and target genes.

The relative expression of ECM genes is represented by $\Delta\Delta C_T$. The median values of $\Delta\Delta C_T$ (n = 5-6) from the co-cultures of aVEC and vVEC are shown in Figure 4.9. The

vVECs co-culturing significantly increased the expression of fibrillin 1 (FBN1) mRNA by VICs, when compared to the control. The median value of aVEC and vVEC co-culturing were 1.902 and 2.100 fold respectively. However, there were no statistical differences between side-specific VEC co-culturing.

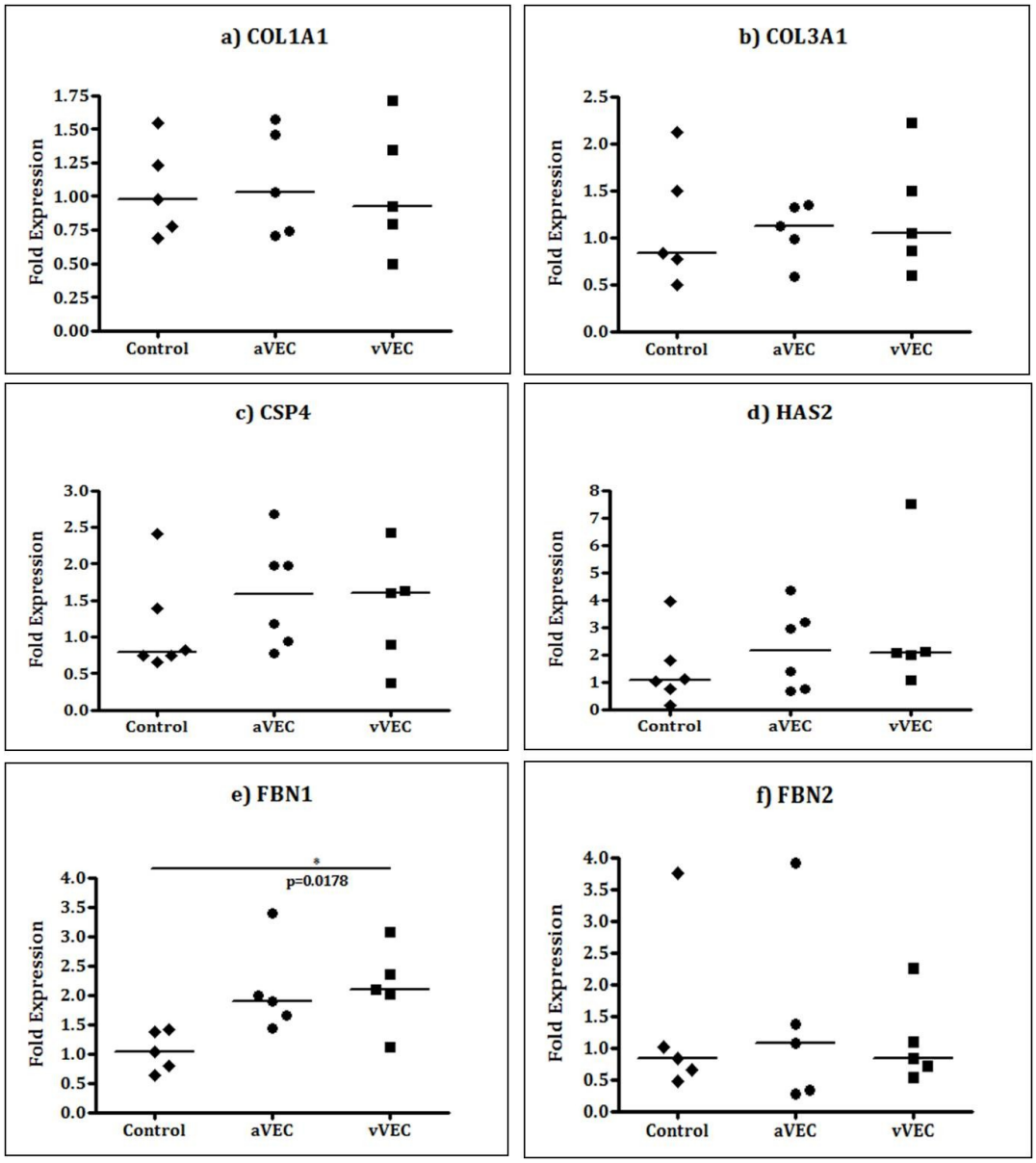


Figure 4.9 Relative gene expression of VICs co-cultured either with aVECs or vVECs. VICs co-cultured with aVECs and vVECs for 48 hours by Transwell, 0.4 μm pore size, were investigated the expression of COL1A1, COL3A1, CSP4, HAS2, FBN1 and FBN2. Relative gene expression ($\Delta\Delta C_T$) of co-culturing was compared to 1 fold expression of the individual controls. The $\Delta\Delta C_T$ among three groups was statistically compared using Kruskal-Wallis and Dunn's Test, n=5-6.

4.4 Discussion

The regulation of side-specific VECs on the production of ECM components by VICs was carried out using a co-culture model. The amount of collagen is increased by the effect of vVEC co-culture, similar to the effect of vVEC-CM, whereas the increased amount of GAGs can be found only by the co-culture of VICs with vVECs. Moreover the investigation of ECM gene transcription reveals the increased expression of FBN1 mRNA by vVEC co-culturing.

Although the conditioned media experiment provided interesting initial data on the potential effects of VECs on the production of ECM by VICs, the presence of any degradable mediators in the EC-CM could be lost over the time period required to collect the media. Therefore the co-culture of VICs and VECs using Transwells was performed. The effect by co-culture for 48 hours was chosen to be comparable with conditioned media experiments. VECs were cultured on the inserted membrane, of 0.4 μm pore size, to make sure that there was no VECs migrated to the bottom of the well where the cultured VICs were.

The phenotypes of VECs were investigated after co-culturing, during which the time the interaction between VECs and VICs is allowed to occur. Although VECs are cultured with low serum media and adjacent to VICs, both aVECs and vVECs maintained their EC phenotypes, as shown by immunostaining of vWF and CD31. However there is no explicit staining of VE-Cadherin by aVECs and vVECs. This may be due to the effect of being in contact with the porous polyester membrane of the inserts.

The negative staining of SMαA by VECs both cultured with and without VICs demonstrates no contamination of VICs in the VEC population. Interestingly, there is an expression of SMαA by aVECs and vVECs, from one animal, after co-cultured

with VICs by (data not shown), suggesting the ability of the two cell types to communicate and potentially initiate EndoMT. The induction of EndoMT involves the putative TGF-β1 signalling pathway. TGF-β specifically induces the translocation of Snail1, not Snail2, into nucleus (Li and Jimenez, 2011) and it, in turn, increases the expression of Snail1 gene. The up-regulation of Snail1 causes the acquisition of mesenchymal phenotype, SMαA, but inhibition of EC phenotype i.e. CD31 and VE-Cadherin (Piera-Velazquez *et al*., 2011).

Investigations of the mechanisms involved in this potential EMT response were outside the scope of this study. However, it is an intriguing observation and suggests that VICs may recruit additional VICs from the VEC population via the release of soluble mediators.

The proliferation of VICs in the presence of VECs on the permeable membrane remained the same as VICs that were cultured with growth-arrested media, for 48 hours, without VECs. The maintained proliferation in the presence of side-specific VECs is consistent with the previous observation that cultured VICs with VEC-CM but in contrast to the observations on the effect of the mixed-VECs, where VICs which were encapsulated in a collagen gel were seen to have reduced amount of cells in the presence of the mixed-VECs on the gel's surface, as compared to the amount of VICs without VEC seeding (Butcher and Nerem, 2006). The lack of influence on the proliferation of VICs assures that the effects of VECs on the modulation of ECM components are independent of an increase in the number of VICs present in the culture.

Co-culturing with vVECs significantly increased the amount of collagen that is secreted into the media by VICs. Although, there is no significant difference between the site-specific VECs, these results are in agreement with the effects seen

with vVEC-CM on collagen content in VIC cultures, suggesting that a stable mediator in both models used mediates this effect.

Nonetheless, the effects of ECs on the collagen production by co-culturing can vary depending on cell types. Coronary artery ECs increased the amount of collagen secreted in the media by SMCs (Myers and Tanner, 1998). Moreover, ECs from the aorta also increased the production of collagen by cardiac fibroblasts (Guarda *et al*., 1993), whereas they reduced the production of collagen by SMCs (Powell *et al*., 1997).

EC-derived paracrine compounds such as ET-1 and NO were shown to have the function of regulating the production of collagen by vascular SMCs and other mesenchymal cells. Exogenous ET-1 had a concentration-dependent effect on increasing the production of collagen by dermal fibroblasts (Kahaleh, 1991). More specifically, it increased the amount of collagen type I that was secreted into the media by vascular SMCs and both secreted collagen type I and III by aorta SMCs (Rizvi *et al*., 1996).

In contrast to ET-1, NO donors reduced the production of collagen by the aorta SMCs, in a dose-dependent manner (Kolpakov *et al*., 1995) and diminished the amount of secreted collagen type I by coronary SMCs (Rizvi and Myers, 1997). In a later study, EC-derived NO was demonstrated to have an inhibitory effect on the production of collagen by coronary artery SMCs. The amount of secreted collagen type I was dramatically increased when the activity of NOS and COX were inhibited in the EC-SMC co-culture (Myers and Tanner, 1998).

The discrete effect on the production/amount of collagen suggests an intricate interaction between several (endothelial) paracrine compounds that can affect both the production and degradation of collagen. Moreover, a particular paracrine factor

can regulate the production of different ECM component differently. The antibody used for neutralising the activity of TGF-β had no effect on the collagen production by SMCs (Powell *et al*., 1997). In contrast to the regulation of collagen, TGF-β modulated the production of GAGs by increasing the amount of s-GAGs produced by SMCs (Ciolino *et al*., 1992; Merrilees and Scott, 1990). Moreover, the exogenous TGF-β can modulate the production of different types of GAGs (HA and s-GAGs) in the concentration-dependent manner (Merrilees and Scott, 1990).

In the study, the vVEC co-culture significantly increases the amount of GAGs secreted in the media by VICs, when compared to the control and the aVEC co-culture. This paracrine effect is consistent with the increasing synthesis of GAGs of the EC-SMC co-culture (Merrilees and Scott, 1981). The increasing amount of GAGs can possibly be as a result of TGF-β produced by co-cultured vVECs; as previously shown in the Chapter 2, VECs can produce TGF-β. However, it is noted that there was no significant difference among the side-specific VECs in terms of TGF-β production.

Since the amount of GAGs measured in the media is the net effect of degradation and synthesis, it is likely that vVECs produce non-stable paracrine factors that affect the homeostasis of GAGs differently from aVECs. As a result, an increasing amount of GAGs is observed when VICs are cultured in the vicinity with vVECs only and this effect is not observed by vVEC-CM.

In contrast to the present study, the co-culturing of venous ECs did not modulate the amount of GAGs produced by myofibroblasts that were encapsulated in a 3-D fibrin gel (Pullens *et al*., 2009). Additionally, under both static conditions and when laminar shear stress was applied, mixed VECs prevented the loss of GAGs in a hydrogel that contained VICs (Butcher and Nerem, 2006). Although these studies did not identify the regulatory function of ECs, they did demonstrate that the

homeostasis of GAGs may not be modulated only by ECs, but also by the unique interaction of cells to the microenvironment.

The influence of VECs on the production of elastin by VICs is also investigated using the co-culturing model. Whereas the amount of secreted elastin cannot be detected by ELISA technique (data not shown), the membrane-bound elastin can be isolated from the VIC culture and quantified by dye-binding method. The amount of elastin produced by VICs is not affected by the VEC co-culturing and VEC-CM (as shown in the previous chapter). This could be a result of a balance effect between synthesis and degradation of elastin by VECs or it could be that VECs do not influence the production of elastin at all. Thus the degradation of radiolabeled elastin that is synthesised before starting the VEC co-culture, along with the incorporation of a radiolabeled component into newly synthesised elastin after the VEC co-culturing begins, should be investigated to address these questions.

There is the possibility that the investigated ECM components are the product of VECs. The measurement of collagen, GAGs and elastin produced by VECs was investigated by the same co-culture system apart from that there was an absence of VICs at the bottom well. The results demonstrated that there are no ECM components measurable with the assays that are produced by VECs and might transfer through the permeable membrane at the bottom wells.

The amount of ECM proteins investigated in these studies may be influenced by VECs affecting either synthesis, degradation or the secretion process. To identify at if synthesis or degradation is modulated by VECs, the inhibition of transcription and translation by Actinomycin D and cycloheximide as well as the production of MMPs and TIMPs by zymography technique could be performed. Although the regulation of the degradation and secretion of the ECM is not pursued in this current study, the modulation of the gene expression is of interest and was investigated.

We chose to look at COL1A1 and COL3A1 encoding collagen alpha-1 type I and III since is found predominantly on aortic side of the valve. In addition, CSPG4 expressing chondroitin sulphate proteoglycan 4, and HAS2, encoding the enzyme to synthesize hyarolunan, is an important gene for GAGs, that are found in the middle part of the valve. Lastly, FBN1 and FBN2 express fibrillin 1 and 2 which are main components of elastic microfibrils found on ventricular portion of the valve.

The semi-quantitative gene expression analysis revealed no significant effect of aVECs and vVECs on the expression of collagen (COL1A1 and COL3A1) and GAG (CSP4 and HAS2) genes by VICs. These results suggest that the increasing amount of collagen and GAG proteins observed in the media are not modulated by VECs at the transcription process and therefore it is unlikely that VEC induce *de novo* synthesis of any of the proteins encoded for by these genes. A greater amount of collagen and GAGs by vVEC co-culture may be regulated by increasing the translation of existing mRNA for collagen and GAGs or by reducing the degradation of ECM proteins in the media. This question remains to be studied.

It is noted that elastin comprises of only 13% of dried weight in the human aortic valve (Bashey *et al*., 1967) and its remodeling process can take up to a year (Starcher, 1986). Moreover, the expressions of elastin and tropoelastin genes are not detectable in porcine tissue because of the lack of species-specific primers. Thus, this study focused on the expression of fibrillins.

Fibrillin is part of the glycoprotein family, which is proposed many functions. It provides a microenvironment which regulates the tropoelastin/elastin cross-linking process (Sabatier *et al*., 2009). Fibrillin 1 (encoded by FBN1 gene) maintains valvular structural integrity by tethering VICs to elastin and collagen (Fedak *et al*., 2002). In contrast, fibrillin 2 (FBN2) is predicted to regulate the assembly of the elastic fibre at the early process (Carta *et al*., 2006).

In the present study, the expression of FBN2 by VICs is not regulated by the presence of VECs. In contrast, the presence of vVECs up-regulates the expression of FBN1 by cultured VICs. These results are coherent to the finding that FBN2 is down-regulated in mature human aortic valves whereas FBN1 is expressed throughout all developmental stages (Votteler *et al*., 2013).

However the discrete results between the sustained amount of elastin protein and the increased expression of FBN1 mRNA maybe a consequence of a different regulatory pathway of elastin and fibrillin. Moreover, the regulation, by side-specific VECs, at the translation of elastin mRNA and the secretion/degradation of elastin proteins remains unclear.

4.4.1 Limitations of the study

There are number of limitations to this study. The mechanisms for the increased production of collagen and GAGs by VICs in co-cultured with vVECs, are not studied. The RT-PCR results do not demonstrate the up-regulation of the genes which are important for collagen and GAG production by aVECs or vVECs. However, the regulation at the translational level of mRNA, secretion and degradation of collagen and GAGs remain to be studied. In order to pursue such questions, VICs will have to be treated with cycloheximide to inhibit the total protein translation. ^{3}[H]proline and ^{35}[S]sulfate can be used to track the secretion of collagen and GAGs, respectively. Lastly, the production, by VICs, of enzymes involved in the ECM degradation such as MMPs and TIMPs could be investigated by zymography.

Moreover, it remains to be elucidated which molecules that the VECs modulate the VIC function. ET-1, NO and TGF-β are EC-derived paracrine agents which modulate the production of ECM by fibroblast and vascular SMCs. Their functions on VICs

production of ECM proteins could be investigated by applying their inhibitors to the VEC-VIC co-cultures. In addition, the levels of these mediators released into the media by VEC could also be determined.

4.5 Conclusion and Future Directions

In conclusion, side-specific VECs elaborate substances that have a different paracrine effect on the functions of VICs that are cultured in vicinity with them for 48 hours. vVECs increased the amount of collagen secreted into the media by VICs, although no side-specific difference was observed. Interestingly, the amount of secreted GAGs was significantly increased only by vVEC co-culture when compared to the control and aVEC co-culture. However, the amount of elastin in the aVEC and vVEC co-culture remained at the same level as the control. In addition, both aVECs and vVECs sustained the proliferation of VICs when they were co-cultured on the permeable membrane. This result suggests that the VEC paracrine effect on the increasing amount of collagen and GAGs is mediated independently of the VIC proliferation. Thus the interaction between VECs and VICs may be important to heart valve structural remodelling.

However, the effect of shear stresses with regard to side-specific VECs needs be investigated to determine whether there is any heterogeneity in the response of VECs to the flow patterns, which are experienced from either side of the valve.

CHAPTER 5 Effect of Shear Stress and VECs

5.1 Introduction

Mechanical shear stress mediates vasotonicity of vessels by regulating structural and functional properties of cells at genetic, molecular and cellular levels. ECs response to shear stress by redistributing cytoskeleton and changing the expression of growth factors and vasotonic molecules, as well as modulate the phenotypes of neighbouring cells (Topper and Gimbrone, Jr., 1999; Davies, 1997). Under both static and shear stress conditions, VECs significantly reduce the expression of SMαA by VICs, as compared to the expression by VICs cultured in a 3-D scaffold alone (Butcher and Nerem, 2006).

The response of ECs to mechanical forces has also been shown to regulate the production of ECM of adjacent tissue. Vascular ECs and mechanical tension stimulate total production of collagen and elastin in the pulmonary arteries (Tozzi *et al*., 1989). The intact-ECs tissue segments which were applied the strain had an increased production of collagen and elastin. Whereas the same effect was not found on the tissue that were removed ECs (Tozzi *et al*., 1989).

VECs on the aortic valve surfaces are also exposed to shear stresses during every cardiac cycle. However, there are few studies investigating the response of VECs to the patterns of shear stress experienced by each surface of the valve and whether these cells have the ability to affect the ECM in the valve.

The effect of laminar shear stress on the production of GAGs has been studied in the porcine aortic valve leaflets *ex vivo* (Weston and Yoganathan, 2001). It was demonstrated that the leaflets which were subjected to the laminar flow maintained the amount of GAG synthesis to a similar extent to that of fresh tissue. In contrast,

the leaflet kept in a static mechanical environment had an increased production of GAGs and total proteins (Weston and Yoganathan, 2001). The importance of interactions between VICs and VECs has also been investigated. Under both static and flow conditions, the presence of VECs maintained the amount of GAGs in the scaffold, containing VIC, at a significantly higher amount than the scaffold without VECs (Butcher and Nerem, 2006).

It is reasonably certain that VECs and VICs respond to their hemodynamic environment, which may influence/determine their ability to interact with VIC and affect the ECM of the valve. Nonetheless, the response of side-specific VECs to the unique shear stress patterns and their effect on VICs function has not been investigated. There is, however, an interesting observation revealing the flow-independent response of VECs from the different aspects of the valve (Sucosky *et al*., 2009). aVECs and vVECs on the valve segments responded differently to the same patterns of shear stress, in terms of the expression of inflammatory molecules (Sucosky *et al*., 2009).

This study has already investigated the ability of VEC to release stable mediators and those with a potentially shorter viability in a co-culture model that affect the ECM production by VICs in culture. This chapter now aims to investigate whether there is a differential response by VECs to the heamodynamic environments experienced by the aortic side (AS) and ventricular side (VS) of that valve that regulates ECM production by the underlying tissue. With reference to levels of collagen, GAGs and elastin, this study aims to expose each side of the valve to the physiological pattern of flow that it experiences as well as the reversed pattern of flow seen by the opposing side. Oscillatory flow, normally generated on the aortic side *in vivo*, is referred as the aortic flow (AF) and, similarly, the laminar shear stress on the ventricular side is referred as the ventricular flow (VF). Modulation of the ECM content using intact valve cusps will be measured after exposing the

surfaces of the valve to the respective patterns of flow using a purpose built bioreactor, Cone and Plate Machine (Sucosky *et al*., 2008).

5.2 Materials and Methods

5.2.1 Porcine aortic valve tissue

Porcine hearts were obtained from the same slaughterhouse (Cheale Meats, UK) as previous experiments. 3 leaflets of aortic valves were dissected under sterile condition with minimal contact to the surface of the valves. All leaflets, separated among animals, were washed with PBS minimum twice. A round-shaped cutter (at the diameter of 10 mm) was then used to dissect the identical portions of the valve from the "belly" area of each leaflet into 2 pieces (Figure 5.1). Tissues were kept with DMEM containing 0.4% FBS under sterile conditions before used for further experiments.

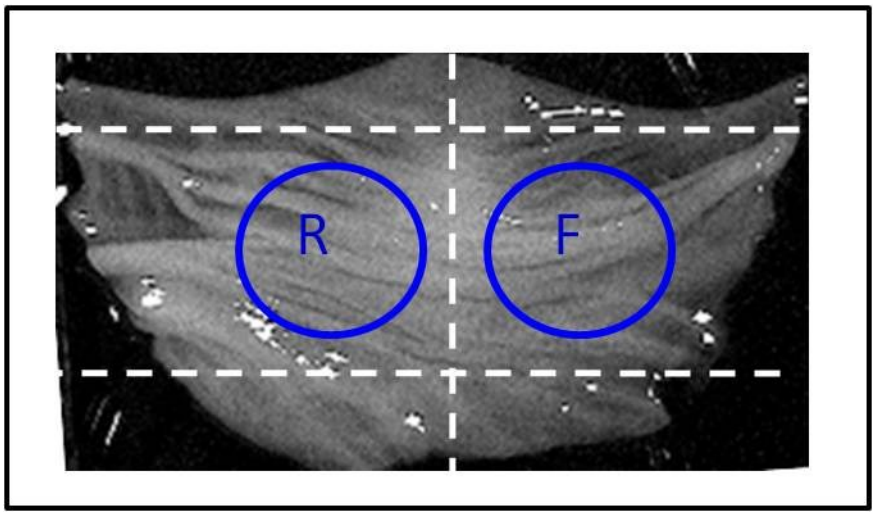


Figure 5.1 The counterpart area of an aortic cusp. The belly from the counterpart areas of the cups were bisected by a rounded-shaper cutter. One piece of the tissue was conducted as a reference and another was to be mounted on the Cone-and-Plate machine to expose to the shear stresses.

5.2.2 ECM production of aortic valves under various mechanical conditions

To understand how mechanical shear stresses and side-specific VECs regulate the production of ECM components, tissues dissected from the same leaflet were cultured under different mechanical conditions. One piece of tissue was used as a reference by statically maintaining the tissue for 48 hours, while another piece of tissue, from the same leaflet, was exposed to VF (laminar shear stress) or AF (oscillatory shear stress), one flow at a time, using a Cone-and-Plate machine. Both pieces of tissue were handled under sterile conditions throughout the experiments and cultured with DMEM containing 0.4% FBS at 37°C, in a 95% air/5% CO_2 incubator. Experiments were performed where the AS or VS were tested in turn, with each side being exposed to both patterns of flow. In addition, the ECM content of fresh tissue (immediately after dissection from the heart) was also investigated to show the respective amounts of ECM prior to any intervention. The experimental design is depicted in the Figure 5.2.

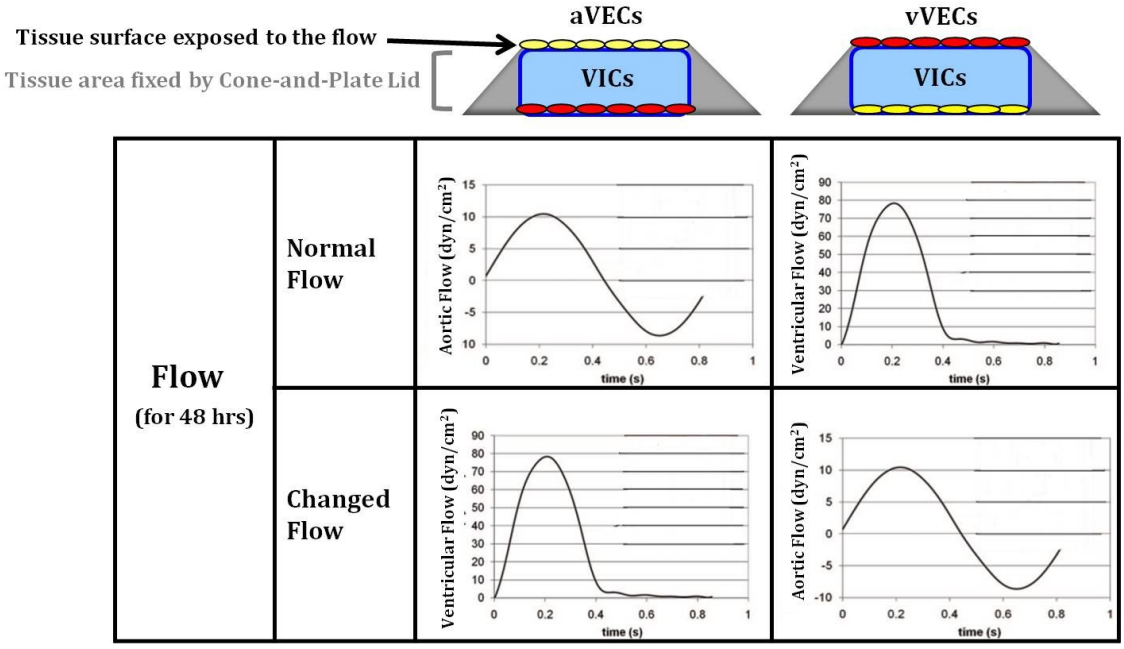


Figure 5.2 Diagram of the experimental design. The cusps were fixed by Cone-and-Plate lid and, thus, exposed only one side to the flow. The tissue on the plate then experienced either their normal or changed flow (from the physiological condition) at a time. The counterpart area of the same cusps were then used as the "fresh tissue" reference or maintained at the static condition for 48 hours and used as another reference (control at static condition).

5.2.2.1 Cone-and-Plate machine

In order to study the effect of shear stress patterns on valve tissue, the Cone-and-Plate device was developed by the Biomedical Engineering Department at the Georgia Institute of Technology. The machine can replicate the oscillatory and laminar shear stress at the same magnitudes and patterns as experienced by the aortic valve *in vivo*. The machine is mainly composed of a cone made of Delrin at 179° cone angle and 80mm in diameter. This sits above a cylindrical plate containing 9 equi-angularly spaced cylindrical wells. Within the wells, cylindrical stainless-steel holders can be placed at different heights in order to be able to mount the valves of varying thickness on the holders and ensure the valve surface is maintained at the same distance from the cone. The different shear stress patterns

can be then generated by a different pattern of rotation of the cone. To fix the position of the tissue, a plate cover with the nine holes (of a diameter of 5-7 mm) openings in identical positions as the tissue is secured over the plate by a number of screws. During their exposure to the flow, tissues were maintained by nutrients with a constant supply of DMEM containing 0.4% FBS from a bottle that allows O_2 and CO_2 exchange to occur (Figure 5.3).

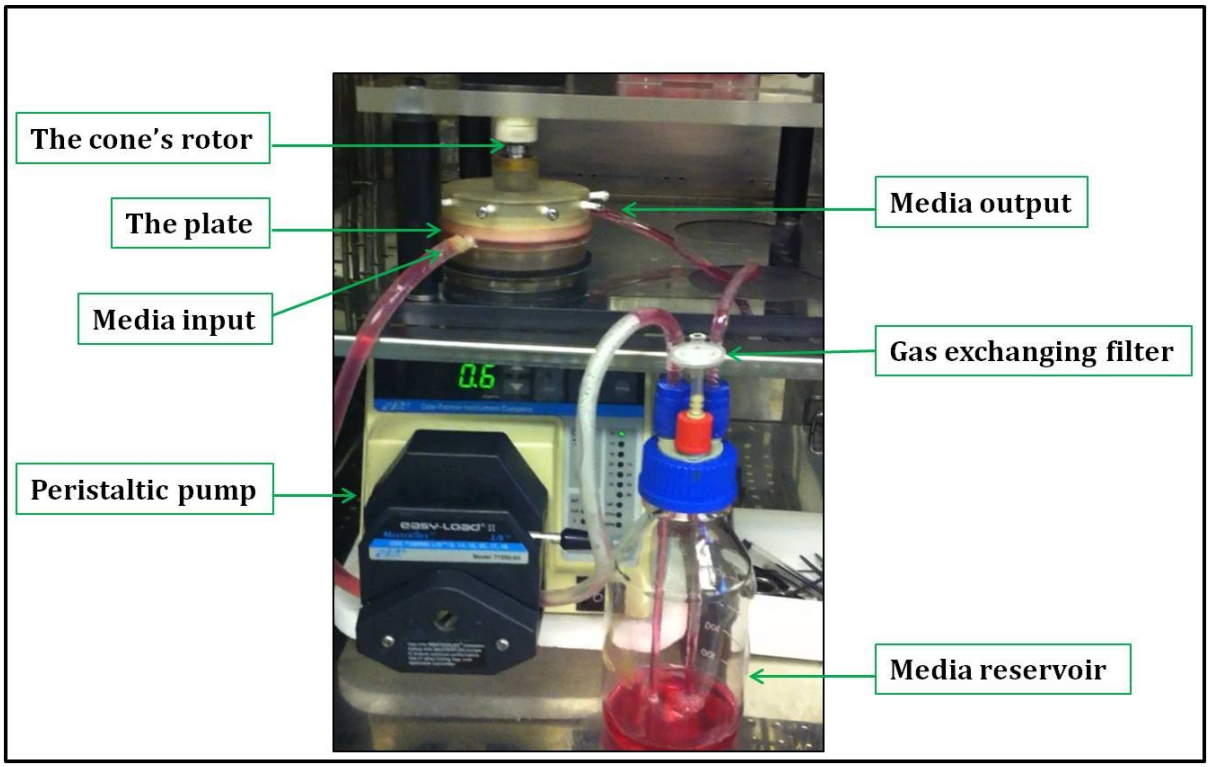


Figure 5.3 The shear stress generating device. 9 pieces of tissue were mounted on "The plate" under the sterile condition and covered by "The cone" which generates the shear stress by the connected rotor. The plate contains the channel of "Media input" that connects to a bottle containing low–serum media, known as the "Media reservoir". The media was circulated by the "Peristalic pump" into the plate through the media input and outward through the "Media output" channel. The pH of the media was controlled by the CO_2 system through the 0.2 μm "Gas exchanging filter".

Nine pieces of tissue were mounted on the aforementioned plate. For each experiment the tissues were carefully placed on either aortic or ventricular side so that only one surface was exposed to the flow. To verify the effect of flow patterns independently from side-specific VECs, an each side of the tissue was also exposed to the flow physiologically seen by the other side of the valve. Tissues were exposed to shear stress for 48 hours, after which they were removed from Cone-and-Plate machine and trimmed with the 5 mm round shape-cutter to ensure that only the tissue area exposed to the flow was collected. Tissue samples were immediately placed in microtubes and dried at 40^0 C on a heating metal overnight. Dried tissues were then cut into small pieces ~1x1 mm inside the microtubes and weighed before extraction and measurement of ECM components.

5.2.2.2 Collagen content of aortic valve tissue

Tissue subjected to either shear stresses or static culture was extracted the collagen by using 10 mg/ml of Pepsin enzyme for 2 days. All dried tissue at a known weight was prepared in a 1.5 ml microtube with 1 ml of Pepsin in 0.5 M acetic then added. The tubes are then shaken vigorously by Multitube Vortexer (Lab-tek International LTD, New Zealand) for 2 days. All microtubes were then spun at 13684 g for 1 minute to remove tissue debris. Supernatant restraining collagen was then transferred into a new microtube and mixed with 100 µl of Acid Neutralising Reagent containing Tris-HCl and NaOH (Sirkol kit). The collagen in the sample was then precipitated and assayed the content by the same protocol as described in section 3.2.3.

5.2.2.3 GAGs content of aortic valve tissue

All samples were dried at 40°C and weighed before GAG solubilisation was started. They were subsequently incubated with 1 ml of 20 mg/ml papain in an extraction buffer (0.2 M sodium phosphate buffer pH 6.4, 800 mg/ml sodium acetate, 400 mg/ml EDTA, sodium salt and 80 mg/ml cysteine HCl). The extraction reaction was performed at 60°C in a water bath while vigorously shaking for few hours. After the tissue was dissolved, all the tubes are spun at 800 g for 1 minute to segregate tissue debris at the bottom. 100 µl (triplicates) of supernatant was then transferred into a new microtube to carry on the measurement of GAGs by Blyscan kit, as described in section 3.2.4.

5.2.2.4 Elastin content of aortic valve tissue

The insoluble elastin obtained in fresh tissue and the tissues that had been subjected to either shear stress or static condition was extracted and converted into a soluble form, α-elastin, by using hot oxalic acid. After cutting it into small pieces and weighing, every piece of dried tissue was added to 1 ml of 0.25 M oxalic acid and heated at 100^0C for 1 hour. The soluble elastin, which was released into the solution, was collected into a new microtube after spinning the residual tissue down at 9503 g for 10 minutes. The extraction was repeated twice and elastin content was subsequently measured by using Fastin kit, as described in section 3.2.5. The amount of elastin from 3 extractions from each sample was then added up to calculate elastin content per mg of dried tissue.

5.2.3 Data and statistic analysis

The amount of collagen, GAGs and elastin was reported in μg per mg of (dried) tissue and represented in the box-and-whiskers plot by Graphpad Prism 5. The band inside the box depicts the median. The top and bottom of the box display the third and the first quartiles, respectively, and the length of the box is the interquartile range (IQR). In these studies, the whiskers illustrate the maximum and minimum of the data set.

The groups of data were analysed statistically by a non-parametric one-way ANOVA or Kruskal-Wallis Test. The multiple pair-wise tests were further evaluated by Dunn's Test. P value less than 0.05, 0.01 and 0.001 are represented by *, ** and ***.

5.3 Results

In these studies, the effect of shear stresses on the modulation of ECM has been investigated. The amount of ECM in the tissue that was cultured under normal shear stresses (AS/AF and VS/VF) was compared to the leaflets kept at static condition or static control (SC), for 48 hours. Additionally, the amount of ECM in fresh tissue (FT) was also referred to those in the native valves. Further insights in the effect of side-specific VECs and the flow patterns were studied by investigating the response of VECs to the reverse pattern of flow (AS/VF and VS/AF).

5.3.1 The effect of shear stresses on the content of collagen

The leaflets that were maintained at SC for 48 hours did not significantly change the amount of collagen from those of FT. The median values of collagen content of FT and SC were 4.632 and 4.948 μg collagen/mg dried tissue, respectively. When the AS was exposed to the AF the amount of collagen significantly increased to 32.56 μg collagen/mg dried tissue as compared to the FT. In contrast, when the VS was exposed to VF there was an increasing trend of collagen content to 13.98 μg collagen/mg dried tissue, but this was not statistically different from FT or SC samples, $p<0.05$ (Figure 5.4).

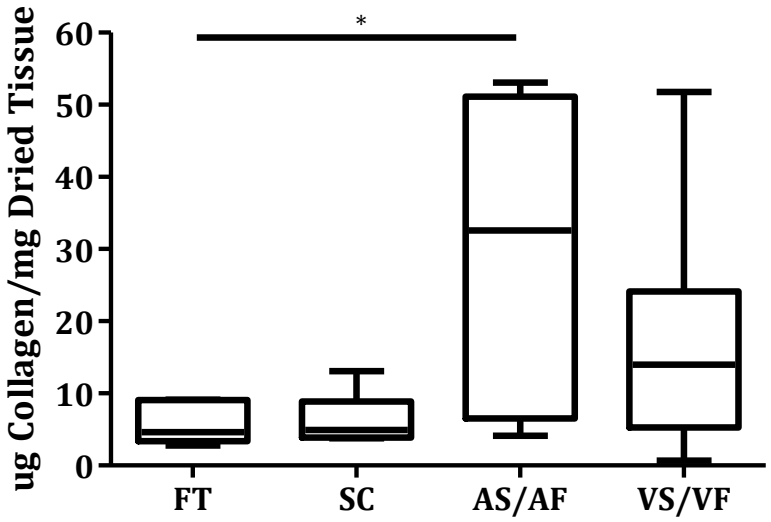


Figure 5.4 The effect of shear stresses on the amount of collagen in the aortic valves. The amount of collagen was reported in μg collagen per mg dried tissue from fresh tissue (FT), static control (SC) for 48 hours and the mimicked physiological flow conditions for 48 hours on the aortic side (AS/AF) and ventricular side (VS/VF). The median values were statistically analysed by Kruskal-Wallis Test and pair-wise compared by Dunn's Test. The amount of collagen in AS/AF samples was significantly higher than FT samples, n numbers of each group were FT=6, CS=6, AS/AF=9 and VS/VF=9.

5.3.2 The effect of side-specific VECs and shear stress patterns on the content of collagen

The side-specific VECs and flow patterns were studied to assess their effect on the content of collagen in the valve, Figure 5.5. The median values of collagen content in the leaflets subjecting their AS to the AF and VF were 32.56 and 16.28 μg collagen/mg dried tissue, respectively. There was a trend of decreasing amount of collagen when the ventricular side was exposed to the flow patterns. The median values of VS/VF and VS/AF were 13.98 and 7.039 μg collagen/mg dried tissue, respectively. However there was no significant different effect, either by specific sides or flow patterns, on the amount of collagen in the aortic valves.

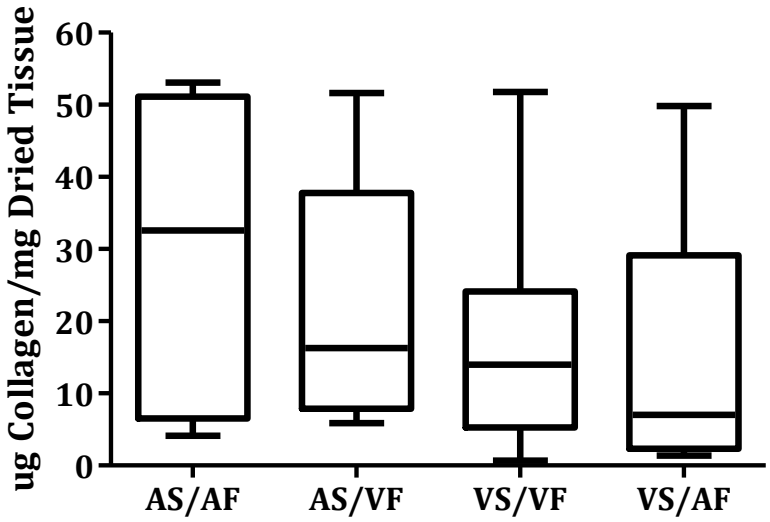


Figure 5.5 The effect of side-specific VECs and flow patterns on the amount of collagen in the aortic valves. The amount of collagen was investigated of the valves that were exposed the surfaces to the normal flow (AS/AF and VS/VF) and changed flow (AS/VF and VS/AF) for 48 hours. The median values were statistically analysed by Kruskal-Wallis Test and pair-wise compared by Dunn's Test. There was no statistically significant difference among all groups, n numbers of each group were AS/AF=9, AS/VF=8, VS/VF=9 and VS/AF=6.

5.3.3 The effect of shear stresses on the content of GAGs

The amount of GAGs was significantly reduced when the leaflets were kept at the static condition for 48 hours as compared to the fresh tissue as demonstrated in the Figure 5.6. The median value of FT was 32.68 μg GAGs/mg dried tissue and it was reduced to 21.37 in the SC samples. The amount of GAGs was increased in the tissues that where the AS was exposed to the AF for 48 hours. The median value of 47.14 μg GAGs/mg dried tissue for AS/AF tissue was significantly higher than that seen in FT and SC ($p<0.05$ and $p<0.001$, respectively). However the amount of GAGs remained non-significantly different to FT or SC in the tissues where the VS was exposed to VF. The median value for VS/VF was 33.33 μg GAGs/mg dried tissue.

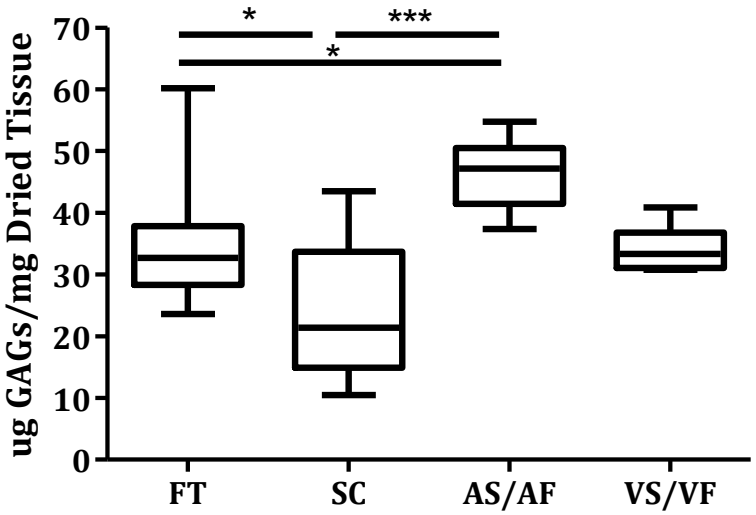


Figure 5.6 The effect of shear stresses on the amount of GAGs in the aortic valves. The amount of GAGs was reported as μg GAGs per mg dried tissue from fresh tissue (FT), static control (SC) for 48 hours and the mimicked physiological flow conditions for 48 hours on the aortic side (AS/AF) and ventricular side (VS/VF). The median values were statistically analysed by Kruskal-Wallis Test and pair-wise compared by Dunn's Test. The amount of GAGs in AS/AF samples was significantly higher than FT samples and SC while those amount in SC samples was significantly reduced from FT, n numbers of each group were FT=24, CS=20, AS/AF=6 and VS/VF=6.

5.3.4 The effect of side-specific VECs and shear stress patterns on the content of GAGs

The effect of flow patterns and side-specific VECs on the amount of GAGs was observed and shown in Figure 5.7. The median value of the AF on the AS was 47.14 μg GAGs/mg dried tissue and it was significant diminished when the same pattern of flow was generated on the VS to 28.39 μg GAGs/mg dried tissue ($p<0.001$). However the effect of VF appears to maintain the amount of GAGs at the non-significantly changed level from other samples in which the median values of VS/VF and the AS/VF were 33.33 and 35.08 μg GAGs/mg dried tissue.

Moreover, the side-specific effect was not prominent. There was no significant difference observed when the AS/AF was compared to AS/VF and the VS/VF was compared to VS/VF, although there was a trend of decreasing amount of GAGs when the AS and VS were exposed to the reverse flow.

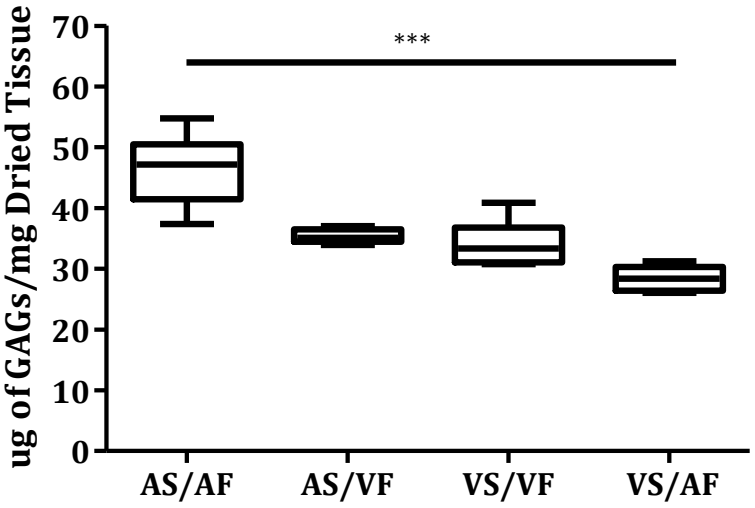


Figure 5.7 The effect of side-specific VECs and flow patterns on the amount of GAGs in the aortic valves. The amount of GAGs was investigated of the valves that were exposed the surfaces to the normal flow (AS/AF and VS/VF) and changed flow (AS/VF and VS/AF) for 48 hours. The median values were statistically analysed by Kruskal-Wallis Test and pair-wise compared by Dunn's Test. The amount of GAGs in VS/AF was significantly optimised from the AS/AF samples. All samples had n numbers = 6.

5.3.5 The effect of shear stresses on the content of elastin

The amount of elastin found in the SC was significantly reduced when compared to those in the fresh tissue ($p<0.0001$) and the leaflet where the VS was exposed to VF ($p<0.01$). The median value of SC was 56.20 μg elastin/mg dried tissue which was decreased from 75.70 μg elastin/mg dried tissue of FT and 74.13 μg elastin/mg dried tissue of VS/VF group (Figure 5.8). Although there was a trend of decreasing amount of elastin in AS/AF group, the elastin content remained non-significantly

different from the controls and had the median value at 62.37 μg elastin/mg dried tissue.

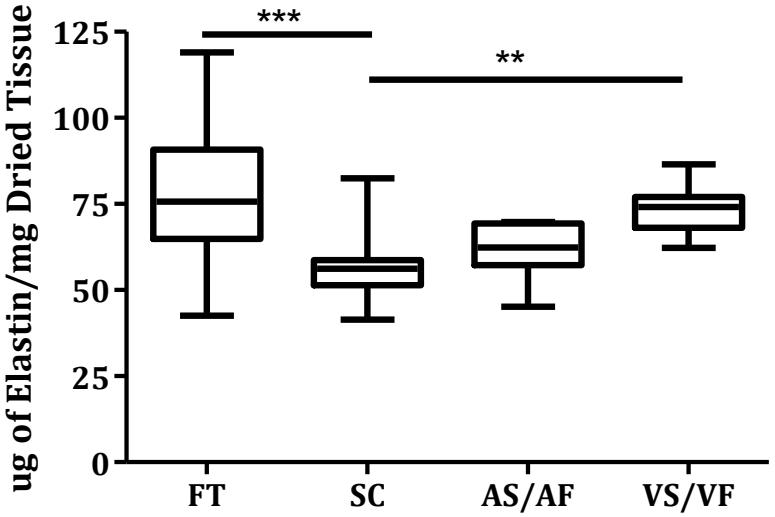


Figure 5.8 The effect of shear stresses on the amount of elastin in the aortic valves. The amount of elastin was reported as μg elastin per mg dried tissue from fresh tissue (FT), static control (SC) for 48 hours and the mimicked physiological flow conditions for 48 hours on the aortic side (AS/AF) and ventricular side (VS/VF). The median values were statistically analysed by Kruskal-Wallis Test and pair-wise compared by Dunn's Test. The amount of elastin in VS/VF samples was significantly higher than SC while those amount in SC samples was significantly reduced from FT, n numbers of each group were FT=28, CS=19, AS/AF=8 and VS/VF=9.

5.3.6 The effect of side-specific VECs and shear stress patterns on the content of elastin

When VF was generated on the AS, it significantly increased the median value of elastin content to 76.18 μg elastin/mg dried tissue as compared 62.37 μg elastin/mg dried tissue to when the AS was exposed to AF ($p<0.05$), as demonstrated in Figure 5.9. The increased amount of elastin when the AS was exposed to VF was similar to when the VS was exposed to VF. However, when AS/AF was compared to VS/VF, there was no significant difference observed. Moreover when the VS was exposed to

AF there was more variance in the measurement of elastin content than the VS/VF group, but the median value of 72.43 μg elastin/mg dried tissue remained non-significantly different from the VS/VF group.

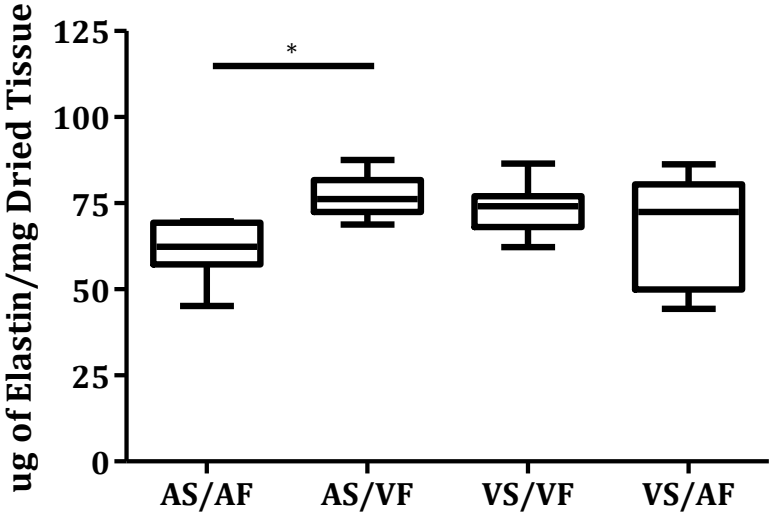


Figure 5.9 The effect of side-specific VECs and flow patterns on the amount of elastin in the aortic valves. The amount of elastin was investigated of the valves that were exposed the surfaces to the normal flow (AS/AF and VS/VF) and changed flow (AS/VF and VS/AF) for 48 hours. The median values were statistically analysed by Kruskal-Wallis Test and pair-wise compared by Dunn's Test. The amount of elastin in AS/VF was significantly increased from the AS/AF samples. n numbers of each samples were AS/AF=8, AS/VF=6, VS/VF=9 and VS/AF=9.

5.4 Discussion

The effects of shear stress and the effect of side-specific VECs to different flow patterns were examined with respect to their ability to influence the ECM components of intact valves. Whereas the particular response of aVECs to the aortic flow increases the production of collagen and GAGs, the ventricular flow maintains the amount of elastin in the valve, in a side-specific independent manner.

Side-specific VECs have been demonstrated (in the previous chapters) to regulate the amount of ECM components produced by VICs*, in vitro* under static conditions. However, the aortic valve resides in a dynamic environment and is exposed to mechanical forces during every cardiac cycle*.* Moreover the effects of stretch and pressure have been demonstrated to affect the production of ECM components in the aortic valve (Balachandran *et al*., 2006; Balachandran *et al*., 2009; Konduri *et al*., 2005). However, the biological response of side-specific VECs to the mechanical shear stress patterns to which it is exposed have not been previously investigated. In these studies, the effects of mechanical shear stresses are incorporated into the *ex vivo* investigations.

The influence of specific flow patterns can be examined by exposing one particular surface of the valves to the shear stresses by using the Cone-and-Plate machine (Sucosky *et al*., 2008). The device can generate both the aortic (oscillatory) and ventricular (laminar) shear stresses and exclude other mechanical forces such as stretch and compression that the valve may experience. For the investigation of the effect of shear stress, fixing of the leaflets on metal bullets using a lid has the advantage of having no increase in pressure and tensile force on the valve specimens. The device allows the study of a single flow effect, in comparison to the native valve where both AF and VF occur continuously on the opposite surfaces of the valves is not possible. However, the regulation of side-specific VECs, which are

independent of shear stress patterns, can be investigated by applying the opposing pattern of flow to either side of the valve.

In these experiments, the variation of ECM components on a valve cusp is minimised by dissecting the pieces from the belly region that are symmetrically adjacent to each other, so that they have very similar fibre structure throughout the area to be exposed to flow or used as the control (Sacks *et al*., 1998).

The importance of mechanical stimulation to maintain the ECM components in the valves is demonstrated by comparing the leaflets that were cultured under static conditions to the fresh tissue. After being kept in a condition which is absent of mechanical forces for 48 hours, the amount of GAGs and elastin were significantly reduced, which is consistent with the observations in studies on valves by other authors (Konduri *et al*., 2005). In contrast, the amount of collagen remains the same extent between SC and FT samples. The similar result can also be observed in other experiments (Balachandran *et al*., 2006; Konduri *et al*., 2005) and is explained by the equal amount and activity of enzymes involving in collagen degradation. The authors found that the production of collagenase and the activities of gelatinase (MMP-2/9) of SC for 48 hours remained the same as the FT (Balachandran *et al*., 2006; Konduri *et al*., 2005).

Collagen is the major ECM component of the fibrosa layer, on the aortic side, of a native aortic valve. The normal physiological flow on the aortic side (AS/AF) is demonstrated in these studies to significantly increase the collagen content during 48 hours of flow incubation. While in static conditions over 48 hours, the amount of collagen in the valves is unchanged as compared to FT.

There appears to be a shift in the balance between synthesis and degradation in the AS/AF samples towards more production and/or less degradation. The greater

amount of the collagen produced by the AS is not evident when the AS is exposed to VF. This may be due to fact that VF on the VS of the porcine aortic valves increased the collagenase activity of MMP-2 and 9, when compared to the control under static conditions (Platt *et al*., 2006b). The expression of MMP-2 and 9 and their collagenase activities, should be investigated further to determine if these effects are due to changes in synthesis or degradation.

In addition to the MMP-2 and 9, cathepsin K and L, which have both collagenase and elastase activities, were reported to be flow-sensitive ECM remodelling enzymes. The production of cathepsin K and L by the ECs from the mouse aorta was increased by aortic flow (Platt *et al*., 2006a; Platt *et al*., 2007). Nevertheless, these enzymes may not prominently involve in the degradation of collagen in the valve tissue for two reasons. Firstly, cathepsin L is an active elastase rather than collagenase, which was confirmed by the prominent loss of elastin degradation but only minor reduction of gelatine degradation activity in siRNA-cathepsin L knockdown cells (Platt *et al*., 2006a). Secondly, the collagenase activity of cathepsin K in response to the aortic flow has the same efficiency as the static control (Platt *et al*., 2007). These observations could explain why the significant reduction of collagen was not observed in the aortic-flow exposure valves during these studies, although cathepsin K and L were reported to be increased by AF when compared to VF.

In addition, the sustained amount of collagen observed in VS/VF samples, the same as FT and SC, suggesting there is an equilibrium between the degradation and production of collagen. These results, however, are different from the *in vitro* observations that revealed the increasing amount of collagen was induced by vVEC-CM and vVECs co-culturing. These might be due to the unique response of VECs to the flow patterns, in terms of collagen production and/or degradation that is not seen in the two static models investigated earlier in this thesis.

It is noted that collagen measured in this study is unlikely to be the covalent cross-linked or mature collagen because the Sirkol dye binds to the $[Gly\text{-}X\text{-}Y]_n$ of the helical structure of collagen. The collagen was released from the valve in a soluble form, by the digestion for 2 days with pepsin, which digests the non helical telopeptide part of the collagen fibre. However the collagen content in FT and CS, in this study is much less than those amount found by other research groups (Balachandran *et al*., 2006; Konduri *et al*., 2005).

Similar to the response on the collagen content, shear stresses is important to maintain the amount of GAGs in the valve. The physiological conditions experienced by the aortic side (AS/AF) can increase the amount of GAGs. The increased amount of GAGs seems to be a specific response of AS to AF and cannot be generated by AF on the VS. It is demonstrated by the significantly reduced amount of GAGs in VS/AF samples in comparison to the effect of AF on the AS.

Steady laminar shear stress maintained the amount of GAGs in the magnitude independent manner, as was previously demonstrated by another laboratory (Weston and Yoganathan, 2001). The side of the aortic valve leaflets that were exposed to the ventricular flow was not identified in their studies. However, the similar results are observed in the current study, in that both VS/VF and AS/VF maintain the amount of GAGs when compared to the native valves.

The responses of the VS to flow patterns are, nonetheless, different from the effect of vVEC co-culturing, which enhanced the content of GAGs to a higher level than VICs culturing alone. The increasing amount of GAGs by static vVECs may be due to the absence of a shear stress mediated effect that causes degradation or inhibition the production of GAGs. However this hypothesis should be further investigated before a firm conclusion can be made.

Elastin is the major ECM component on the ventricularis layer on the ventricular side of the aortic valve. These studies reveal the regulation of VF on the amount of elastin in the side-specific VECs independent manner. The VF on the VS maintains the elastin content at the same level as the native valves. Thus it is not surprising that the amount of elastin produced by *in vitro* VICs remains unchanged by culturing VICs with static VECs and VEC-CM.

Mechanical shear stress plays an important role in the regulation of the elastin content in the valves. The amount of elastin is significantly reduced when mechanical forces are absent for 48 hours, but can be restored to the same level as FT by applying VF on the VS. However, this response is not observed by the AF effect on the AS. This may be attributed to the activities of shear-sensitive elastase, cathepsin K and L, since their activities are inhibited by ventricular flow but increased by the aortic flow (Platt *et al*., 2006b; Platt *et al*., 2006a; Platt *et al*., 2007). This is further supported by the evidence that the VF effect on the amount of elastin is independent of side-specific VECs.

The content of elastin when VF is applied to the AS is significantly increased (when compared to the AF on the AS) up to the similar level that can be found when VF is applied to the VS. This suggests that aVECs response to the VF is to increase the inhibition activity of elastases relative to the AF. This is similar to the reaction of mouse vascular ECs to AF and VF (Platt *et al*., 2006a; Platt *et al*., 2007). vVECs, however, do not significantly reduce amount of elastin in response to the AF. The ability to decrease the elastin content was not seen in all valve tested. Thus, the number of the experiments and the time that the valves are exposed to flow patterns should be increased to confirm the response of the VS to AF.

5.4.1 Limitations of the study

There are some experiments which could not be performed in this study due to the limited availability of equipment and time. The magnitude and patterns of shear stresses that were generated by cone-and-plate machine were designed to mimic the physiological paradigm. However, the actual flow rate in this system was not pursued because of the lack of the required equipment to make these measurements in our laboratory. For example, an ultrasonic flow probe and a digital flow meter would be needed to measure the actual flow rate and pressure in the bioreactor. The study has therefore relied on the published work that documents the development and capability of the Cone-and-Plate machine (Sucosky *et al*., 2008).

It would have been ideal for the effect of flow patterns on one side of the valve to be investigated from the same leaflet and exposed to both AF and VF on the same day. As only one cone-and-plate is available (i.e. the ability to expose a single chamber to a single pattern of flow) this was not possible. Steps were taken however to try and pair the observations by taking identical samples from each valve, one which acted as the SC and the other exposed to flow.

The ECM components observed in these studies could be the total amount produced by VECs and VICs. Moreover, it could not exclude the effect of VECs on the side that did not get exposed to the flow. For that reason, the localisation of collagen, GAGs and elastin, including flow-sensitive ECM remodelling enzymes, should have been observed in order to investigate the response of VECs and VICs to the shear stresses in further detail. In these studies, the localisation of collagen type I and III only in the AS/AF samples were performed (data not shown) due to the limited time for observations to be made.

In addition, synthesis and degradation of the ECM components from each condition were not investigated. RT-PCR of collagen, GAG, elastin and matrix remodelling enzymes could also be performed to examine the newly synthesis of the ECM components at the transcription level. Incubation of the valve cusps with radiolabeled proline, acetate and valine could also be employed as a more sensitive measure of the synthesis of collagen, GAGs and elastin, respectively. The production of matrix remodeling enzymes, MMPs, TIMPs, and cathepsins, involved in the degradation of ECM components could be confirmed by immunostaining. Moreover, their activities could be examined in different ways. For example, the activities of gelatinase and elastase could be measured by zymography or fluorescein-conjugated gelatin and elastin. Specific activity of cathepsin L can be further investigated by detection the fluorescent product AMC (amino-4-methylcoumarin), which is linked to the synthetic substrate, specific to cathepsin L, and commercially available. This is clearly where future work needs to be performed to accurately determine the balance between synthesis and degradation under each of the flow conditions and on each of side of the valve.

5.5 Conclusions and Future Directions

These studies demonstrate that side-specific VECs respond differently to the same shear stress patterns and such interactions are important to the regulation of ECM components, although the molecular mechanism regulating this process still requires further study.

Shear stress plays an important role in the regulation of collagen, GAGs and elastin, as was demonstrated by the decreasing amount of ECM from the leaflets in the absence of any mechanical forces when compared to the fresh valve. The specific responses of AS to the AF to increase the content of collagen and GAGs cannot be replicated by a single effect of cell type or flow pattern. The AS ECs do not enhance the amount of collagen and GAGs in response to the VF. Moreover, the AF on VS does not induce the induction of the ECM component, but instead reverses the effect of the AS/AF. In contrast to the response on collagen and GAGs, VF is a crucial factor in maintaining the amount of elastin in the valve, independent of the specificity of VECs.

Although AS ECs have a unique interaction with the AF, they tend to have a similar ECM regulation as VS ECs when they response to the VF. In contrast, VS ECs do not respond to the changed flow or AF in a similar manner as AS ECs.

Thus, side-specific VECs and their responses to the particular patterns of shear stresses are important to the regulation of ECM components. Such understanding is imperative to the creation of tissue-engineered heart valves that must be created from the "right" cells to produce the specific ECM components and, in turn help develop and maintain the integrity and durability of the valves.

CHAPTER 6 Adipose-Derived Stem Cells as a Potential Source of Valve Endothelial Cells for Heart Valve Tissue Engineering

6.1 Background

As part of TEHV, EC source is required that will match side-specific VECs in terms of the ability to respond to the flow patterns and regulate the specific ECM components as previously shown in chapter 5.

Adipose-derived stem cells (ADSCs) are potentially a suitable cell for use in regenerative medicine and tissue engineering applications. Abdominal ADSCs can be isolated in abundant quantities with a minimally invasive procedure (e.g. via liposuction) and can be autologously acquired from a patient; thus minimising the chance of immunogenic rejection. More importantly, ADSCs have the potential to differentiate into many types of cells, for example, cardiomyocytes, endothelium, myocytes, chondrocytes, neuronal-like cells and osteoblasts (Gimble *et al*., 2007).

ADSCs have been successfully differentiated into ECs by many stimulants. These include matrix coated culture plates, media for culturing ECs, VEGF and shear stress (Bassaneze *et al*., 2010; Cao *et al*., 2005; Martinez-Estrada *et al*., 2005; Zhang *et al*., 2011). The ADSCs that express fetal liver kinase (FLK-1, a receptor for VEGF) were differentiated into ECs by culturing on the collagen IV-coated flasks supplemented with the endothelial growth media 2 (EGM2) (Martinez-Estrada *et al*., 2005). The similar EC differentiation method was carried on by Cao *et al.* (Cao *et al*., 2005) by culturing ADSC FLK-1+ cells on a Matrigel coated plate with EGM2 in addition of 50

ng/ml of VEGF. The ADSC FLK-1+ cells were able to express CD31, VE-Cadherin and NOS III as well as take-up LDL.

The effect of shear stress on the EC differentiation by ADSCs was also investigated (Bassaneze *et al*., 2010). After exposure to the laminar shear stress, the ADSCs significantly increased the production of NO and VEGF but failed to express EC markers e.g. vWF, CD31 and FLK-1. Recently, the effect of EGM2 and shear stress was revealed to increase the production of NO and EC markers by ADSC CD31-/CD45- cells (Zhang *et al*., 2011).

This study aims to differentiate ADSCs into VECs by shear stress and VEGF, and use the differentiated ADSCs as a potential source of VECs for tissue engineering of heart valves. It is hypothesised that the aortic and ventricular shear stress patterns can differentiate ADSCs into side-specific VECs. Furthermore, the differentiated ADSCs can regulate the function of VICs to produce ECM components. Thus, the ADSCs that express CD34 will be isolated and cultured on gelatin coated plates with EGM2. Subsequently, the ADSCs will be supplemented with VEGF in low-serum EGM2 and exposed to the AF and VF using the Cone-and-Plate machine. The EC differentiation of the ADSC cells will be assessed by their ability to uptake low-density lipoprotein (LDL) and express of EC markers. The side-specific phenotypes of the EC-differentiated cells will be investigated by the differential expression of EC markers, vWF, CD31 and NOS III. Their expressions previously suggested the distinct phenotypes among aVECs and vVECs in porcine (Simmons *et al*., 2005). In addition, the response of the differentiated ADSCs to flow patterns will compared to the characters of native side-specific VECs, in terms of the regulation on ECM contents, as demonstrated in chapter 5.

6.2 Materials and Methods

6.2.1 Isolation of Adipose derived stem cells from adipose tissue

The abdominal adipose tissue was obtained from 3 patients who underwent abdominal liposuction. ADSCs were isolated from the adipose aspirate as previously described (Colazzo *et al.*, 2011). The isolation involved mixing the adipose aspirate with an equal volume of 0.9% NaCl and allowed to settle for 5 minutes. The fat phase was separated and set at the top of the aqueous/blood layer. This step was repeated until the fat phase turned a more yellow colour. The fat tissue was then digested by one volume of 0.15 % (w/v) of collagenase A (Roche) in saline and mixed well at 37°C for 1 hour. The collagenase activity was then inactivated by adding FBS for one in tenth volume of digested tissue. After the digestion, the bottom phase now contained the stromal vascular fraction (SVF) cells and was then collected in the 50 ml tubes through a sterile mesh to remove tissue debris. The SVF cells were then centrifuged at 800g for 5 minutes. Cell pellet was re-suspended with the saline and filtered through the 100 μm and 40 μm cell strainers thereafter. SVF cells were then spun again and the pellet was re-suspended in the 160mM of NH_4Cl for 10 minutes to break down remaining red blood cells. The lysed cells in the supernatant were discarded after the centrifugation at 800 g for 5 minutes. The SVF cell pellet was re-suspended in a small amount of EGM2 and the total number of cells was quantified. ADSCs were then immediately isolated from the mixture of cells in the SVF by CD34 immunoselection.

6.2.2 Isolation of CD34+ cells and culture of adipose derived stem cells

Cells that expressed CD34 were isolated from the populations of cells by using anti-mouse IgG Microbeads (MACS Miltenyi Biotec). ADSC CD34+ cells were labelled with monoclonal mouse IgG1 anti-human CD34 (Cell Signaling, UK) before being allowed to bind with the anti-mouse IgG Microbead in the magnetic column. The heavy and light chain of IgG1 anti-CD34 binds to the Microbead and thus separates the bound cells from unbound non-CD34 labelled cells. Since this method is based on the binding of the antibody on the cell surface and the Microbeads, a buffer containing Ca^{2+} and Mg^{2+} is not recommended. Moreover, the isolation was performed quickly and the cells kept cold all the times.

The Buffer was prepared by mixing 2mM EDTA and 0.5% BSA in PBS pH7.2 (Sigma-Aldrich) and kept cold. ADSCs were washed with PBS and isolated by centrifugation at 400g for 5 minutes at 4˚C. The cell pellet was re-suspended in 100µl of the buffer and 1µl of the antibody against CD34 was added. The antibody was left to bind on the cell membrane at 4˚C for 1 hour and the unbound antibodies were washed thereafter. 2ml of the buffer (for less than 10^7 cells) was added to the reaction and centrifuge at 300g for 5 minutes at 4˚C. The supernatant was discarded and the cell pellet was re-suspended with 80µl of the buffer. 20µl of the Microbeads were added (for less than 10^7 cells) and mixed well. The Microbeads and CD34 antibody were incubated at 4˚C for 15 minutes to allow binding between the antibody and Microbeads. The non-bound Microbeads, which had no cells binding on them, were then washed off with 2ml of the buffer by centrifugation at 300 g for 10 minutes, at 4˚C. In a meantime, a magnetic column was placed in the magnetic field and rinsed with 500 µl of cold buffer. After the isolation and removal of the "free-of-cells" Microbeads, the pellet was re-suspended in 500µl of the buffer. The suspension was

then applied on the column. The flow-through solution which contained CD34 negative cells was removed and the column was washed with 500 µl of the cold buffer for 3 times. After washing, 1ml of cold buffer was added to the column and it was removed from the magnetic field stand. The ADSCs that expressed CD34 and were bound to the Microbeads in the column were washed away from the column by pushing the plunger into the column. The "plunged" cells or CD34+ cells were collected in a 15ml tube and then centrifuged at 400g for 5 minutes. The pellet containing the CD34+ cells was then re-suspended with EGM2 media and cultured in a culture flask coated with 1% of gelatine which was maintained in a CO_2 incubator.

6.2.3 Differentiation of adipose derived stem cells by VEGF and shear stresses

ADSC CD34+ cells at were cultured with complete EGM2 until passage 4-5. To start the EC differentiation, 2.0 x 10^4 cells were seeded on a 13 mm coverslip (Fisher Sciencetific, UK) in a 24 well plate. The culturing of the cells was continued with complete EGM2 until reaching 80% confluence. The media was then changed to low serum EGM2 to growth arrest overnight prior to commencing the differentiation protocol.

Aortic and ventricular shear stresses were applied on the cells to attempt to differentiate them into ECs. The flow patterns were generated by the Cone-and-Plate machine, as described in the section 5.2.2.1. The only difference in this experiment from *ex vivo* study was the plate. Rather than using the bullets and lid to fix tissue, the cells on the coverslips were mounted on a plate which contained 9 recessed wells which was then placed in the flow chamber. The cells were then exposed to either AF or VF in 0.4% FBS with EGM 2 media supplemented with

50ng/ml of VEGF (Cell Signalling, UK) for 4 days for which used as the optimal flow incubation time by Bassaneze and colleagues (Bassaneze *et al*., 2010). ADSCs that were maintained in a static environment with 50ng/ml of VEGF in low serum EGM2 served as the control.

6.2.4 Endothelial cell function: Acetylated LDL-uptake

Differentiated ADSC CD34+ cells on the coverslips were removed from the Cone-and-Plate machine and placed into 24-well plates. All the washing and incubation was performed in the plate. Both the static controls and differentiated cells were washed with PBS twice before being incubated with Alexa Fluor® 594 Acetylated LDL or AcLDL (Molecular Probes™, UK). The AcLDL was prepared at 3 μg/ml in 0.4% FBS with EGM2. 500 μl of the media containing AcLDL was added to every well and the incubation was then carried on in a 95% air/5% CO_2 incubator at 37^0 C for 4 hours with protection from exposure to light. Following this incubation all samples were washed 3 times with PBS to remove the excessive AcLDL that had not been taken up by the cells. They were then fixed with 4% PFA for 10 minutes. The cell membrane was permeabilised by incubation with 0.5% Triton X in PBS for 3 minutes to allow the staining of cell nucleui. The nucleui was stained twice with DAPI for 5 minutes. Finally the coverslips were the mounted on the slides with Permaflour (Beckman Coulter, UK). The amount of LDL taken up by cells was photographed using a fluorescent microscope (Nikon, Japan) and the intensity of the fluorescence was analysed by Image J.

6.2.5 Immunocytochemistry of endothelial makers

Endothelial and VIC markers expressed by the ADSC CD34+ cells after their differentiation was investigated using ICC. The staining protocol has been described in section 2.2.4 with the exception that human specific primary antibodies were employed for CD31 and NOS III rather than the antibodies used to investigate porcine tissue. The mouse anti-human CD31 (Dako, Denmark) was prepared at 1:40 dilution and the rabbit anti-human NOS III (Thermo Scientific, UK) was used at 1:50.

6.2.6 Data Analysis

The intensity of fluorescent staining was analysed by Image J. The threshold of intensity was set for each staining and applied for every group of samples. For every analysis, the area of fraction of the target protein staining (red or green colour) was normalised with the area fraction of nucleus (blue colour). Furthermore, mean values (of the area fraction) of each sample was calculated from 2-5 optical fields and plotted on a graph using Excel programme. The error bars represented SEM.

The data from all sample groups was statistically analysed by Prism 5 using the Kruskal-Wallis test, which considers median variance as the criteria. The pair-wise comparison was further assessed by Dunn's Multiple Comparison Test. P value less than 0.05, 0.01 and 0.001 are represented by *, ** and ***.

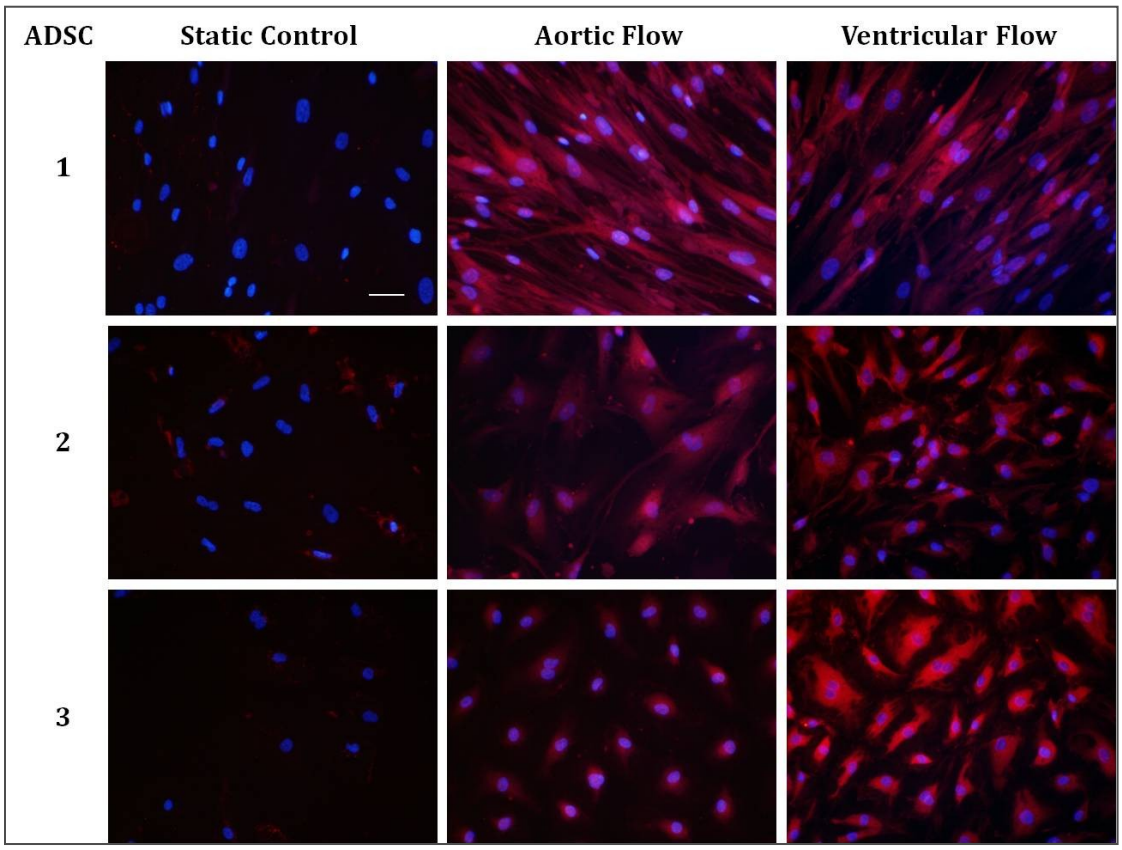
6.3 Results

The effect of different shear stress patterns on the EC differentiation of human ADSCs was investigated by ICC. The ADSCs were maintained in complete EGM2 being exposed to AF and VF for 4 days. During the 4 days of flow, ADSCs were supplemented with 50ng/ml of VEGF in EGM2 containing 0.4% FBS. ADSCs which were maintained under static conditions in EGM2 containing 0.4% FBS and 50ng/ml VEGF served as the control.

The ability of cells to absorb AcLDL was investigated in ADSCs that were exposed to AF, VF or kept under static conditions. Both AF and VF patterns led to increase uptake of AcLDL, as compared to the static control (Figure 6.1). ADSCs under static conditions had nearly none of the staining of LDL (median value was at 0.2286). In comparison, the AF and VF exposed cells increased the absorption and, thus, the staining of LDL to 5.675 and 5.031, respectively. However there was no difference observed between cells exposed to AF or VF ($p=0.0665$, $n=3$).

The expression of vWF, an EC marker, was also observed in ADSCs after they were exposed to the respective flow patterns. As depicted in Figure 6.2, VF tended to increase the expression of vWF for all 3 ADSC samples; the median value of area fraction was 6.234. However, there was no significant difference ($p=0.0509$) when compared to the static control or the AF treated samples. Both static conditions and the AF did not induce the expression of vWF and had the area fraction of vWF staining lower than nucleus staining as demonstrated by the value < 1. The median values of the control and AF samples were at 0.08409 and 0.3740, respectively.

a)



b)

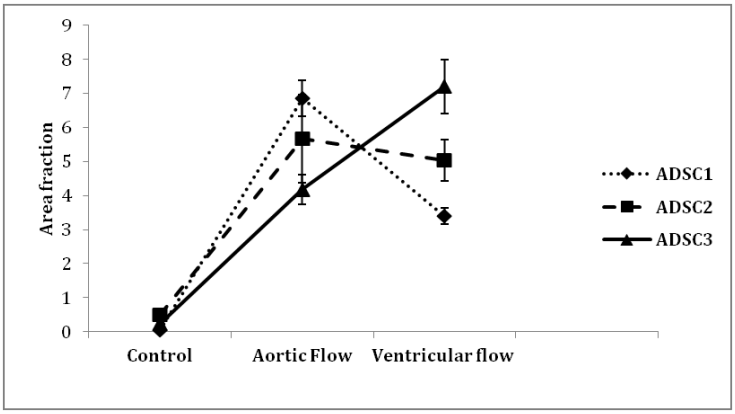
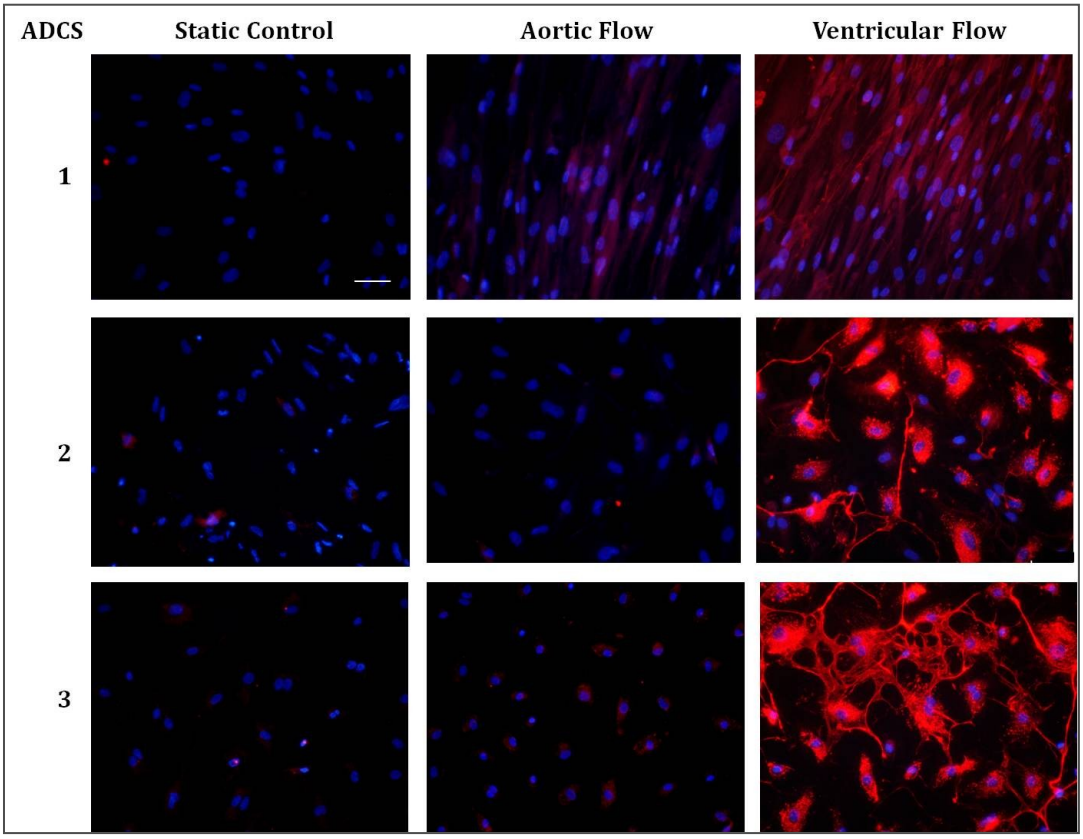


Figure 6.1 Effect of shear stress patterns on AcLDL uptake. a) Immunocytochemistry of differentiated ADSCs stained for AcLDL, in red. Human ADSCs were cultured with 50 ng/ml of VEGF in (0.4% FBS) EGM2 during the maintenance under either static culture (left panel), aortic flow (middle panel) or ventricular flow (right panel) for 4 days. b) Fluorescent quantitative analysis represented mean values of the area fraction and the error bars depicted the SEM. The area fraction was calculated from 2-5 optical fields of each sample. The mean values, n=3, among groups were statistically analysed by Kruskal-Wallis and pair-wise compared by Dunn's Test.

a)



b)

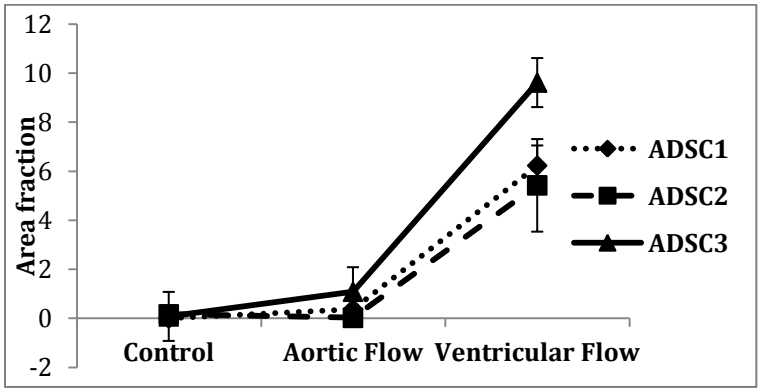


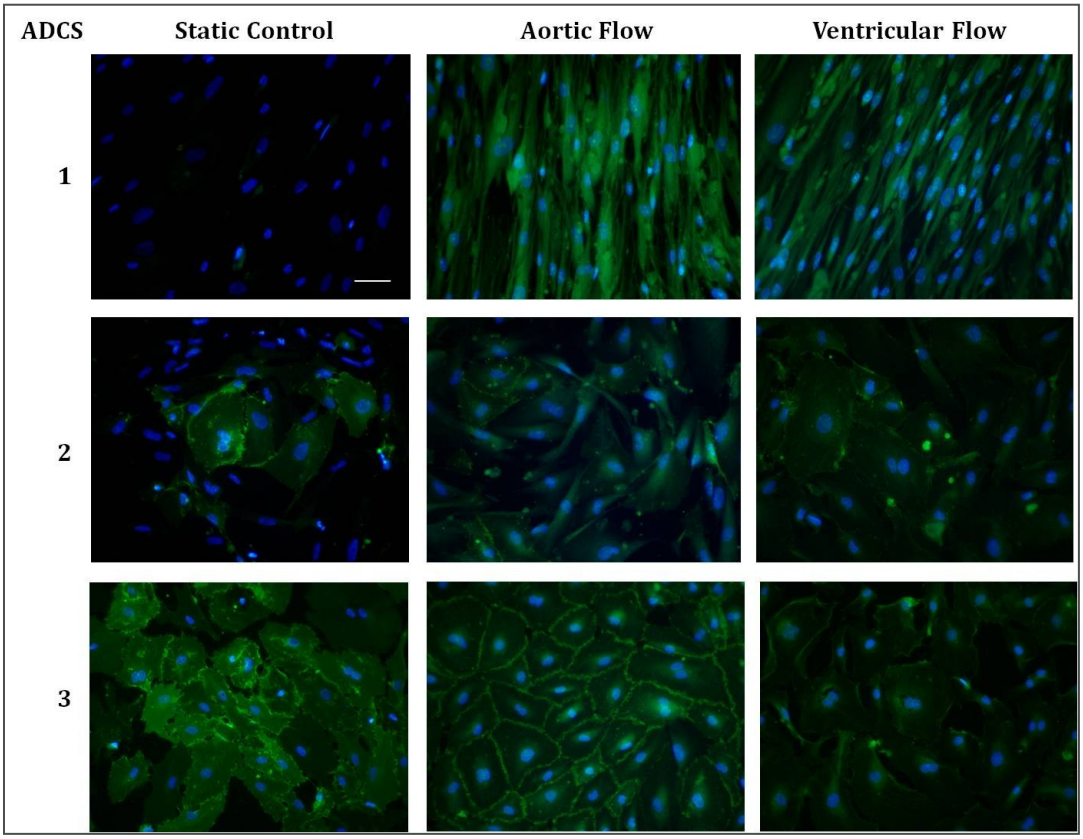
Figure 6.2 Effect of shear stress patterns on the expression of vWF. a) Immunocytochemistry of differentiated ADSCs stained for vWF, in red. Human ADSCs were cultured with 50 ng/ml of VEGF in (0.4% FBS) EGM2 during the maintenance under either static culture (left panel), aortic flow (middle panel) or ventricular flow (right panel) for 4 days. b) Fluorescent quantitative analysis represented mean values of the area fraction and the error bars depicted the SEM. The area fraction was calculated from 2-5 optical fields of each sample. The mean values, n=3, among groups were statistically analysed by Kruskal-Wallis and pair-wise compared by Dunn's Test.

The expression of CD31 on the cell membrane was also observed in differentiated ADSCs. The ICC of the cells from the control and flow-treated samples suggested a different base line expression of CD31 (Figure 6.3). Whereas ADSCs from donor 1 and 2 had low level of CD31 expression at the static control and increased expression of CD31 in response to flow, the cells isolated from the third donor expressed highest level of CD31 under static conditions that were maintained by AF but decreased by VF effect.

Although the area fraction of CD31 expression by the AF effect the third donor remained the same as the static condition, CD31 staining revealed more uniformity around the cell membrane by the effect of AF. There was no statistically significant difference among any culturing conditions ($p=0.4911$, $n=3$). The median values of control, AF and VF groups were 0.08478, 9.089 and 5.742, respectively.

The expression NOS III, an endothelial specific isoform of NOS, was also investigated in ADSCs that were exposed to different patterns of flow. As shown in the Figure 6.4, AF and VF induced shear stress led to the increased expression of NOS III by the cells exposed the flow for 4 days. The median value of the amount of staining by the static control increased from 0.3110 to 4.347 after exposure to VF, and to 10.25 following exposure of AF. This increased expression by the effect of AF was significantly different from the control ($p=0.0390$). Although there was a trend that the VF also induced the expression of NOS III in the ADSC cells to the higher level than that of the control, there was no statistical difference between these two groups.

a)



b)

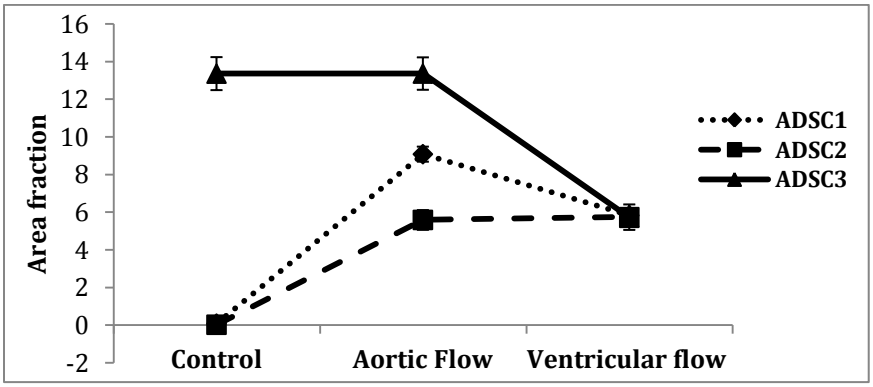
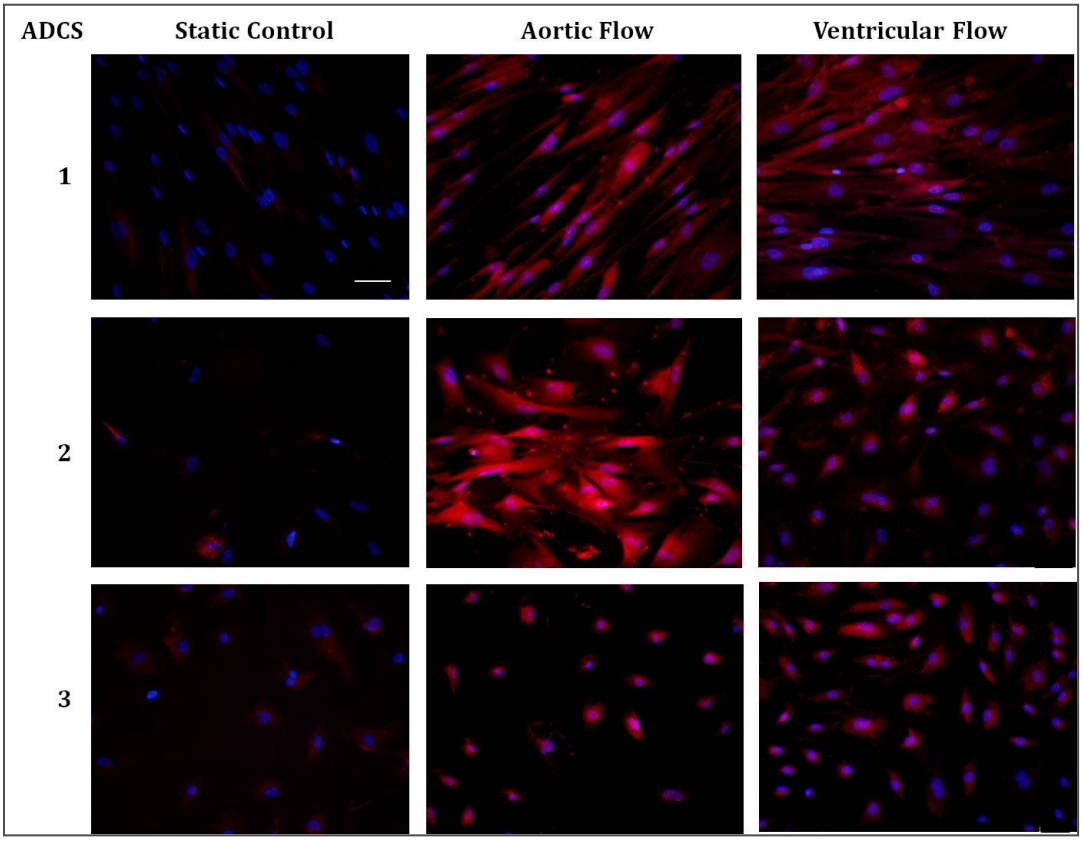


Figure 6.3 Effect of shear stress patterns on the expression of CD31. a) Immunocytochemistry of differentiated ADSCs stained for CD31, in red. Human ADSCs were cultured with 50 ng/ml of VEGF in (0.4% FBS) EGM2 during the maintenance under either static culture (left panel), aortic flow (middle panel) or ventricular flow (right panel) for 4 days. b) Fluorescent quantitative analysis represented mean values of the area fraction and the error bars depicted the SEM. The area fraction was calculated from 2-5 optical fields of each sample. The mean values, n=3, among groups were statistically analysed by Kruskal-Wallis and pair-wise compared by Dunn's Test.

a)



b)

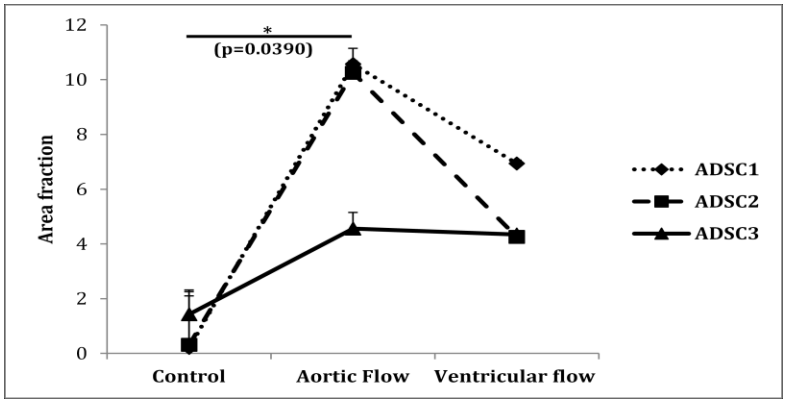
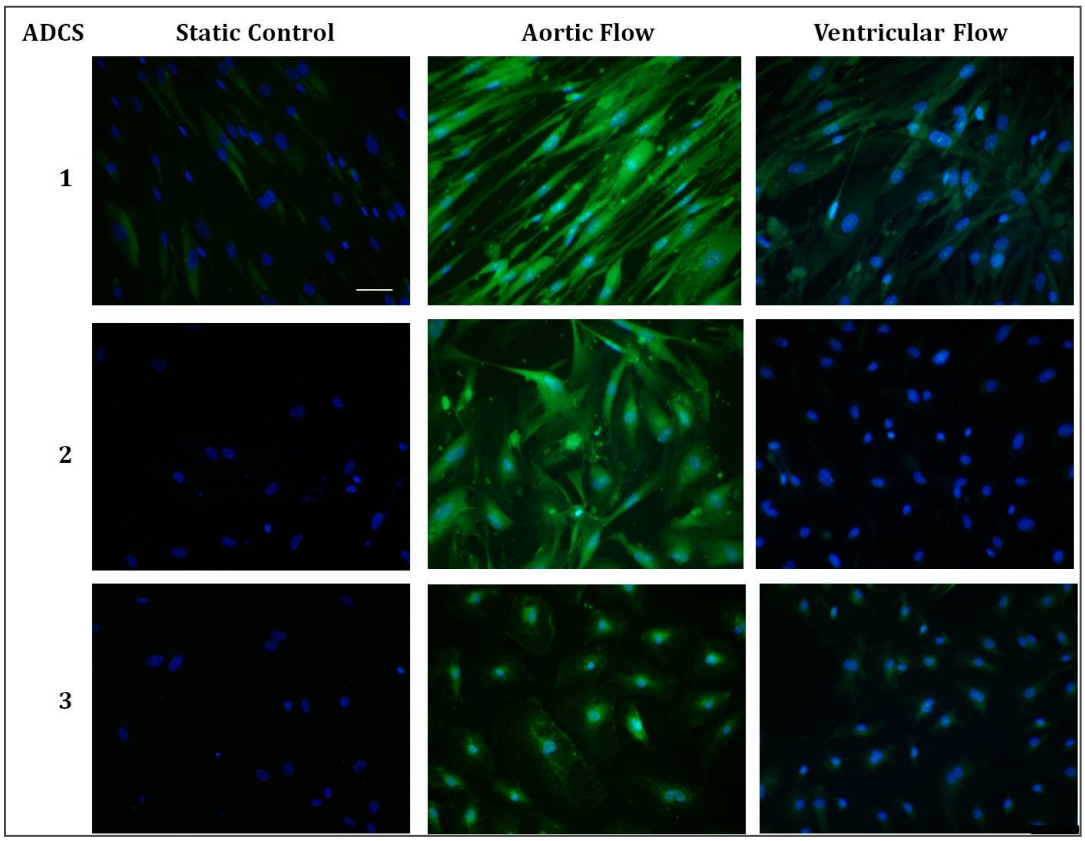


Figure 6.4 The effect of shear stress pattern on the expression of NOS III. a) Immunocytochemistry of differentiated ADSCs stained for NOS III, in red. Human ADSCs were cultured with 50 ng/ml of VEGF in (0.4% FBS) EGM2 during the maintenance under either static culture (left panel), aortic flow (middle panel) or ventricular flow (right panel) for 4 days. b) Fluorescent quantitative analysis represented mean values of the area fraction and the error bars depicted the SEM. The area fraction was calculated from 2-5 optical fields of each sample. The mean values, n=3, among groups were statistically analysed by Kruskal-Wallis and pair-wise compared by Dunn's Test.

The expression of SMαA was also investigated to assess the effect of shear stress on mesenchymal differentiation, as shown in the Figure 6.5. The AF significantly induced the expression of SMA by ADSCs, as represented by the increasing median value of the staining to 6.526. In comparison, the cells which were maintained in static conditions and those exposed to the VF show little or no expression of SMαA with median values at 0.04306 and 0.5834, respectively. It was noted that the staining of SMαA in the AF samples did not appear as mature fibres.

a)



b)

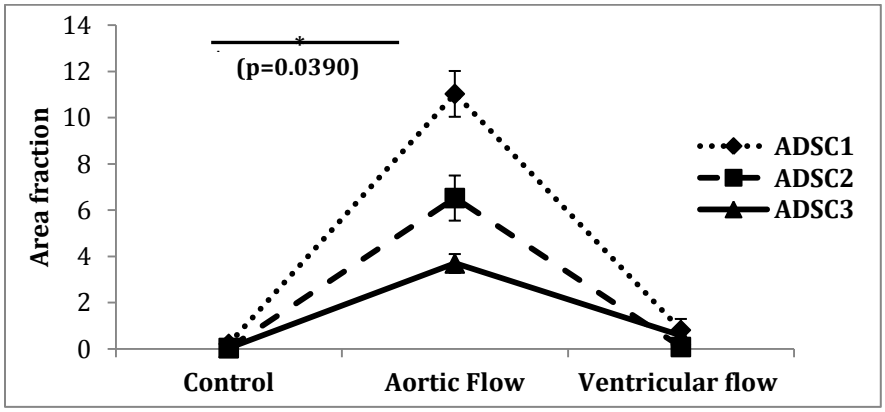


Figure 6.5 The effect of shear stress patterns on the expression of SMαA. a) Immunocytochemistry of differentiated ADSCs stained for SMαA, in red. Human ADSCs were cultured with 50 ng/ml of VEGF in (0.4% FBS) EGM2 during the maintenance under either static culture (left panel), aortic flow (middle panel) or ventricular flow (right panel) for 4 days. b) Fluorescent quantitative analysis represented mean values of the area fraction and the error bars depicted the SEM. The area fraction was calculated from 2-5 optical fields of each sample. The mean values, n=3, among groups were statistically analysed by Kruskal-Wallis and pair-wise compared by Dunn's Test.

6.4 Discussion

The effect of the shear stress patterns and VEGF on the differentiation of ADSCs into VECs is established in these studies. Shear stresses increased the expression of endothelial cell markers and functional properties associated with ECs. Moreover, the AF significantly enhances the expression of NOS III and SMαA by ADSCs, whereas the VF failed to stimulate the same effects.

In these studies, the ADSCs were isolated by CD34 immunoselection before the culturing with EGM2 on a gelatin coated plate, for a number of reasons. Firstly, ADSCs can be obtained from the SVF, of homogenised adipose tissue, which also contains other cells e.g. pre-adipocytes, macrophages, fibrobasts, pericytes, ECs and hematopoietic cells (Zimmerlin *et al*., 2010). Although ADSCs share the expression of protein markers with mesenchymal stem cells and pericytes, a glycoprotein CD34 is expressed only by (primary) ADSCs and absent in other mesenchymal and pericyte cells in the SVF (Mizuno *et al*., 2012; Lin *et al*., 2010). The positive immunoselection of CD34+ cells maximises the number and purity of the stem cell population from the heterogeneous SVF cells (Gimble *et al*., 2007).

Additionally, it has been demonstrated that CD34 is quickly lost in the cultured ADSCs by passage 3, although it is highly expressed in the primary ADSCs (Helder *et al*., 2007; Ning *et al*., 2006; Mitchell *et al*., 2006). However, it has also been revealed that the loss of CD34 expression by cultured ADSCs does not affect the differentiation potential (Suga *et al*., 2009). Thus the expression of CD34 by ADSCs at later passages was not pursued in these studies.

After being selected for CD34+ cells, the ADSCs are differentiated into VECs by combined stimulators i.e. endothelial media, VEGF and shear stress. It has been demonstrated that the culturing of ADSCs with EGM2 alone did not induce ADSCs to

express EC markers; however, those cells in the EGM2 were readily transformed into ECs when the VEGF or laminar shear stress was applied (Zhang *et al*., 2011). In contrast, ADSCs in DMEM (Bassaneze *et al*., 2010) and endothelial cell growth supplement media (Fischer *et al*., 2009) had a restricted expression of EC markers after being exposed to shear stress alone. Similarly, the static controls in this study, the ADSCs with VEGF in low-serum EGM2, do not take-up LDL nor express EC markers, with the exception of cells from one patient who had constitutive expression of CD31. Moreover, those cells which were maintained at the same conditions as the controls respond to shear stresses by subsequently being able to taking-up LDL and expression of EC markers.

The modified LDL (acetylated at the lysine residue of the apoprotein unit or AcLDL) can be taken-up by ECs and macrophages. Thus, the positive staining of AcLDL in the cytoplasm of cells can be used to characterise ECs. In these studies, there is a strong trend suggesting that both AF and VF induce the LDL up-take by ADSCs when compared to the static control, although this difference was not statistically significant, most likely due to the small number of experiments that were performed. A similar effect of shear stress to stimulate the up-take LDL by ADSCs has been demonstrated previously (Fischer *et al*., 2009).

In addition to the function of the LDL uptake, the effect of the differential flow patterns on the expression of EC markers is also investigated.

It has been reported that porcine aVECs expressed higher levels of mRNA of vWF, CD31 and eNOS than vVECs (Simmons *et al*., 2005). However, in this study, there is a prominent trend suggesting that the VF increase the expression of vWF by ADSCs, similar to the effect of shear stress reported on CD31-/CD45- ADSC (Zhang *et al*., 2011). Thus the sustained expression of vWF stimulated by AF, which is contradictory to the comparable cells (aVECs) reported by Simmons and colleagues,

suggests a complicated regulation on the production of vWF rather than a single effect from the flow pattern.

There is no obvious effect of AF and VF on the production of CD31 by ADSCs. While the ADSCs isolated from 2 patients respond to the shear stress in the similar manner, in that they increase the expression of CD31, there was a discrepancy with the data collected from the cells of the other donor. The expression of CD31 by the ADSC of this patient indicates the biological variability that may exist with these cells, and hence the different potential of ADSCs to be derived into ECs. Moreover, it is unlikely to be the expression by ECs that might contaminate in the ADSC population, as demonstrated by the fact that the ADSCs from this patient do not express other EC markers i.e. vWF and NOS III under static conditions. In addition, in the mixture of SVF cells, percentages of CD31+, vWF+ and FLK-1+ cells maintained stability over passages (Mitchell *et al*., 2006). Thus, if there is a presence of ECs in the ADSC culture, the positive staining of vWF and NOS III should be concomitant with the CD31.

Endothelial NO plays important role in the regulation of the mechanical properties (El-Hamamsy *et al*., 2009) and the prevention of calcification (Richards *et al*., 2013) of the aortic valve. The production of NOS III is thus considered as a functional marker of VEC-derived ADSCs. In this study, the AF significantly increases the expression of NOS III by ADSCs. This effect is comparable with the results demonstrating the higher mRNA expression of NOS III by VECs on the aortic side (Simmons *et al*., 2005). Nonetheless, these observations are contradictory to the research carried out on the human valves, which revealed the higher expression of NOS III on the ventricular side of the valve and its function to protect the calcification on that side (Richards *et al*., 2013).

There is a trend that the VF also increases the expression of NOS III by ADSCs; however it is not conclusive in this study, that the expression of NOS III by ADSCs is flow-pattern dependent. In addition, it has been demonstrated that the expression of NOS III is complicatedly regulated by flow patterns (Ziegler *et al*., 1998b). Whereas laminar shear stress increased NOS III mRNA expression, oscillatory shear stress increased the activity of promoter of NOS III.

The ADSCs also have the potential to derive into SMC lineage. It has been demonstrated that the ADSCs readily expressed SMαA when treated with TGF-β (Lee *et al*., 2006). In these studies, ADSCs significantly increase the expression of SMαA when they are exposed to the AF, whereas the VF does not induce the same effect. Interestingly, the expression of SMαA is co-expressed with NOS III. This may suggest the potential to commit to EndoMT by EC-derived from ADSCs, similar to the ability of VECs that can transdifferentiate into mesenchymal cells and are considered as the source of VICs (Paruchuri *et al*., 2006; Paranya *et al*., 2001). Nonetheless, this raises the concern that the ADSCs that are differentiated by AF could participate in the pathological development of valve disease as evidenced by the expression of SMαA by VICs of calcified valves (Latif *et al*., 2014).

Although these studies reveal the different expression of NOS III by the different flow patterns and this is similar to the differential expression by aVECs and vVECs (by Simmons and colleagues), the absolute marker for side-specific VECs has not been established yet. Thus the differentiation of ADSCs into side-specific VECs remains unclear. Moreover, the ability of EC-derived ADSCs to regulate the production of ECM by VICs requires further investigation.

6.4.1 Limitations of the study

There are few technical issues in these studies. The limited number of the Cone-and-Plate flow chambers available did not allow the differentiation to be performed by exposing two shear stress patterns at a time. Thus, the ADSCs at different cultures/passages were prepared. Due to this limitation, the ADSCs were cultured under static conditions and used as the control separately for each differentiation.

The isolation of ADSCs from the SVF (by selecting CD34+ cells) did not exclude the CD31+/CD34+ cells which are identified as capillary ECs. The exclusion of capillary ECs could be done by another immunoselection of CD31 after CD34+ selection. However, it has been demonstrated that the human ADSCs at primary culture contained only 10% of CD31+ cells that also included CD31+/CD34- cells (Planat-Benard *et al*., 2004). Thus the exclusion of CD34+/CD31+ cells was not done in order to minimise the time of isolation and time that ADSCs were in the harsh condition (4^0C without serum), during isolation. It, in turn, increased the survival of CD34+ ADSCs to be further cultured.

The quantification of LDL-uptake activity and the expression of EC markers by Image J is a semi-quantitative method. The attempt to quantify the production of EC markers was performed by using Western Blots. However, the blotting step was interfered by the grease which was used for mounting the coverslips in the flow chamber of the Cone-and-Plate machine. The grease contamination in the protein solution prohibited the transfer of proteins on the membrane. Further experiments are required to vary the speed and time of centrifugation to remove grease before Western Blot analysis is performed.

The numbers of cell isolates could have been increased from more donors/patients. In addition, the experiments to evaluate the ECM regulation by EC-derived ADSCs in

a co-culture with VICs could have also been evaluated. However those plans could not be accomplished due to the considerable of time was spent to develop the Western blot technique for samples exposed to the flow by Cone-and-Plate machine.

6.5 Conclusions and Future Directions

The ADSCs have the potential to differentiate into VECs, as demonstrated by the expression of EC and mesenchymal markers, when stimulated by EGM2, VEGF and shear stress. However, a marker of side-specific VECs has not yet been established, making it is difficult to judge if the AF and VF stimulate differentiation into cells that resemble VECs from each side of the valve. The aortic shear stress significantly increases the expression of NOS III and SMαA by ADSCs, whereas the ventricular shear pattern does not statistically induce the same effect.

Although the ability to uptake LDL and expression the EC markers by EC-derived ADSCs is demonstrated in these studies, their ability to regulate the production of ECM by VICs remains un-studied. In order to evaluate if differential flow patterns can induce the differentiation of ADSCs into side-specific VEC, their effects on the ECM production by VICs need to be compared to the effects seen by aVECs and vVECs already presented in this thesis. To address this question, the ADSCs will need to be seeded onto 3-D scaffolds containing VICs and continuously exposed to the different patterns of flow in a similar manner to the experiments in Chapter 5. Alternatively, the effects of condition media could be assessed or they could be co-cultured with VICs as described in Chapters 3 and 4. This information will be of use in choosing a cell type and conditioning protocol for the development of tissue engineered heart valves.

Chapter 7 Discussion

The aims of this thesis were to demonstrate if there is a side-specific marker or functional response that differentiates between cells on each side of the valve. From the functional perspective the thesis focused on the potential role that side-specific VECs play a role in the regulation of specific ECM proteins in the aortic valve. Moreover, it sets out to determine if the response of side-specific VECs to the patterns of shear stress generated on each side of the aortic valve, is also important for the ECM remodeling.

This thesis established the method to isolate and culture VECs from the different aspects of valve individuals; allowing the investigation of communication and response between side-specific VECs and VICs practical. While there was no appreciable difference in phenotypic markers expressed by the cells, aVECs and vVECs show a differential effect on the content of collagen and GAGs produced by VICs. The effect on collagen content is presumably mediated by stable factors that accumulate in vVEC conditioned media whereas GAGs content is affected by labile mediators differentially released by VECs under static condition. When the effect of shear stress patterns was addressed, the AS shows a unique response to the AF such that this interaction influences the total amount of collagen and GAG in the valve tissue and it cannot be replicated by the VS exposed to the same pattern of flow. In contrast to collagen and GAGs, elastin content is affected by the pattern of shear stress in a specific VEC-independent manner. Taken together, these findings reveal the importance of cell sources and shear stress patterns on the regulation of the ECM components. These observations are of value TEHV projects, in meeting the challenge of re-generating a heart valve that contains native valves ECM structure and biomechanical properties. Thus, the differentiation of ADSCs into VECs that have side-specific characters to regulate the production of ECM was attempted by

applying different shear patterns. Our data suggest that ADSCs are the potential source of VECs which can be induced to have EC functions and produce the EC functional markers. However the effect of AF and VF on the differentiation of ADSCs and their ability to regulate the ECM contents needs further investigation.

VECs contribute an important role to maintain valve integrity, in a similar way as vascular ECs elsewhere. For instance, VECs regulate inflammatory reactions between blood and the valve (Muller *et al*., 2000), deliver nutrients to VICs underneath (Tompkins *et al*., 1989) and regulate phenotypes of the underlying VICs (Butcher and Nerem, 2006). Moreover, they release the vasodilators and vasoconstrictor agents to adjust the contraction of the valve (Ku *et al*., 1990; Pompilio *et al*., 1998; Peltonen *et al*., 2009) as well as to regulate valve mechanical properties (El-Hamamsy *et al*., 2009). In addition to the regulation of valvular homeostasis, VECs are also thought to be VIC progenitor cells and replenish VICs via the activation of EndoMT (Wylie-Sears *et al*., 2011; Paruchuri *et al*., 2006; Paranya *et al*., 2001).

Although VECs share some function with vascular ECs, the studies at genetic and molecular level reveals different transcription profiles (Farivar *et al*., 2003) and the unique signalling pathways (Butcher *et al*., 2004) between VECs and the nearby ECs from the aorta. Additionally, there are an increasing number of studies demonstrating the phenotypic heterogeneities of VECs from the aortic and ventricular side of the aortic valve. These include a differential gene transcription profile (Simmons *et al*., 2005), differential production of NOS III and Cx43 proteins (Richards *et al*., 2013; Inai *et al*., 2004) and the differential expression of miRNA-70 in a flow-pattern independent manner (Holliday *et al*., 2011). More importantly, AS and VS differentially respond to the same patterns of shear stress, in terms of production of inflammatory molecules (Sucosky *et al*., 2009) suggesting the unique

inherited properties of aVECs and vVECs which are independent of the flow patterns.

The protocol to isolate and maintain VECs and VICs from the same valve was validated in this study, in the attempt to investigate and compare the intrinsic phenotypes of side-specific VECs without shear stress effect. aVECs and vVECS are isolated by the method which is adapted from collagenase and single colony expansion (Butcher *et al*., 2004). Being isolated by collagenase in a separate isolation chamber and expanded as a cluster, mimics the physiological environment of ECs (as opposed to trying to grow populations of cells from a single VEC). VECs were cultured in conditions that reduce the chance of contamination of VECs from another side of the valve, but increases VEC growth; thus, in turn, increasing the efficiency of VEC isolation. Moreover, aVECs and vVECs maintain EC phenotypes over passages in the similar manner as those found *in situ* in the current study.

Besides the vasodilators and constrictors that have been investigated, this thesis reveals further function of aVECs and vVECs to release and modulate the cytokines which are involved in inflammatory responses and remodeling of ECM components. Those stable molecules which are increased in the media cultured with aVECs and vVECs sides, at 48 hours under the resting condition, are IL-1β, IL-8 and TGF-β. In addition, IFN-γ and TNF-α which are present in the media appear to be metabolised by culturing with VECs.

It has been reported that IL-1β and TGF-β increase the production of collagen. The amount of collagen that was secreted into the media was increased in the peritoneal mesothelial cells treated with IL-1β (Yang *et al*., 1999). The treatment with IL-1β increases TGF-β mRNA expression by the mesothelial cells (Yang *et al*., 1999). Endothelial progenitor cells that are treated with TGF-β augment the production of collagen type I and III (Sales *et al*., 2006). The production of collagen and MMP-2 is

increased by stretched vascular SMC in the TGF-β dependent manner (O'Callaghan and Williams, 2000). Moreover, TGF-β is postulated to reduce the synthesis of proteases involving in the degradation of ECM, thus increasing in the effect on matrix protein synthesis (Roberts *et al*., 1992).

However, IL-1β and TGF-β have also been shown to reduce the production of GAGs. For example, the synthesis and expression of sulfate-GAGs are reduced by IL-1β treated fibroblasts (Qwarnstrom *et al*., 1993; Qwarnstrom *et al*., 1989). The release of GAGs from cartilage explants into the media was also reduced by the treatment with either IL-1β or TGF-β (Zanni *et al*., 1995).

Interestingly, the increase in the amount of GAGs was found only when VICs were co-cultured with vVECs. This effect could be a result of a short-life mediator which might be produced and degraded during the 48 hours of incubation. In the CM, it is likely that there is only the effect of molecules which are stable after 48 hours e.g. IL-1β and TGF-β and those molecules can attenuate the effect of GAG production when they are left to incubate with VICs. This can explain why the increasing amount of GAGs was not found by VICs cultured with vVEC-CM. Additionally, the modulation of GAGs was also not observed by aVECs which suggests the differential effect of aVECs and vVECs on the interaction with VICs at resting condition.

In contrast to collagen and GAGs, the amount of elastin was not affected by cultured VECs so that it leads to the hypothesis that elastin may be modulated by shear stress.

It has been proposed that ECs transmit shear stress into biological response through various mechanotransducers; for example, shear-sensitive ion channels (K^+/Cl^- channels), integrin binding proteins and glycocalyx (a thin layer of PGs around cell membrane) (Tarbell *et al*., 2005). Beyond being able to response to

shear stress, ECs can distinguish different types of flow and respond to them differentially (Barakat and Lieu, 2003). For instance, ECs from the aorta differentially express TGF-β and NOS III mRNA in response to the different flow patterns of laminar and oscillatory shear stress (Lum *et al*., 2000; Ziegler *et al*., 1998a). Umbilical vain ECs increased the production of antioxidant (Cu/Zn superoxide dismutase) and anti-inflammatory factors following the exposure to laminar waveform whereas pro-oxidant (NADH oxidase) was increased by the effect of oscillatory waveform (De Keulenaer *et al*., 1998; Weinberg *et al*., 2010). Furthermore, ECs have been shown to discriminate between steady laminar from oscillatory shear stress through the Na-K-Cl co-transporter protein (Suvatne *et al*., 2001). Whereas the laminar flow exposed ECs increased both amount and activity of the co-transporter, oscillatory flow did not have an effect on the co-transporter level.

VECs from the opposite sides of the aortic valve have been demonstrated to have a differential response to different flow patterns. Whereas AS that was exposed to VF increased in the production of inflammatory molecules (VCAM-1, ICAM-1, TGF-β1 and BMP-4), VS did not respond to the VF in the same manner (Sucosky *et al*., 2009). Moreover, VS did not induce the production of inflammatory factors by the AF which is believed a cause inflammation and calcification on the fibrosa of the valve. Taken all together, this information suggests that VECs are not only able to discriminate the flow patterns but aVECs and vVECs also show a unique response to the different flow types.

This thesis further aimed to investigate the effect of different flow patterns on the response of VECs, from the different aspect of the valve, in terms of the regulation of ECM remodelling. It has been demonstrated that mechanical force is important to maintain the amount of GAGs and elastin as compared to the effect of static condition. Particularly, the laminar waveform plays a crucial role on the modulation

of elastin in the valve in side-independent manner. This can explain the absence of the effect of VEC-CM and VEC co-culture on the elastin content when VECs are maintained under static condition. Furthermore, the unique response of AS to the AF to increase the amount of collagen and GAGs in the valve cannot be replicated when the VS that was exposed to the same flow. In contrast, vVECs (instead of aVECs) under static culture were able to affect the content of collagen and GAGs, produced by VICs, as previously shown by the CM and co-culture experiments. This may be due to a differentially expression of shear-sensitive channels e.g. K^+/Cl^- ion channels or a differential sensitivity to the flow between aVECs and vVECs Thus the regulation function of VECs under static culture is distinct from those exposed flow patterns.

In addition to the shear stress experienced by VECs on the surface, the valves are subject to other mechanical forces every cardiac cycle. Those are cyclic stretch and pressure and have been demonstrated to regulate the ECM in the valve (Balachandran *et al*., 2006; Konduri *et al*., 2005; Merryman *et al*., 2006; Xing *et al*., 2004). This study additionally highlights the crucial role of flow patterns and their responses by side-specific VECs on the modulation of ECM components. It thus provides the fundamental implication to the regeneration of a functional heart valves in a laboratory; leading to the production of a living valve that possesses biomechanic properties and capability to adapt to the hemodynamic environment.

Up to date, surgical replacement, either mechanical or biological prostheses is considered to be the only treatment for aortic valve disease. The ability of the valve substitutes to grow in paediatric patients cannot be achieved by mechanical or bioprosthetic valves since neither contains living cells. Moreover, the adaptive remodelling of mature heart valves during pregnancy implies the crucial requirement of valve substitutes to be able to continuously adapt to the changing haemodynamic environment (Wells *et al*., 2012). The significant ability of a living

valve is further demonstrated by Ross operation in which the pulmonary valve is grafted to the aortic position. The replacement with the viable valve allows the valvular adaptation to occur at both the cellular and tissue level (Ikhumetse *et al*., 2006; Rabkin-Aikawa *et al*., 2004a) and can improve the survival rate of the recipients (El-Hamamsy *et al*., 2010). All these data imply the important properties of the living valve substitutes for TEHV.

The original purpose of the last chapter in this thesis was to generate EC that would act as side-specific VECs and be able to function and respond to the heamodynamic environment by regulating ECM components. However, due to the time limitations, ADSCs are only derived into VECs by shear stresses generated on the aortic valve *in vivo* i.e. AF and VF.

ADSCs are differentiated into VECs by both AF and VF. After shear stress exposure, they increased the LDL uptake activity and the production of EC markers such as vWF and CD31. Furthermore, they also responded to the different flow patterns differentially. Those responses are the production of an EC functional marker, NOS III and the myofibroblast SMαA, which was increased after being exposed to the AF. Although the co-expression of NOS III and SMαA by AF-differentiated ADSCs suggests the ability to commit EndoMT and act as a potential source of VICs, similar to the previous finding (Colazzo *et al*., 2011), this raises the concern of disease development by SMαA expression cells (Aikawa *et al*., 2006; Latif *et al*., 2014; Rabkin *et al*., 2001). Overall, this data suggest the potential of ADSCs to be used for TEHV. Moreover, the function of VEC-derived ADSCs that are differentiated by different types of flow need to be further investigated for their ability to modulate the ECM components through the response of VICs.

It is noted that although there is an increasing research on the heterogeneous phenotypes of side-specific VECs, the markers to identify their distinction have not

been established. Thus, the identification of which VEC phenotypes differentiated ADSCs correspond to is not clear possible.

It is likely that side-specific VECs exert a different function to modulate the ECM components, as demonstrated in this thesis, rather than a different expression of a cell surface. In addition, the unique physiological function between aVECs and vVECs has been revealed, in a flow pattern-independent manner.

Nearly to the end of this thesis, I have contributed a project to demonstrate differences in the mechanical properties of VECs on the AS and VS of valves. This effect was related to differences in actin expression by aVEC and vVECs. *In situ* analysis demonstrated that actin expression was significantly higher in vVECs, as compared to aVECs. This characteristic remained when VECs were cultured for several passages and exposed to the reverse pattern of their respective flow. The stiffness of vVECs in flow-independent manner was further confirmed by *ex vivo* analysis of the valves that were exposed a surface to both physiological and reverse patterns of flow. The results also showed that the stiffness of vVECs was remained even if they are exposed to the other side pattern of flow (Chester un-published observations). The possibility exists that differences in cell mechanics may be a differential marker that can distinguish between aVECs and vVEC. However, the relevance of this difference to cell function is currently under investigation.

In summary, this thesis reveals the function of aVECs and vVECs as well as their unique response to the shear stress to modulate the ECM components produced by VICs. Moreover this finding is important for the development of TEHV.

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Review Article

Hemodynamics and Mechanobiology of Aortic Valve Inflammation and Calcification

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I am hereby asking for your permission to re-print the picture on my thesis and will not use it for other purposes.

Sincerely,

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