Endothelial Cell Impact on Smooth Muscle Cell Properties: Role of Hemodynamic Forces

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Declaration

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I would like to dedicate this thesis to my parents who have always been there for me (both emotional and financial) and have always believed in me.

Abstract

The vascular endothelium is a dynamic cell monolayer located at the interface of the vessel wall and bloodstream, where it regulates the physiological effects of humoral and hemodynamic stimuli on vessel tone and remodelling. Hemodynamic forces are of particular interest and include shear stress, the frictional force generated by blood as it drags against the endothelium, and cyclic strain, transmural pressure due to the pulsatile nature of blood flow. Both forces can profoundly modulate vascular endothelial metabolism and function and, within normal physiological ranges, typically impart an atheroprotective effect which disfavours pathological remodelling of the vessel wall. Changes to arterial wall architecture (i.e. remodelling) are a key feature of vascular diseases (e.g. atherosclerosis) and often stem from disruption of normal blood flow patterns, leading to vascular endothelial dysfunction and dysregulation of the underlying smooth muscle cell layer. The focus of the PhD project was to investigate hemodynamic challenge of vascular endothelial cells impacts smooth muscle cells. In order to assess the hemodynamic challenge of vascular endothelial cells, shear stress and cyclic strain were applied to BAECs. Both forces resulted in morphological realignment of cells along with a clear realignment for the actin cytoskeleton in the direction of flow. Furthermore, ZO-1 localisation also increased at the cell-border. We next investigated how hemodynamic challenge of vascular endothelial cells putatively impacts vascular smooth muscle cell growth properties. Four experimental models were employed namely; laminar shear stress, turbulent shear stress, pulsatile shear stress with co-culture and cyclic strain using in vitro hemodynamic modelling. Laminar shear stress, pulsatile shear stress with co-culture and cyclic strain of endothelial cells resulted in a decrease in BASMC proliferation with a parallel increase in apoptosis. Turbulent shear resulted in the opposite effect caused a slight increase in BASMC proliferation with no effect on apoptosis. This indicated that physiological forces impart an atheroprotective effect. In the hemodynamic models, BAECs and BASMCs were not in physical contact. This suggested that BAECs secreted factor(s) acting directly on the BASMC (or indirectly) on the BAECs were responsible for these effects. As the BAECs and BASMCs were not in physical contact this suggested that BAECs secreted factor(s) acting directly on the BASMC (or indirectly) on the BAECs were responsible for these effects. To investigate the endothelial signalling pathways and effectors putatively mediating these effects specific pharmacological inhibitors were employed. The results revealed that an integrin-Rac1 pathway possibly upstream of NO production may be mediating this endothelial regulatory response under LSS. We investigated the impact of LSS-derived BCM on the expression of cell cycle associated genes within the smooth muscle cells both single gene- and microarray-based RealTime PCR methodologies. Our results highlighted key CDK, cyclins and other cell cycle regulatory proteins. This study confirms the importance of hemodynamic challenge on the endothelium and the putative interactions between endothelial and smooth muscle cells in vascular remodelling.

Abbreviations

Ap–1	Activating protein-1
ADP	Adenosine Diphosphate
ANG	Angiotensin
ATP	Adenosine triphosphate
BAEC	Bovine aortic endothelial cell
BASMC	Bovine aortic smooth muscle cell
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDK	Cyclin dependent kinase
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CVD	Cardiovascular disease
DMSO	Dimethyl sulfoxide
DNA	Deoxy nucleic acid
ECM	Extracellular matrix
ECS	Extracapillary space
EDTA	Ethylenediaminetetraacetic acid
ENOS	Endothelial nitric oxide synthase
ERK	Extracellular regulated kinase
ET-1	Endothelin-1
FAK	Focal adhesion Kinase
FCS	Fetal calf serum

FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde phosphate dehydrogenase
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HBSS	Hank's balanced salt solution
ICAM-1	Intracellular adhesion molecule-1
JNK	c-jun N-terminal kinases
LSS	Laminar shear stress
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MT-MMP	Membrane-type MMP
NADPH	Nicotinamide adenine dinucleotide phosphate
NFkB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
OxLDL	Oxidised low density lipoprotein
PAI-1	Plasminogen activator inhibitor-1
PBD	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PECAM1	Platelet endothelial cell adhesion molecule

PI-3 kinase	Phosphoinositide 3 kinase
PTK	Protein tyrosine kinase
PTX	Pertussis toxin
RAS	Rennin angiotensin system
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	Replication protein A
RTK	Receptor tyrosine kinase
SUMO-1	Small ubiquitin-like modifier activating enzyme-1
TF	Tissue factor
TFIIH	Transcription factor II H
TGF-β	Transforming growth factor-β
TLDA	TaqMan® low density array
TNF-α	Tumour necrosis factor-alpha
t-PA	Tissue plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
ZO-1	Zonula occludens-1

Units

%	Percent
μg	Microgram
μL	Microlitre
μm	Micrometer
bp	Base pair
cm	Centimeter
Da	Dalton
g	Gram
h	Hour
kDa	Kilodalton
min	Minute
mL	Millilitre
mm	Millimeter
mM	Millimolar
ng	Nanogram
^{o}C	Degrees celsius
RPM	Revolutions per minute
sec	Second
U	Enzyme activity units
v/v	Volume per volume
w/v	Weight per volume

Publications

Peer Reviewed Papers:

Killeen MT, Murphy RP, Cummins PM. Cyclic strain and shear stress of vascular endothelial cells modulates vascular smooth muscle cell proliferation and apoptosis in a reciprocal manner. *Am J Physiol Heart Circ Physiol* 2008; In Preparation.

Fitzpatrick PA, Murphy RP, Guinan AF, Killeen MT, Walsh TG, Tobin NP, Pierotti AR, Cummins PM. Shear stress regulates neprilysin expression in vascular endothelial cells via a reactive oxygen species-dependent pathway. *Arterioscler Thromb Vasc Biol* 2008; In Revision.

Cummins PM, von Offenberg Sweeney, Killeen MT, Birney YA, Redmond EM, Cahill PA. Cyclic strain-mediated matrix metalloproteinase regulation within the vascular endothelium: A force to be reckoned with. *Am J Physiol Heart Circ Physiol* 2007;**292:**H28-H42.

Poster Presentations:

Killeen MT, Murphy RP, Cahill PA, Cummins PM. Cyclic strain of vascular endothelial cells reduces smooth muscle cell proliferation, possibly via an MMP-2 dependent mechanism. *8th Annual Conference on Arteriosclerosis, Thrombosis, and Vascular Biology* 2007; Chicago IL, USA.

Fitzpatrick PA, Killeen MT, Birney YA, Glucksman MJ, Cahill PA, Cummins PM. Shear stress regulation of NEP is mediated via reactive oxygen species in endothelial cells. *7th Annual Conference on Arteriosclerosis, Thrombosis, and Vascular Biology* 2006; Denver CO, USA.

Figures and Tables

CHAPTER 1

Introduction

1.1 Introduction

Cardiovascular disease (CVD) includes dysfunctional conditions of the heart, arteries and veins, which supply oxygen to all parts of the body. Different forms of CVD include atherosclerosis, stroke, hypertension and intimal hyperplasia. CVD is the leading cause of death in the United States and most European countries. In the United States, after age 40, 49% of men and 32% of women develop coronary heart disease (Yu *et al*., 2007). CVD is also the main cause of mortality in Ireland accounting for over 36% of all deaths (approximately 10,000 deaths per year) (Irish Heart Foundation). The largest number of these relate to coronary heart disease and heart attacks with 22% of all deaths occurring under 65 (premature death). Ireland also suffers from one of the highest mortality rates in Europe with 52 premature deaths per 100,000 as compared with the EU average of 42 (Irish Heart Foundation). Over €169 billion is spent every year on cardiovascular disease in Europe. There are significant variations in different countries with relation to healthcare budgets on the treatment of CVD. Many European countries including the UK and Germany invest around 15-17% of their healthcare budget while Ireland invests a mere 4.4% of the total healthcare budget (Leal *et al.,* 2006). In view of these facts a Cardiovascular Health Strategy was implemented in Ireland in 1999 by the government to reduce CVD mortality rates by 2009 at a cost of €220m. To date, only €60m has been invested. Ireland also has a shortage of cardiologists with only 11 per million compared to an EU average of 35 per million. While the trend is going in the right direction, these reports clearly indicate that further investment and research is required to support this strategy (Irish Heart Foundation).

Whilst significant progress has been made over the last 30 years in the reduction of CVD, it is still extremely prevalent in our society. Diagnostic and treatment facilities have greatly improved and huge efforts have gone into public health awareness programmes. Risk factors such as smoking, diet, cholesterol, obesity sedentary lifestyle, high blood pressure and elevated cholesterol levels are all causative factors in CVD (gender, age, genetics and ethnicity are also contributing factors).

In order to understand the cellular basis of CVD, we need to examine the physiological, cellular, and genetic processes that regulate the vascular endothelium and ultimately, vascular remodelling. Among the physiological factors, hemodynamic forces associated with blood flow (i.e. shear stress and cyclic circumferential strain) play a pivotal role in vascular remodelling events. Indeed, under normal physiological conditions these forces impart an "atheroprotective" effect that disfavours pathological remodelling of the vessel wall. When these forces become perturbed or attenuated however, we get pathological vessel remodelling. *It is our purpose in this thesis to examine how these hemodynamic forces impact the endothelium with consequences for the functional properties of the underlying smooth muscle cells and ultimately for vessel remodelling.*

1.2 Vascular Remodelling

Vascular remodelling can be described as any enduring change in the size or composition of a blood vessel. Remodelling of the blood vessel may occur to accommodate and adapt to changes in hemodynamic forces or as a response to inflammation or injury. Two basic types of vessel remodelling include normal or

"healthy" remodelling (e.g. angiogenesis, vasoconstriction and vasodilation) and abnormal or "unhealthy" remodelling (e.g. atherosclerosis and restenosis), the latter stemming from endothelial dysfunction. When the endothelium becomes dysfunctional (diet, injury, abnormal blood flow), normal biochemical processes within the vessel wall become impaired. Among the ensuing changes, one typically observes a decrease in synthesis and release of endothelial derived nitric oxide (NO), a potent vasodilator. These changes lead to loss of remodelling control, pre-disposing the vasculature to inflammatory diseases such as atherosclerosis. The following sections will examine in greater detail the effects of hemodynamic forces on the endothelium under both physiological and pathological circumstances.

1.2.1 Blood Vessel Structure

Blood vessels function to transport blood throughout the body. The arterial wall comprises of three layers: the *tunica intima*, which is the innermost layer and consists of a single layer of endothelial cells; the *tunica media*, which is the middle layer and consists of smooth muscle cells, connective tissue membrane and elastic fibres providing support and tone for the vessel wall; and the *tunica adventitia*, the outermost layer comprised primarily of fibroblasts and connective tissue (Li *et al*., 2007). Both arteries and veins are comprised of the same three layers with less smooth muscle and connective tissue present in veins, thus making them thinner and containing less blood pressure than in arteries (Fig. 1.1). Blood vessel remodelling is pivotal to the development of many CVDs.

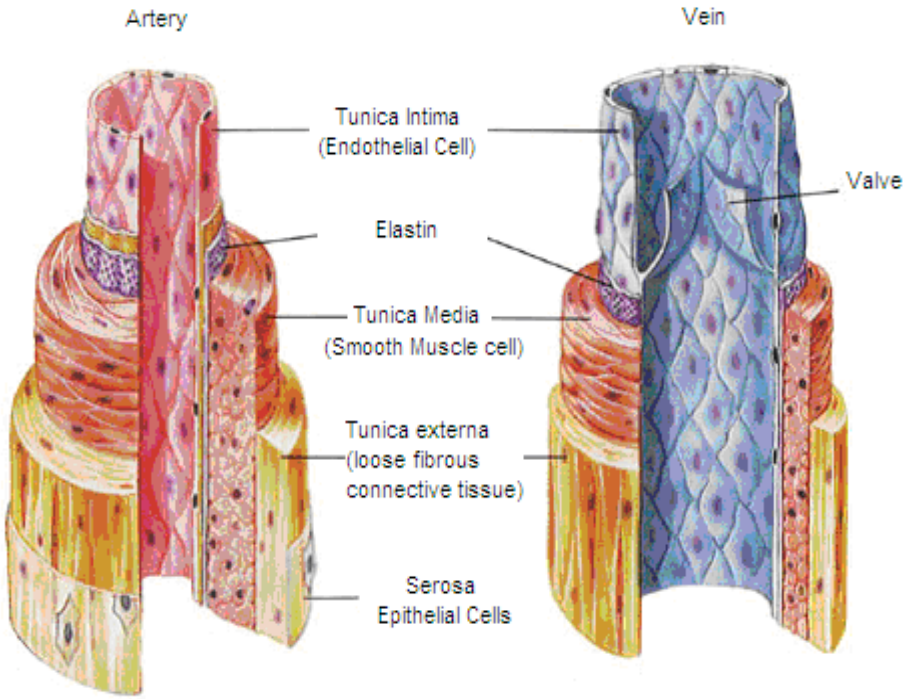


Fig. 1.1: Human Blood Vessels. Blood vessels function to transport blood throughout the body. They are comprised of three main layers: *tunica intima*, which is the innermost layer comprising the endothelium; *tunica media* is the middle layer that makes up the bulk of the vessel wall thickness; and *tunica adventitia* is the outermost layer. (Fox Stuart 1 Human Physiology 4th Brown Publishers).

1.2.2 Vascular Endothelial Cells (ECs)

The endothelial cell appears flat and cobble stone-like in shape. They form a single cell monolayer or "endothelium" that lines the lumen of blood vessels. The endothelial cell membrane contains many complex proteins that behave as receptors or ion channels, as well as caveolae which regulate the passage of fluid and macromolecules between the luminal and cellular compartments. The cortical web surrounding the endothelial monolayer contributes to cell shape and elasticity by responding to changes in intravascular pressure in addition to anchoring membrane proteins (Esper *et al*., 2006). Endothelial permeability (barrier function) is maintained

by the regulated apposition of tight/adherens junction protein complexes between adjacent endothelial cells. They regulate the passage of solutes and macromolecules between the blood and sub-endothelial space. Regulation of this is crucial for vascular homeostasis and is a central mechanism underlying vascular remodelling processes (Balda and Matter, 1998). Actin stress fibres that cross the cytoplasm adapt the shape of cells in response to hemodynamic forces such as blood flow and vessel wall distension. When flow increases, shear stress increases, causing cells to flatten and align in the direction of blood flow. As flow decreases, cells lose their realignment, increase in volume and appear like "cobble stones" (Esper *et al*., 2006). As will be discussed in detail later, the primary function of the endothelium is to maintain the health of the blood vessel by serving as a dynamic signalling interface between the vessel wall and circulating blood conditions.

1.2.3 Vascular Smooth Muscle Cells (SMCs)

Smooth muscle cells are spindle–shaped cells which can contract and relax and thus are the driving force behind vasoconstriction and vasodilation. In a relaxed state they are 20-500 microns in length and 5 microns wide. SMCs are characterised by their organised appearance and are arranged in sheets or bundle of cells. The cytoplasms of SMCs are inter-connected by gap junctions to allow different molecules and ions to pass between cells. Gap junctions are composed of connexons which connect across the intercellular space and provide communication to regulate simultaneous contraction of the medial SMCs (Yeh *et al*., 1997; Lash *et al*., 1990). The cellular cytoskeleton comprises actin filaments and a contractous protein called myosin. These filaments along with intermediate filament proteins (desmin and

vimentin) provide an organised cytoskeleton (Giuriato *et al*., 1992). SMC actin filaments are coupled to cell-ECM focal adhesions and cell-cell junctions. The former focal adhesion and intercellular junctions provide actin cytoskeleton attachment sites to both extracellular connective tissue and neighbouring cells. Cells are therefore mechanically coupled to one another such that cytoskeletal contraction of one cell induces contraction in an adjoining cell.

SMC contraction is caused by the sliding of myosin and actin filaments over each other. Intermediate filaments (desmin and vimentin) are also involved in pulling the cell ends, thus causing the cell to shorten. Myosin undergoes a conformational change producing globular heads which protrude and attach cross-bridges with the actin filaments causing the filaments to move. The heads then release the actin filament and adopt their original configuration. They can also bind to another part of the actin molecule and drag it along further - a process known as cross-bridge cycling. This process can only occur when the myosin heads have been activated to allow cross-bridges to form (Kamm and Stull, 1985). The myosin heads are comprised of heavy and light protein chains. When the light chains are phosphorylated by an enzyme called myosin light-chain kinase (MLCK), they become activated and contraction begins. MLCK only works when the SMC is stimulated to contract. Stimulation causes an increase of calcium into the SMC either from the extracellular space or endoplasmic reticulum. Calcium binds to calmodulin forming a calcium-calmodulin complex. This in turn activates MLCK allowing contraction to begin (van Lierop *et al.*, 2002). To reverse contraction, myosin light-chain phosphatase dephosphorylates the myosin light chains (Kamm and Stull, 1985).

1.2.4 Endothelial Homeostasis

As already mentioned the vascular endothelium is a monolayer of endothelial cells lining the vessel. The endothelium is strategically located between the bloodstream and the vessel wall, thereby playing a pivotal role in vascular homeostasis (Sagripanti *et al*., 2000). It regulates the physiological (and pathological) input of mechanical and humoral stimuli on underlying SMCs in the medial layer of the vessel wall. In this way, it regulates vessel tone and remodelling and modulates biological processes in immune and inflammatory processes. Under normal conditions, the endothelium displays anti-platelet, anti-coagulant and fibrinolytic properties, thus providing a non-thrombogenic surface for blood flow. A subtle balance between endothelium-derived relaxing and contracting factors helps maintains vascular homeostasis.

Among the physiological stimuli that impact upon the endothelium, mechanical or hemodynamic forces associated with blood flow are of central importance. These include cyclic strain, which is caused by a transmural force acting perpendicularly to the vessel wall (stems from the "pulsatile" nature of cardiac output), and shear stress, the frictional force created by blood flow as it "drags" against endothelial cells in vessels. These biomechanical forces have a profound effect on the endothelium and can regulate cellular fates including morphology, function (e.g. proliferation, migration, angiogenesis, barrier etc.), gene expression patterns and the synthesis/secretion of various biomolecules essential for the regulation of vessel remodelling processes (Traub and Berk, 1998).

1.2.5 Endothelial Dysfunction

Most common cardiovascular diseases such as atherosclerosis, restenosis and cerebral ischemia are preceded by endothelial dysfunction. This occurs when endothelial homeostasis is compromised by pathological stresses, which may include oxidative stress, hyperlipidemia, hypertension, inflammation and abberant blood flow (Melo *et al*., 2004). Endothelial dysfunction is often characterised by reduced NO production, an increase in contracting factors such as endothelin-1 (ET-1) and angiotensin-II (ANG-II) and elevated oxidative damage. This biochemical imbalance leads to an increase in vascular tone, platelet adhesion, barrier failure, inflammation and ultimately, undesireable changes in smooth muscle cell proliferation and migration (Bonetti *et al*., 2003).

As pointed out, NO production represents just one of several proposed mechanisms whereby physiological levels of shear stress may protect against endothelium dysfunction and aberrant wall remodelling. It regulates the surrounding environment by inhibiting the activation of growth factors and displaying anti-migratory/anti-proliferative effects on underlying SMCs. NO also displays anti-inflammatory properties within the endothelium by inhibiting cytokines and cell adhesion molecules that attract immune cells, and is partly mediated through NO-dependent inhibition of nuclear transcription factor-kappa B (NF-κB) (Cannon *et al*., 1998).

Nitric oxide (NO) is a free radical when released from endothelial cells. Three nitric oxide synthase (NOS) isoforms have been identified: neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, whilst inducible NOS

(iNOS) is regulated by cytokine stimulation. In endothelial cells, NO is synthesised from L-arginine by eNOS, a process which requires O_2 and nicotinamide adenine dinucleotide phosphate (NADP). Both shear stress and cyclic strain are potent stimuli for NO synthesis. Shear stress for example, activates the opening of specialized Ca^{2+} and K^+ ion channels in the endothelial cell resulting in hyperpolarisation. This leads to an increase in intracellular Ca^{2+} and eNOS activation. Due to its low molecular weight and lipophilic properties, NO easily diffuses across the endothelial cell membrane to the smooth muscle cell (Esper *et al*., 2006). NO stimulates soluble guanylyl cyclase, which in turn increases cyclic GMP. The increase in intracellular cGMP causes a decrease in intracellular Ca^{2+} and dephosphorylation of myosin light chains, leading to smooth muscle cell relaxation, as shown in Fig. 1.2 (Lusher *et al*., 1990).

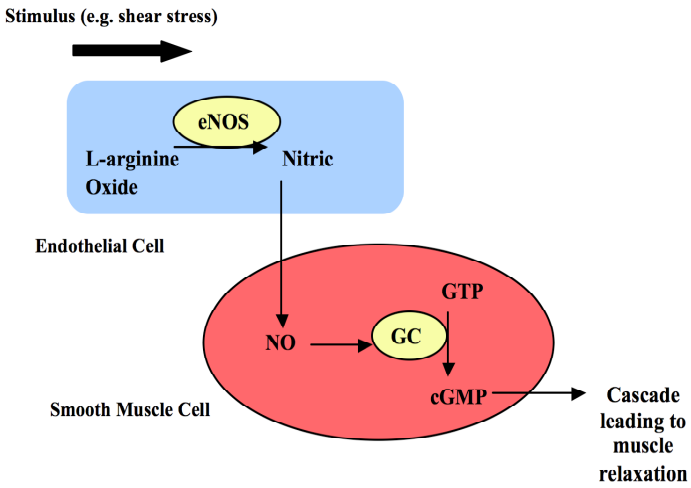


Fig. 1.2: Nitric Oxide pathway. Nitric oxide from the endothelium enters smooth muscle cells where it activates guanylyl cyclase (GC). GC converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). The increase in cGMP decreases Ca^{2+} resulting in smooth muscle cell relaxation.

Depending on NO release and the site of activation of cGMP, various functional outcomes may arise. For example, in vascular SMCs, induction of cGMP decreases intracellular calcium resulting in vasodilation (Moncada *et al*., 1991). In platelets, increased cGMP decreases platelet activation and adhesion to the endothelium

(Radomski *et al.,* 1993). NO also inhibits the activation of growth factors, as well as displaying anti-inflammatory properties by inhibiting cytokines and cell adhesion molecules that attract immune cells and prevents excessive exocytosis of inflammatory mediators via blockade of Weibel-Palade body secretion (i.e. Ang-II, ET-1, von Willebrand Factor, p-selection). In this way, NO production and release represents one of several potential mechanisms whereby physiological levels of shear stress protect the endothelium against dysfunction. In areas of vessel curvature or bifurcation, where the normally atheroprotective "laminar" shear stress becomes attenuated and/or "turbulent", vessel homeostasis becomes imbalanced. In these flow compromised areas, NO production is greatly reduced and an inflammatory process leading to abberant wall remodelling ensues. These hemodynamically sensitive locations within the vasculature are therefore obvious sites for development of atherosclerotic plaques (Esper *et al.,* 2006). Fig. 1.3 highlights this concept.

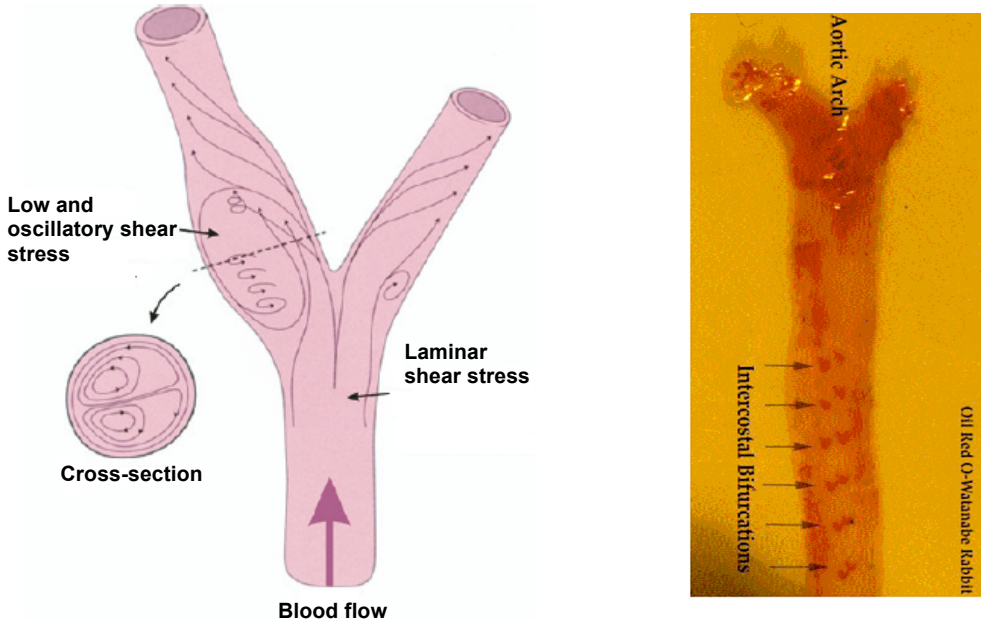


Fig. 1.3: Laminar versus turbulent shear stress – consequence for endothelium dysfunction. Steady laminar shear stress promotes release of factors from endothelial cells that inhibit thrombosis and adverse remodelling events. Low shear stress and turbulence favour pro-thrombotic, pro-migratory, and pro-proliferative effects contributing to adverse remodelling in diseases such as atherosclerosis. (Chatzizisis *et al.*, 2007)

1.2.6 Endothelial-Dependent Remodelling: Angiogenesis

Angiogenesis, a physiological form of endothelial-dependent remodelling, is a process involving the formation of a new blood vessel from pre-existing vessels. During angiogenesis, new capillaries are formed by "sprouting" from existing microvessels in response to paracrine factors released from neighbouring cells. This process is essential in many physiological functions including embryogenesis, reproductive development, vascular wound healing, and repair. Angiogenesis occurs by sequential events in response to physiological stimuli. These are tightly regulated by angiogenic stimulators and inhibitors. The balance between angiogenic stimulators and inhibitors is crucial as excessive stimulation can cause a shift in the balance resulting in an "angiogenic switch". This switch can lead to endothelial dysfunction, manifested as unhealthy or unwanted angiogenesis, the latter characteristic of pathologies such as cancers, dysfunctional uterine bleeding, inflammatory and immune diseases (Carmeliet *et al.*, 2005).

Angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) activate receptors on endothelial cells that line the pre-existing blood vessels. This results in the degradation of their basement membrane through the release of proteases including matrix metalloproteinases (MMPs), plasminogen activators, tryptases and cathepsins. The endothelial cells subsequently proliferate into the surrounding interstitial matrix and connect to neighbouring vessels forming capillary sprouts. These sprouts extend towards the angiogenic stimulus by the migration of endothelial cells, a process which employs vascular cell adhesion molecule-1

(VCAM-1), intracellular adhesion molecule-1 (ICAM-1) and integrins. The sprouts finally differentiate and form loops to become new vessel lumen (Rosen *et al*., 1997), which is demonstrated in Fig. 1.4.

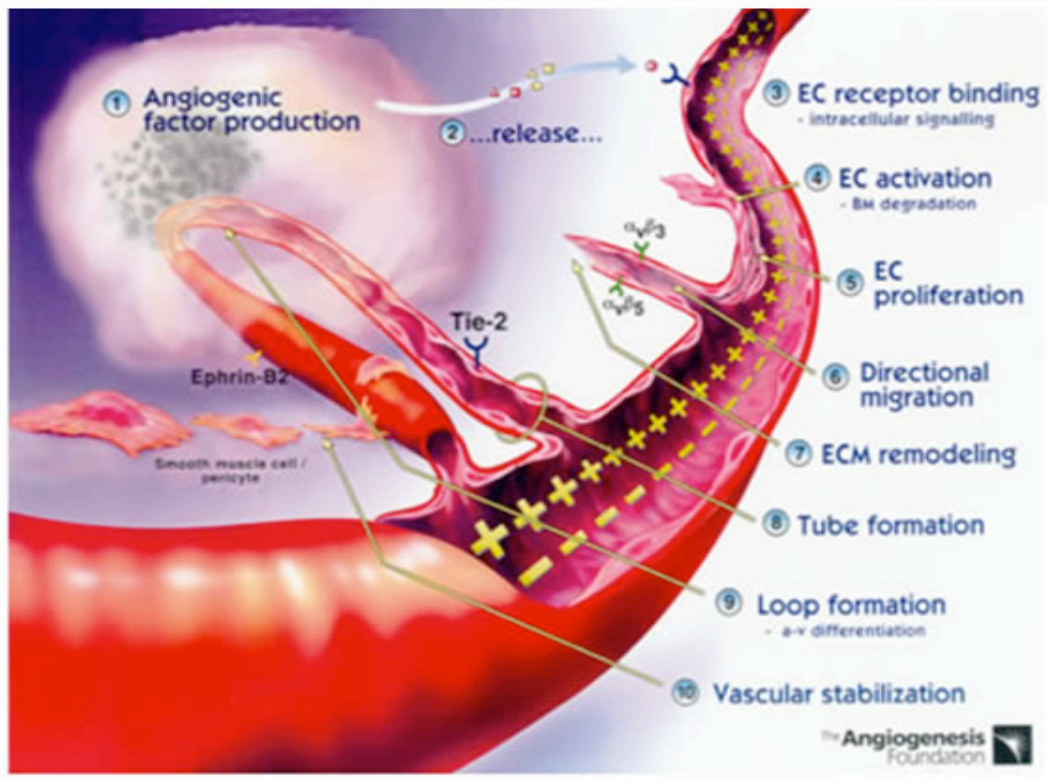


Fig. 1.4: Angiogenesis signalling pathway. Angiogenesis occurs by sequential events in response to stimuli, which are regulated by angiogenic stimulators and inhibitor. [http:www.angio.org]

VEGF and bFGF are key regulators in angiogenesis. bFGF stimulates endothelial cells, smooth muscle cells and fibroblasts, increasing proliferation, migration and differentiation. It also stimulates VEGF synthesis in tumour cell lines (Klein *et al* 1997; Tsai *et al*., 1995). VEGF is more selective, stimulating endothelial cells only. VEGF responses are mediated primarily by cell surface VEGF receptors. These receptors activate a tyrosine kinase signalling cascade that in turn activates several angiogenic processes such as EC barrier destabilization, proliferation, and migration (Hyder *et al*., 1999). Cytokines such as platelet derived growth factor (PDGF) and

transforming growth factor-β (TGF-β) indirectly induce VEGF and bFGF expression by vascular smooth muscle cells rather then directly stimulating endothelial cell proliferation. Both cytokines up-regulate VEGF and bFGF transcription in vascular smooth muscle cells. Hypoxia, another potent angiogenic stimulus increases VEGF expression but not bFGF (Brogi *et al*., 1994). Angiogenesis inhibitors target VEGF and VEGF receptors causing arrest of endothelial cell proliferation and preventing vessel growth. They also induce regression of existing vessels by increasing endothelial cell apoptosis (Carmeliet *et al*., 2005).

As mentioned, unhealthy angiogenesis may also contribute to the progression of pathologies such as atherosclerosis. In normal arteries, a dense network of capillaries known as the "vasa vasorum" supply blood to the outer adventitial and medial layers, while in diseased arteries these networks become mores extensive and disorganised and extend into the intimal layer of the atherosclerotic plaque. These can cause haemorrhages, blood clotting and decreased blood flow to the heart muscle, leading to myocardial infarction (Isner, 1999).

1.2.7 Endothelial-dependent remodelling: Atherogenesis

Atherosclerosis is characterized by progressive deleterious remodelling of the vessel wall. Atherosclerotic lesions occur at specific mechanically sensitive locations within the vasculature. There are five major regions of arterial plaque formation; (i) coronary arteries, (ii) major branches of the aortic arch, (iii) major branches of the abdominal aorta, (iv) visceral extremity branches of the abdominal aorta and (v) lower extremity branches of the abdominal aorta. Hemodynamic forces, in particular shear

stress, play a fundamental role in this localisation. In response to normal levels of shear and the laminar shear characteristic of linear stretches of vessel, shear imparts an atheroprotective effect on the endothelium, preventing the formation of fatty streaks associated with plaque development (Traub and Berk, 1998). However, in certain bifurcated and curved regions of vessel, flow becomes turbulent/attenuated and both cyclic strain and shear stress are dramatically altered. This results in increased ROS levels and decreased nitric oxide production, the former caused by a reduction in antioxidants, leading to endothelial "activation", one of the initial events in the endothelial dysfunction cascade leading to atherosclerotic plaque development. This is further exacerbated by reduction in endothelial barrier integrity and increased platelet adhesion and neutrophil infiltration into the sub-endothelial space, in essence leading to an inflammatory reaction (Cunningham *et al.,* 2005).

During this inflammatory response, low density lipoprotein (LDL) accumulates in the sub-endothelial matrix and is oxidised, becoming trapped within the vessel wall. Oxidised LDL stimulates the production of pro-inflammatory components such as adhesion molecules (ICAM-1, PCAM-1, VCAM-1, P-selectin and E-selectin), growth factors (macrophage colony-stimulating factor) and chemotactic proteins (monocyte chemotactic protein-1, MCP-1). These pro-inflammatory molecules, in particular ICAM-1 and MCP-1, act as potent chemoattractants for monocytes/macrophages (Kume et al., 1992). Monocytes "roll" along the endothelium allowing them to slow down and firmly attach to the endothelial surface. After adhering, monocytes enter the arterial wall through cell junctions by diapedesis. Once in the sub-intimal space, monocytes transform into active macrophages. Substantial uptake of LDL by macrophages results in foam cell formation within atherosclerotic lesions, an effect

that is mediated by scavenger receptors SR-A and CD36 (Febbraio *et al*., 2000; Suzuki *et al*., 1997). Furthermore, macrophages along with T-cells secrete growth factors and cytokines, stimulating smooth muscle cell migration and proliferation into the intima, generating lipid deposits and calcification. These characteristic stages of lesion development ultimately yield an atherosclerotic plaque with a thin fibrous cap, which is shown in Fig. 1.5 (Quinn *et al*., 1987).

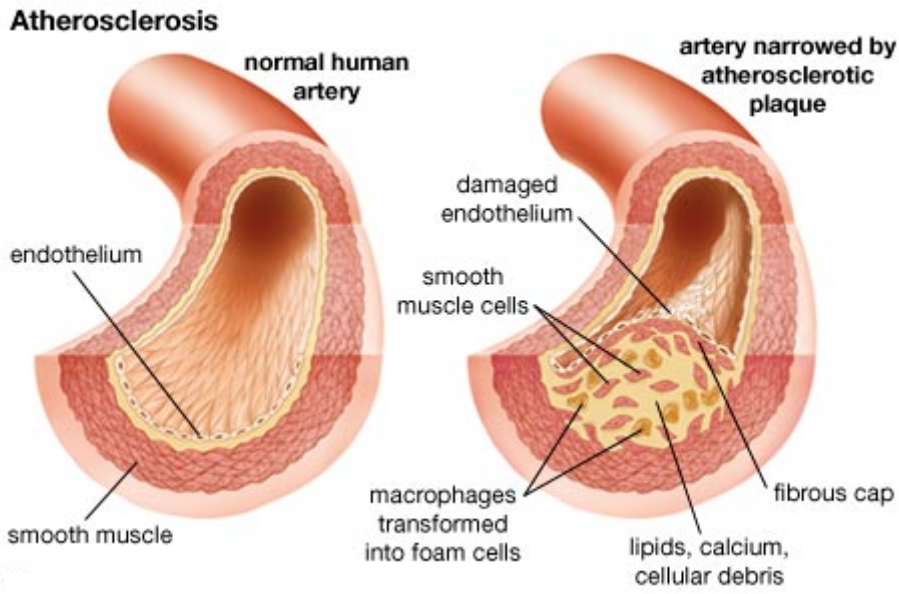


Fig. 1.5: Diagram of the atherosclerotic plaque formation. Endothelial dysfunction leads to the initiation of atherosclerosis. Over time, lipid accumulation, SMC migration and proliferation, and matrix synthesis within the lesion narrow the arterial lumen. [http:www.britannica.com/eb/art-95216]

There are three main determinants of a plaque's vulnerability to rupture: (i) The core size and consistency- a larger core of soft lipid-rich atheromatous gruel is highly unstable, (ii) Thickness of the fibrous plaque- thinning of the cap increases a plaque's ability to rupture, and (iii) Cap inflammation and repair- a cap which is slow to heal due to decreased SMC presence, or an inflamed cap due to macrophage influx, is more likely to rupture (Pasterkamp and Virmani, 2002). Studies have shown that SMC proliferation accounts in part for lesion formation in its early stages with many

advanced plaques comprising a SMC-rich fibrous cap overlying a necrotic core (Clowes and Schwartz, 1985). SMC have been considered as directly responsible for generating the atherosclerotic plaque via proliferation, migration from the medial layer, and synthesis of matrix proteins (Ross, 1993). However, recent reports have shown the beneficial protective role of SMC in atherosclerosis as studies have found that plaques which undergone rupture have a paucity of SMCs compared to stable lesions. Also the fibrous cap of advanced plaques is thinned from the loss of SMCs (Weissberg *et al*., 1996; Davies *et al*., 1993). Therefore, it is now widely accepted that SMC proliferation is deleterious in the *early* steps of atherosclerotic lesion formation, but SMCs protect the integrity responsible for promoting plaque stability and that a lack of SMC proliferation in established plaques is associated with the increased incidence of plaque rupture (Newby and Zaltsman, 1999). Atherogenesis typically does not manifest itself until middle and old age, however it does begin early in life. Moreover, whilst plaque localization is clearly localized to hemodynamically unstable regions of the vasculature, other risk factors such as smoking, diet, genetics, stress and injury can influence the rate and extent of plaque initiation and progression.

1.3 Factors Impacting Endothelial Homeostasis and Dysfunction

Multiple factors, both internal and external, can impact endothelial homeostasis and dysfunction through regulation (and dysregulation) of vascular cell processes including apoptosis, proliferation, and migration. The following subsections will examine these factors in more detail.

1.3.1 External Factors

Endothelial dysfunction is a key early step in the development of a variety of diseases such as atherosclerosis. External factors such as infection, diet, genetics, stress, smoking and sedentary lifestyle impact endothelium health (dos Santos *et al*., 2008). Active smoking as well as passive smoking significantly impairs the endothelium as the degree of dysfunction is associated with the extent of exposure. The exact cause of the deleterious effect of smoking on endothelial function remains unclear but several potential mechanisms may be involved including direct toxicity to endothelial cells, increased levels of oxidative stress, platelet activation and oxidative modification of lipoproteins (Celermajer *et al*., 1996). Active smoking is associated with an increased tendency towards thrombosis which may increase the risk of myocardial infarction and ischemic stroke (Barbash *et al*., 1993; Mueller *et al*., 1992). The average increase in coronary atherosclerosis in smokers is approximately 25% (McGill *et al*., 1988). Diet and obesity are also involved in endothelial dysfunction. Studies have shown that obesity is accompanied by increased triglycerides which lead to ADP deficiency a factor in the production of ROS. Oxidative stress in turn can reduce NO through peroxynitrite formation leading to endothelial dysfunction. Obesity also leads to increased pro-inflammatory cytokines, prostaglandins, and elevated LDL. Indeed, LDL reduction by diet can greatly reduce hyperglycemia and lower oxidative stress to improve endothelial function (Gielen and Hambrecht, 2004). Exercise also reduces cardiovascular events as regular exercise has been shown to improve glycemic control and prevent overt type diabetes in patients. It increases glucose uptake in skeletal muscle, thereby lowering glucose levels and improving insulin action leading to improved vasoreactvity (Tuomilehto *at al*., 2001; Goodyear

et al., 1991). Genetics and stress are also significant risk factors. Polymorphisms of the activin-receptor-like-kinase 1 (ALK1) gene encoding transforming growth factor-β (TGF-β) were detected in endothelial cells of patients with pulmonary hypertension (Trembath, 2001). Moreover, animal studies using rats have shown that chronic social stress by crowding impaired endothelial function via reduced vascular NO synthesis and altered vascular function (Bernatova and Csizmadiova, 2006). Mental stress is known to provoke myocardial ischemia (Deanfield *et al*., 1984) and is linked to increased morbidity and mortality (Ghiadonia *et al.*, 2000; Bosma *et al*., 1997). It is clear therefore that external factors can impair endothelium homeostasis resulting in initiation and progression of vascular diseases such as atherosclerosis. Treatment of these external factors can modify their impact on the endothelium and associated cardiovascular disorders.

1.3.2 Hemodynamic Factors

As mentioned earlier, a central feature of endothelial dysfunction is abberant blood flow leading to improper hemodynamic stimulation. Blood vessels are constantly subjected to mechanical or hemodynamic forces. These include cyclic strain, a transmural force acting perpendicular to the vessel wall, which stems from the "pulsatile" nature of cardiac output and shear stress, the frictional force created by blood flow "dragging" against the endothelium (Lehoux *et al*., 2003) (Fig. 1.6). Both forces are involved in regulating vascular cell processes including apoptosis, proliferation, migration and morphological characteristics. Under normal conditions, hemodynamic forces impart an "atheroprotective" effect on the endothelium that disfavours pathological changes in vessel structure.

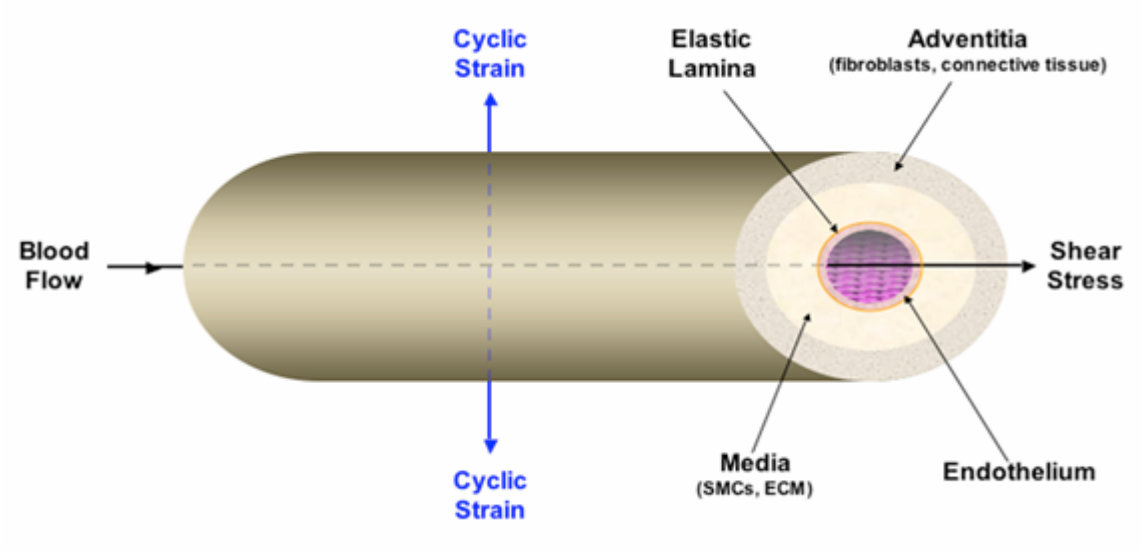


Fig. 1.6: Biomechanical stimulation of a blood vessel. Hemodynamic forces associated with blood flow, namely cyclic strain and shear stress, play a pivotal role in the physiological control of vascular tone, remodelling and the initiation and progression of vascular pathologies [Cummins *et al.*, 2007].

1.3.2.1 Cyclic Strain

Cyclic strain causes the arterial wall to rhythmically distend and relax to the cardiac cycle, leading to outward stretching of both vascular endothelial cells and smooth muscle cells (Kakisis et al., 2004). In maintaining this normal circumferential stress, according to Laplace's equation, the wall tension can be described by T=Pr/h where P is blood pressure, r is vessel radius and h is thickness of the wall (Lehoux et al., 2003).

Numerous studies have shown that cyclic strain has a profound effect on endothelial metabolism and can induce qualitative and quantitative changes in gene expression and cell fate. In addition to affecting the expression and/or activation of numerous signalling molecules associated with mechanotransduction, cyclic strain has been shown to modulate the expression and activation of numerous classes of effector

genes in vascular endothelial cells including those regulating; (i) Vessel diameter - NO, eNOS, cyclooxygenase-2 (COX II), endothelin-1 (ET-1), and thimet ologopeptidase (Coen *et al*., 2004, Cotter *et al*., 2004), (ii) Cell-cell communication and barrier function - zonula occludens (ZO-1), occludin and ICAM-1 (Collins *et al*., 2006; Pradhan *et al*., 2004), (iii) Proliferation - PDGF and VEGF (Sumpio *et al*., 1998; Zheng *et al*., 2001), and (iv) Migration/angiogenesis - MMP-2, MMP-9, MT1-MMP, uPA, and RGD-dependent integrin (von Offenberg Sweeney *et al*., 2005; Yamaguchi *et al*., 2002).

Vascular cells respond to cyclic stretch both morphologically and phenotypically. Cyclic strain has an immediate effect on vascular cells. After exposure for 15 min, the actin fibres morphologically align perpendicularly to the force vector, subsequently followed by phenotypic or cell fate changes (Iba *et al*., 1991). Induced endothelial alignment from cyclic strain is regulated by focal adhesion kinase (FAK) and paxillin (Yano *et al*., 1996; Naruse *et al*., 1998), whereas the smooth muscle cell response is mediated by NO and NADPH signalling (Standley *et al*., 2002; Chen *et al*., 2003).

Physiological levels of cyclic strain (5-10%) on aortic endothelial cells have been shown to increase EC proliferation (Iba *et al.*, 1991; Li and Sumpio, 2005) and migration (von Offenberg Sweeney *et al*., 2005), whilst reducing EC apoptosis (Haga *et al*., 2003; Liu *et al*., 2003). Studies by von Offenberg Sweeney *et al*. for example have linked cyclic strain to endothelial migration and angiogenesis. Their studies showed a two-fold increase in migration and tube formation in response to chronic equibiaxial cyclic strain (5%, 24 h) of endothelial cells, events which were Giα subunit-dependent and protein tyrosine kinase-independent (von Offenberg Sweeney

et al., 2005). Moreover von Offenberg Sweeney and others have demonstrated that cyclic strain of endothelial cells up-regulates MMP-2 and MMP-9 expression, activity and secretion, thereby implicating a role for these enzymes in strain-dependent vascular remodelling (von Offenberg Sweeney *et al.*, 2004; von Offenberg Sweeney *et al.*, 2005). In response to physiological levels of cyclic strain, apoptosis is suppressed in vascular endothelial cells by the activation of Akt (Chen *et al.*, 2001). This anti-apoptotic effect was dependent on the activation of PI3-Kinase (PI3K) leading to the phosphorylation of Bad (Bayer *et al.*, 1999). By contrast, higher "pathological" levels of cyclic strain (15-20%) induced apoptosis in endothelial cells (and smooth muscle cells), a process which involved an increase in integrin expression and activity (Wernig *et al.*, 2003), as well as TNF-α clustering (Sotoudeh *et al.*, 2002). This in turn lead to p38 and JNK phosphorylation, with subsequent p53 activation and ultimately the up-regulation of pro-apoptotic Bax (Mayr *et al.*, 2002). Elevated cyclic strain also induces production of reactive oxygen species (ROS) resulting in direct oxidative damage on the endothelium (Mayr *et al.*, 2002).

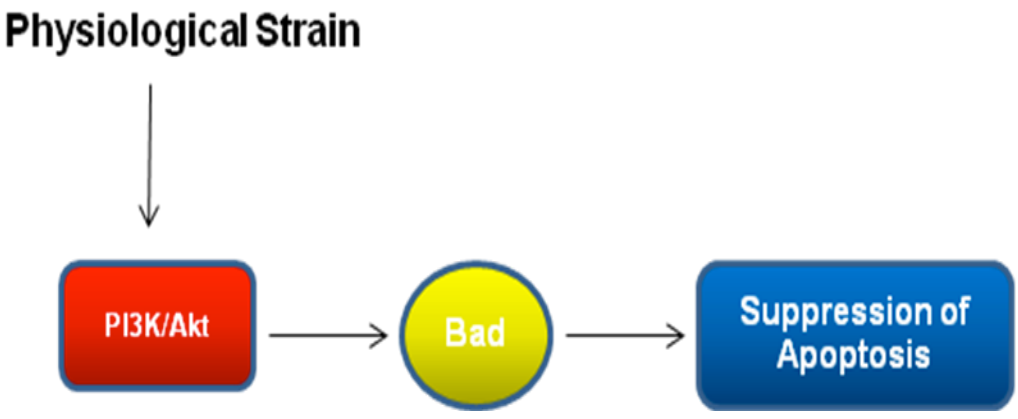


Fig. 1.7: Cyclic strain suppresses apoptosis in endothelial cells. Physiological levels of cyclic strain (5-10%). PI3K/AKT activation leads to Bad phosphorylation suppressing apoptosis.

Interestingly, literature indicates a large diversity in the effects of cyclic strain applied directly to SMCs. Differences in species, vascular bed, and extracellular matrix type all contribute to variable observations (Kakisis *et al*., 2004). Exposure of rabbit aortic SMCs to cyclic strain showed an increase in proliferation (Birukov *et al*., 1995), whilst both inhibition and enhancement of proliferation has been reported in bovine aortic smooth muscle cells (Dethlefesen *et al*., 1996; Mills *et al*., 1997). PDGF plays a vital role in stretch-induced proliferation, usually with increased levels of both PDGF-A and PDGF-B mRNA observed in SMCs (Wilson *et al*., 1993; Li *et al*., 1997). The extracellular matrix (ECM) also plays a role in stretch-induced proliferation. Exposure of canine coronary SMCs grown on elastin to cyclic strain resulted in reduced proliferation, while no change in proliferation was observed with cells grown on collagen (Spofford *et al*., 2003). Smooth muscle cells exposed to cyclic strain underwent enhanced apoptosis via a p53 dependent pathway (Mayr *et al.,* 2002). Moreover cyclic strain has also been implicated in a paracrine regulatory axis between endothelial cells and smooth muscle cells. Recent studies by von Offenberg Sweeney *et al.* have shown that cyclic strain-induced endothelial MMP-2 significantly reduced smooth muscle cell migration. Moreover, the effects of MMP-2 appeared to be mediated through a putative interaction with an EC signalling system (von Offenberg Sweeney *et al*., 2004).

These studies clearly demonstrate the importance of cyclic strain in regulating vascular cell function by inducing changes in gene transcription and effector activation, thereby leading to changes in cell fate. Cyclic strain also functions by increasing endothelial sensitivity to shear stress resulting in a lowered threshold level of shear required to provoke regulatory effectors. It should be noted that both cyclic

strain and shear stress "jointly" impact endothelial homeostasis within the vessel wall (Zhao *et al.,* 1995), although most studies choose to investigate them as "independent variables".

1.3.2.2 Shear Stress

The endothelium is exposed to shear stress due to blood flow dragging against the vessel wall. Shear rate within a vessel can be described by the equation: $\tau = 4\mu Q/\pi r^3$ where τ is shear stress (dynes/cm^2), μ is blood viscosity (dynes sec/cm^2), Q is flow rate (mL/sec), and r is the vessel radius (cm). The term r is raised to the third power. Thus, where Q is constant, a small change in r will result in a large change in τ. Shear stress acts directly on the endothelium and under physiological conditions, the mean shear stress remains constant at 10-15 dynes/cm^2, promoting an anti–inflammatory, anti-thrombotic, anti-coagulative and pro-fibrinolytic state. The atheroprotective impact of shear is also manifested through anti-apoptotic effects on endothelial cells.

At biomechanically sensitive regions of the vasculature, such as branch points or bifurcations, the steady laminar flow of blood is disrupted, leading to turbulent flow patterns. The central wall of the bifurcation is subjected to high shear stress while the lateral wall experiences re-circulation vortexes resulting in flow reversal and shear stress attenuation (Lehoux *et al*., 2003) (Fig. 1.3). Whilst steady laminar shear promotes anti-thrombotic effects, endothelial cell survival and NO production, all conducive with atheroprotection, shear attenuation and turbulence reverse these effects, resulting in pro-inflammatory events and endothelial dysfunction (Traub and Berk, 1998).

Laminar shear stress of endothelial cells leads to reorganization of actin-containing stress fibres and gross morphological realignment in the direction of the flow vector, both occuring in tandem with changes in gene and protein expression. Shear stress regulates expression of various endothelial proteins including growth factors (TGF-β and PDGF) (Negishi *et al.,* 2001; Resnick *et al*., 1993), adhesion molecules (ICAM-1 and VCAM-1) (Chiu *et al*., 2003), vasodilators (NO), vasoconstrictors (ET-1) (Kuchan *et al*., 1993) and coagulation factors (Lin *et al*., 1997).

Endothelial cell response to shear stress is differentially mediated through activation of G-proteins, ion channels, integrins, and receptor tyrosine kinases. These mechanosensors, which are discussed in detail in the next section, activate second messenger systems to impact target gene expression and cell function. MAPKs (extracellular signal regulated kinase ERK1/2, for example) are serine/threoine kinase second messengers activated in response to mechanical stimuli in endothelial cells. ERK1/2 plays a major role in endothelial mechanotransduction as it can target various substrates including other protein kinases (Raf and Mek-1), transcription factors (c-myc, c-jun and c-fos) and enzymes (cPLA2) (Berk *et al*., 1995). Another shear-sensitive member of the MAPK family is JNK, which is reported to have an apoptotic effect on endothelial cells. Unsurprisingly, studies have shown that shear stress blocks JNK activity (Surapisitchat *et al*., 2001).

Shear stress of endothelial cells invariably leads to activation of eNOS and NO production, key players in the atheroprotective effects of shear on the endothelium. Moreover, under laminar flow conditions, endothelial ROS production (e.g. free radicals, non-radicals, and hydrogen peroxide) is balanced by cellular antioxidant

generation, preventing oxidant damage to the cell. As with NO, ROS are also important modulators of signalling cascades, gene transcription, cell growth and apoptosis, although the precise mechanisms are still unknown (Chen and Keaney, 2004; Ulrich and Bachschmid, 2000).

In conclusion, hemodynamic forces such as cyclic strain and shear stress can induce qualitative and quantitative changes in vascular cell gene expression and function via complex signalling pathways, with important consequences for vessel remodelling and overall health. The ability of vascular cells (and particularly endothelial cells) to detect and respond to changes in their hemodynamic environment is therefore of relevance to vascular pathology and involves a process referred to as "mechanotransduction". This will be discussed in greater detail in the next section.

1.4 Endothelial Mechanotransduction

Vascular endothelial cells and smooth muscle cells employ various mechanisms to detect mechanical stimuli such as cyclic strain and shear stress and convert then into chemical signals via mechanoreceptors. These include G-proteins, integrins, ion channels, and receptor tyrosine kinases (Papadaki and Eskin, 1997). These mechanisms can illicit signalling cascades, ultimately leading to transcription factor regulation and associated consequences for gene expression and cellular functions (Kakisis *et al*., 2004). A fuller understanding of endothelial mechanosensitivity and hemodynamic regulation requires an appreciation of these signalling mechanisms.

1.4.1 Heterotrimeric G-proteins

Heterotermic G-proteins comprise of 3 subunits - α, β, and γ. Several isoforms of each subunit exist resulting in dozens of possible trimer combinations. The specific combination of subunits affects not only the receptor it binds but also which downstream target is affected. They provide a means to transduce various extracellular stimuli into specific cellular responses. In this way, they play a vital role in endothelial-mediated regulatory processes (Redmond *et al.,* 1998). This diversity of G-protein subunit combinations helps to transduce and integrate a multitude of external signals. G-proteins are defined based on the sequence and function of their α subunits and fall into four main categories; Gαs, Gαq, Gαi and Gα12. G-proteins are typically (but not exclusively) associated with a GPCR, which is located at the cell surface. Upon activation by mechanical or humoral factors the GPCR may undergo a conformational change resulting in activation of the bound G-protein. This promotes the exchange of bound GDP (guanine diphosphate) for GTP (guanine triphosphate) on the α subunit, subsequently causing the bound heterotrimeric molecule (inactive) to be released from the receptor and to dissociate into an active α monomer and βγ dimer. Both the α and βγ subunits can interact with distinct downstream effectors such as adenylyl cyclase, phosphodiesterases, phospholipase C, Src, and ion channels. These effectors in-turn regulate the intracellular concentrations of secondary messengers such as cAMP, cGMP, IP3, DAG, arachidonic acid, sodium, potassium, and calcium ions, ultimately culminating in a physiological response (e.g. regulation of gene transcription). The α subunit "self-deactivates" via an intrinsic GTPase activity and re-associates with the βγ subunit to repeat the G-protein cycle. This is illustrated in Fig. 1.7 (Simon *et al.,* 1991; Morris *et al*., 1999).

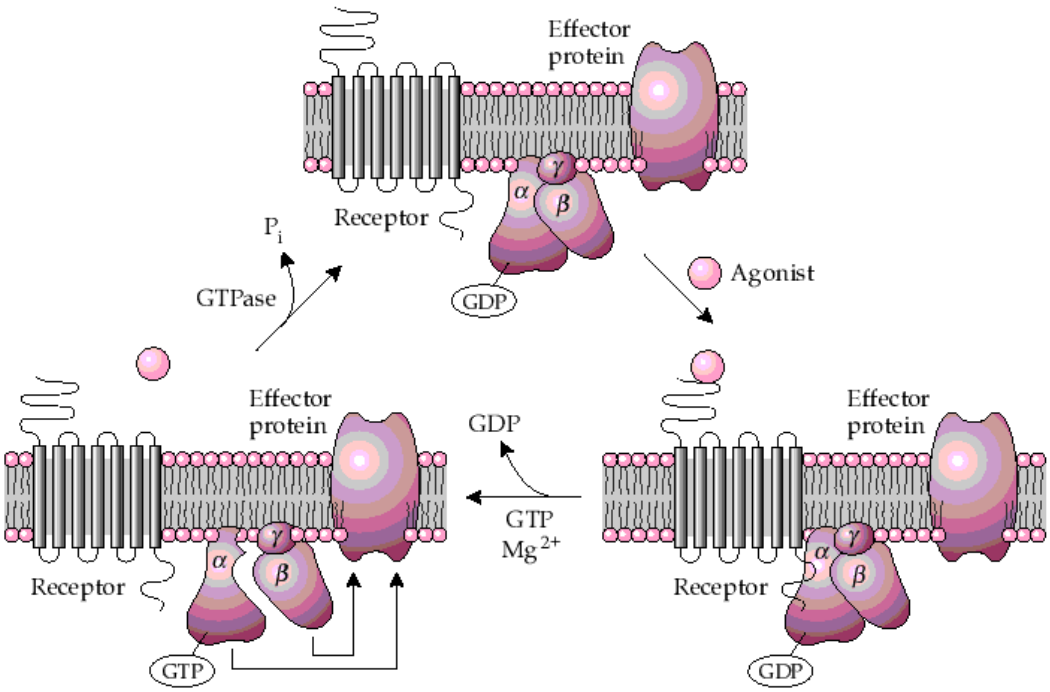


Fig. 1.8: Mechanism of G-Protein Action. Following cell stimulation (i.e. Agonist), the activated GPCR catalyses GDP/GTP exchange at the α subunit, promoting dissociation of the inter-generic complex. Both α and βγ subunits subsequently interact with downstream effectors. Following intrinsic hydrolysis of GTP by the α subunit, the α and βγ subunits re-associate into functional heterotrimer.

Studies have reported shear stress-dependent G-protein activation by showing that antisense Gαq oligonucleotides could block shear-induced ras-GTPase activity (Gudi *et al*., 2003). Similarly, pertussis toxin (a Giα subunit inhibitor) has been shown to prevent shear stress activation of ERK1/2 (Jo *et al*., 1997). G-protein activation was also detected in response to cyclic strain, a process dependent on both force magnitude and strain rate, confirming the role of G-proteins in mechanotransduction (Clark *et al*., 2002). Moreover, work conducted in this laboratory has shown that cyclic strain can regulate mRNA expression and enzymatic function of the zinc metallopeptidases, thimet oligopeptidase and neurolysin, in BAECs via alternate Gi protein signalling pathways (Cotter *et al*., 2004).

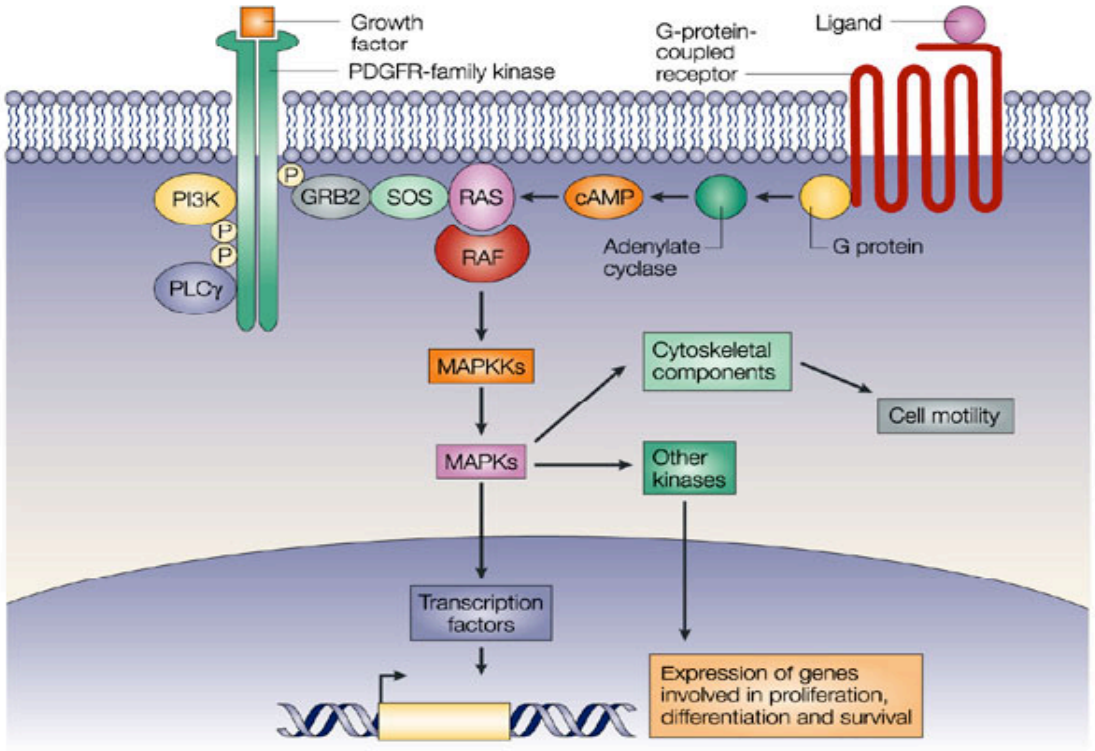


Fig. 1.9: G-protein signalling cascade. External Stimuli such as mechanical or humoral can activate G-proteins, which ultimately results in the phosphorylation of MAPKs, which phosphorylate and regulate the activities of substrates such as transcription factors, cytoskeletal components and other kinases, which in turn regulate cell survival, proliferation, differentiation and motility. In addition to stimulating MAPK signalling, platelet-derived growth factor receptor (PDGFR)-family kinases also activate other signalling pathways — such as those involving phosphatidylinositol 3-kinase (PI3K) and phospholipase-Cγ (PLCγ) — which also lead to changes such as increased proliferation and survival (not shown). (Dibb et al., 2004).

1.4.1.1 Small G proteins (GTPases)

Distinct from heterotrimeric G-proteins, the small G-protein superfamily contains over 150 members. They serve as signal transducers regulating a diverse range of cellular functions (Heo and Meyer, 2003). They are monomeric guanine nucleotide proteins with molecular masses of 20-25 kDa. Structurally and functionally, they can be classified into 5 sub-families, namely; (i) Ras (Ras, Rap and Ral), (ii) Rho (Rho, Rac and cdc4), (iii) ARF (Arf1-Arf6, Arl1-Arl7 and Sar), (iv) Rab (>60 members eg.

Rab5) and (v) Ran. In general, the Ras GTPase family controls cell fate and differentiation, the Rho GTPase family regulates actin cytoskeletal dynamics and cell migration, the Rab GTPase family controls vesicular trafficking and the Ran GTPase family controls microtubule organisation and nuclear transport (Lundquist *et al*., 2006). They function as molecular switches in a manner dependent on their guanine nucleotide bound forms by cycling between a GDP-bound "inactive" state and GTP-bound "active" state, with GDP/GTP exchange mediated by guanine nucleotide exchange factors (GEFs). Their activity is time-limited by GTPase activating proteins (GAP), proteins which accelerate the intrinsic GTPase activity and deactivation. Active GTP-bound GTPases subsequently interact with effectors that mediate downstream cellular responses (Kono *et al*., 2008), as illustrated in Fig. 1.8.

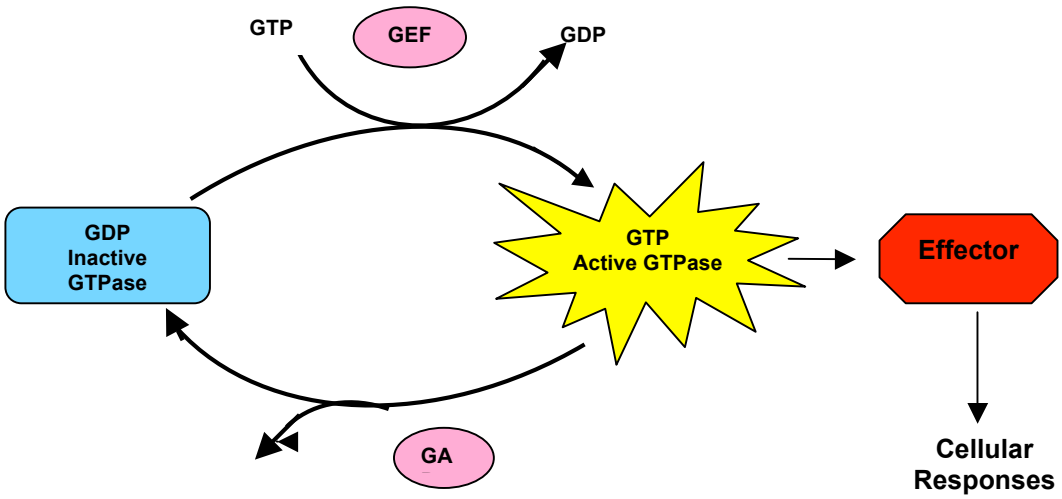


Fig. 1.10: GTPase Cycle. In resting cells, GTPases exist in the GDP-bound inactive form. Following cell stimulation, guanine nucleotide exchange factors catalyze the exchange of GDP with GTP. The activated GTPase then interacts with effectors to elicit downstream cellular responses. GTPase activating proteins (GAPs) stimulate the intrinsic activity of GTPases and convert GTP-bound active form back to inactive GDP-GTPases.

Shear stress induces a rapid activation of Ras GTPase which in turn regulates ERK1/2 and JNK (Tseng *et al*., 1995; Li *et al*., 1996). Activation of Rho, Rac and Cdc42 has also been observed in endothelial cells in response to shear stress (Wojciak-Stothard and Ridley, 2003). Furthermore, Rac1 GTPase activation in response to shear stress has been shown to contribute to reactive oxygen species (ROS) production in endothelial cells (Tzima *et al*., 2002; Yeh *et al*., 1999). As we can see small GTPases serve as mechanotransducers which modulate downstream signalling pathways regulating diverse and cellular events such as proliferation, apoptosis, migration and cytoskeleton rearrangement, however many other GTPase functions are still being elucidated.

1.4.2 Integrins

Integrins exist as αβ heterodimers that connect the cellular cytoskeleton to the ECM, forming a signalling interface as shown in Fig. 1.9 (Ernstrom *et al*., 2002). Each combination of subunits has its own binding specificity and signalling properties. As multiple forms of each subunit exist, subunit combinations can give rise to at least 24 different integrins, 16 of which are associated with the vasculature (Lehoux *et al*., 2003). Each subunit has a large extracellular domain, a transmembrane spanning region and a short cytoplasmic domain. The extracellular domain binds directly to extracellular matrix proteins such as fibronectin, laminin, collagen, vitronectin, fibrinogen and osteopontin. The cytoplasmic domain associates with intracellular signalling molecules and cytoskeletal proteins to regulate cellular events involving signal transduction and cytoskeletal organisation (Davies, 1995; Wilson *et al*., 1995).

Integrin signalling is crucial in development, maintenance and function of the vascular system. Integrins have the ability to act as a bridge between the ECM and the cellular cytoskeleton, enabling them to transmit mechanical and biochemical signals in either direction (inside-out and outside-in signalling). Integrin signalling may involve changes in integrin affinity and/or avidity, the former referring to changes in integrin conformation and the latter referring to changes in integrin clustering at focal adhesion sites (Giancotti and Ruoslahti, 1999; Schoenwaelder and Burridge, 1999). This can subsequently result in the activation of signalling cascades to regulate biological processes such as cell migration, growth, adhesion, inflammation and differentiation (Fig. 1.9).

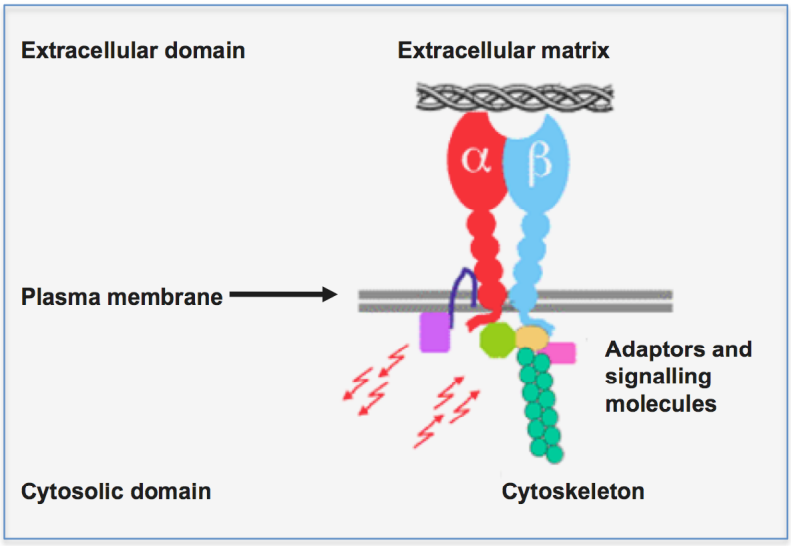


Fig. 1.11: Integrin structure. Integrin αβ heterodimer linked to the actin cytoskeleton via adaptor and ECM proteins.

Integrins play an important role in the mechanosensor/mechanotransduction process in vascular endothelial cells (and SMCs). Both shear stress and cyclic strain are known to activate integrins, increasing their affinity for extracellular proteins. Both subunits interact with intracellular signalling molecules and cytoskeletal proteins

to regulate cellular responses (Shin *et al*., 2003). These responses vary depending on which integrin is activated. For example, cyclic strain of vascular smooth muscle cells grown on vitronectin and fibronectin results in increased proliferation, which can be inhibited by either anti-β5 or anti-αvβ3 antibodies. However, SMCs grown on elastin or laminin do not proliferate under the same conditions (Wilson *et al*., 1995; Reusch *et al*., 1996). In endothelial cells, the work of Chen *et al*. has elegantly demonstrated the shear-dependent activation of integrins leading to their association with the adaptor protein, Shc (Chen *et al*., 1999). Increased binding of WOW-1, an antibody specific for activated αvβ3, has also been shown under shear in endothelial cells (Tzima *et al*., 2001). Shear induced activation of integrins leads to increased activation of Fak, c-Src (a non-receptor tyrosine kinase) and adaptor molecules such as Shc (Fig. 1.10). These mediate the activation of ERK1/2, a member of the MAPK kinase family (Lehoux *et al*., 2003, Chen *et al*., 1999), as well as transcription factors such as NF-κB and AP-1.

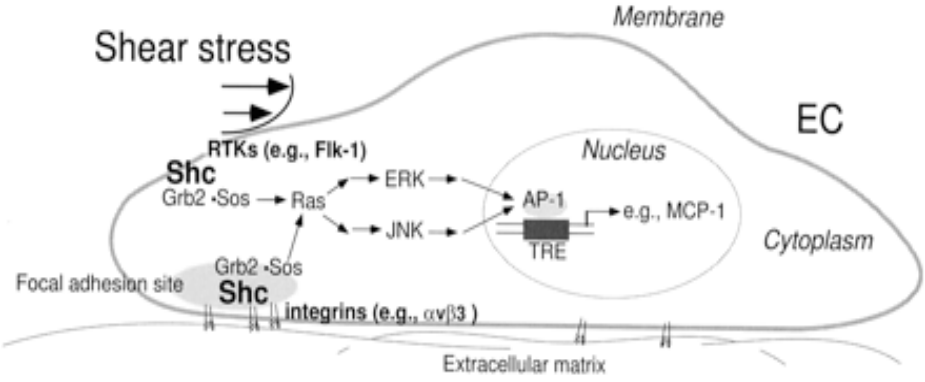


Fig.1.12: Integrin/RTK-dependent mechanotransduction in ECs in response to shear stress. Integrins (αvβ3) and receptor tyrosine kinases (RTKs, e.g. Flk-1) in ECs convert mechanical stimuli such as shear into chemical signals by associating with Shc. Ras activation by the Shc·Grb2·Sos complex activates ERK and JNK pathways leading to transcriptional activation of AP-1/TRE-mediated gene expression (Chen *et al.*, 1999).

Importantly, blockade of integrins using neutralizing antibodies or RGD blocking peptides abrogates these shear-dependent effects (Bhullar *et al*., 1998). Selective blockade of integrin αvβ3 prevented shear stress activation of ERK, JNK, and NF-κB, while RGD peptide abolished the anti-apoptotic effect of shear stress (Katsumi *et al*., 2004). Finally, with respect to cyclic strain, work in our laboratory has demonstrated that integrin blockade with cRGD peptide reduced strain-induced endothelial tube formation (von Offenberg Sweeney *et al*., 2005).

1.4.3 Protein tyrosine Kinases (PTKs)

Tyrosine phosphorylation is an important mechanism in signal transduction with PTKs playing key roles in various cellular responses (e.g. proliferation, migration, differentiation, and survival). There are two distinct categories of PTKs: RTKs (59 members) and non-receptor PTKs (32 members).

1.4.3.1 Receptor Tyrosine Kinases (RTKs)

Members of the RTK family include insulin receptor (IR), epidermal growth factor receptor (EGFR), and PDGF. RTKs consist of an extracellular ligand binding domain, a single transmembrane helix, a cytoplasmic domain containing the catalytic core and additional regulatory sequences. The transmembrane domain anchors the receptor in the plasma membrane, while the extracellular domains bind growth factors. Upon stimulation by humoral or mechanical factors, RTK dimerization occurs. This causes catalytic tyrosine kinase domain activation leading to

autophosphorylation. This in turn leads to the stabilization of the active conformation and the creation of phosphotyrosine docking sites for other intracellular signal transduction proteins (Fig. 1.11). These may include GTPase RAS, PI3-kinase, phospholipase C and adaptor proteins such as Shc and Grb2 (Lemmon *et al*., 1994). Numerous studies have shown an increase in RTK activities in endothelial cells which have been exposed to mechanical stimuli such as cyclic strain and shear stress (Sadoshima *et al*., 1997; Ishida *et al*., 1996). RTKs also play key roles in shear stress-mediated regulation of endothelial cell shape and stress fibre formation (Chien *et al*., 1998).

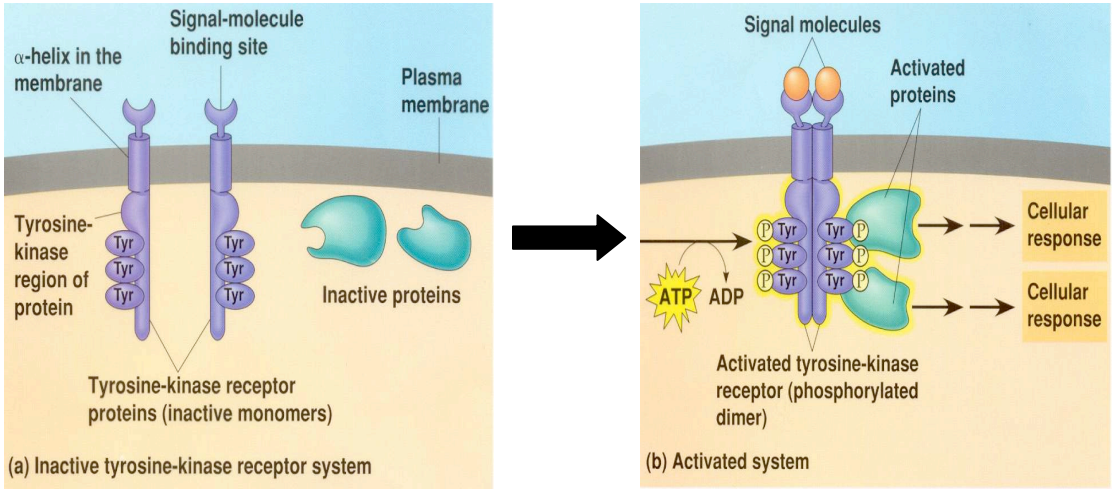


Fig. 1.13: Receptor tyrosine kinase activation. RTKs become activated by ligand binding (or mechanical factors) causing a conformational change in the receptor and dimerization/autophosphorylation of a tyrosine(s) on the cytosolic domain of the receptor. This results in the recruitment of other signalling proteins involved in the signalling cascade [http://www.uic.edu].

1.4.3.2 Non-Receptor PTKs

Non-receptors PTKs unlike RTKs are located in the cytoplasm, nucleus, or anchored to the plasma membrane. They consist of 8 families: Src, JAK, ABL, FAK, FPS, Csk, Syk and Btk. Each family contains several structurally diverse members

involved in mediating different aspects of cell function (e.g. Src/cell proliferation, FPS/differentiation, ABL/growth inhibition, and FAK/cell adhesion) (Schesslinger, 2000). Shear stress has been shown to rapidly activate FAK and Src in endothelial cells (Schaller and Parsons, 1994).

1.4.4 Ion Channels

Ion channel activation is one of the most rapid responses to hemodynamic challenge, occurring within seconds or minutes of stimulation. These include shear stress-induced K^+ channels and stretch-activated cationic channels (Baraket *et al*., 2006). K^+ channel activation leads to cell membrane hyperpolarisation and can be activated by shear rates as low as 0.1 dynes/cm². Blockade of this channel in endothelial cells by either barium chloride or tetraethylammonium results in the attenuation of shear-dependent increases in NO, eNOS and TGF-β (Oleson *et al*., 1988). Cl^- channels are activated independently of K^+ channels and induce cell membrane depolarisation following the initial hyperpolarisation by K^+. This suggests that K^+ channels achieve maximal activation before the Cl^- channels. Ion channel activation also varies in response to shear stress magnitude. For example, high shear stress results in Ca^{2+} influx and cytoskeletal/morphological reorganisation whilst low shear stress does not elicit any of these responses in endothelial cells (Barakat *et al*., 2006). Studies by Ohno *et al*. have also shown that shear induction of TGF-β could be attenuated by K^+ channel blockade, implying a role for the latter in flow-stimulated gene expression in endothelial cells. They also demonstrated that shear stress elevation of cGMP is mediated by K^+ channels (Ohno *et al*., 1995). Interestingly, this shear-sensitive K^+ channel appears to be endothelial cell specific, as it was not found

in vascular smooth muscle cells (Papadaki and Eskin, 1997). From these reports it is clear that ion channels serve as mechanosensors, however, the force-dependent activation process itself remains unclear.

1.4.5 Endothelial Cell Receptors

Cell surface receptors have also been shown to play an important role in the mechanotransduction process in vascular endothelial cells. In direct response to shear stress, PECAM1 has been implicated in activation of a Src family kinase. VE-cadherin is also required in this pathway by associating with VEGFR1. Once this occurs, active Src kinase transactivates VEGFR1 leading to PI3K activation resulting in downstream signalling such as integrin activation and Akt-dependent phosphorylation of eNOS. This model of integrin activation by PI3K could explain why integrin antibodies such as anti-$\alpha_v\beta_3$ (Bhullar *et al.*, 1998) inhibit responses to flow (Hahn and Schwarz, 2009). Previous studies by Chen *et al.* have also demonstrated that shear stress (12 dynes/cm²) induced a rapid and transient tyrosine phosphorylation of Flk-1 and its concomitant association with the adaptor protein Shc (Chen *et al.*, 1999).

1.5 Endothelial Cell-Smooth Muscle Cell Interactions

Vascular endothelial and smooth muscle cells are the major cellular components of the vessel wall and as such both direct and indirect EC-SMC interactions play an important role in maintaining normal vessel homeostasis. Evidence has demonstrated that cell-cell interactions can regulate cell functions with respect to growth, migration

and differentiation, as well as affect the expression of regulatory molecules such as fibrinolytic factors, coagulation factors and angiogenic factors (Yoshida *et al*., 1996; Helenius *et al*., 2004). In this regard, *in vivo* studies have shown direct physical contact between endothelial cells and smooth muscle cells via "myoendothelial bridges" (Spagnoli *et al*., 1982; Little *et al*., 1995).

In vitro EC-SMC "static" co-culture models (i.e. non-contact indirect interaction models) are relatively commonplace in literature. By contrast, EC-SMC "hemodynamic" co-culture models, which specifically explore the impact of endothelial hemodynamic challenge (e.g. shear) on SMC remodelling, are relatively few. EC-SMC co-culture has previously been demonstrated to enhance SMC adhesion and spreading, a mechanism shown to be dependent on β1-integrin and the activation of P13K/Akt (Wang *et al*., 2007). EC-SMC co-culture also induced SMC differentiation, again mediated by the PI3K/Akt pathway (Brown *et al*., 2005). In a recent hemodynamic study model, EC-SMC co-culture induced ICAM-1, VCAM–1, and E-selectin gene expression in endothelial cells, an effect which could be inhibited by endothelial shear stress (Chiu *et al*., 2003). Moreover, endothelial shear decreased SMC migration and MMP-2 activity in an EC-SMC co-culture system apparently consistent with the atheroprotective effect of shear stress (Sakamota *et al*., 2006). Consistent with this latter paper, a related study by von Offenberg Sweeney *et al.* demonstrated that cyclic strain of BAECs could significantly reduce BASMC migration in a non-co-culture model. This effect was found to be mediated by strain-induced endothelial MMP-2. Moreover, MMP-2 appeared to act indirectly in these events through a putative interaction with a BAEC signalling system (von Offenberg Sweeney *et al.*, 2004b).

These studies clearly indicate that bi-directional cross-talk (with consequences for cellular functions) exists between endothelial cells and smooth muscle cells, which in-turn may be regulated by the magnitude and nature of hemodynamic challenge to the endothelium. *This phenomenon is undoubtedly of vital importance to endothelial-dependent remodelling and vessel health and will form the basis for the experimental objectives of this thesis*. In view of the regulatory complexities involved, the following sub-sections will take a closer look at the vascular signaling mechanisms central to the regulation of cell functions such as proliferation, migration etc.

1.5.1 Proliferation

The cell cycle consists of sequential events that occur in cell replication. It comprises of four distinct phases; G_1 phase, S phase, G_2 phase and M phase. G_1-S-G_2 phases are collectively known as interphase. In addition, cells that have temporarily or reversibly ceased dividing are in a quiescent state (the G_0 phase). During G_1 phase, the cell grows and becomes larger and the various factors needed to replicate DNA are assembled. The S phase involves DNA synthesis, after which the cell enters the G_2 phase where it continues to grow and produce new proteins. The M phase (mitosis) involves cell division after which the cells return to the G_1 phase, the cell cycle completed. Cells in the G_1 phase do not always complete the cycle and may enter a quiescent or G_0 state. Regulation of the cell cycle is crucial to prevent any uncontrolled replication and involves detecting and repairing any genetic damage. Cell cycle check points at the G_1/S phase and G_2/M phase ensure an orderly cell cycle progression. G_1/S progression is a rate-limiting step in the cell cycle and is also

known as a restriction point (Vermeulen *et al.,* 2003). The cell cycle is illustrated in Fig. 1.12.

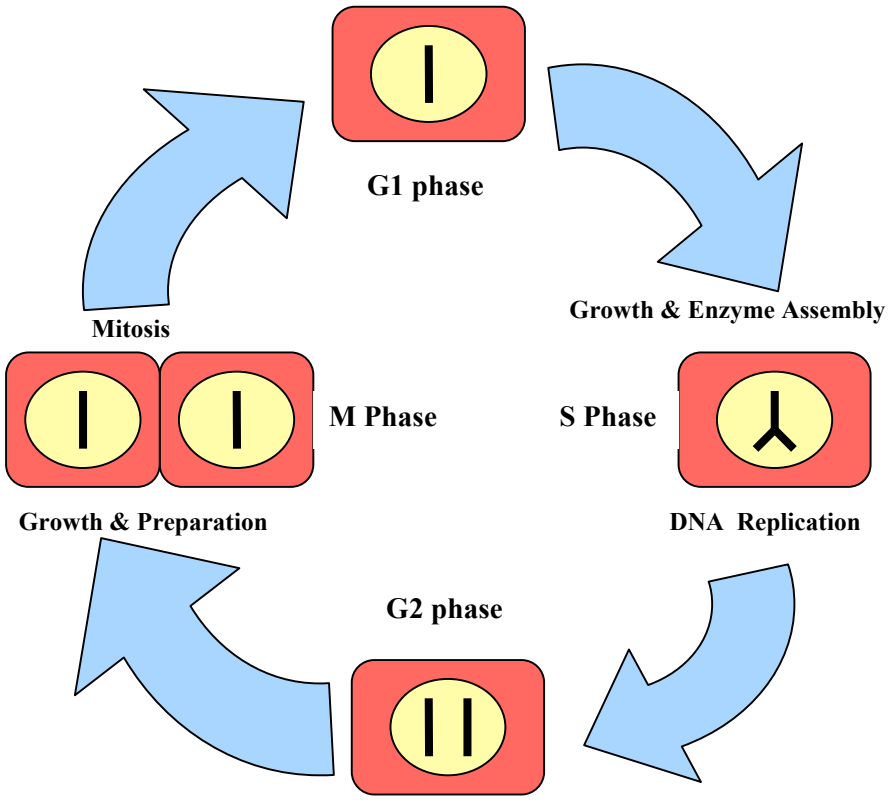


Fig. 1.14: The Cell Cycle. The first phase of the cell cycle (G_1) involves cell growth and preparation for DNA replication. It then enters the S phase where DNA synthesis occurs (chromosomes formed). During G_2 phase, cell growth continues in preparation for cell division. The cell finally enters the M Phase where mitosis occurs (chromosome are separated and divided into daughter cells). The cell returns to the G_1 phase and cell cycle completion.

Regulation of the cell cycle progression relies on two classes of molecules; cyclin dependent kinases (CDKs) and cyclins. Cyclins serve as regulatory subunits, whilst CDKs function as catalytic subunits. Once bound together, they become activated and phosphorylate a variety of (largely unknown) target proteins, thereby coordinating entry into the successive phases of the cell cycle (Table 1.1). Whilst cyclin levels change with the different phases of the cell cycle, levels of CDKs tend to remain stable (Koledova and Khalil, 2006). In response to extracellular signals such as

growth factors, cyclin D is typically the first cyclin to be activated, with low levels produced during early G_1 phase and increasing towards G_1/S phase. It binds to CDK4 and CDK6 forming active complexes which are involved in early G_1 phase, as well as phosphorylating retinoblastoma susceptibility protein (Rb). This latter protein controls the expression of molecules needed to progress from G_1 to S phase by binding to E2F transcription factors and blocking transcription. Rb phosphorylation causes it to dissociate from the E2F/DP1/Rb complex, thereby activating E2F, which in turn activates transcription of genes such as thymidine kinase, DNA polymerase, cyclin A and cyclin E (all required for entry into the S phase). CDK2 is sequentially activated by cyclin E (E1 and E2), forming an active complex pushing the G_1/S transition and is rapidly deactivated once the cell enters S phase. Within the S phase, cyclin A levels increase and activate CDK2 forming a cyclin A-CDK2 complex to initiate the G_2/M phase transition (possibly to facilitate in DNA replication) (Sherr, 1993). Cell cycle progression through mitosis is regulated by the cyclin B-CDK1 complex. Cyclin B is degraded during anaphase, regulating cellular progression out of mitosis and back to the G_1 phase (King *et al.*, 1994).

CDK	Cyclin	Cell Cycle Phase	Species
CDK1	B	G2/M phase	Vertebrates
CDK1	A	G2/M phase	Vertebrates
CDK2	A	S phase	Vertebrates
CDK2	E	G1/S transition	Vertebrates
CDK4	D	G1 phase	Vertebrates
CDK6	D	G1 phase	Vertebrates

Table 1.1: CDKs and Cyclins. A range of typical CDK/cyclin complexes associated with cell cycle phase transitions in vertebrates.

Cyclin-CDK complexes are regulated by a variety of specific CDK inhibitor proteins that bind to and inactivate CDKs. There are two main groups; *Ink4* family (p15*ink* and p16*ink4*) and the *KIP/Cip* family (p27*Kip*1, p21*Cip*1/*Waf*1 and p75*Kip*2). The *Ink4* family inhibit CDK4/6 activity, whilst the *KIP/Cip* family inhibit the cyclin E-CDK2 complex (Sherr and Roberts, 1999), thus halting the cell cycle in the G_1 phase.

SMC proliferation plays a crucial role in various diseases such as cancer, diabetes and atherosclerosis. In this regard, endothelium-derived components are frequently proposed to regulate SMC proliferation. In *vivo* studies have shown that L-arginine, a metabolic precursor of endothelial NO, prevented intimal thickening after balloon angioplasty (Tarry and Makhoul, 1994). Studies have also shown that NO inhibits G_1/S transition by inhibiting CDK2-mediated Rb phosphorylation possibly via p21 (Ishida *et al*., 1997). NO stimulates generation of cyclic adenosine 3'5'-monophosphate (cAMP) and cyclic guanosine 3'5'- monophosphate (cGMP) in SMCs, second messengers known to mediate changes in cell proliferation. Studies have shown that cAMP suppresses induction of cyclin D/A expression and may cause up-regulation of p27*Kip*1 levels in SMCs. This in turn decreases CDK2/4 activities contributing to cell cycle arrest (Vadiveloo *et al*., 1997; Fukumoto *et al*., 1999; Kronemann *et al*., 1999). cGMP partially delays cell cycle progression by suppressing cyclin D and CDK4 (Koyoma *et al*., 2001). TGF-β and PKC are also known to inhibit cell proliferation by preventing Rb phosphorylation G_1/S phase transition. By contrast potent growth factors such as PDGF and VEGF induce mitogenic signalling pathways such as mitogen-activated protein kinase (MAPK/ERK1/2) pathway and

phosphatidylinositol3-kinase (PI3K) pathway in SMCs, thereby promoting cell proliferation (Bornfeldt and Krebs, 1999).

As we can see the cell cycle is a staggeringly complex process involving specific cyclin-CDK interactions with consequences for downstream CDK substrates and the associated regulation of a variety of cellular processes including transcription, translation, DNA synthesis, and mitosis. Dysregulation of this cycle is therefore a pivotal feature of diseases characterised by unwanted cell proliferation, which include endothelial dysfunction-based diseases such as atherosclerosis and restenosis.

1.5.2 Apoptosis

Apoptosis is a carefully regulated process that occurs in normal development and homeostatic maintenance in all tissues including cells of the vascular wall. It is distinguished from necrosis at the morphological and biochemical level. During apoptosis the cell shrinks and loses contact with neighbouring cells. Its chromatin condenses around the nuclear membrane with subsequent plasma membrane blebbing resulting in the cell being fragmented into apoptotic bodies (membrane enclosed structures). These bodies are then digested by macrophages without causing an inflammatory response. In contrast, necrosis results from damaging external stimuli causing severe swelling and loss of membrane integrity ultimately resulting in disruption of the cell. This allows cellular contents to leak into the extracellular environment and elicit an inflammatory response (Leist and Jaattela, 2001). Apoptosis can be triggered by a number of stimuli such as microbial infection, death receptor activation, ionizing radiation and cellular stress. Cellular stress typically activates an

"intrinsic" pathway, whilst death receptors activate an "extrinsic" signalling pathway. Moreover, both pathways converge to activate the caspase system (Danial and Korsmeyer, 2004).

The extrinsic pathway is activated by death receptors - cell surface receptors belonging to the tumour necrosis family (TNF). They consist of an intracellular domain and a death domain (DD) in their cytoplasmic tail essential for initiating apoptosis. Apoptotic signals are transmitted upon binding to their respective ligands. The most characterised death receptor ligands are TNF receptor-1 (TNF-1) Fas ligand (CD95) and TNF-Related Apoptosis Inducing Ligand (TRAIL), either of which can initiate a caspase cascade within seconds, culminating in apoptosis as shown in Fig. 1.13 (Putcha *et al*., 2002).

Cellular stress (viral infection, oxidative stress, humoral factors, radiation, and toxic chemicals) may initiate the intrinsic pathway through the mitochondrion. Apoptotic proteins cause the mitochondria to undergo morphological change causing swelling (either through membrane pore formation or by increased permeability) allowing apoptotic effectors to leak out. These effectors include cytochrome c, apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspases (SMAC) and other pro-apoptotic molecules (Gupta, 2003). The membrane pore is formed by pro-apoptotic (Bad, Bax, Bid and Bak) and anti-apoptotic (Bcl-2 and Bcl-XL) proteins. Pro-apoptotic proteins act as sensors to cellular damage, relocating to the surface of the mitochondrion where anti-apoptotic proteins are located, disrupting pore formation dynamics and leading to release of other pro-apoptotic molecules

(Adams and Cory, 2001). These factors amplify the apoptotic signal and activate the caspase cascade.

Caspases are a family of cysteine proteases and are central to apoptotic execution of apoptosis. They exist as inactive zymogens within the cell and are activated following induction of apoptosis. They can be divided into two groups; initiator caspases (caspase-2, -8, -9, -10) and effector caspases (caspase-3, -6, -7). Induction of apoptosis results in activation of an initiator caspase causing downstream activation of effector caspases. These then cleave key cellular protein targets leading to biochemical and morphological changes, which are described below;

(i) Inactivation of enzymes involved in DNA repair: Caspase-3 prevents DNA repair by preventing cleavage of poly (ADP-ribose) polymerase or PARP.

(ii) Breakdown of structural nuclear proteins: Caspase-6 causes degradation of laminin, an intra-nuclear protein that maintains nuclear shape and interaction between chromatin and nuclear membrane. Its degradation by caspase-6 results in chromatin condensation and nuclear fragmentation, known characteristics of apoptosis.

(iii) Fragmentation of DNA: Caspase-activated DNase (CAD) causes fragmentation of DNA into nucleosomal units. It is cleaved by caspase-3 releasing it from the inactive complex (ICAD).

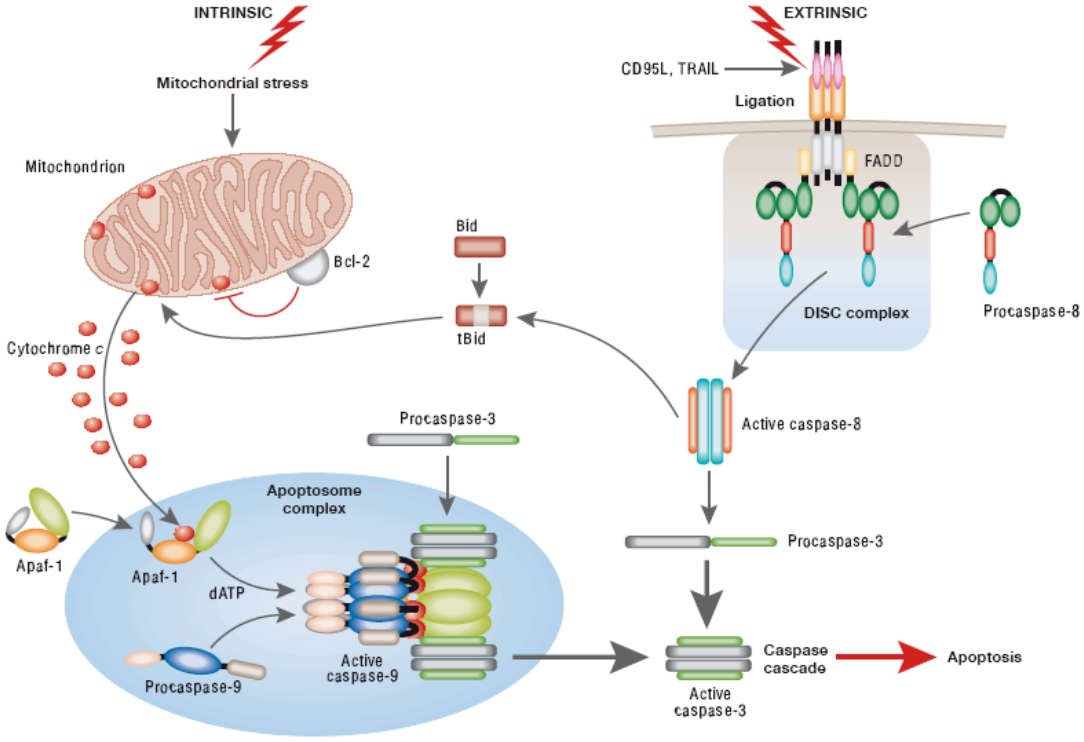


Fig. 1.15: The extrinsic and intrinsic pathways to caspase activation. The extrinsic pathway is activated by triggering of death receptors such as Fas-associated death domain protein (FADD), which form a death-inducing signalling complex (DISC). This recruits and activates the initiator caspase-8. In the intrinsic pathway, cellular stress induces apoptosis by mitochondrial perturbation and the release of proteins such as cytochrome c. The Bcl-2 family regulates this release with anti-apoptotic and pro-apoptotic members. Cytochrome c binds to apoptotic factor1 (Apaf1) causing a formation of the Apaf/caspase-9 apoptosome complex and activates initiator caspase-9. Activated initiator caspases-8/-9 in turn activate the effector caspases-3/-6/-7 which cleave key cellular substrates resulting in the biochemical and morphological changes associated with apoptosis (Mac Farlane and Williams, 2004).

NO inhibits apoptosis by directly inhibiting caspases through nitrosylation (Bennett *et al.*, 1999) and by inactivating the caspase cascade via downstream mediators such as cGMP (Pollman *et al.*, 1996), tumour suppressor protein p53 (Messmer *et al.*, 1994) and Fas (CD95). Interestingly, NO also down-regulates anti-apoptotic Bcl-2 (Xie *et al.*, 1997; Hata *et al.*, 1996) and up-regulates pro-apoptotic Bax, the latter promoting apoptosis via induction of p53 (Ghatan *et al.*, 2000). Within SMCs, studies have shown that cAMP inhibits apoptosis via PKA (Orlov *et al.*, 1999). Other studies suggest that SMC apoptosis may occur via signalling through

death receptors as delivery of exogenous Fas ligand has been shown to induce SMC apoptosis and reduced neointima formation (Sata *at al*., 1998). Importantly, elevated SMC apoptosis is also associated with rupture-prone atherosclerotic plaques. Apoptosis, influenced through both intrinsic and extrinsic pathways to regulate SMC growth, is therefore critical in the regulation of vessel remodelling events and vascular health.

1.5.2 Migration

Cell migration is a feature of numerous physiological processes. It is a dynamic cyclical process where the cell extends a protrusion and adheres to the extracellular matrix (ECM). The generation of the contractile force by the actin-myosin cytoskeleton moves it forward towards the protrusion and attachments at the cell rear release as the cell continues to move forward. These steps involve the continuous rearrangement of cytoskeletal elements and dynamic cell-ECM interactions. The cycle is initiated by external signals which activate a complex signalling cascade involving a variety of different molecules (Gerthoffer *et al*., 2007).

In order for a cell to migrate, it must become polarized by reorganisation of the microtubule cytoskeleton. Establishment of this polarity along with its maintenance is governed by a set of inter-linked positive feedback loops involving integrins, microtubules, phosphoinositide 3-kinases (PI3Ks) and the Rho family of GTPases. One of the first molecules to become polarized is chemotactic agent phosphatidyl-inositol triphosphate (PIP3). Accumulation of PIP3 (by PI3Ks) at the leading edge activates the Rho family GTPases (Rac, Cdc42) resulting in actin polymerization

(Rodriguez *et al.*, 2003). Cdc42 is a key regulator of polarity as cells lose their ability to respond to a chemotactic gradient and migrate randomly. Cdc42 activates downstream targets Par6 and PKC, which localize the microtuble-organizing centre (MTOC) and Golgi apparatus in front of the nucleus, thereby establishing cell polarity (Etienne-Manneville and Hall, 2002; Rodriguez *et al.*, 2003).

The cycle is initiated by external signals (chemotactic stimuli), which are sensed and converted to polarized internal responses in the cell membrane. Small GTPases (RhoA, Rac1 and Cdc42) act together to co-ordinate cell migration. Rac1 acts at the front of migrating cells, stimulating actin-mediated membrane protrusion of the leading cell toward the chemotactic stimuli, or along varying adhesiveness within the ECM. Rac1 and Cdc42 activate SCAR/WAVE and WASP proteins, which in-turn activate the Arp2/3 complex, generating cellular protrusions which contract at the front of the cell and propel the cell towards the stimulus (Raftopoulou and Hall, 2004). Protrusions can take the form of lamellipodia with a branching dendritic network of actin filaments, or filopodia where parallel bundles of filaments are formed. Cdc42 is also involved in the directionality of movement and can contribute to cell speed by enhancing Rac1-mediated membrane protrusion at the front of cells (Nobes and Hall, 1995). RhoA and its effector ROCK act primarily at the rear of cells to induce forward movement of the nucleus and cell body and mediate detachment of the back of the cell from the ECM. ROCK regulates myosin II causing the release of adhesive connections in the rear of the cell and retraction of the tail completes the cycle. Cell migration is regulated by growth factors (PDGF), cytokines, extracellular matrix components, and hemodynamic forces (shear stress and cyclic strain). Cell

migration coordinates a variety of intracellular activities with Rho-GTPases playing key roles (Ridley, 2001).

1.5 Summary

The vascular endothelium is a dynamic cellular interface between the vessel wall and bloodstream. The unique position of the endothelium allows it to regulate vessel tone and remodelling in response to numerous physiological and pathological stimuli. Central among these stimuli are blood flow-associated hemodynamic forces (cyclic strain and shear stress). Endothelial cells detect hemodynamic forces by converting them into chemical signals via mechanoreceptors (e.g. G-proteins, integrins, ion channels, protein tyrosine kinases, caveolae etc.). These in turn initiate signalling cascades leading to the modulation of transcriptional and post-translational mechanims to bring about changes in endothelial cell functions. In this way, blood flow can regulate endothelial cell metabolism and can induce qualitative and quantitative changes in gene expression, ultimately impacting on vessel wall homeostasis (including smooth muscle cell properties).

1.6 Thesis Overview

The data presented in the following chapters examines how hemodynamic challenge of vascular endothelial cells putatively impacts on vascular smooth muscle cell proliferation and apoptosis in different *in vitro* model systems as a paradigm for their regulatory co-interaction *in vivo*. Specifically, it details the role played by laminar shear stress (and to a lesser extent, cyclic strain) in this context, as well as the

possible endothelial signalling pathways and effectors involved in mediating the observed effects. It also investigates the impact of this hemodynamic regulatory paradigm on the expression of cell cycle-associated genes within smooth muscle cells. Our findings are presented in the following manner:

Chapter 3: Vascular Cell Characterisation Studies

Chapter 4: Endothelial Cell Hemodynamic Challenge – Impact on Smooth Muscle Cell Growth

Chapter 5: Endothelial Laminar Shear Stress – Signalling Studies and Smooth Muscle Cell Cycle-Associated Gene Expression Changes

CHAPTER 2

Materials & Methods

2.1 Materials

All reagents used in this study were of the highest purity commercially available and were of cell culture standard when applicable.

Acros Organics (New Jersey, USA)

Formaldehyde (37%)

AGB Scientific (Dublin, Ireland)

Whatmann chromatography paper

Amersham Pharmacia Biotech (Buckinghamshire, UK)

Anti-mouse 2^0 antibody, HRP-conjugated

Anti-rabbit 2^0antibody, HRP-conjugated

ECL Hybond nitrocellulose membrane

ECL hyperfilm

Rainbow molecular weight marker, broad range (6-175kDa)

Bachem UK Ltd. (St. Helens, UK)

Cyclic RGD peptide

Bio-Rad Laboratories (California, USA)

Script™ cDNA synthesis kit

Bio Sciences Ltd (Dun Laoghaire, Ireland)

DEPC-treated water

Trizol® reagent

Calbiochem (Bad Soden, Germany)

Genistein

GM6001

MMP-2 inhibitor (cis-9-Octadecenoyl-N-hydroxylamide)

MMP-9 inhibitor I

NF023

PD98059

Pertussis toxin

TGF-βR1 antagonist

Cayman Chemical Company (Michigan, USA)

Anti-rabbit eNOS polyclonal IgG

Coriell Cell Repository (New Jersey, USA)

Bovine Aortic Endothelial Cells

Bovine Aortic Smooth Muscle Cells

DakoCytomation (Glostrup, Denmark)

Anti-rabbit von Willebrand Factor polyclonal IgG

Fluorescent Mounting Media

Dunn Labortechnik GmBH (Germany)

Bioflex® plates (Pronectin®-coated)

Gibco (Scotland, UK)

UltraPURE™ Distilled Water DNAse-/RNAse-Free

Invitrogen (Groningen, The Netherlands)

Vybrant™ Apoptosis Assay Kit #2

Vybrant™ CFDA-SE cell tracer kit

Molecular Probes (Oregon, USA)

Alexa Fluor® 488 $F(ab')_2$ fragment of goat anti-mouse IgG (H+L)

Alexa Fluor® 488 $F(ab')_2$ fragment of goat anti-rabbit IgG (H+L)

MWG Biotech (Milton Keynes, UK)

Primer sets for; GAPDH, eNOS, MMP-2, CDK1, CDK2, CDK4, CDK5, Cyclin D1, Cyclin A, Cyclin E, and p27*Kip*

(note: primer sequences are shown in text)

Nalgene (New York, Ireland)

Cryogenic vials

Cryo freezing container

PALL Corporation (Dun Laoghaire, Ireland)

Biotrace nitrocellulose membrane

Pierce Chemicals (Cheshire, UK)

BCA protein assay kit

Supersignal West Pico chemilumenescent substrate

Promocell (Heidelberg, Germany)

Human Aortic Endothelial Cells

Human Aortic Smooth Muscle Cells

Endothelial Cell Basal Media

Smooth Muscle Cell Growth Media

basic fibroblast growth factor

Fetal calf serum

Endothelial Cell Growth-Stimulating Factor

Epidermal Growth Factor

Fetal calf serum

Hydrocortisone

Insulin

Trypsin Neutralizing Solution

SABiosciences (Frederick, MD, USA)

RT² First Strand Kit

RT² SYBR Green/ROX qPCR Master Mix

Human Cell Cycle RT² Profiler™ PCR Array

Sarstedt (Drinagh, Wexford, Ireland)

1.5 ml micro tube with safety cap

10, 200, and 1000 μl pipette tips

15 and 50 ml falcon tubes

2, 5, 10, and 25 ml serological pipettes

6-well tissue culture plates

96-well tissue culture plates

T-25 tissue culture flasks

T-75 tissue culture flasks

T-175 tissue culture flasks

P100 tissue culture petri dishes

Scientific Imaging Systems (Eastman Kodak Group, Rochester, NY)

Kodak 1D image analysis software

Sigma Chemical Company (Poole, Dorset, England)

2-propanol

Ammonium persulfate

Agarose

β-mercaptoethanol

Bisacrylamide

Bovine serum albumin

Brightline haemocytomoeter

Bromophenol blue

CHAPSO

Chloroform

Dimethylsulfoxide

Ethylenediaminetetraacetic acid

Ethidium bromide

Fetal calf serum

Gelatin

Hanks balanced salt solution

Hydrochloric acid

Isopropanol

Lauryl sulfate (i.e. sodium dodecyl sulphate (SDS))

Methanol

Mineral oil (molecular grade)

N-Acetyl-Asp-Glu-Val-Asp-pNitroanilide

p-Nitroaniline

Penicillin-streptomycin (100x)

Phosphate buffered saline

Polyacrylamide

Ponceau S solution

Potassium chloride

Potassium phosphate

Potassium phosphate-dibasic trihydrate

Potassium hydroxide

RPMI 1640 tissue culture media

Sodium chloride

Sodium orthovanadate

Sodium phosphate-dibasic anhydrous

Sodium phosphate-monobasic anhydrous

Tetramethylethylenediamine

Triton® X-100

Trizma base

Trypsin-EDTA (10x)

Tween® 20

Qiagen (West Sussex, U.K.)

SYBR Green® PCR Kit

Spectrum Laboratories Inc., (Santa Clara, Ca, US)

Cellmax™ Perfused Transcapillary Co-Culture Capillaries

Zymed Laboratories (CA, USA)

Anti-mouse ZO-1 monoclonal IgG

2.2 Cell Culture Methods

All cell culture procedures were carried out under clean, sterile conditions using a Bio Air 2000 MAC laminar flow unit. Cells were routinely examined using an Olympus CK30 phase-contrast microscope.

2.2.1 Culture of Bovine Aortic Endothelial Cells

Differentiated Bovine Aortic Endothelial Cells (BAECs) were obtained from Coriell Cell Repository, New Jersey, USA. (Catalogue No. AG08500). The cells were derived from a one year old male Hereford cow (thoracic aorta removed immediately post-mortem on 10/22/85). BAECs were cultured in RPMI 1640 media supplemented with 10% fetal calf serum (FCS) and antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin). Cells were cultured in T25 cm^2, T75 cm^2, and T175 cm^2 flasks, as well as 6-well plates, and grown in a humidified atmosphere of 5% CO_2/95% air at 37°C. In the case of cyclic strain experiments, cells were grown on Bioflex® 6-well culture plates, which have a flexible Pronectin®-bonded silicon membrane growth surface. All experiments were carried between cell passage 5 – 13.

2.2.2 Culture of Bovine Aortic Smooth Muscle Cells

Differentiated Bovine Aortic Smooth Muscle Cells (BASMCs) were obtained from Coriell Cell Repository, New Jersey, USA. (Catalogue No. AG08504). The cells were derived from a one year old male Angus breed (thoracic aorta removed immediately post-mortem on 10/22/85). BASMCs were cultured in RPMI 1640 media

supplemented with 10% fetal calf serum (FCS) and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin). Cells were routinely cultured in T25 cm^2, T75 cm^2, and T175 cm^2 flasks, as well as 6-well plates and grown in a humidified atmosphere of 5% CO_2/95% air at 37°C. All experiments were carried out between cell passage 5 – 13.

2.2.3 Trypsinisation of BAECs and BASMCs

BAECs and BASMCs are adherent cell lines and trypsinisation was required for their sub-culture. Growth media was removed from the flasks and the cells were washed in Hank's Balanced Salt Solution (HBSS) to remove α-macroglobulin, a trypsin inhibitor present in FCS. An appropriate volume of trypsin/ethylenediamene tetracetic acid (10% v/v trypsin/EDTA in HBSS) was subsequently added to the cells and incubated for 1 – 2 min at 37°C until the cells were rounded, but not fully detached and the flask then tapped briefly to detach them from the growth surface. Trypsin was inactivated by the addition of complete growth media containing FCS and the cells were removed from suspension by centrifugation at 1,000 g for 5 min. Cells were then counted using a bright-line haemocytometer for incorporation into experiments, sub-cultured at a 1:5 spit ratio, or cryopreserved.

2.2.4 Culture of Human Aortic Endothelial Cells

Differentiated Human Aortic Endothelial Cells (HAECs) were purchased from Promocell (Heidelberg, Germany). The cells were derived from a 22 year old male human. HAECs were cultured in Endothelial Cell Basal Media (Promocell) supplemented with 5% fetal calf serum (FCS), 5% Endothelial Cell Growth-

Stimulating Factor (ECGS), 10 ng/ml Epidermal Growth Factor, 1 µg/ml Hydrocortisone, and antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin). Cells were routinely cultured in T25 cm^2, T75 cm^2, and T175 cm^2 flasks, as well as 6-well plates, and grown in a humidified atmosphere of 5% CO_2/95% air at 37°C. All experiments were carried out on between cell passage 5 – 9.

2.2.5 Culture of Human Aortic Smooth Muscle Cells

Differentiated Human Aortic Smooth Muscle Cells (HASMCs) were purchased from Promocell, Heidelberg, Germany. The cells were derived from a 22 year old male human. HASMCs were cultured in Smooth Muscle Cell Growth Media (Promocell) supplemented with 5% fetal calf serum (FCS), 0.5 ng/ml Epidermal Growth Factor, 2 ng/ml basic fibroblast growth factor 5 µg/ml insulin, and antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin). Cells were cultured in T25 cm^2, T75 cm^2, and T175 cm^2 flasks, as well as 6-well plates, and grown in a humidified atmosphere of 5% CO_2/95% air at 37°C. All experiments were carried out between cell passage 5 – 7.

2.2.6 Trypsinisation of HAECs and HASMCs

HAECs and HASMCs are adherent cell lines and trypsinisation was required for their sub-culture. Growth media was removed from the flasks and the cells were washed in HBSS. An appropriate volume of 10% v/v trypsin/EDTA (in HBSS) was added to the cells and incubated for 2 – 5 min at room temperature until the cells were rounded, but not fully detached, and tapped briefly to detach them from the growth

surface. Trypsin Neutralizing Solution (Promocell) was added and the cells were removed from suspension by centrifugation at 1,000 g for 5 min. Cells were then counted using a bright line haemocytometer for incorporation into experiments, sub-cultured at a 1:3 split ratio, or cryopreserved.

2.2.7 Cryogenic Preservation and Recovery of Cells

For long-term storage, BAECs, BASMCs, HAECs and HASMCs were stored in liquid nitrogen in a cryofreezer unit (Thermoylen Locator Jr. Cryostorage System). Cells to be stored were centrifuged after trypsinisation and the supernatant was removed. The resultant pellet was resuspended in 1 ml of freezing media as follows: BAECs and BASMCs – RPMI 1640 supplemented with 20% v/v FCS, 10% dimethylsulphoxide (DMSO), and antibiotics. HAECs and HASMCs – Growth media containing 5% FCS, 10% DMSO and antibiotics. The cell suspension was transferred to sterile cryogenic vials and frozen in a -80^0C freezer at a rate of -1^0C/minute in a Nalgene cryo freezing container. Following overnight freezing at -80^0C, the cryovials were transferred to a liquid nitrogen cryofreeze unit. Cells were recovered by rapid thawing and resuspended in 5 ml growth media followed by centrifugation at 1,000 g for 5 min to remove DMSO. The resultant pellet was resuspended in fresh growth media and transferred to a culture flask. Media was removed the following day, cells washed in HBSS and fresh growth media added.

2.2.8 Cell Counting

Cell counting was performed using a bright-line haemocytometer slide to control cell seeding densities and to monitor BASMC proliferation rates post-treatment. Trypan blue staining was used to determine cell viability. 20 µl of trypan blue was added to 100 µl of cell suspension and incubated for 2 min at room temperature. 20 µl of this suspension was loaded to the counting chamber of the haemocytometer and the cells were visualized by light microscopy (Fig. 2.1). Viable cells excluded the dye and thus appeared colourless, whilst dead cells stained blue. The number of viable cells was calculated using the following equation:

Average Cell No. x 1.2 (dilution factor) x 1 x 10^4 (area under coverslip mm^3) = Viable cells/ml

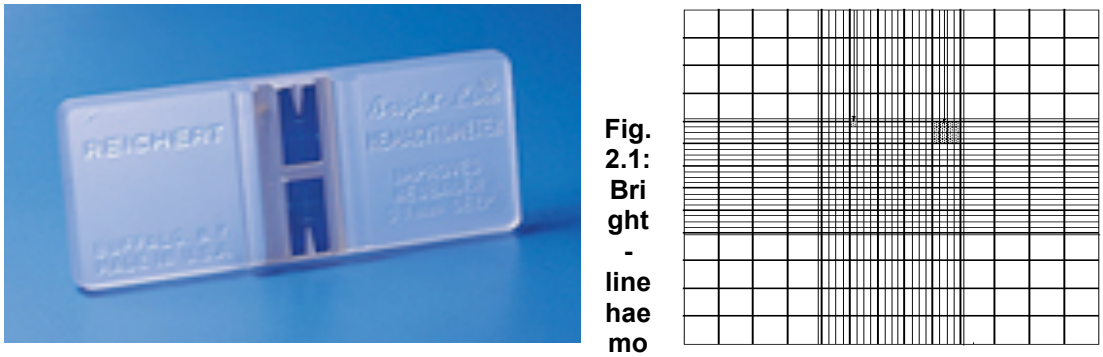


Fig. 2.1: Bright-line haemocytometer slide. The slide contains a very shallow well or chamber with a grid etched on it. A liquid suspension of cells is place on the grid and is viewed using a light microscope. The number of cells per grid can be counted to calculate the number of cells per ml of suspension.

2.2.9 Hemodynamic Force Studies

2.2.9. Laminar Shear Stress: Orbital Rotation

For laminar shear stress studies, BAECs and HAECs were seeded at 2 x 10^5 cells/cm^2 in 6-well plates and allowed to grow to confluency. Following this, media

was removed and replaced with 4 ml of fresh growth media and plates subjected to laminar shear stress on an orbital rotator (Stuart Scientific Mini Orbital Shaker OS) as previously described (Colgan *et al*., 2007). Suitable rotational speeds were selected to produce a shear stress range from 0-10 dynes/cm^2 (0-24 h) (Fig. 2.2), according to the equation described by Hendrickson and co-workers (Hendrickson *et al.*, 1999);

$$\tau = \alpha\sqrt{\rho n(2\pi f)^3}$$

Where τ = shear stress (dynes/cm^2)

α = radius of rotation (cm)

ρ = density of liquid (g/L)

n = viscosity (7.5 x 10^{-3} dynes/cm^2 at 37°C)

f = rotations per second

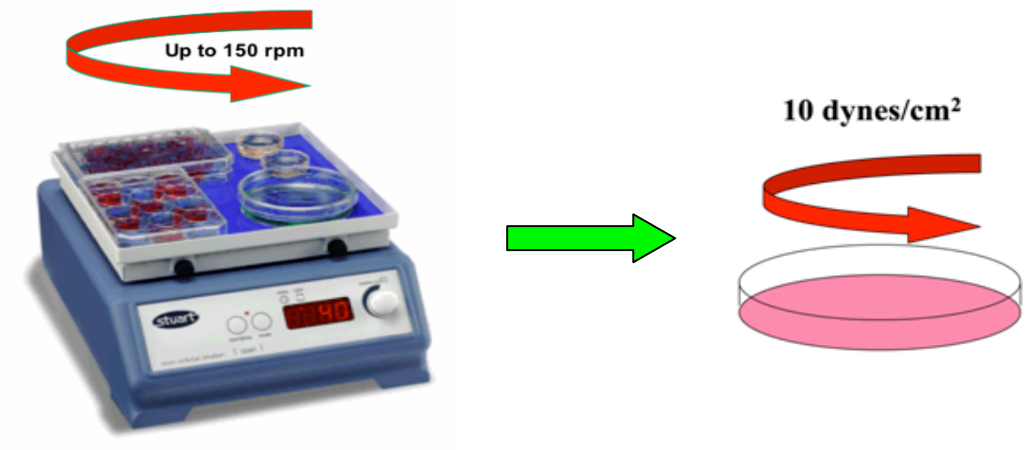


Fig. 2.2: Stuart orbital rotator. Apparatus used for non-pulsatile laminar shear stress studies.

Post-shear, BAEC-conditioned media (BCM) was typically harvested and incubated with BASMCs in order to monitor effects on BASMC apoptosis and proliferation.

2.2.9.2 Pulsatile Shear Stress: CELLMAX® Artificial Capillary System

The CELLMAX® Artificial Capillary System (Spectrum Labs, CA) is an automated closed perfusion system comprising a bundle of 50 semi-permeable, Pronectin®-coated polypropylene capillaries. Media from a reservoir is pumped through the capillary bundle at a chosen flow rate via gas permeable silicone rubber tubing. As the gear pump rotates, the motor shaft forces the pump pins to depress the pump tubing on the capillary module, thereby forcing growth media to flow in a pulsatile fashion through the flow path and the capillary bundle. An electronic control unit housed outside the humidified incubator regulates pump flow rate and pulse amplitude. The co-culture system itself is housed in a humidified CO_2 incubator. Prior to cell seeding and shearing studies, the system is pre-equilibrated by purging the system with normal growth media (RPMI 1640 containing 10% FCS and antibiotics) and then circulating media through the system at high flow (20 dynes/cm²) for 3 days (Fig 2.3).

For co-culture preparation, trypsinised BAECs (T75 flask of confluent BAECs) were seeded into the intralumenal space (ILS) of the capillary bundle using a "double-syringe" method (described by the manufacturers), and the displaced media was discarded. The cells were gently titurated three times across the bundle to ensure an even distribution. Cells were then allowed to adhere to inner capillary surfaces for 3 h, after which the pump was set to low flow (0.3 ml/min; pulse pressure of 6 mmHg; shear stress of 0.5 dynes/cm^2) and returned to the incubator for 3 days. BASMCs were then seeded into the extra-capillary space (ECS), again using the double-syringe method. Cells were allowed to attach to the outer capillary surface for 3 h before

restoring the media at low flow for a further 5 days. BAEC and BASMC attachment periods (3 h) were both conducted in low serum RPMI (1% FCS) to enhance cell attachment to capillary walls. During cell growth to confluency, growth media was adjusted to 20% FCS. The number of cells that did not adhere following low flow restoration were routinely counted to monitor adherence efficiency post-seeding. At confluency, the media flow rate was gradually "ramped up" to high flow (20 dynes cm^2) over 5 h. After completion of the experimental time-course (5 days), cells were harvested from ILS and ECS compartments by first washing the cells with HBSS solution using the double-syringe method, and removing the remaining cells by treatment with 0.125% trypsin-EDTA (3-5 min at 37°C). The circulating media was also harvested at the end of each experiment. For mono-culture preparations (i.e. endothelial cell-free controls), only BASMCs were seeded into the ECS compartment and BAECs excluded from the ILS compartment.

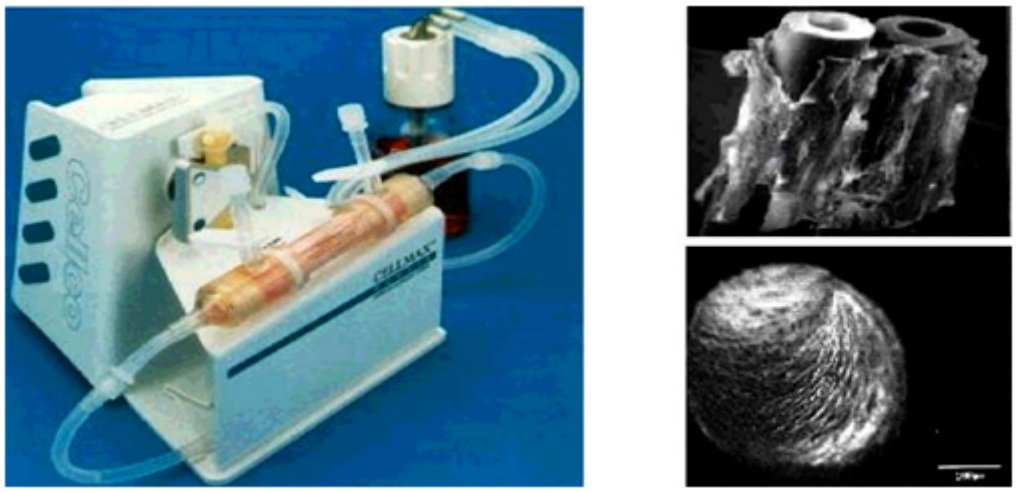


Fig. 2.3: CELLMAX® Artificial Capillary System. Electron micrograph of extracapillary vascular smooth muscle cell (top right) and intraluminal endothelial cell growth (bottom right) within the capillary bundle of the Cellmax® Artificial Capillary System (left).

2.2.9.3 Cyclic strain: Flexercell® Tension Plus™ FX-4000T™ System

For cyclic strain studies, BAECs were seeded onto 6-well Bioflex® plates. Bioflex® plates have a flexible ECM-coated silicon membrane bottom which can be deformed by microprocessor-controlled vacuum (Dunn Labortechnik GmbH, Germany) and allowed to come to confluency. Media was then replenished and the cells were exposed to equibiaxial cyclic strain. A Flexercell® Tension Plus™ FX-4000T™ system (Flexercell International Corp., Hillsborough, NC) was used to apply physiological equibiaxial cyclic strain to each plate (0-10% strain, 60 cycles/min, 0-24 h, cardiac waveform) as previously described by others in our laboratory (Collins *et al.*, 2006; von Offenberg Sweeney *et al.*, 2004) (Fig. 2.4).

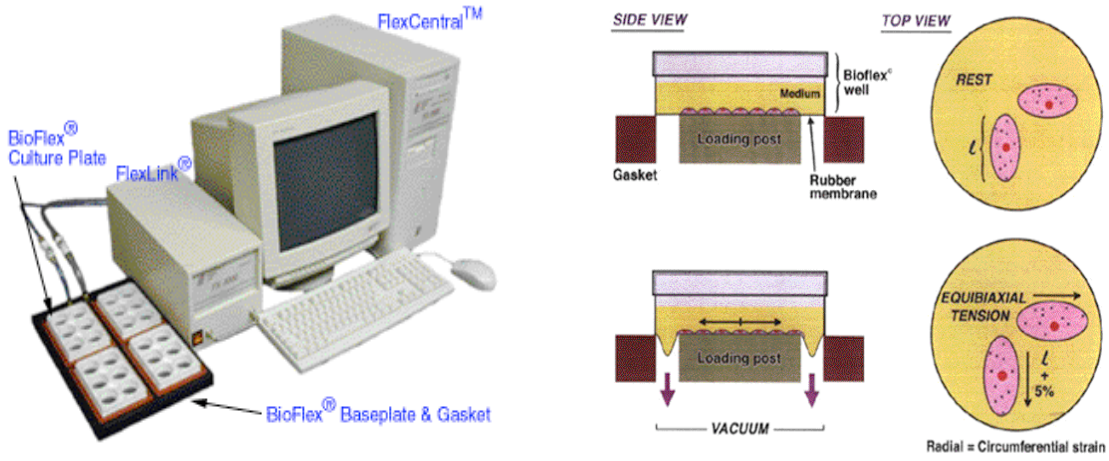


Fig. 2.4: Flexercell® Tension Plus™ FX-4000T™ system. The system is used to apply physiological equibiaxial cyclic strain to cells.

2.2.10 Inhibitor Studies

For pharmacological inhibition studies, BAECs were grown in 6-well plates until approximately 70-80% confluent, after which the growth media was removed and the cells were rinsed in HBSS. Inhibitors were reconstituted in a suitable diluent. Working concentrations were then prepared in RPMI 1640 supplemented with 10% FCS and antibiotics. Cells were then exposed to inhibitors for 1 h prior to, subsequently for the duration of, hemodynamic challenge. The following pharmacological inhibitors were used (concentrations were gained from current literature and manufacturer's recommendations);

Apocynin (10 μM - NAD(P)H oxidase)

Cyclic RGD peptide (100 μM, cRGD - β1/β3 Integrins)

Pertussis toxin (100 ng/ml, PTX - Heterotrimeric Gα-subunit)

Genistein (50 μM - Protein Tyrosine Kinases)

GM6001 (10 μM - Matrix Metalloproteinases)

L-NAME (1 mM - eNOS)

MMP-2/-9 Inhibitors (100 μM)

NSC23766 (50 μM - Rac1)

ALK5 Inhibitor I (20 μM - TGF-β R1)

2.2.11 Preparation of Whole Cell Lysates

BAECs and BASMCs and were washed in HBSS before being harvested using either a cell scraper (for 6-well plate studies) or by trypinization (for perfused capillary studies) and centrifuged at 1,000 g for 5 min. The cells were lysed in a modified RIPA buffer (50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM

sodium pyrophosphate, 1 mM sodium orthovanadate, 100 mM NaF, and 1% Triton X-100 supplemented with a 1/100 dilution of Sigma protease and phosphatase inhibitor cocktails). Cells were rotated gently for 1 h at 4°C and subsequently centrifuged at 13,000 g for 20 min at 4°C. The supernatant fraction was stored at -80°C.

2.3 Biochemical Methods

2.3.1 Bicinchoninic Acid (BCA) protein microassay

In this assay, Cu^{2+} reacts with protein under alkaline conditions to produce Cu^{+}, which in-turn reacts with BCA to produce a coloured product. Two separate reagents were supplied in the commercially supplied available kit (Pierce Chemicals); **(A)** an alkaline bicarbonate solution and; **(B)** a copper sulphate solution. 1 part solution B is mixed with 49 parts solution A. 200 µl of this mixture is then added to 10 µl of protein lysate or BSA standards (standard curve in the range 0-2 mg/ml). The 96-well plate is incubated at 37°C for 30 minutes and the absorbance read at 560 nm using a Bio-TEK® EL*x*800 microtitre plate reader.

2.3.2 Western Blotting

SDS-PAGE was carried out on BAEC and BASMC lysates as described by Laemmli *et al*. (Laemmli, 1970) using 10% polyacrylamide gels. Gels were prepared as outlined below in Table 2.1;

Recipe	Resolving gel (10%)	Stacking gel (5%)
Upper Tris Buffer	0 ml	0.75 ml
Lower Tris Buffer	1.5 ml	0 ml
40% Acrylamide	1.5 ml	0.375 ml
10% w/v SDS	6 µl	30 µl
10% w/v Ammoniun Persulfate	30 µl	15 µl
Distilled water	3 ml	1.85 ml
TEMED	7 µl	7 µl

Table 2.1: SDS-PAGE gel formulations. Resolving gel buffer and stacking gel buffers have a pH of 8.8 and 6.8, respectively.

The resolving gel was poured first, overlaid with ethanol and allowed to polymerize for 20 min. The ethanol was removed and the stacking gel was overlaid onto the resolving gel, a comb inserted and the gel allowed to polymerize for 20 min. Combs, clamps, and gaskets were then removed and the gel plates inserted into the electrophoresis chamber. The chamber was subsequently filled with reservoir buffer (25 mM tris, 192 mM glycine, 0.1% SDS, pH 8.3).

The protein concentration of each sample was determined by BCA assay to facilitate equal protein loading/well. 4X loading buffer (4 g SDS, 20 ml glycerol, 2 ml β-marcaptoethanol, 0.04 g bromophenol blue and 24 ml 0.25 M TrisCl, made up to a final volume of 50 ml, pH 6.8) was added to the volume-adjusted samples and boiled at 95ºC for 5 min and immediately placed on ice. Samples were then loaded onto the gel and the samples were separated electrophoretically at 150 V, 90 mA and 150 W for 3 h.

Following electrophoresis, the gel was removed and soaked for 10 min in transfer buffer (25 mM tris pH 8.3, 192 mM glycine, 20% methanol and 0.1% w/v SDS).

Nitrocellulose membrane and Whatmann filter paper were cut to the same size as the gel and soaked in transfer buffer. Proteins were transferred to the membrane for 1 h at 100 V, 500 mA and 150 W using an Atto semi-dry transfer system. Following transfer, the membrane was soaked in Ponceau S Solution for 5 min to monitor and normalize for variations in protein loading. The membrane was subsequently washed gently in a solution of 1X PBS and 0.05% Tween-20 (PBS-Tween). Following washing, the membrane was blocked for 2 h in blocking solution (5% BSA solution). Membranes were washed again in PBS-Tween and incubated overnight in eNOS primary antibody (Anti-rabbit eNOS polyclonal IgG at 1/2000 dilution (Cayman Chemical Company) at 4ºC. The membranes were washed vigorously for 30 minutes in PBS-Tween and then incubated with a suitable HRP-linked secondary antibody for 2 h (Anti-rabbit 2^0antibody, HRP-conjugated at 1/5000 dilution) (Amersham Pharmacia Biotech)) with subsequent washing for 30 min in PBS-Tween. Membranes were developed by incubation in West Pico Supersignal reagent (Pierce) according to the manufacturers instructions. Blots were developed by autoradiography (Amersham Hyperfilm) to visualise bands. The images were captured using a Kodak DC290 digital camera and a quantitative comparison between bands carried out using Kodak 1D (version 3.5.4) densitometry imaging software.

2.3.3 Immunocytochemistry

Both endothelial and smooth muscle cells (bovine and human) were prepared for immunocytochemical analysis to visually monitor the expression and/or subcellular localization of marker proteins, as previously described by Groarke *et al.*, with minor modifications (Groarke *et al.*, 2001). Cells were washed twice in PBS and fixed with

3% formaldehyde for 15 min. Cells were subsequently washed in PBS, permeabilised for 15 min with 0.2% Triton X-100, and blocked for 30 min in 5% BSA solution. Following this, cells were incubated for 2 h with the appropriate primary antibody or stain (Table 2.2). Cells were then incubated for 1 h with 1:400 dilution of either AlexaFluor-488 anti-mouse or anti-rabbit secondary antibody. Cells were also stained with nuclear DAPI staining by incubating cells with 0.5 x 10^{-6} µg/ml DAPI for 3 min. Cells were sealed with coverslips using DAKO mounting media (DAKO Cytomation, Cambridgeshire, UK) and visualized by fluorescent microscopy (Olympus BX50).

Primary Anti-serum/Stain	Company	Concentration/Dilution	Time (h)
Rhodamine-Phalloidin	Molecular Probes	1:200	1
SMC α-actin	Sigma	1:400	3
von Willebrand Factor	DakoCytomation	1:1000	3
Zonula Occludens-1	Zymed	0.5 µg/ml	3

Table 2.2 Immunocytochemical reagents. Staining and antisera information for immunocytochemical imaging.

2.3.4 Caspase-3 Assay

Caspase-3 assay is a colorimetric assay that detects p-nitroanilide (pNA) release after Caspase-3 cleaves the bond from the labelled substrate, Ac-DEVD-pNA. The absorbance of pNA was measured using a microtiter plate reader at 405 nm. Substrate (Ac-DEVD-pNA) is mixed with 10X Assay Buffer (20 mM Hepes, 0.1% CHAPSO, 5 mM DTT, 2 mM EDTA, and 10% DMSO) to give a 2 mM concentration. 10 µl of this substrate is added to 25 µl of cell lysate and diluted in 1X Assay Buffer (20 mM HEPES, 0.1% CHAPSO, 5 mM DTT, and 2 mM EDTA) to give a final volume of 100 µl. The samples were incubated in a 96-well plate for 45 min and the absorbance

measured at 405 nm. Appropriate negative controls and blanks were included in the assay. A pNitroanilide (pNA) standard curve (0-200 µg/µl), prepared under identical assay conditions, allowed for Caspase-3 activity to be calculated for each sample.

2.4 Molecular Methods

2.4.1 RNA Isolation

Post-treatment, BASMCs were harvested for gene expression analysis. Trizol® is a ready-to-use reagent for the isolation of total RNA, DNA, and/or protein from cells and tissues. Based on a method developed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Trizol® maintains the integrity of the RNA, whilst disrupting the cells and dissolving the cellular components. Post-treatment, cells were washed and lysed directly in 6-well plates by the addition of 1 ml Trizol® per $cm^{2.}$ A pipette was used to titurate samples a number of times to ensure complete homogenization. The samples were subsequently incubated at room temperature for 5 min. 0.2 ml chloroform was added per ml of Trizol® used and mixed vigorously for 15 sec before being incubated for 5 min at room temperature. Samples were then centrifuged at 12,000 g for 15 min at 4°C. The mixture separated into a lower red phenol-chloroform phase, an interphase, and an upper colourless aqueous phase. RNA remained in the aqueous phase, which was carefully removed and transferred to a fresh tube. The RNA was precipitated out of solution by the addition of 0.5 ml of isopropanol per 1 ml of Trizol® used. Samples were incubated for 15 min at room temperature and then centrifuged at 12,000 g for 10 min at 4°C. The RNA precipitate forms a gel-like pellet

on the side of the tube. The supernatant was removed and the pellet washed in 1 ml of 75% ethanol per ml of Trizol® used followed by centrifugation at 7,500 g for 5 min at 4^0C. The resultant pellet was air-dried for 5-10 min before being resuspended in DEPC-treated water. The sample was then stored at -80^0C.

2.4.2 Spectrophotometric Analysis of Nucleic Acids

RNA concentration was determined by measuring the absorbance at 260nm. DEPC water in a quartz cuvette was used to blank the spectrophotometer. The RNA concentration was determined as follows;

$$ABS_{@260nm} \times 40 \times \text{dilution factor} = [RNA]\ (\mu g/ml)$$

The purity of RNA samples was also established by reading the absorbance at 260 nm and 280 nm and then determining the ratio between the two (A_{260}/A_{280}). Pure RNA has a ratio of 2.0, lower ratios indicate the presence of proteins, higher ratios imply the presence of organic reagents.

2.4.3 RealTime PCR analysis

2.4.3.1 Reverse Transcription

cDNA was synthesized from messenger RNA (mRNA) using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, California, USA). The RT reaction was prepared as described in Table 2.3. Using a PCR Sprint Thermo Cycler (Thermo

Electron Corporation, Massachusetts, USA), the RT reaction was subjected to one cycle consisting of three phases; (i) 25°C for 5 min, (ii) 42°C for 30 min, and (iii) 85°C for 5 min. The resultant cDNA was stored at -20/-80°C until required for PCR.

RT Cocktail	**Volume**
5X Buffer	4 μl
Reverse Transcriptase	1 μl
RNA	1 μg
RNase-free water to a final volume	20 μl

Table 2.3: RT Recipe.

2.4.3.2 RealTime PCR

RealTime PCR was carried out using a Rotor-GeneRG-3000^{TM} LightCycler (Corbett Research, Australia) using SYBR Green PCR chemistry. SYBR Green is a fluorescent dye which, when bound to the minor groove of double-stranded DNA, emits fluorescence. The fluorescence increase during amplification is directly proportional to the amount of cDNA product formed, thus allowing for quantitative comparisons between genes of interest. cDNA was amplified for the target sequences of interest namely MMP-2, eNOS, CDK1, CDK2, CDK4, CDK6, Cyclin A, Cyclin D1, Cyclin E, p27*kip*, and GAPDH (the latter housekeeping gene included for normalization purposes). Primer sequences of interest are detailed below in Table 2.4.

Target gene	Primer Sequence	Annealing Temp	Product Size
CDK1	For: 5'-CAGACTAGAAAGTGAAGAGG-3' Rev: 5'-CTTCTGGAGATCTATACCAG-3'	55ºC	415 bp
CDK2	For: 5'-CATTCCTCTTCCCCTCATCA-3' Rev: 5'-AGCTCCGTCCATCTTCATCC-3'	55ºC	491 bp
CDK4	For: 5'-CCTTCCCGTCAGCACAGTTC-3' Rev: 5'-CAGAGCGTAACCACCACAGG-3'	55ºC	206 bp
CDK6	For: 5'-TTGGATAAAGTGCCAGAACC-3' Rev: 5'-TTTCCTAGTTGGTCGACATC-3'	55ºC	354 bp
Cyclin D1	For: 5'-CTGGCCATGAACTACCTGGA-3' Rev: 5'-CCACTTGAGCTTGTTCACCA-3'	57ºC	332 bp
Cyclin A	For: 5′-AGACCCTGCATTTGGCTGTG-3′ Rev: 3′-ACAAACTCTGCTACTTCTGG-5′	55ºC	294 bp
Cyclin E	For: 5'-TTCTGGATTGGCTGATGGAG-3' Rev: 5'-AAGCAGCGAGGACACCATAA-3'	57ºC	462 bp
p27*kip*	For: 5'-CGGGTTAGCGGAGCAGTG-3′ Rev: 5'-AGGCTTCTTGGGCGTCTG-3′	57ºC	253 bp
MMP-2	For: 5'-TGGCAACCCCGACGTGG-3′ Rev: 5'-AGGCTTCTTGGGCGTCTG-3′	55 ºC	534 bp
eNOS	For: 5'-CCGTGTCCAACATGCTGCT -3′ Rev: 5'-ACCTCGCATTACCATACACA -3′	55ºC	396 bp
GAPDH	For: 5'-AGGTCATCCATGACCACTTT-3' Rev: 5'-TTGAAGTCGCAGGAGACAA-3'	55ºC	337 bp

Table 2.4: Primer sequences for RealTime PCR.

Each PCR reaction was set up in triplicate (Table 2.5);

PCR Mix	Volume
SYBR Green (total reagent)	12.5 μl
DEPC Water	8.5 μl
Forward Primer	1.0 μl
Reverse Primer	1.0 μl
cDNA	2.0 μl
Total	25 μl

Table 2.5: PCR Recipe..

The following PCR cycle program was used for product amplification with the appropriate annealing temperature;

Denature		95^0C	15 min	
Cycling	Denature	95^0C	20 sec	45 cycles
	Annealing	59^0C	30 sec	
	Extension	72^0C	30 sec	
Hold		60^0C	1 min	
Melt		50 – 100^0C		

Melt curve analysis was routinely carried out to rule out non-specific primer-dimer products. For quantification purposes, glyceraldehyde phosphate dehydrogenase (GAPDH) a house-keeping gene, was included for normalization purposes. Agarose gel electrophoresis was also carried out to confirm fragment sizes of various primers.

2.4.4 PCR Microarray

This specific analysis profiles the expression of 84 genes relevant to cell cycle regulation. The PCR Microarray system includes the RT² First Strand Kit which generates the CDNA (SABiosciences, Frederick, MD, USA) and the Human Cell Cycle RT² Profiler™ PCR Array (SABiosciences, Frederick, MD, USA).which examines the expression of the relevant genes. The real-time PCR instrument employed was the ABI-7900HT Fast RT-PCR system (Applied Biosystems, Warrington, UK)

2.4.4.1 Reverse Transcription

The RT² First Strand Kit (SABiosciences, Frederick, MD, USA) was used to generate cDNA and contains a genomic DNA elimination step. A genomic DNA elimination mixture (Table 2.6) was prepared for each RNA sample;

DNA Elimination	Volume
Total RNA	1 μg
GE (5X gDNA Elimination Buffer)	2 μl
RNase-free water to a final volume	10 μl

Table 2.6: DNA Elimination Recipe. This step removes any residual genomic DNA from the RNA sample.

The samples are incubated at 42ºC for 5 min and immediately placed on ice for at least 1 min. The following RT recipe (Table 2.7) was then prepared for each sample to give a final volume of 10 μl;

RT Cocktail	Volume
BC3 (5X RT Buffer 3)	4 μl
P2 (Primer and External Control Mix)	1 μl
RE3 (RT Enzyme Mix 3)	2 μl
RNase-free water	3 μl
Total volume	10 μl

Table 2.7: RT Recipe.

Samples were centrifuged briefly and RT Cocktail and Genomic DNA Elimination Mixture were combined to give a final volume of 20 μl. The samples were incubated

at 42ºC for 15 min and then heated at 95ºC for 5 min to stop the reaction. The resultant cDNA was stored at -20 ºC.

2.4.4.2 PCR Microarray

The Human Cell Cycle RT² Profiler™ PCR Array (SABiosciences, Frederick, MD, USA) profiles the expression of 84 genes key to cell cycle regulation in a 96-well format. The array monitors the expression of genes that positively and negatively regulate the cell cycle, DNA replication, checkpoints, and arrest. Five house-keeping genes and three RNA and PCR quality controls were also included. The real-time PCR instrument employed was the ABI-7900HT Fast RT-PCR system with variable Taqman® Low Density Array platform (compatible with the Human Cell Cycle RT² Profiler™ PCR Array). For each sample, a PCR reaction mix was prepared and aliquoted out onto a separate plate at 25 μl per well (Table 2.8 - volumes shown are PCR reaction mix per well);

PCR Mix	Volume
RT² SYBR Green/ROX qPCR Master Mix	12.5 μl
ddwater	10.5 μl
cDNA	1.0 μl
Total	22.5 μl

Table 2.8: PCR Recipe.

The following PCR cycle program was then used for product amplification;

Denature		95^0C	10 min	
Cycling	Denature	95^0C	15 sec	} 40 cycles
	Annealing	60^0C	60 sec	
	Extension	60^0C	30 sec	
Hold		60^0C	1 min	
Melt		50 – 100^0C		

A brief diagrammatic overview of the RT^2 PCR microarray procedure is shown below in Fig. 2.6;

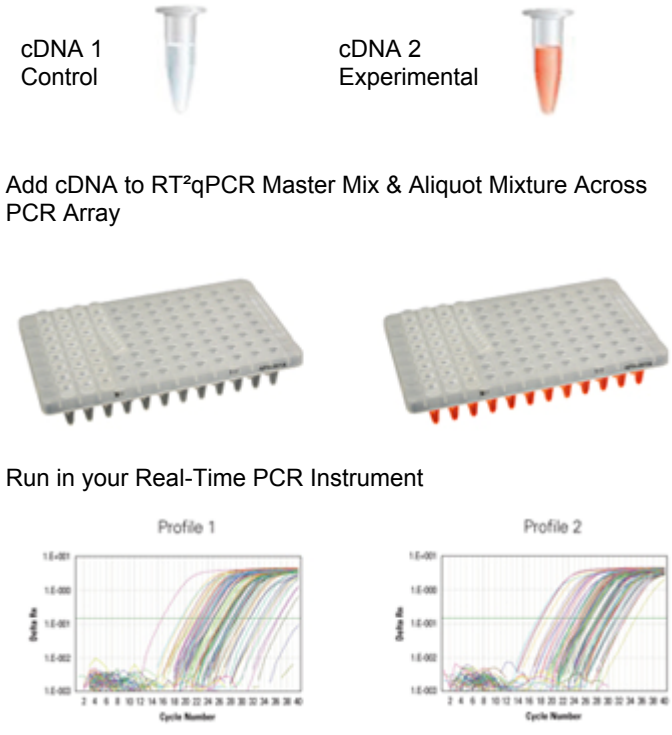


Fig. 2.6: RT^2 PCR Array. The array combines the real-time PCR performance with the ability of the microarrray to detect the expression of 84 key related genes to cell cycle regulation.

2.5 Fluorescence Activated Cell Sorting (FACS) Analysis

Flow cytometry is a process used to characterise the properties of fluorescently labelled cells. It provides information about cell size, granularity, and the phenotypic expression of protein markers on or in a particular cell, as well as the proliferative, apoptotic, and necrotic state of cells in response to different experimental treatments.

2.5.1 Apoptosis

A Vybrant™ Apoptosis Assay Kit #2 was used to monitor apoptosis in BASMCs. The kit contains a Alexfluor488-conjugated recombinant Annexin V. The Alexafluor488 dye is an almost perfect spectral match to Fluorescein IsoThioCyanate (FITC), but it creates brighter and more photostable conjugates. The kit also includes red-fluorescent Propidium Iodide (PI) nucleic acid-binding dye. PI stains necrotic cells (but not live apoptotic cells) with red fluorescence, binding tightly to the nucleic acids in the cell. After staining a cell population with Alexafluor488-conjugated Annexin V and PI in the provided binding buffer, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence. These populations can easily be distinguished using a flow cytometer with the spectral line of an argon-ion laser set for 488 nm excitation.

BASMCs were seeded onto 6-well plates (5×10^4 cells/cm^2) and allowed to grow for 24 h. Cells were then quiesced for 48 h and incubated with BCM harvested from hemodynamic experiments. Post-treatment (4 and 6 days treatment with BCM), BASMCs were washed once in PBS, harvested by trypsinization and pelleted by

centrifugation (1,000 g for 5 min). Cells were washed again in 1 ml PBS (containing 0.1% BSA), and resuspended by gentle pipetting. Cells were subsequently pelleted by centrifugation and re-suspended in 200 μl of 1X Annexin-binding buffer. Propidium Iodide (0.4 μl from 100 μg/ml working solution) and 1 μl Alexafluor488 Annexin V were added to the cell suspension and incubated at room temperature for 15 min. Cells were then placed on ice pending flow cytometry analysis (Becton Dickinson FACSCaliber).

2.5.2 Proliferation

The succinimidyl ester of carboxyfluorescein diacetate (CFDA-SE) was used to monitor proliferation in BASMCs. CFDA-SE is a dye that passively diffuses into the cytoplasm of cells. CFDA-SE couples to both intracellular and cell-surface proteins by reaction with lysine side chains and other available amine groups. When cells divide, CFDA-SE labelling is distributed equally between the daughter cells, resulting in halving of the mean cellular fluorescence. As a result, each successive generation in a population of proliferating cells is marked by a halving of cellular fluorescence intensity that is readily detected by flow cytometry (Molecular Probes).

BASMCs were seeded onto 6-well plates (5 x 10^4 cells/cm^2) and allowed to grow for 24 h. Cells were washed once with PBS and 1 ml of 5 μM carboxy-fluorescein diacetate succinimidyl ester (CFDA-SE, prepared in PBS) was added to each well for 10 min at 37°C. Following incubation, CFDA-SE was replaced with fresh media and the cells were allowed to recover for 6 h before 48 h quiescence. Cells were then treated with BCM harvested from hemodynamic experiments. Post-treatment (4 and 6

days treatment with BCM), BASMCs were harvested by trypsinization/centrifugation and washed twice with 1 ml PBS (containing 0.1% BSA). Cells were resuspended in 1 ml PBS (containing 0.1% BSA) and then placed on ice pending flow cytometry analysis. Proliferation was also routinely monitored by cell counting over a 7-day BCM treatment period using a Sigma bright-line haemocytometer.

2.6 Statistical Analysis

Results are expressed as mean±SEM. Experimental points were performed in triplicate with a minimum of three independent experiments (n=3). Means were compared by Student's unpaired *t*-test or Two-Way ANOVA with replication, where applicable. A value of $P \leq 0.05$ was considered significant.

CHAPTER 3

Vascular Cell Characterisation Studies

3.1 Introduction

Studies have shown that both laminar shear stress and cyclic strain have a profound effect on morphological and phenotypic changes in endothelial cells (Iba *et al*., 1991). Endothelial cells can sense the direction and magnitude of blood flow and are able to adapt their shape and cytoskeletal structure in response to this. Hemodynamic forces have also been shown to modulate the expression and/or activation of several effector genes such as eNOS, matrix metalloproteinases (e.g. MMP-2), and zonula occludens (ZO-1), the latter known to be involved in endothelial tight junction assembly (Collins *et al*., 2006; von Offenberg Sweeney *et al*., 2004; Colgan *et al*., 2007).

The aim of this chapter was to assess the characteristics of the procured bovine and human vascular cells (BAEC/BASMC, HAEC/HASMC) wrt marker protein expression and morphological response under static and force conditions.

3.2 Results

3.2.1 BAEC/BASMC Characterisation

Our initial studies examined the gross morphology of static BAECs and BASMCs using light microscopy. Both cell types were cultured in RPMI 1640 supplemented with FCS and antibiotics. BAECs displayed a characteristic "cobblestone" morphology, whilst BASMCs displayed a "spindle" morphology (Fig. 3.1 A,B). BAECs were then examined immunocytochemically for EC-specific von Willebrand Factor (vWF), whilst BASMCs were monitored for expression of SMC-specific α-actin (Fig. 3.1 C,F). Both BAECs and BASMCs were positive for their individual marker. Negative controls included primary antibody exclusion (Fig. 3.1 D,E).

3.2.2 HAEC/HASMC Characterisation

Gross morphology of static HAECs and HASMCs was also examined using light microscopy. HAECs were cultured in Promocell Endothelial Media supplemented with 5% FCS, 5% ECGS, 1 μg/ml hydrocortisone, and antibiotics. HASMCs were cultured in Promocell Smooth Muscle Cell Media supplemented with 5% FCS, 0.5 ng/ml epidermal growth factor, 2 ng/ml bFGF, 5 μg/ml insulin, and antibiotics. HAECs displayed a cobblestone morphology, whilst HASMCs displayed a "spindle" morphology (Fig 3.2 A,B). HAECs were then examined immunocytochemically for EC-specific vWF, whilst HASMCs were monitored for expression of SMC-specific α-actin (Fig. 3.2 C,F). Both HAECs and HASMCs were positive for their individual markers. Negative controls included exclusion of primary antibody (Fig. 3.2 D,E).

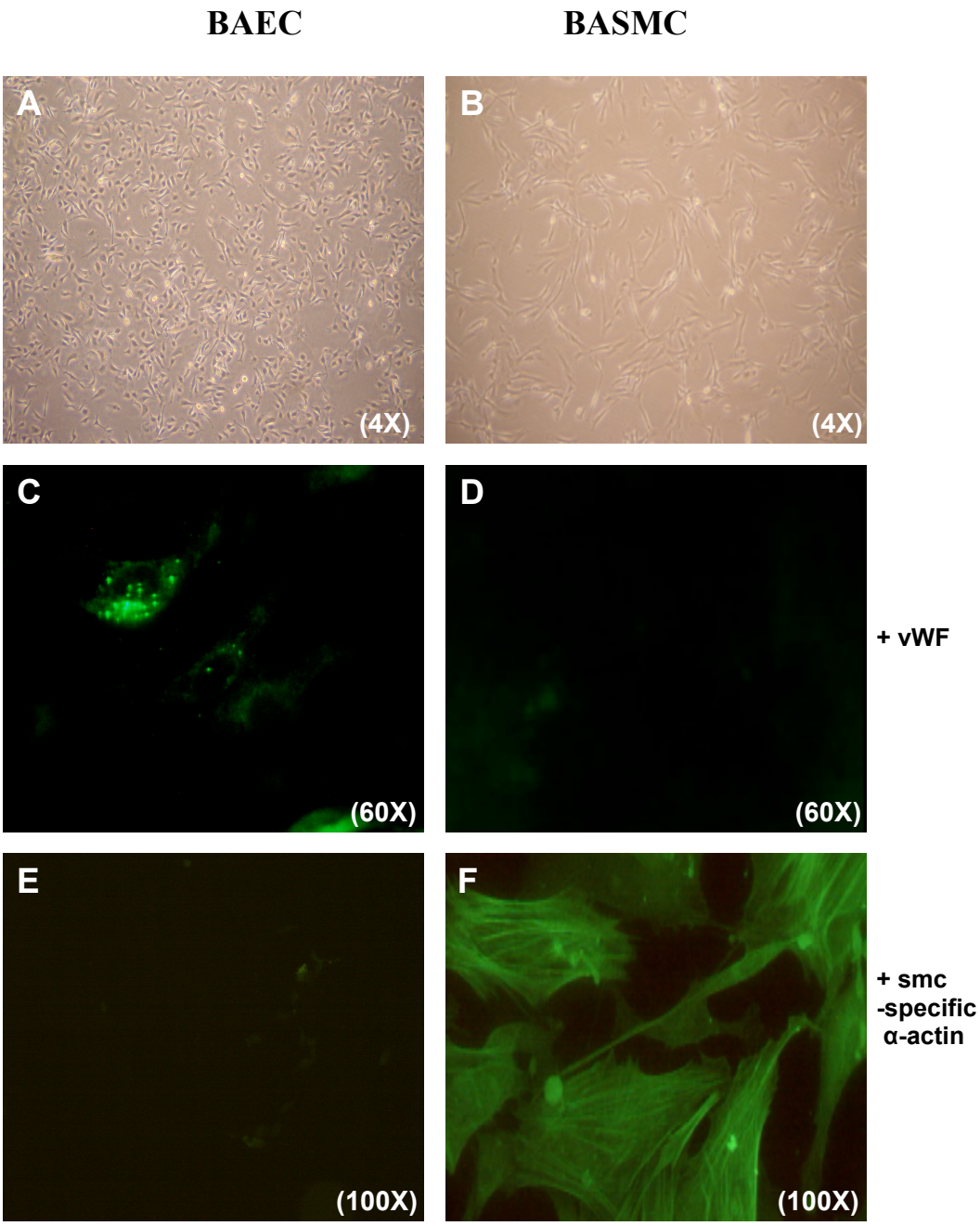


Fig. 3.1: BAEC and BASMC marker protein expression. Brightfield image of static; (A) BAECs and (B) BASMCs. Immunocyochemical expression of; (C) endothelial cell-specific vWF and (F) smooth muscle cell-specific α-actin (F). Controls involving incubation without primary anti-serum show no fluorescence (D, E). Images are representative of 3 experiments.

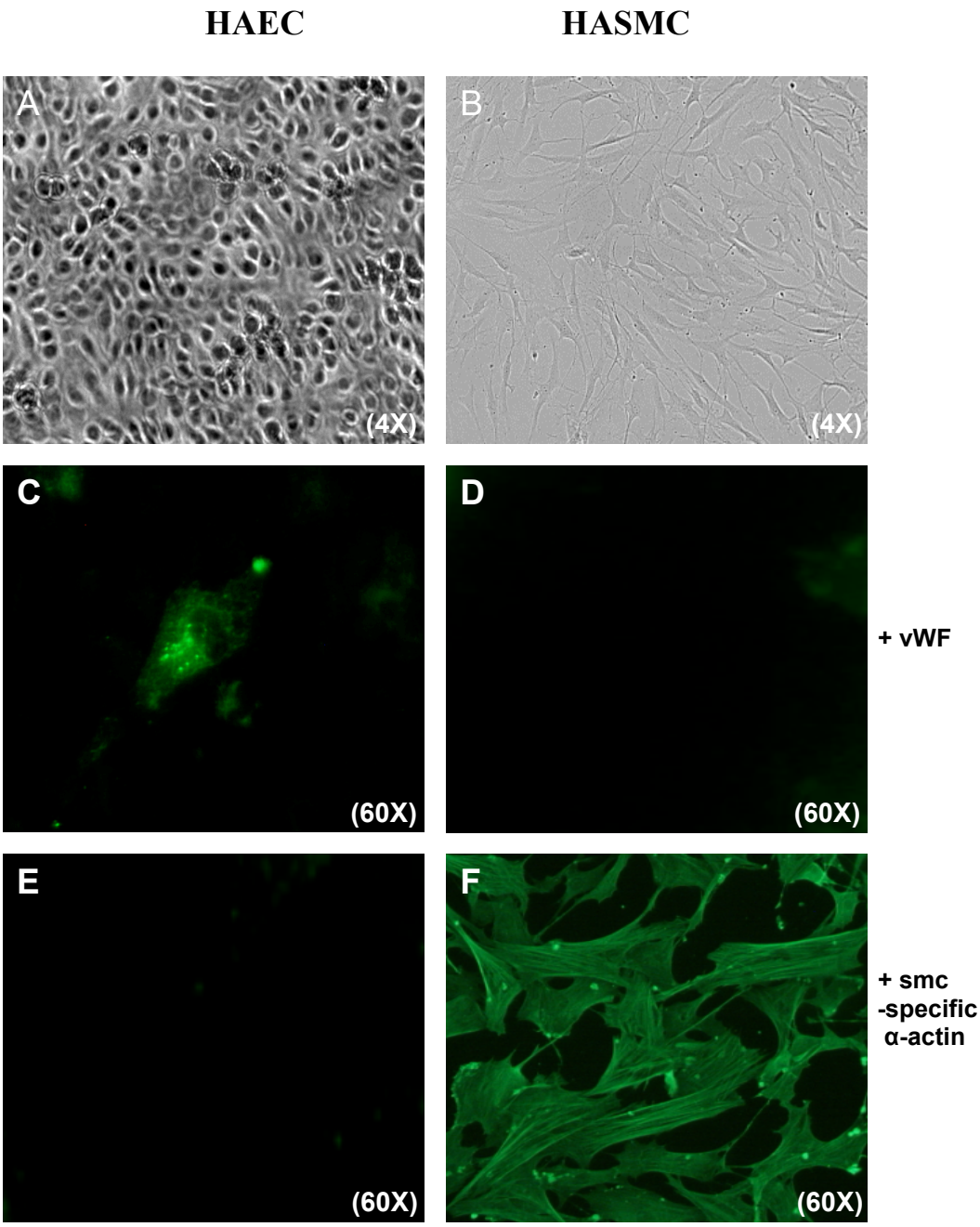


Fig. 3.2: HAEC and HASMC marker protein expression. Brightfield image of static; (A) HAECs and (B) HASMCs. Immunocyochemical expression of; (C) endothelial cell-specific vWF and (F) smooth muscle cell-specific α-actin (F). Controls involving incubation without primary anti-serum show no fluorescence (D, E). Images are representative of 3 experiments.

3.2.3 BAEC Responsiveness to Shear Stress

The effects of shear stress (10 dynes/cm^2, 24 h) on BAEC morphology were examined in order to ensure they were capable of sensing and responding to a defined level of hemodynamic stimulation. Post-shear, cells were monitored for changes in shear-dependent indices such as cellular realignment, cytoskeletal remodelling, barrier function and eNOS expression.

Following exposure to laminar shear stress (orbital rotation model), BAECs demonstrated gross morphological realignment in the direction of the shear vector. This contrasts with static cells which were more random and multi-directional (Fig. 3.3 A, B). Cells were also examined by fluorescence microscopy for morphological changes in the actin cytoskeleton using Rhodamine-Phalloidin (i.e. stains for F-actin). Static cells expressed F-actin in a disorganised "stress fibre" fashion. Sheared cells by contrast, displayed a clear realignment of the cytoskeleton in the direction of flow with a "cortical actin" ring clearly visible along the cell periphery (Fig 3.3 C,D) conducive to enhanced barrier function. Shear-dependent tight junction assembly, an important index of endothelial barrier function (Colgan *et al.*, 2007), was also monitored following BAEC shear. Static cells displayed a highly "discontinuous" pattern of zonula occludens-1 (ZO-1) immunoreactivity along the cell-cell border, whilst localization for this tight junction-protein appeared somewhat more defined and continuous in sheared endothelial cells (Fig 3.3 E,F).

To further examine the effects of shear stress on BAECs, the regulatory effect on eNOS mRNA and protein expression levels were examined. Several studies have

shown that shear stress of endothelial cells increases both eNOS mRNA and protein (Li *et al*., 2003; Davis *et al*., 2001; Nadaud *et al*., 1996). Following exposure of BAECs to shear stress (10 dynes/cm^2, 24 h), eNOS mRNA and protein levels were examined. eNOS mRNA levels, as measured by RealTime PCR, increased by 2.4±0.2 fold compared to control (Fig. 3.4 A). eNOS protein levels increased by 1.58±0.1 fold compared to control, as observed by Western immunoblotting (Fig. 3.4 B).

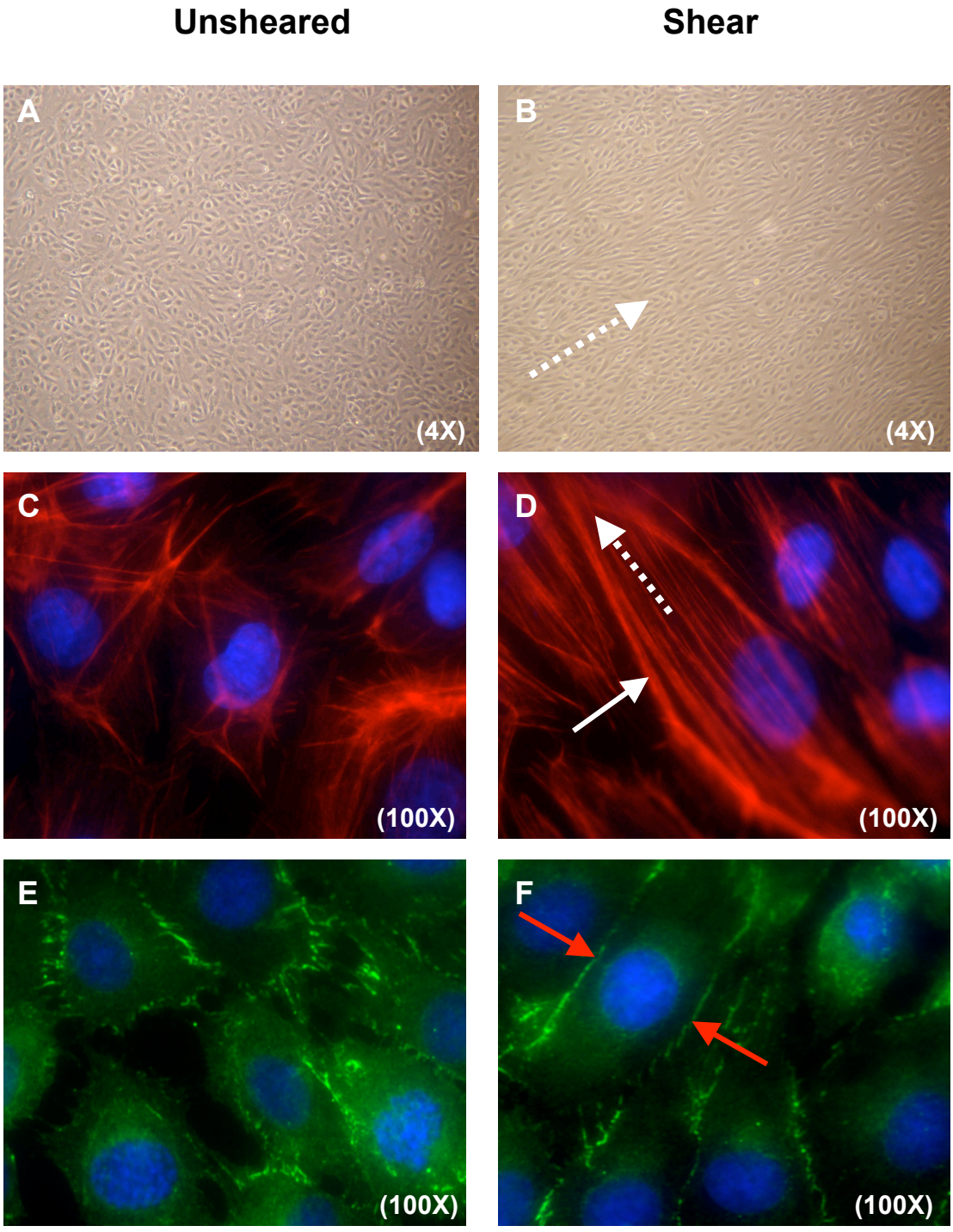


Fig. 3.3: BAEC responsiveness to shear stress. Following BAEC exposure to shear stress (10 dynes/cm^2, 24 h), changes in cellular morphology and cytoskeletal remodelling were monitored. Brightfield image of; (A) unsheared control cells and (B) sheared cells. Rhodmaine-Phalloidin staining for F-actin in; (C) unsheared control cells and (D) sheared cells. Dotted arrows indicates direction of shear vector. Solid white arrow indicates cortical actin formation. Cell-cell border localization of ZO-1 in; (E) unsheared control cells and (F) sheared cells. Red arrows displays ZO-1 cell-cell border localization. DAPI nuclear staining is shown in blue. Images are representative of 3 experiments.

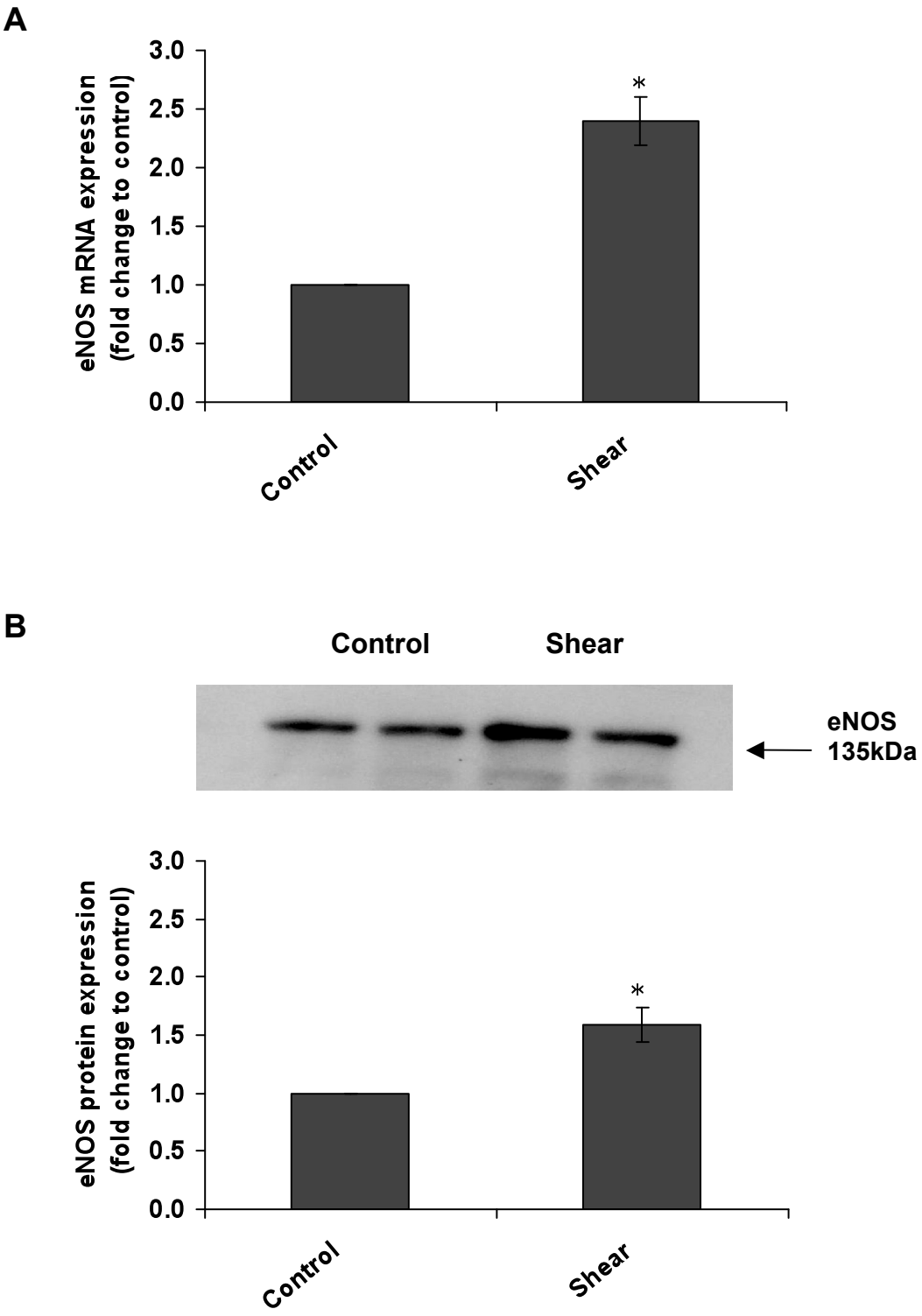


Fig. 3.4: Shear-dependent up-regulation of eNOS mRNA and protein expression. Following BAEC exposure to shear stress (10 dynes/cm^2, 24 h), changes in eNOS mRNA and protein levels were monitored; (A) eNOS mRNA and (B) eNOS protein. Histogram in (A) represents fold change in mRNA expression compared to unsheared control (2.4±0.2 fold). Histogram in (B) represents fold change in Western immunoblot band intensity compared to unsheared control (1.58±0.1 fold) (immunoblot is shown above histogram). Histograms are averaged from three independent experiments (±SEM). *P≤0.05 relative to unsheared control.

3.2.4 BAEC Responsiveness to Cyclic Strain

Following BAEC exposure to equibiaxial cyclic strain (10%, 24 h, cardiac waveform), cells were examined for changes in cytoskeletal reorganisation, barrier function and MMP-2 expression. Static cells expressed actin in a disorganized state, whilst cells exposed to cyclic strain appeared to display higher cortical actin realignment at the cell-cell contacts (Fig. 3.5 A,B). Furthermore, barrier function was investigated in response to cyclic strain. It revealed that static cells displayed a jagged localization pattern for tight junction-associated ZO-1 along the cell-cell border (Fig. 3.5 C). Cells exposed to cyclic strain however, showed a more pronounced pattern of ZO-1 immunoreactivity along the cell-cell border (Fig. 3.5 D).

To further examine the effects of cyclic strain on BAECs, the regulatory effect on MMP-2 mRNA expression levels were examined. Following exposure to cyclic strain as described above, MMP-2 mRNA expression was monitored by Real-Time PCR. Fig. 3.6 illustrates that 10% strain increased MMP-2 mRNA expression levels by 1.7±0.37 fold compared to unstrained controls.

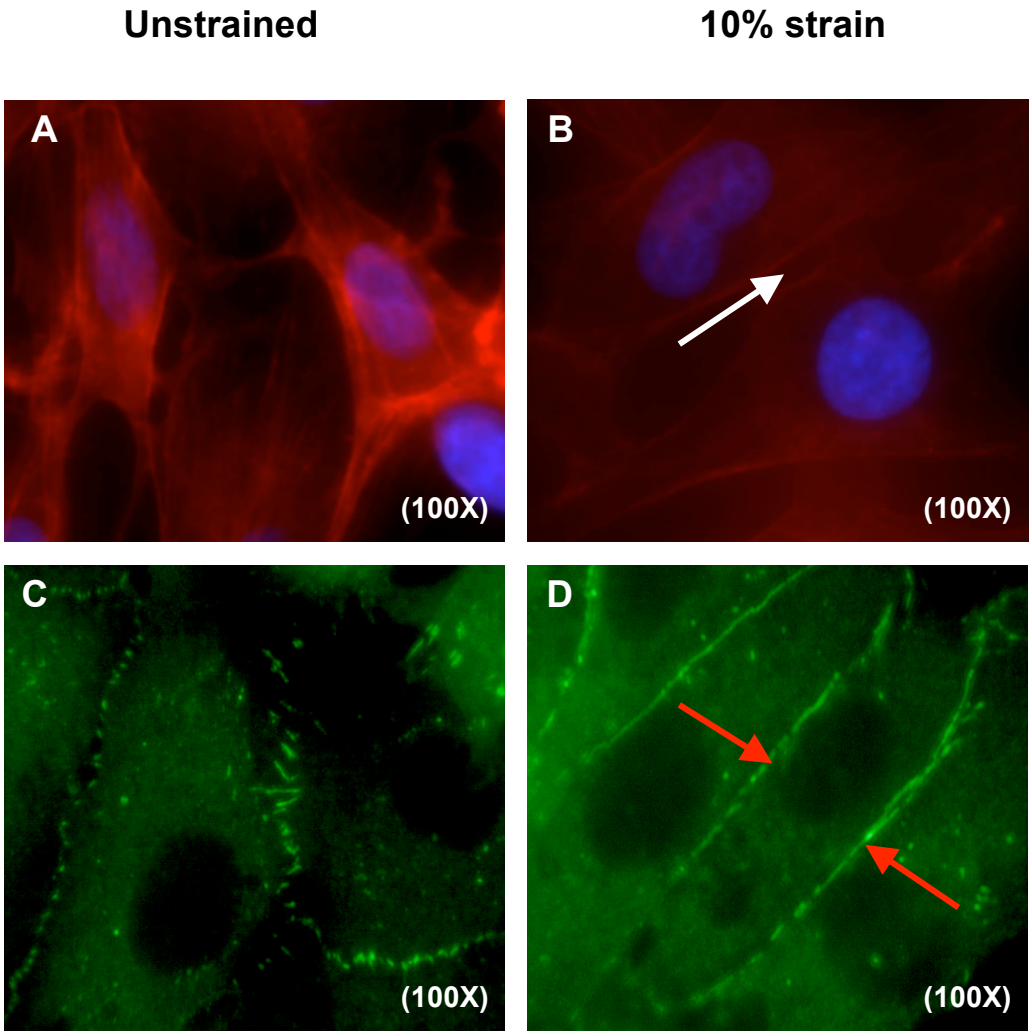


Fig. 3.5: BAEC response to cyclic strain. Following BAEC exposure to cyclic strain (5-10%, 24 h), changes in cellular morphology and cytoskeletal remodelling were monitored. Rhodmaine-Phalloidin staining for F-actin in; (A) unstrained control cells and (B) strained cells. Solid white arrow indicates cortical actin formation. Cell-cell border localization of ZO-1 in; (C) unstrained control cells and (D) strained cells. Red arrows displays ZO-1 cell-cell border localization. DAPI nuclear staining is shown in blue. Images are representative of 3 experiments.

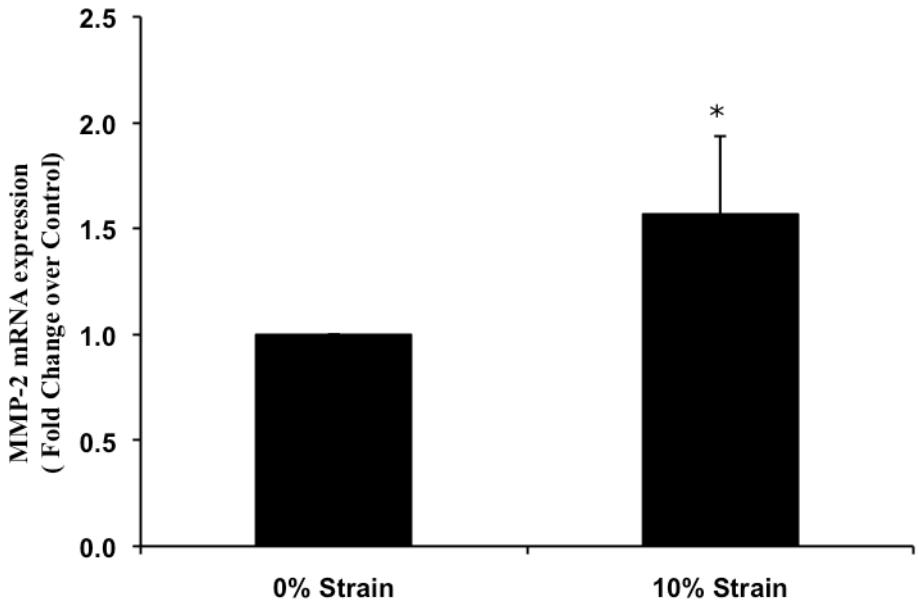


Fig. 3.6: Strain-dependent up-regulation of MMP-2 mRNA expression. Following BAEC exposure to equibiaxial cyclic strain (10% strain, 24 h), changes in MMP-2 expression levels were monitored. Histogram represents fold change in mRNA expression level (1.7±0.37 fold) compared to unstrained control (0%) and is averaged from three independent experiments (±SEM). *$P \leq 0.05$ relative to unstrained control.

3.3 Discussion

Our initial studies investigated the basic characteristics of endothelial and smooth muscle cell lines obtained from commercial sources. Both vascular endothelial and smooth muscle cells were found to exhibit expected morphological characteristics under static conditions and to express cell-specific markers (e.g. vWF and α-actin), the latter monitored by immunocytochemistry.

The responsiveness of BAECs to shear stress and cyclic strain, the hemodynamic forces under study, were also examined. Exposure of BAECs to laminar shear stress (10 dynes/cm^2, 24 h) induced a gross morphological realignment of cells in the direction of flow. In parallel with this, F-actin realignment and cortical actin formation in response to shear were also observed, indicating the importance of cytoskeletal reorganisation in flow-dependent endothelial responses (Colgan *et al*., 2007; Birukov *et al.*, 2002). Furthermore, we monitored endothelial barrier function in response to shear stress. ZO-1, a tight junction-associated protein, appears highly discontinuous with long finger-like projections in unsheared cells. Following shear however, it becomes more continuous and linearly distributed along the cell-cell border, consistent with tight junction assembly and up-regulation of barrier function (Colgan *et al.*, 2007). Regulation of endothelial barrier function by circulatory stimuli such as shear is crucial for maintaining homeostasis, as barrier disruption can lead to vascular diseases (Colgan *et al*., 2007). We also investigate the effect of shear stress on eNOS, a well characterized shear stress-responsive enzyme in endothelial cells. After 24 h of shear stress, both mRNA and protein expression levels of eNOS

increased significantly, an effect that has been observed in both *in vitro* and *in vivo* models (Searles *et al*., 2006).

Endothelial cells exposed to cyclic strain (10%, 24 h) also exhibited a degree of actin remodelling, as F-actin stress fibre reduction and cortical actin formation was observed in response to strain. Cyclic strain also enhanced localization of endothelial ZO-1 at the cell-cell border. These findings correlate to other results from our laboratory, which demonstrate that physiological levels of cyclic strain can up-regulate the expression and assembly of tight junction proteins (occludin and ZO-1) in BAECs (Collins *et al*., 2006). We also investigated the response of MMP-2 mRNA after exposure of BAECs to cyclic strain (10%, 24h). MMPs are involved in ECM degradation and vessel remodelling. Studies have shown that cyclic strain of vascular endothelial cells can up-regulate MMP-2 mRNA (Grote *et al*., 2003; von Offenberg Sweeney *et al*., 2004). This correlates with our own findings demonstrating cyclic strain-induced up-regulation of MMP-2 mRNA. It should also be noted, previous studies from our laboratory have shown that cyclic strain increases BAEC migration and tube formation (von Offenberg Sweeney *et al.*, 2005), with apparent roles for MMP-2 and MMP-9 in these events. Physiological levels of cyclic strain have also been shown to increase proliferation, whilst reducing apoptosis (Iba *et al.*, 1991; Li and Sumpio, 2005; Haga *et al*., 2003; Liu *et al*., 2003).

In conclusion this chapter has confirmed morphological characteristics of the commercially procured vascular endothelial and smooth muscle cells (bovine and human) to be used for subsequent investigations. It also confirmed the expression of

cell-specific markers for endothelial and smooth muscle cultures. Finally, it demonstrated the "mechano-responsiveness" of endothelial cells (specifically BAECs) with respect to cellular morphology, cytoskeletal remodelling, and mechano-sensitive gene (eNOS, MMP-2) expression. The following chapters will investigate how variable hemodynamic challenges to endothelial cells putatively impacts smooth muscle cell growth properties.

CHAPTER 4

EC Hemodynamic Challenge –

Impact on SMC Growth Properties

4.1 Introduction

Vascular smooth muscle cells and endothelial cells are the major cellular components of the vessel wall. Vascular endothelial cells, which provide an interface between circulating blood and the vessel wall, are constantly subjected to mechanical forces associated with blood flow and play a pivotal role in maintaining vessel structure and function. The endothelium is exposed to two principal hemodynamic forces, laminar shear stress and cyclic strain. Shear stress is the frictional force generated by blood flow and cyclic strain is the transmural pressure exerted perpendicularly to the direction of flow (the latter stemming from the pulsatile nature of cardiac output). Under physiological conditions hemodynamic forces impart an atheroprotective effect. However, alteration in the magnitude and pattern of hemodynamic challenge can favour endothelial dysfunction leading to pathological changes in vessel structure associated with vascular diseases such as atherosclerosis. Endothelial cells (and SMCs) can detect and respond to hemodynamic forces. Indeed, the previous chapter demonstrated endothelial cell responsiveness to both shear stress and cyclic strain. Moreover, force-dependent (and independent) EC-SMC interactions likely play an important role in cellular communication in the regulation of vascular remodeling. In this regard, a number of studies have demonstrated the importance of endothelial cells in regulating smooth muscle cell functions such as proliferation, migration and apoptosis.

The aim of this chapter was to investigate how different types of hemodynamic challenge to BAECs may impact on BASMC proliferation and apoptosis.

In order to investigate this, a number of model systems were employed. These included; (i) orbital rotation model (laminar shear stress), (ii) CELLMAX® Artificial Capillary System (pulsatile shear), and a (iii) Flexercell® Tension Plus™ FX4000T™ System (cyclic strain). Initially, we examined how physiological levels of laminar shear stress on endothelial cells impact on smooth muscle cell proliferation and apoptosis in a force- and time-dependent manner using an established orbital rotation model to generate BAEC-conditioned media (BCM) for studies with BASMCs. As a control experiment, we also examined turbulent shear using an improvised model (back-forth shaking). A perfused transcapillary co-culture system (CELLMAX® Artificial Capillary System) was also employed to investigate how pulsatile laminar shear of BAECs impacts on BASMC proliferation and apoptosis. With this system, BAECs were seeded into the intra-lumenal space (ILS) of the Pronectin®-coated capillary bundle and BASMCs seeded onto the extracapillary surface (ECS). This model much more accurately mimics the three-dimensional hemodynamic environment of the blood vessel, allows for application of higher shear rates (with pulsatility) than orbital rotation, and enables dual direction cell-cell communication (Redmond *et al*., 1995). In addition to shear stress the endothelium is chronically exposed to cyclic circumferential strain. Like shear it plays an important role in physiological control of vascular tone, remodelling, and associated pathologies. Previous studies in our lab have demonstrated that incubation of BASMCs with cyclic strain-derived BCM significantly decreased BASMC migration via a putative mechanism involving MMP-2 (von Offenberg Sweeney *et al*., 2004). We therefore decided to revisit this hemodynamic model as part of our studies using the Flexercell® Tension Plus™ FX4000T™ System.

4.2 Results

4.2.1 Laminar shear-derived BCM decreases BASMC proliferation in a time- and force-dependent manner

We first examined the effect of BAEC-conditioned media (BCM) on BASMC proliferation following BAEC exposure to laminar shear stress (LSS) of varying magnitude. BAECs were exposed to varying levels of laminar shear stress (0, 2.5, 5, and 10 dynes/cm²) for 24 h. BASMCs were subsequently incubated with shear-derived BCM and their proliferation monitored. Hemocytometer cell counts clearly indicated shear-dependent decreases in BASMC proliferation by 12±0.5% and 20±0.7% at 5 and 10 dynes/cm², respectively ($P \leq 0.05$) (Fig. 4.1 A,B). Shear-dependent decreases in BASMC proliferation were also confirmed by FACS analysis (Fig. 4.1C).

We next investigated if this anti-proliferative effect on BASMCs was dependent on the duration of BAEC shear. The effect of BCM on BASMC proliferation following BAEC exposure to constant laminar shear stress (10 dynes/cm²) of varying duration (24 and 48 h) was examined. Hemocytometer cell counts showed a shear-dependent decrease in BASMC proliferation by 19±1.6% and 34±0.7% at 24 and 48 h, respectively ($P \leq 0.05$) (Fig 4.2 A,B). A temporal response was also confirmed by FACS analysis (Fig 4.2 C).

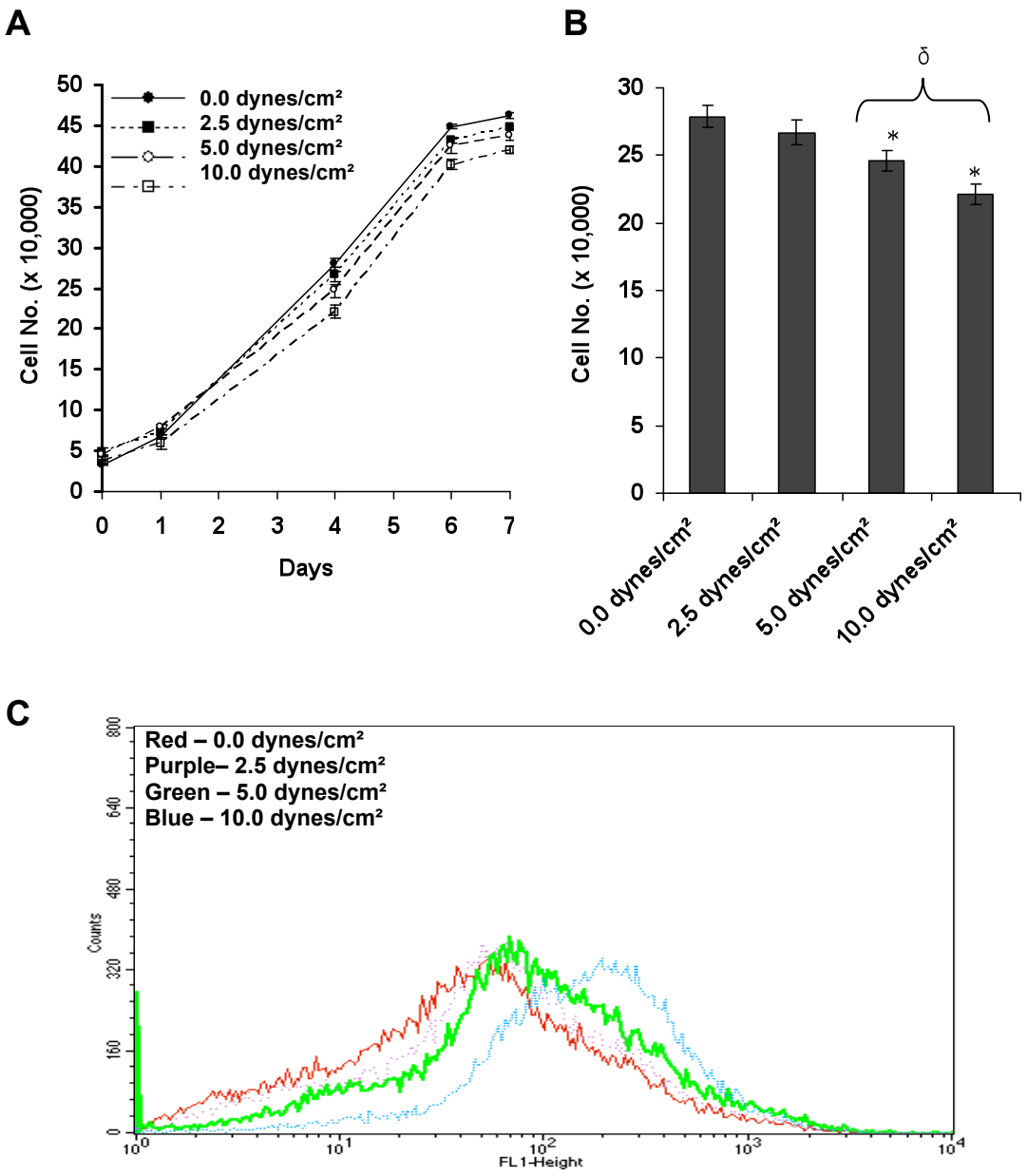


Fig. 4.1: Force-dependent effect of shear-derived BCM on BASMC proliferation. BAECs were exposed to varying levels of laminar shear stress (0-10 dynes/cm^2, 24 h). BCM was harvested and incubated with static BASMCs for up to 7 days to monitor effects on proliferation. Effects of BCM on BASMC proliferation as shown by cell counts; (A) over 7day period and (B) specifically highlighted for day 4. Day 4 shows decreases in BASMC proliferation by 12±0.5% and 20±0.7% at 5 and 10 dynes/cm², respectively. Corresponding FACS analysis taken for day 4 is also shown (C). Proliferation decreases as the peaks shift to the right. All data are averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared control. $^{\delta}P$≤0.05 versus 5 dynes/cm^2**.** FACS scan are representative of 3 experiments.

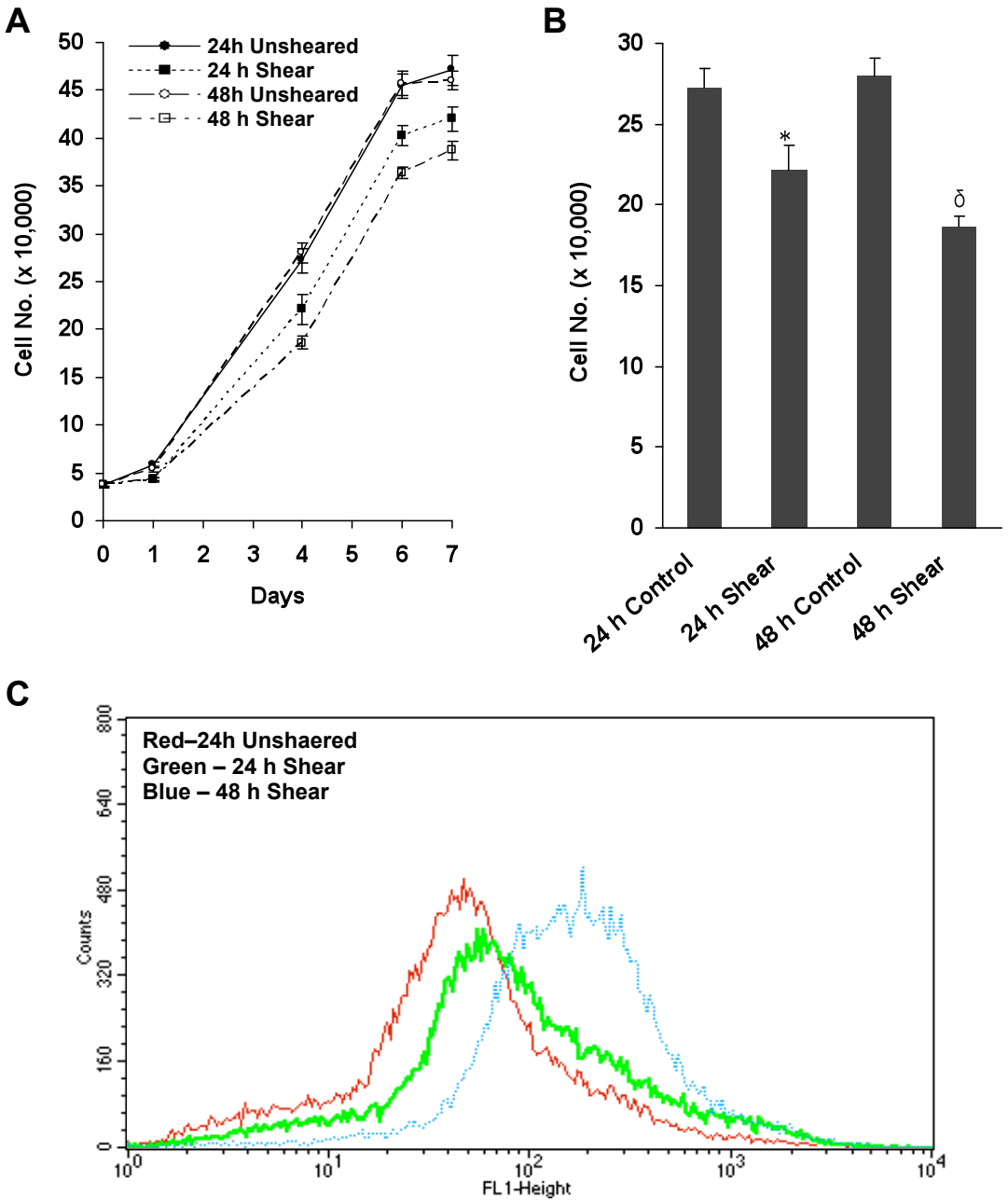


Fig. 4.2: Time-dependent effect of shear-derived BCM on BASMC proliferation. BAECs were exposed to a constant level of laminar shear stress (10 dynes/cm^2) for varying times (0, 24, and 48 h). BCM was harvested and incubated with static BASMCs for up to 7 days to monitor effects on proliferation. Effects of BCM on BASMC proliferation as shown by cell counts; (A) over 7day period and (B) specifically highlighted for day 4. Day 4 shows a decrease in BASMC proliferation by 19±1.6% and 34±0.7% at 24 and 48 h, respectively. Corresponding FACS analysis taken for day 4 is also shown (C). Proliferation decreases as the peaks shift to the right. All data are averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared control. $^{\delta}P$≤0.05 versus 24 h shear. FACS scan are representative of 3 experiments.

4.2.2 Laminar shear-derived BCM increases BASMC apoptosis in a time- and force-dependent manner

We next determined the impact of BCM on BASMC apoptosis following BAEC exposure to laminar shear stress of varying force (0-10 dynes/cm²) for 24 h. Our results indicated an increase in apoptosis from 3.84±0.4% in unsheared controls to 5.94±0.3% and 9.24±1.1% at 5 and 10 dynes/cm², respectively ($P \leq 0.05$) (Fig. 4.3 A,B). Results also indicated an apparent dose-dependent increase in caspase-3 activity (monitored in BASMC lysates as an alternate apoptotic index) (Fig. 4.3 C). Necrotic levels in all samples were very low ranging from 0-0.5%.

We next investigated if the pro-apoptotic effect on BASMC was dependent on the time of BAEC shear. To this end, the effect of BCM on BASMC apoptosis following BAEC exposure to constant laminar shear stress (10 dynes/cm²) of varying duration (24 and 48 h) was examined. Our results showed increases in apoptosis by 1.24±0.8 fold and 1.97±0.8 fold after 24 and 48 h of shear, respectively, relative to unsheared 24 and 48 h controls ($P \leq 0.05$) (Fig. 4.4 A, B). Time-dependent increases in BASMC caspase-3 activity were also observed after 24 and 48 h shear, as shown in Fig. 4.4 C. Necrotic levels in all samples were very low ranging from 0-0.8%.

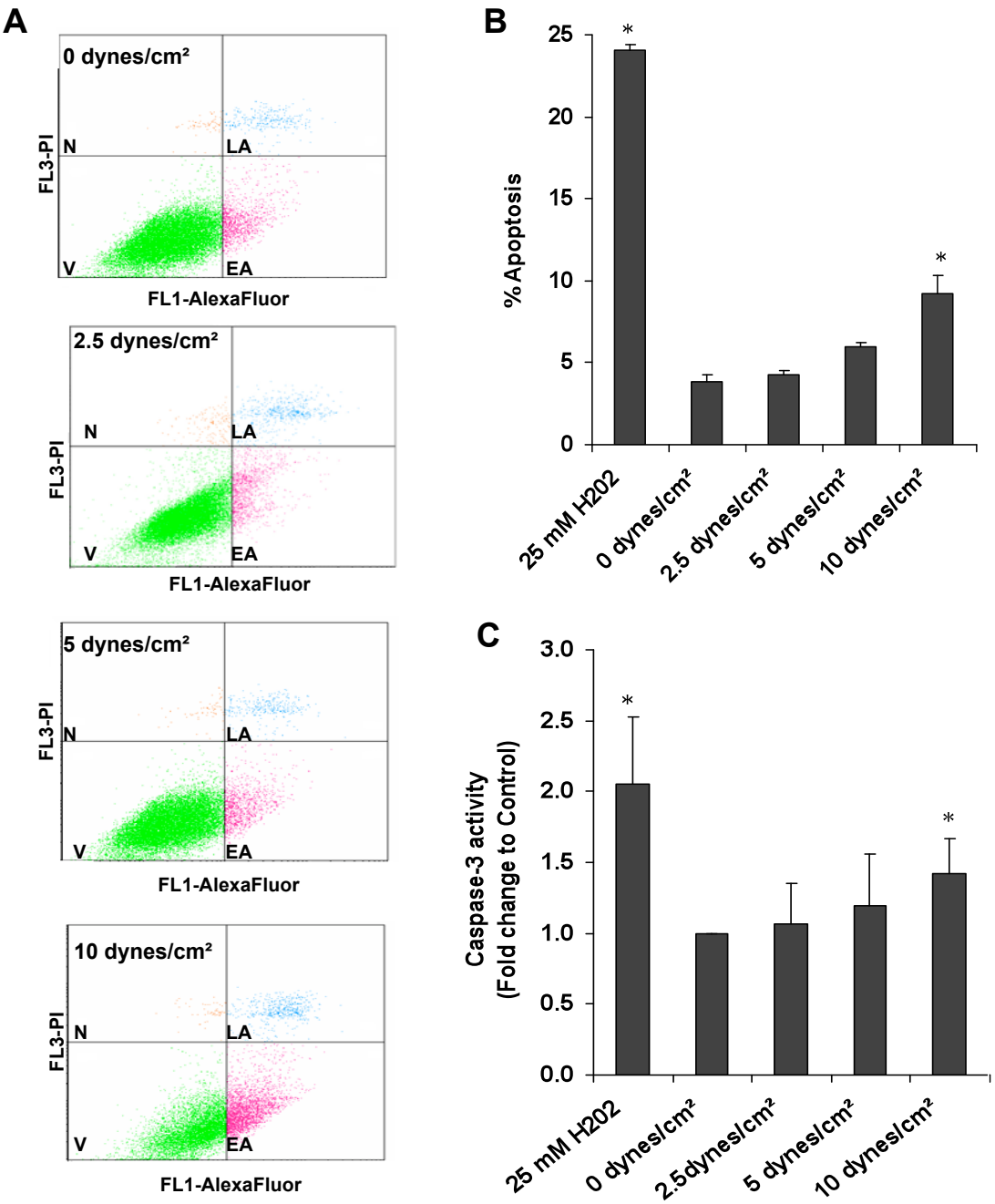


Fig. 4.3: Force-dependent effect of shear-derived BCM on BASMC apoptosis. BAECs were exposed to varying levels of laminar shear stress (0-10 dynes/cm^2, 24 h). BCM was harvested and incubated with static BASMCs for up to 4 days to monitor effects on apoptosis. Effect of BCM on BASMC apoptosis as shown by FACS analysis (A) with corresponding histogram at day 4 (B). An increase in apoptosis was observed from 3.84±0.4% in unsheared controls to 5.94±0.3% and 9.24±1.1% at 5 and 10 dynes/cm², respectively. FACS measures EA (early apoptosis) and LA (late apoptosis). Caspase-3 activity is shown in (C). 25 mM hydrogen peroxide (H_2O_2) is included in (B, C) as a positive control for apoptotic induction. All data are averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared control. FACS scans are representative of 3 experiments.

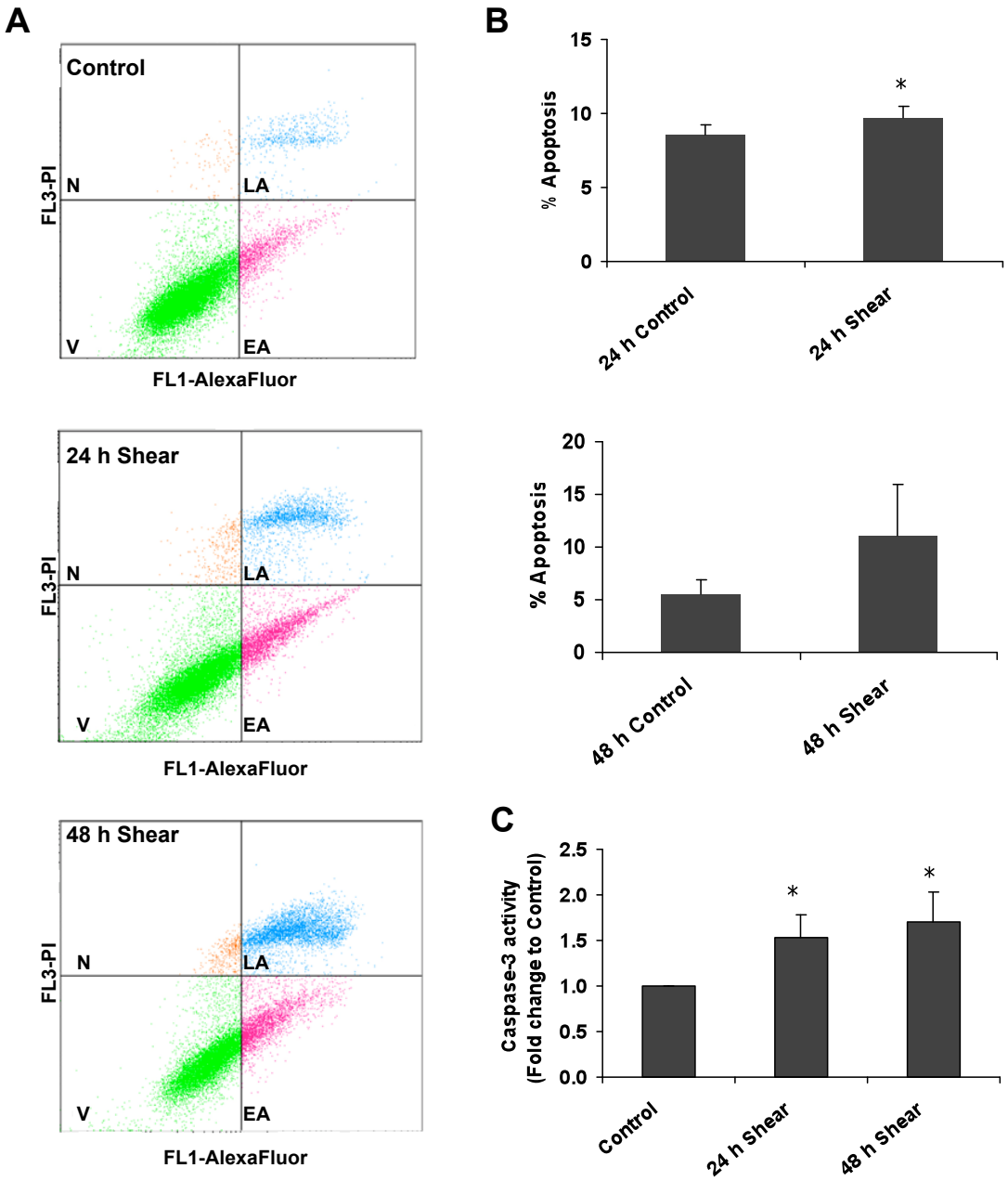


Fig. 4.4: Time-dependent effect of shear-derived BCM on BASMC apoptosis. BAECs were exposed to constant laminar shear (10 dynes/cm^2) for 24 and 48 h. BCM was harvested and incubated with static BASMCs for up to 4 days to monitor effects on apoptosis. Effect of BCM on BASMC apoptosis as shown by FACS analysis (A) with corresponding histogram at day 4 (B) (Note: 48 h shear control is not shown). An increase in apoptosis was observed by 1.24±0.8 fold and 1.97±0.8 fold after 24 and 48 h. FACS measures EA (early apoptosis) and LA (late apoptosis). Caspase-3 activity is shown in (C). All data are averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared control**.** FACS scans are representative of 3 experiments.

4.2.3 Putative impact of turbulent shear stress (TSS) on BASMC proliferation and apoptosis

As a control experiment, we also looked at the effect of TSS-derived BCM (24 h) on BASMC proliferation and apoptosis. In the absence of a cone-plate viscometer, a relatively crude model (back-forth shaking) was used to generate a moderate level of turbulence. Unlike laminar shear stress, turbulent shear typically induces an "atherogenic" effect *in vivo* (Traub and Berk, 1998). In response to TSS, BASMCs displayed an increase in proliferation by 10±0.4% ($P \leq 0.05$) (Fig. 4.5 A,B), and a small decrease, albeit statistically insignificant, in apoptosis (from 4.27±0.6% to 3.86±0.7%) (Fig 4.5 C,D).

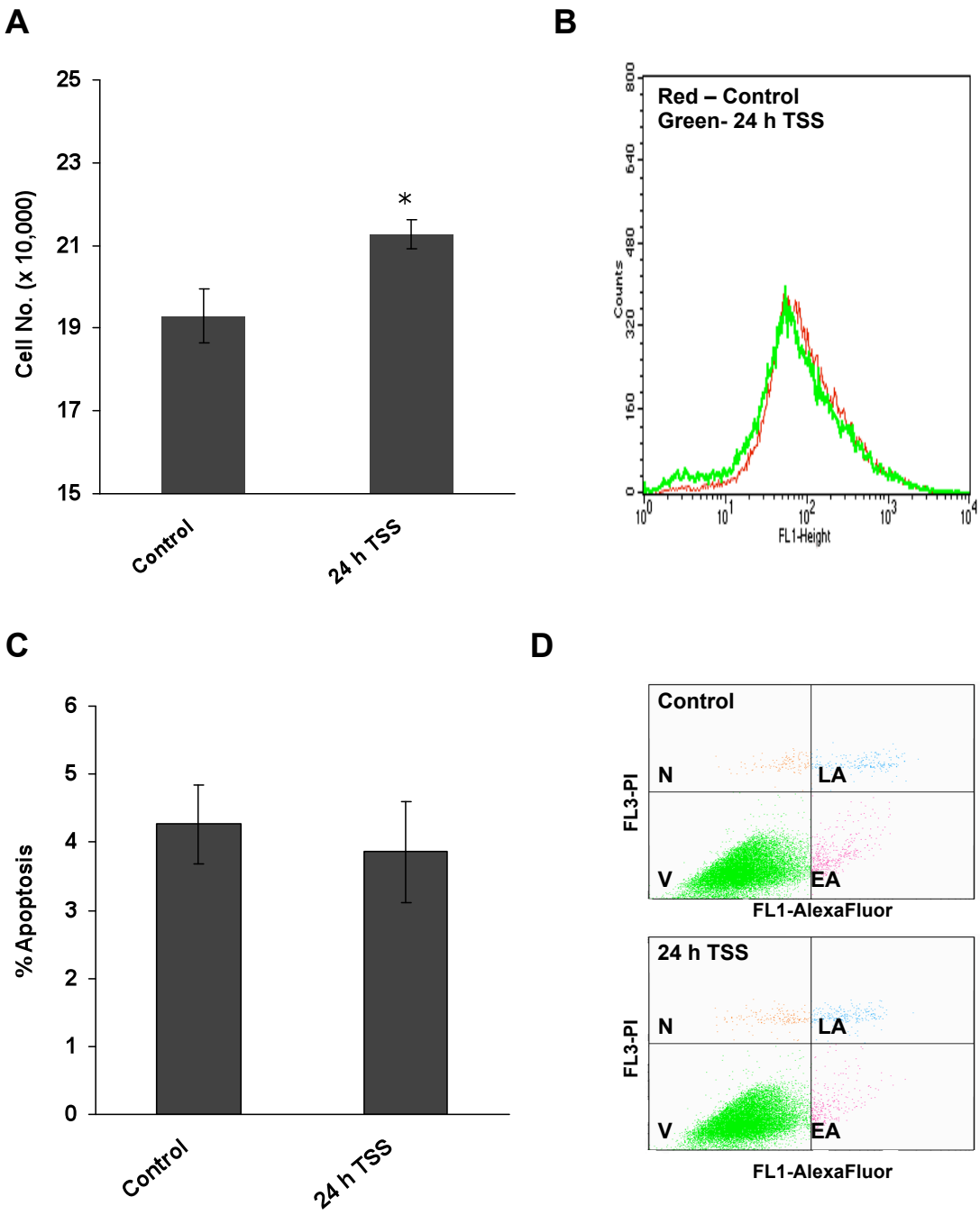


Fig. 4.5: Effect of TSS-derived BCM on BASMC proliferation and apoptosis. BAECs were exposed to a moderate level of TSS (24 h). BCM was harvested and incubated with static BASMCs for up to 7 days to monitor effects on proliferation and apoptosis. Effect of BCM on BASMC proliferation at day 4 as shown by cell counts (A) with corresponding FACS analysis at day 4 (B) (Increase by 10±0.4%). FACS measures EA (early apoptosis) and LA (late apoptosis). Effect of BCM on BASMC apoptosis as shown by histogram (C) with corresponding FACS analysis at day 4 (D) (from 4.27±0.6% to 3.86±0.7%). All data are averaged from three independent experiments ±SEM. *$P \leq 0.05$ versus non-turbulent control. FACS scans are representative of 3 experiments.

4.2.4 BAEC pulsatile shear decreases BASMC proliferation in a perfused co-culture model

For these studies, BAECs were seeded into the intra-lumenal spaces of the capillary bundles, whilst BASMCs were seeded onto the extracapillary surface of the capillary bundle, thereby mimicking the 3-D hemodynamic environment of a blood vessel. In this model, direct EC-SMC contact was prevented by the thickness of the capillary wall (~150 μM), although the porous nature of the wall allowed for humoral cross-talk between the two cell lines. Following a short ramping-up period, intra-luminal media flowrate was adjusted to give a shear level of 20 dynes/cm^2 (high shear) and maintained at this level for 5 days. Parallel low shear co-cultures were also maintained at 0.3 dynes/cm^2 (just enough media flow to ensure adequate perfusion). BASMC-only controls were included in all experiments (i.e. BAECS were excluded from the ILS in order to correct for the putative effects of hydrostatic pulse-pressure on BASMC proliferation and apoptosis).

Post-shear (5 days), BASMCs were harvested for analysis of proliferation in normal growth media. Using hemocytometer cell counting, we observed a significant decrease (approx. 42±0.9% at day 4) in the proliferation rate of harvested BASMCs maintained under high shear co-culture with BAECs ($P \leq 0.05$) (Fig. 4.6 A,B). This decrease was also confirmed by FACS analysis of harvested BASMCs (i.e. BASMCs were pre-labelled with carboxyfluorescein diacetate (CFDA-SE) *before* being seeded into the extra-capillary space) (Fig. 4.6 C). Noteworthy, in the absence of BAECs, we observed a slight increase in BASMC proliferation under high flow (also determined by direct FACS analysis) (Fig. 4.6 D).

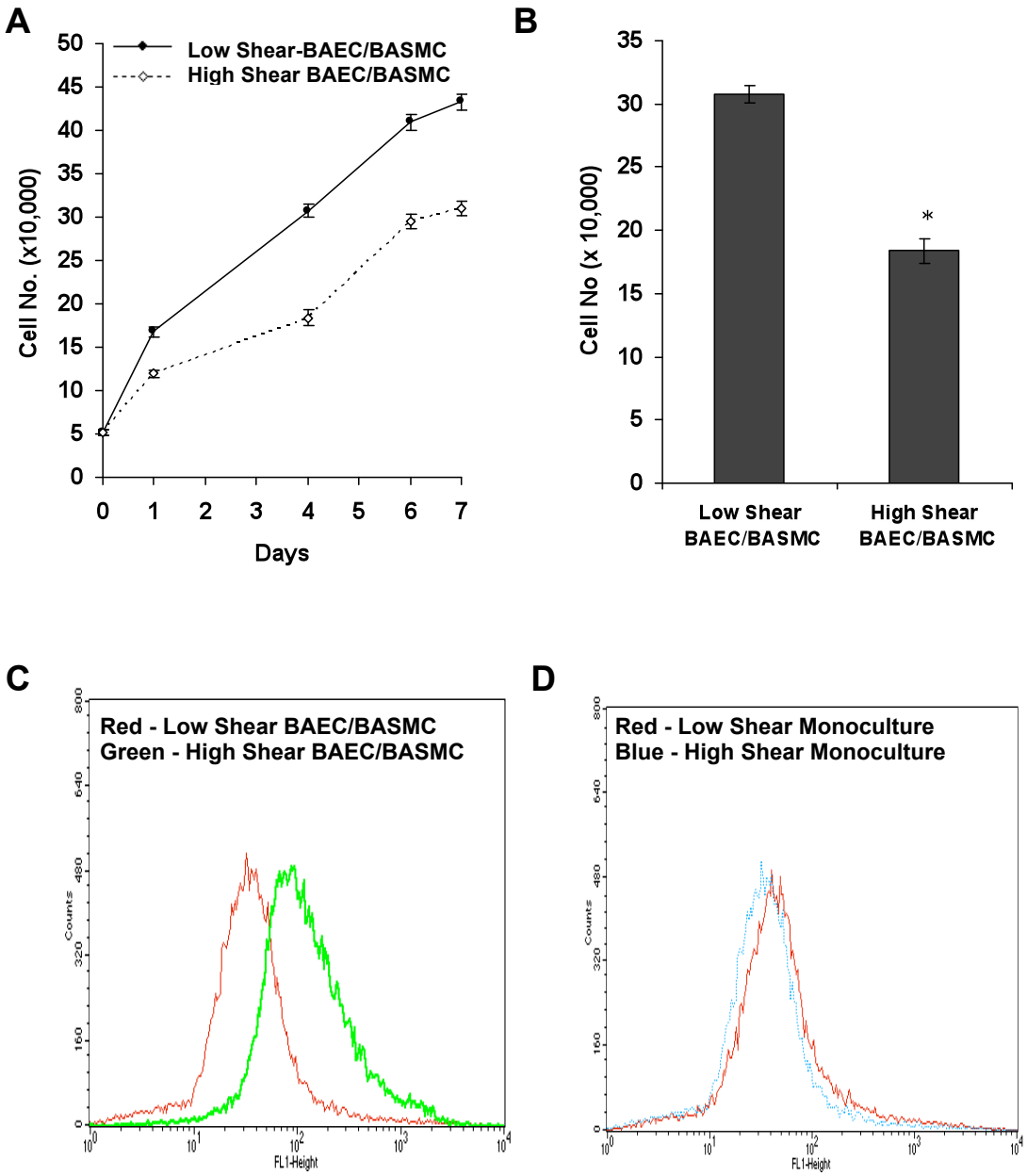


Fig. 4.6: BAEC/BASMC co-culture: Impact of pulsatile shear on BASMC proliferation. BASMCs co-cultured with BAECs under low (0.3 dynes/cm^2) and high shear (20 dynes/cm^2) conditions for 5 days. Post shear, harvested BASMCs were seeded onto 6-well plates (5x10^4 cells/well) and monitored for proliferation by cell counts for up to 7 days. Effects of shear on BASMC proliferation; (A) monitored over 7 days and (B) highlighted specifically for day 4 (decrease of 42±0.9%). Post-shear, CFDA-SE pre-labelled BASMCs were also harvested for direct FACS analysis of the co-culture model (C) and mono-culture (D). Proliferation decreases as the peaks shift to the right. All data are averaged from three independent experiments ±SEM. *$P \leq 0.05$ versus low shear co-culture. FACS scan is representative of 3 experiments.

4.2.5 BAEC pulsatile shear increases BASMC apoptosis in a perfused co-culture model

We next examined how shear stress of BAECs impacts on BASMC apoptosis. BAECs were seeded into the intra-lumenal spaces of CELLMAX® Pronectin-coated capillary bundles, whilst BASMCs were seeded onto the extracapillary surface of the capillary bundle, thereby mimicking the 3-D hemodynamic environment of a blood vessel. Following a short ramping-up period, intra-luminal media flowrate was adjusted to give a shear level of 20 dynes/cm^2 (high shear) and maintained at this level for 5 days. Parallel low shear co-cultures were also maintained at 0.3 dynes/cm^2 (just enough media flow to ensure adequate perfusion). BASMC-only controls were included in all experiments (i.e. BAECS were excluded from the ILS in order to correct for the putative effects of hydrostatic pulse-pressure on BASMC proliferation and apoptosis).

Post shear, BASMCs were harvested and monitored for apoptosis in normal growth media. Using FACS analysis, we observed an increase in apoptosis (2.8±0.6% to 6.9±0.5%) for BASMCs maintained under high shear co-culture with BAECs ($P \leq 0.05$) (Fig. 4.7 A,B). A significant increase in apoptosis was also confirmed by monitoring caspase-3 activity in harvested BASMCs ($P \leq 0.05$) (Fig. 4.7 C). Noteworthy, in the absence of BAECs, we observed similar increases in BASMC apoptosis as monitored by FACS analysis and caspase-3 assay (data not shown).

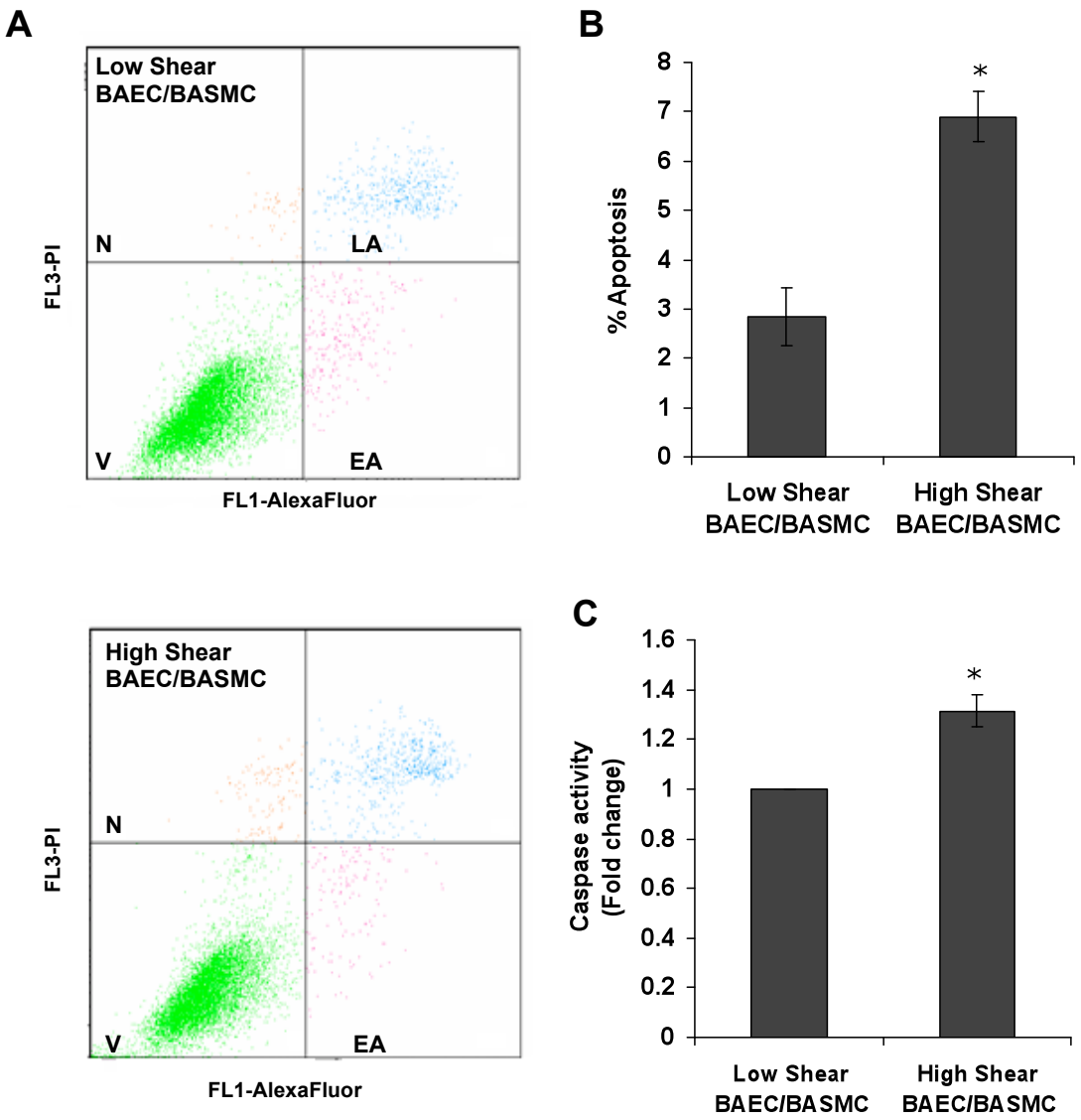


Fig. 4.7: BAEC/BASMC co-culture: Impact of pulsatile shear on BASMC apoptosis. BASMCs co-cultured with BAECs under low (0.3 dynes/cm^2) and high shear (20 dynes/cm^2) conditions for 5 days. Post shear, harvested BASMCs were seeded onto 6-well plates and monitored for apoptosis for up to 7 days. Effects of shear on BASMC apoptosis; (A) by FACS analysis with (B) corresponding histogram at day 4 (an observed increase in apoptosis - 2.8±0.6% to 6.9±0.5%). FACS measures EA (early apoptosis) and LA (late apoptosis). Caspase-3 activity is shown in (C). All data are averaged from three independent experiments ±SEM. *P≤0.05 versus low shear co-culture. FACS scans are representative of 3 experiments.

4.2.6 Pulsatile shear-derived BCM decreases BASMC proliferation

We next investigated the effect on BASMC proliferation of BCM (obtained from low (0.3 dynes/cm^2) and high shear (20 dynes/cm^2) CELLMAX® media reservoirs following "co-culture" experimentation). BCM was harvested and subsequently incubated with BASMCs. Proliferation was then monitored over 7 days by hemocytometer cell counts and FACS analysis. There was a shear-dependent increase in proliferation by 10%±0.75% compared to low shear. The pulsatile shear-dependent increase in proliferation was reversed by 12.5±1.03% in BASMCs in the presence of BAECs at high shear stress ($P \leq 0.05$) (Fig. 4.8 A,B, C).

4.2.7 Pulsatile shear-derived BCM increases BASMC apoptosis

In tandem with the above study, the effect on BASMC apoptosis of BCM (obtained from low - 0.3 dynes/cm^2 and high - 20 dynes/cm^2 CELLMAX® media reservoirs following co-culture experimentation) was also investigated. BCM was harvested and subsequently incubated with BASMCs.Apoptosis was then monitored by FACS analysis. The results showed a slight increase in BASMC apoptosis in response to high pulsatile shear derived BCM from a monoculture (2.42±0.63% compared to control 1.34%±0.52%) (data not shown). The presence of BAECs did not reverse this effect, but in a similar manner increased BASMC apoptosis as measured by FACS (2.42±1.17% compared to control 1.34%±0.52%) as shown in Fig. 4.9 A, B. This effect was also determined by Caspase-3 assay showing that PLS BCM in both the presence and absence of BAECs increased apoptosis in BASMC by (1.17±0.17 fold and 1.13±0.06 fold) as shown in Fig. 4.9 C.

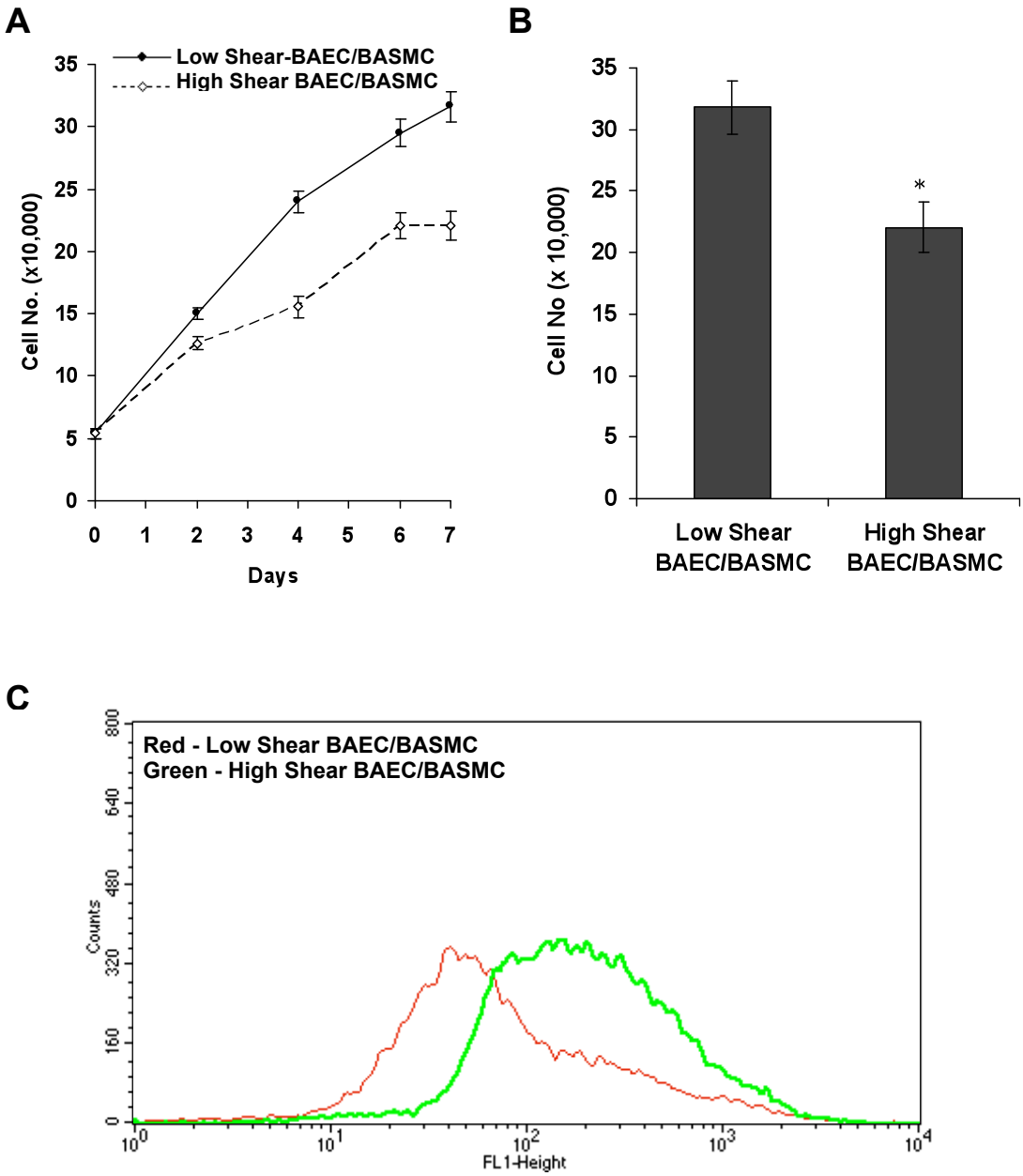


Fig. 4.8: BAEC/BASMC co-culture: Impact of pulsatile shear-derived BCM on BASMC proliferation. BASMCs co-cultured with BAECs under low (0.3 dynes/cm^2) and high shear (20 dynes/cm^2) conditions for 5 days. Post-shear, BCM was harvested and incubated with static quiescent pre-labeled BASMCs for up to 7 days to monitor proliferation. Effects of BCM on BASMC proliferation as shown by cell counts; (A) monitored over 7 days, and (B) highlighted specifically for day 4 (decrease by 12.5±1.03%). Corresponding FACS analysis specifically highlighted for day 4 is also shown (C). Proliferation decreases as the peaks shift to the right. All data are averaged from three independent experiments ±SEM. *P≤0.05 versus low shear co-culture. FACS scan is representative of 3 experiments.

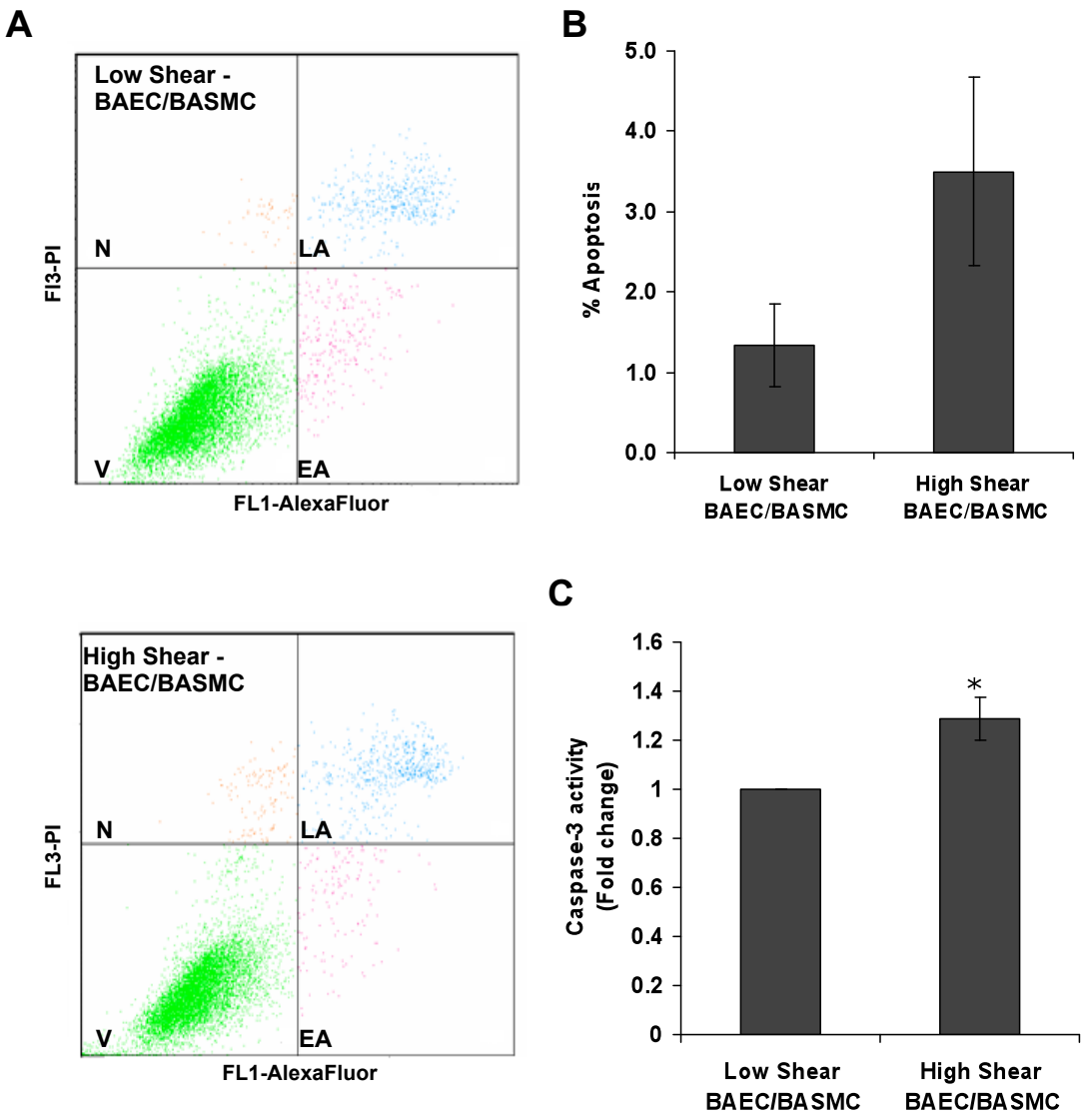


Fig. 4.9: BAEC/BASMC co-culture: Impact of pulsatile shear-derived BCM on BASMC apoptosis. BASMCs co-cultured with BAECs under low (0.3 dynes/cm^2) and high shear (20 dynes/cm^2) conditions for 5 days. Post-shear, BCM was harvested and incubated with static quiesced BASMCs for up to 7 days to monitor apoptosis. Effects of BCM on BASMC apoptosis; (A) by FACS analysis with (B) corresponding histogram at day 4. Increase in apoptosis by 2.42±1.17% compared to control 1.34%±0.52%. FACS measures EA (early apoptosis) and LA (late apoptosis). Caspase-3 activity is shown in (C). All data are averaged from three independent experiments ±SEM. *P≤0.05 versus low shear co-culture. FACS scans are representative of 3 experiments.

4.2.8 Cyclic strain-derived BCM decreases BASMC proliferation in a force-dependent manner

In this chapter, we investigated the effect of BCM on BASMC proliferation following BAEC exposure to equibiaxial cyclic strain of varying magnitude. BAECs were exposed to varying levels of cyclic strain (0-10% strain, 24 h) and the subsequent conditioned media (BCM) harvested and incubated with BASMCs. BASMC proliferation was monitored by hemocytometer cell counts and FACS analysis as previously described. Our results by hemocytometer cell counts indicated a significant decrease in BASMC proliferation for *both* 5 (16±2.06% at day 4) and 10% strain (15±2.56% at day 4) ($P \leq 0.05$) (Fig. 4.10 A,B). FACS analysis yielded similar findings (Fig. 4.10 C).

4.2.9 Cyclic strain-derived BCM increases BASMC apoptosis in a force-dependent manner

We next examined the impact of BCM on BASMC apoptosis following BAEC exposure to equibiaxial cyclic strain of varying magnitude (0-10% strain, 24 h). BASMCs were incubated with harvested BCM and monitored for apoptosis by FACS analysis. Our results indicate slight increases in apoptosis to 3.43±1.1% and 4.03±0.9% at 5% and 10% strain, respectively ($P \leq 0.05$) (Fig. 4.11). Necrosis levels in all samples were very low ranging from 0-0.9%.

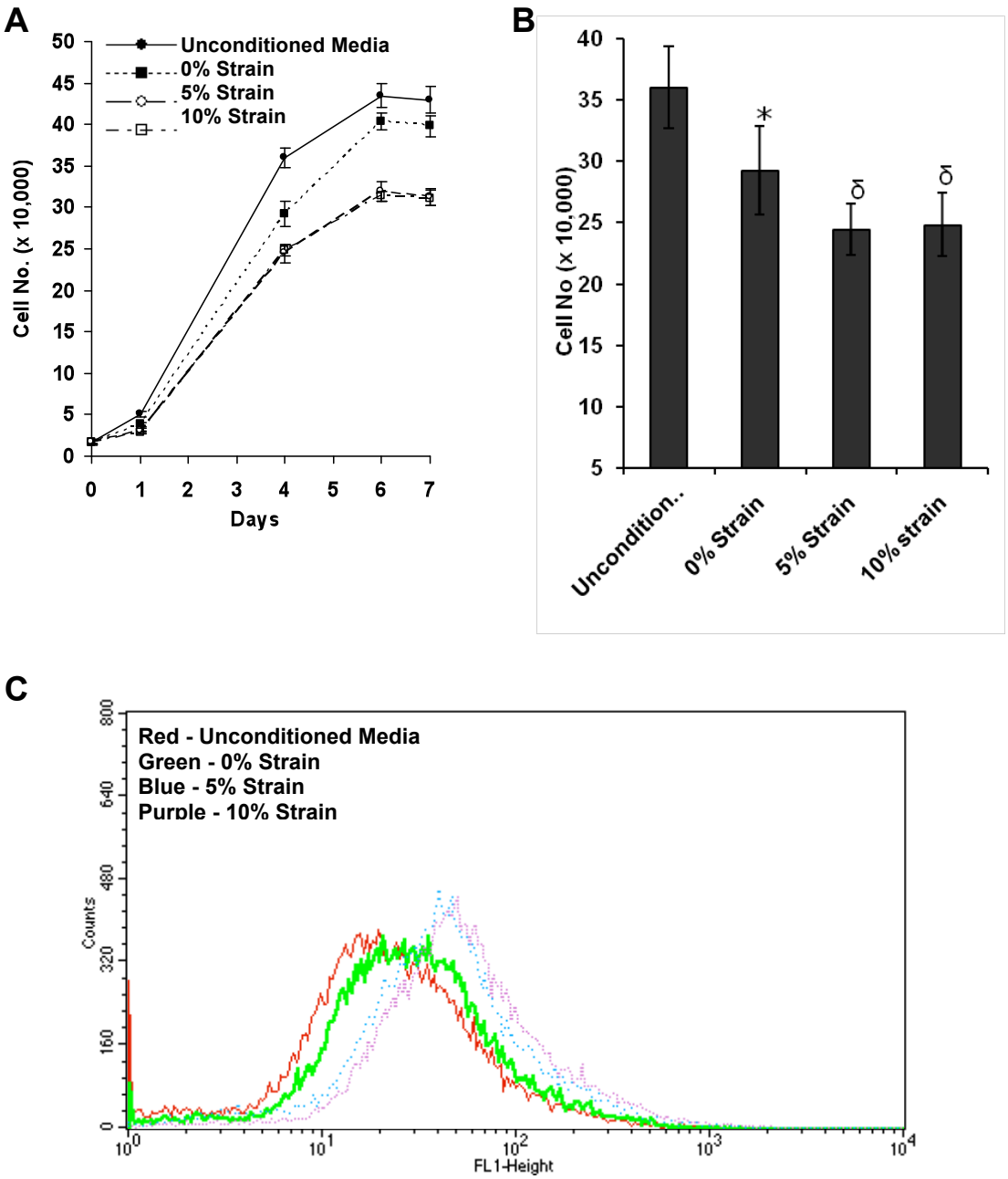


Fig. 4.10: Force-dependent effect of cyclic strain-derived BCM on BASMC proliferation. BAECs were exposed to varying levels of cyclic strain (0-10%, 24 h). BCM was harvested and incubated with static BASMCs for up to 7 days to monitor effects on proliferation. Effects of BCM on BASMC proliferation as shown by; (A) cell counts over 7-day period and (B) specifically highlighted for day 4 - decreases at 5% (16±2.06% at day 4) and 10% strain (15±2.56% at day 4). Corresponding FACS analysis specifically highlighted for day 4 is also shown (C). Proliferation decreases as the peaks shift to the right. All data is averaged from three independent experiments ±SEM. *$P \leq 0.05$ versus unsheared control of 3 experiments.

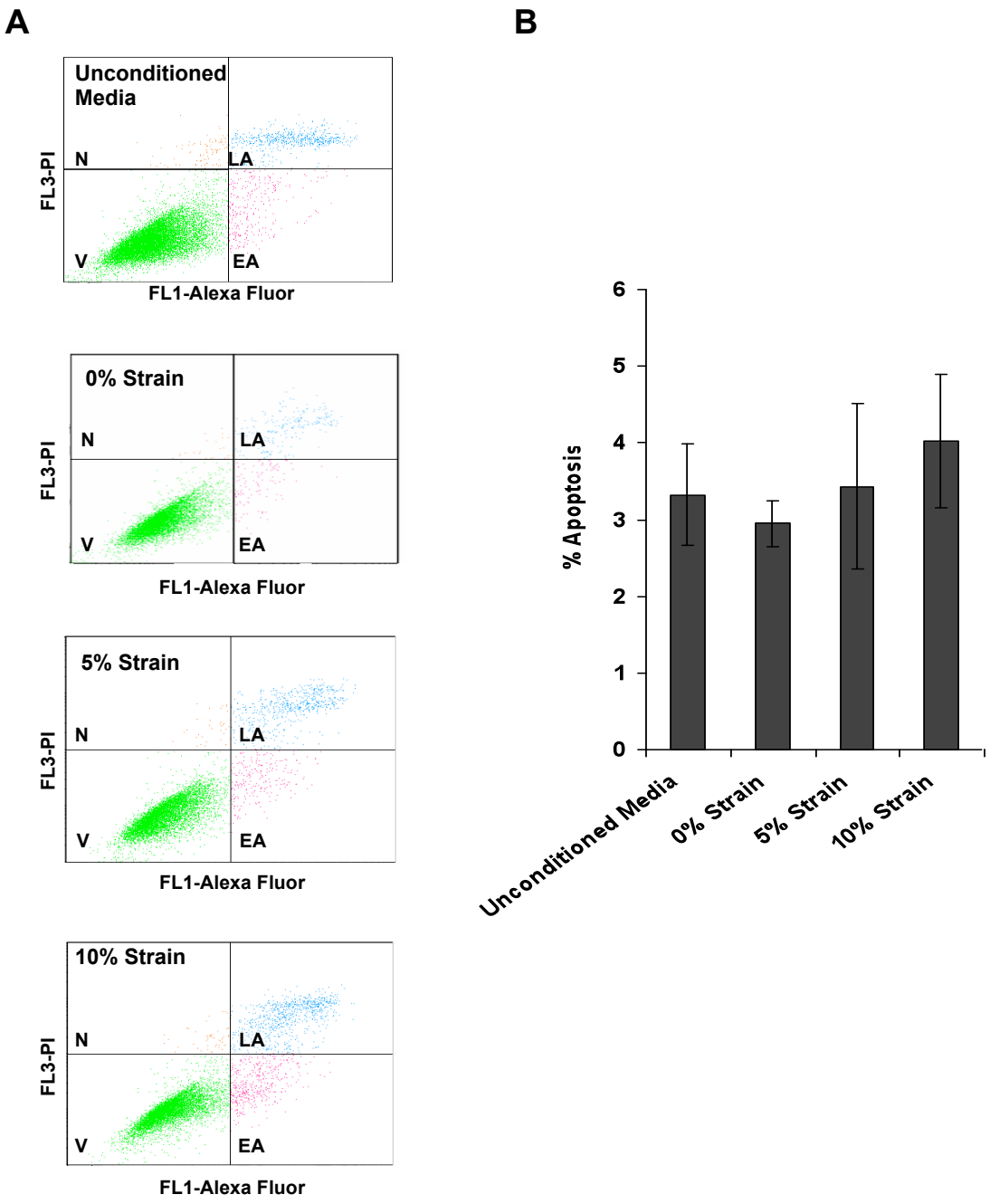


Fig. 4.11: Force-dependent effect of cyclic strain-derived BCM on BASMC apoptosis. BAECs were exposed to varying levels of cyclic strain (0-10%, 24 h). BCM was harvested and incubated with static BASMCs for up to 4 days to monitor effects on apoptosis. Effect of BCM on BASMC apoptosis as shown by; (A) FACS analysis with corresponding histogram at day 4 (B) (increases in apoptosis observed to 3.43±1.1% and 4.03±0.9% at 5% and 10% strain). FACS measures EA (early apoptosis) and LA (late apoptosis). All data is averaged from three independent experiments ±SEM. **P*≤0.05 versus 0% strain.

4.3 Discussion

Hemodynamic forces play an important role in physiological control of vascular tone, remodelling and associated pathologies. Moreover, these forces indirectly impact vascular smooth muscle cell fate decisions by regulating vascular endothelial signalling events. Disruption in blood flow can perturb the normal hemodynamic challenge to the vascular endothelial cell and result in adverse remodelling of the vessel wall. Suitable communication between endothelial and smooth muscle cells is therefore essential for maintaining proper vessel homeostasis. SMC proliferation and apoptosis are particularly important during vascular development. Indeed, dysregulated smooth muscle cell proliferation and apoptosis is an important contributor to the pathogenesis of several cardiovascular disease states such as atherosclerosis.

The first goal in this chapter was to ascertain, using a number of different experimental approaches, how shear stress challenge to endothelial cells impacts on BASMC proliferation and apoptosis. Initial investigations clearly demonstrated that LSS-derived BAEC-conditioned media (BCM) (0-10 dynes/cm^2, 24 h) decreased BASMC proliferation in a force-dependent manner. Whilst a shear rate of 2.5 dynes/cm^2 had no significant effect on BASMC proliferation, higher shear rates (5-10 dynes/cm^2) led to progressively higher decreases in BASMC proliferation. Inhibition of BASMC proliferation by LSS-derived BCM (10 dynes/cm^2, 0-48 h) was also found to be time-dependent. These findings are somewhat consistent with previous studies in our lab which have demonstrated an anti-migratory effect (approx. 35%) of cyclic strain BAEC-conditioned media on vascular smooth muscle cells (Von Offenberg

Sweeney *et al.*, 2004). In parallel with a decrease in BASMC proliferation, we also observed a BCM-dependent increase in BASMC apoptosis (both time- and force-dependent). Collectively, these findings point to a relationship between the magnitude and time of LSS applied to BAECs and the anti-proliferative/pro-apoptotic effects on BASMCs.

As a control experiment, we looked at the effect of turbulent shear stress (TSS) of BAECs on BASMC proliferation and apoptosis. LSS typically induces an atheroprotective effect *in vivo*, whilst TSS has the opposite effect (Traub and Berk, 1998). In areas of vascular bifurcation and curvature, where blood flow shear stress is low or oscillatory, vessel homeostasis becomes imbalanced leading to endothelial dysfunction. These areas are key locations for deleterious remodelling of the underlying medial smooth muscle cells leading to development of atherosclerotic plaques (Esper *et al*., 2006). Our studies have found that following exposure of BAECs to an improvised form of moderate turbulent shear for 24 h, the resultant BCM increased BASMC proliferation slightly without effecting BASMC apoptosis. Unsurprisingly, these TSS findings are in contrast with our observations of the effects induced by LSS-derived BCM, and are consistent with the notion that hemodynamic shear can serve as an *independent variable* to modulate the influence of endothelial cells on the proliferative and apoptotic profiles of underlying smooth muscle cells within the vessel wall. Moreover, these findings correlate somewhat with previous reports indicating that direct application of TSS to SMCs promotes SMC proliferation (Haga *et al*., 2003). Turbulent shear has also been shown to directly increase endothelial cell proliferation in contrast to the inhibitory effect of laminar shear (Kraiss *et al*., 2001; Dardik *et al*., 2005; Lin *et al*., 2000).

The orbital rotation/BCM model we employed had certain limitations; there was non-physiological geometry, it had a maximum shear capacity of only 10 dynes/cm^2, and it was a non co-culture format. Therefore we decided to employ a far more superior model system to investigate EC-SMC communication. A novel transcapillary co-culture system (CELLMAX® Artificial Capillary System) was employed to verify our previous BCM findings and to investigate how pulsatile shear stress of BAECs may impact on BASMC proliferation and apoptosis in a hemodynamic *co-culture* format. The advantage of this system is that it closely reflects the three-dimensional hemodynamic environment of a blood vessel (i.e. BAECs were seeded into the intra-lumenal capillary space of the Pronectin®-coated capillary bundles, whilst BASMCs were seeded onto the outer capillary walls), creating a more realistic mimic of EC-SMC co-regulation during hemodynamic challenge experiments. Higher physiological shear rates are also possible with this system.

Our initial findings with this system demonstrated that BASMC mono-cultures (i.e. BASMCs cultured alone on CELLMAX capillaries, in the absence of intra-luminal BAECs, under low and high shear) increased their proliferation under high pulsatile shear, most likely due to the elevated hydrostatic pulse pressure (Birney *et al.*, 2004). We next investigated the effect of BAEC/BASMC co-culture on BASMC proliferation following BAEC exposure to pulsatile shear at low and high rate for 5 days. BASMCs (pre-labeled with CFDA-SE) were then harvested and; (i) immediately subjected to FACS analysis and (ii) incorporated into cell count assays over 7 days to monitor their proliferation. Shear-derived BCM was also harvested from the media reservoir and incubated with static quiescent BASMCs (prepared separately) to monitor impact on proliferation. In each instance the high shear was

found to significantly reduce BASMC proliferation. As there was no direct EC-SMC contact in this co-culture model (and as BCM from the media reservoir was seen to exhibit an anti-proliferative effect), this likely points to the shear-induced endothelial release of a paracrine anti-proliferative factor(s). The identity of these factors has yet to be definitively established (although possible candidate molecules will be examined in the next chapter). These results support a role for the endothelium in "protecting" the underlying SMCs against hemodynamic forces. Such endothelium-mediated regulation of the smooth muscle cell can also be seen in other studies. Noteworthy is an early EC-SMC parallel plate co-culture study by Nackman *et al.* in which flow over ECs was seen to decrease SMC proliferation, as measured by [^{3}H]-thymidine uptake. Unfortunately, this study had a number of serious limitations to enable one to draw firm conclusions (e.g. lack of any SMC-only mono-culture flow controls, endothelial de-nudation from the membrane during flow, lack of any flow-dependent dose response effect etc.) (Nackman *et al.*, 1998). Another study by Sakamoto et al. has shown that during EC-SMC co-culture, shear stress decreases SMC migration and MMP-2 activity via flow-induced endothelial cell NO (Sakamoto *et al*., 2006). *In vitro* co-culture studies, using the CELLMAX® Artificial Capillary System, have also shown that pulsatile shear stress increases SMC migration, which is reversed by flow-induced endothelial cell PAI-1 and PDGF (Redmond *et al*., 2001; Palumbo *et al*., 2002). Pulsatile shear stress using the CELLMAX® Artificial Capillary System also regulates eNOS, cyclooxygenase, and G-protein expression in co-cultured SMCs and ECs (Redmond *et al*., 1998; Hendrickson *et al*., 1999). Furthermore, studies have shown that under hemodynamically "static" conditions, EC-SMC co-culture enhances vascular smooth muscle cell adhesion and spreading via

activation of β1-integrin and phosphatidylinositol 3-kinase/Akt pathways (Wang *et al.*, 2007).

Interestingly, whilst the anti-proliferative effect of high pulsatile shear on BASMCs was BAEC-dependent, the pro-apoptotic effects appeared to be BAEC-independent. This contrasts with the BAEC-dependent pro-apoptotic effects seen with orbital shear-derived BCM, although this may be explained by the presence of pulsatile pressure in the CELLMAX® model (which is absent in the orbital rotation model). Recent findings from our lab have already shown that pulsatile shear stress increased SMC apoptosis in an endothelial-independent manner (Birney *et al.*, 2004). It is therefore tempting to speculate that both conditioned media (BCM) and pulse pressure may be inducing the *same* pro-apoptotic pathway in a non-additive manner, with either stimulus inducing pathway saturation. Further experimentation will be required to properly address this issue in greater detail.

We next investigated how cyclic strain of BAECs may impact on BASMC proliferation. Our results showed that BCM harvested from endothelial cells exposed to cyclic strain significantly decreased BASMC proliferation. This is consistent with our earlier results showing anti-proliferative effects on BASMCs when BAECs are exposed to laminar shear stress (pulsatile and non-pulsatile). Moreover, it supports our hypothesis that hemodynamic challenge to the endothelium under physiological condition contributes to BASMC quiescence and medial layer integrity. Our cyclic strain results are also consistent with the findings of von Offenberg Sweeney and co-workers who demonstrate anti-migratory effects of BCM on BASMCs following chronic 5% strain (von Offenberg Sweeney *et al.*, 2004). Noteworthy, previous studies have also presented reports on the effects of cyclic strain applied directly to

SMCs on their growth *in vitro*. In this regard, findings have been conflicting. Depending on the species of SMC, the extracellular matrix environment, and the type of cyclical strain regime applied, SMCs can either increase (Birukov *et al*., 1995; Wilson *et al*., 1993) or decrease (Morrow *et al*., 2005; Schulze *et al*., 2003; Hipper and Isenerg 2000) their proliferative capacity. With regard to the latter, cyclic strain has been shown to decrease DNA synthesis of SMC and to inhibit SMC cycle progression at the G_1/S phase (Hipper and Isenerg, 2000; Chapman *et al*., 2000).

Cyclic strain-derived BCM also enhanced BASMC apoptosis. This is again consistent with our previous results showing pro-apoptotic effects on BASMCs when BAECs are exposed to laminar shear stress (pulsatile and non-pulsatile). It also concurs with several other studies that report how direct application of cyclic strain to SMCs induces SMC apoptosis *in vitro* both (Morrow *et al*., 2005; Kakasis *et al*., 2004; Mayr *et al*., 2002) and *in vivo* (Mayr *et al*., 2000; Wernig *et al*., 2002).

In conclusion, we have shown that hemodynamic challenge of BAECs impacts BASMC apoptosis and proliferation via paracrine factor(s). Using both endothelial-conditioned media and EC-SMC co-culture approaches, laminar shear stress of BAECs *in vitro* was observed to decrease BASMC proliferation, whilst increasing apoptosis. By contrast, moderate turbulent shear stress of BAECs increased BASMC proliferation with no effect on apoptosis. Similarly, treatment of BAECs with equibiaxial cyclic strain also decreased BASMC proliferation, whilst enhancing BASMC apoptosis. These findings confirm that blood flow-associated forces can serve as *independent variables* to modulate the influence of endothelial cells on the proliferative and apoptotic profiles of underlying smooth muscle cells within the

vessel wall, likely contributing to the atheroprotective influence of hemodynamic forces under defined physiological ranges.

CHAPTER 5

EC Laminar Shear Stress – EC Signalling and SMC Cell Cycle Gene Expression

5.1 Introduction

Both shear stress and cyclic strain, blood flow-associated stimuli that impact on the endothelium, profoundly modulate vessel wall homeostasis and under normal physiological conditions, impart an atheroprotective effect that disfavours pathological changes in vessel wall structure. Vascular endothelial cells detect these forces via mechanosensors (e.g. G-proteins, integrins, PTKs and ion channels), which in turn can initiate complex signalling cascades within endothelial cells, culminating in cellular responses often manifested as altered migratory, proliferative and apoptotic profiles in both endothelial *and* smooth muscle cells (i.e. both autocrine and paracrine regulation). Moreover, interactions between the endothelial cell and the extracellular matrix (ECM) are critical in determining the vascular endothelium response to these forces.

In the previous chapter, we confirmed that *in vitro* hemodynamic challenge of BAECs regulated BASMC proliferation and apoptosis. ***This chapter will specifically focus on those endothelial signalling mechanisms activated in response to LSS which may putatively mediate the "anti-proliferative" effects of BCM observed on SMCs. For these studies, specific pharmacological inhibition strategies will be employed to block selected endothelial signalling mechanisms. As the bulk of our studies have been done using a BAEC/BASMC cell culture model, we also conduct an investigation to determine the applicability of this regulatory paradigm to an equivalent human vascular cell model (HAEC/HASMC). Finally, in an effort to shed further light on the regulatory events taking place within the SMC phase, the***

impact of LSS-derived BCM on expression of smooth muscle cell cycle-associated genes will be examined.

5.2 Results

5.2.1 Integrin blockade: cRGD

In order to determine the involvement of endothelial integrins in modulating BCM-dependent effects on BASMC proliferation, BAECs were pre-treated with a pharmacological inhibitor of integrins, cyclic RGD (cRGD) peptide (100μM), prior to and for the duration of applied shear stress (10 dynes/cm^2, 24 h) (Buckley *et al.*, 1999). Static BASMCs were incubated with the harvested BCM and monitored for proliferation by FACS analysis and cell counts as previously described. LSS-derived BCM decreased BASMC proliferation by 18±2.1% relative to control, by BAEC pre-incubation with cRGD ($P \leq 0.05$) (Fig. 5.1 A-C). A baseline inhibitory effect of cRGD peptide (7±1.7%) was also noted ($P \leq 0.05$).

5.2.2 Rac1 blockade: NSC23766

To ascertain the role of Rac1 GTPase in modulating BCM-dependent effects on BASMC proliferation, NSC23766, a specific pharmacological inhibitor for Rac1, was employed (Gao *et al.*, 2004). BAECs were pre-treated with 50 μM NSC23766 prior to and for the duration of shear stress (10 dynes/cm^2, 24 h). Static BASMCs were subsequently incubated with harvested BCM and monitored for proliferation by FACS analysis and hemocytometer cell counts. LSS-derived BCM decreased BASMC proliferation by 19±0.8% relative to control ($P \leq 0.05$) (Unsheared). In the presence of NSC23766, this effect was fully recovered to baseline proliferation levels. A baseline inhibitory effect of NSC23766 (5±0.9%) was also noted (Fig. 5.2 A-C).

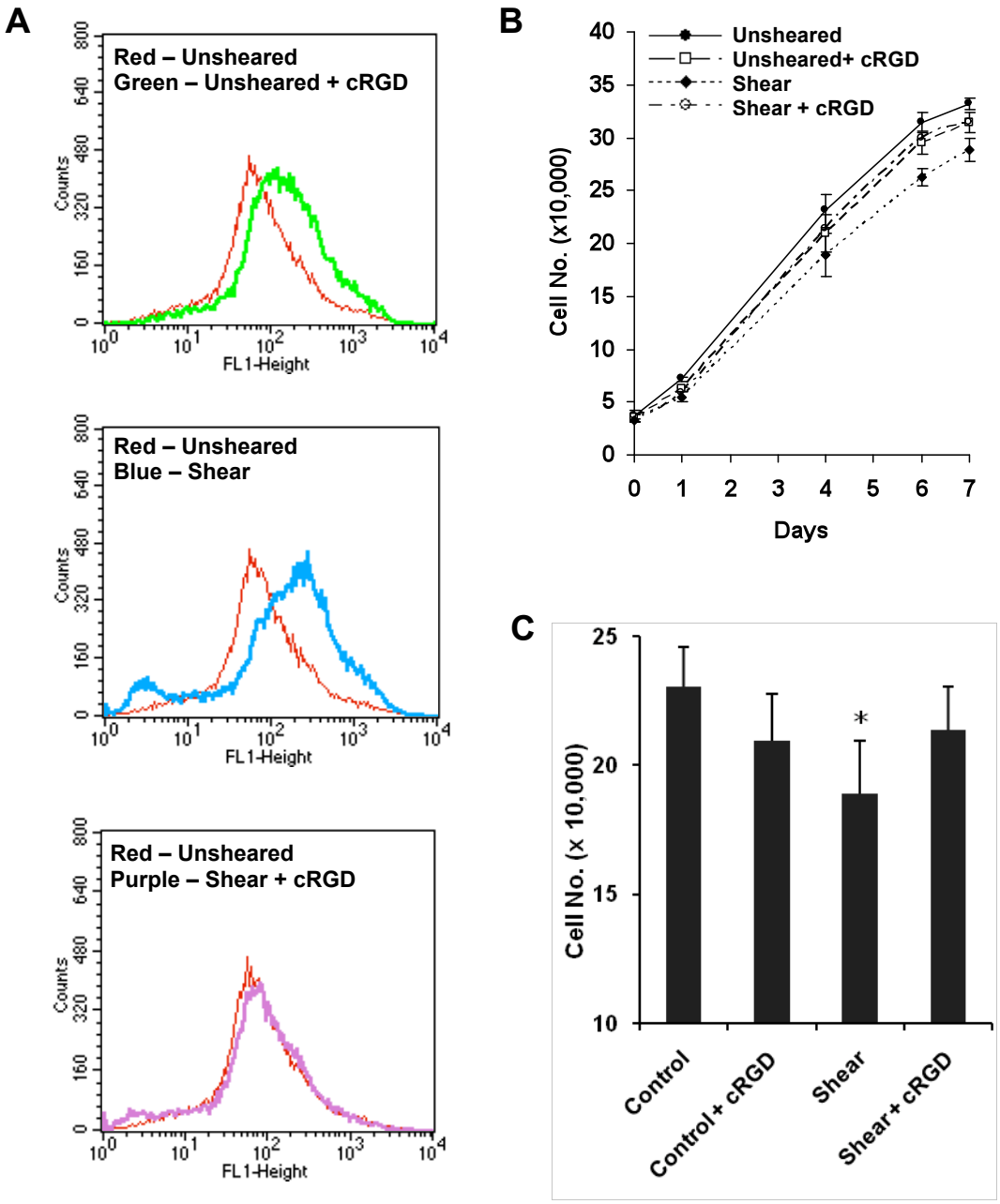


Fig. 5.1: Effect of LSS-derived BCM on BASMC proliferation following BAEC integrin blockade. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence or presence of cRGD. The harvested BCM was incubated with static quiescent BASMCs and proliferation was monitored by; (A) FACS analysis at day 4 (18±2.1% - proliferation decreases as the peaks shift to the right) and (B) hemocytometer cell counting over 7 days. Histogram in (C) shows cell counts specifically for day 4. Histograms were averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared controls. δP≤0.05 versus shear. FACS scans are representative of 3 experiments.

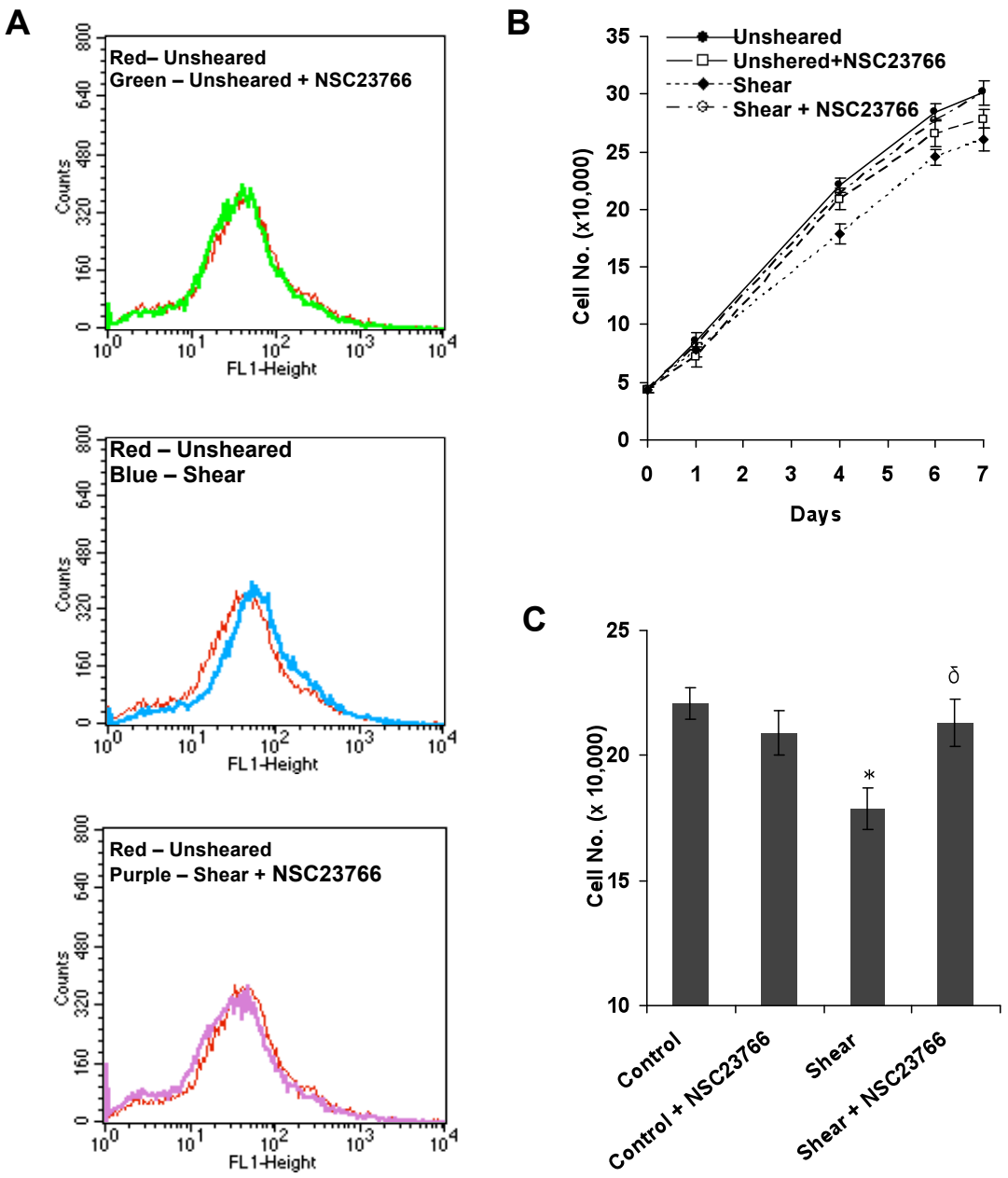


Fig. 5.2: Effect of LSS-derived BCM on BASMC proliferation following BAEC Rac1 blockade. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence or presence of NSC23766. The harvested BCM was incubated with static quiescent BASMCs and proliferation was monitored by; (A) FACS analysis at day 4 (19±0.8% - proliferation decreases as the peaks shift to the right) and (B) hemocytometer cell counting over 7 days. Histogram in (C) shows cell counts specifically for day 4. Histograms were averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared controls. δP≤0.05 versus shear. FACS scans are representative of 3 experiments.

5.2.3 eNOS blockade: L-NAME

To examine the role of NO, L-NAME a specific pharmacological inhibitor was employed. BAECs were pre-treated with 1 mM L-NAME prior to and for the duration of shear stress (10 dynes/cm^2, 24 h). Static BASMCs were incubated with the harvested BCM and monitored for proliferation by FACS analysis and hemocytometer cell counts. LSS-derived BCM decreased BASMC proliferation by 12±1.0% relative to control (unsheared), an effect that was reversed by BAEC pre-incubation with L-NAME ($P \leq 0.05$) (Fig. 5.3 A-C). A slight baseline inhibitory effect of L-NAME (3±1.2%) was also observed (albeit statistically insignificant).

5.2.4 TGF-β blockade: ALK5 R1 Antagonist

The role of TGF-β in modulating BCM-dependent effects on BASMC proliferation was investigated using ALK5 R1 Antagonist (20 μM), a specific pharmacological inhibitor for TGF-β. Static BASMCs were incubated with the harvested BCM and monitored for proliferation by FACS analysis and hemocytometer cell counts. LSS-derived BCM decreased BASMC proliferation by 14±1.3% relative to control (unsheared), an effect that could be fully attenuated by BAEC pre-incubation with ALK5 R1 Antagonist ($P \leq 0.05$) (Fig. 5.4 A-C). A slight baseline inhibitory effect of ALK5 R1 Antagonist (1±0.9%) was also observed (albeit statistically insignificant).

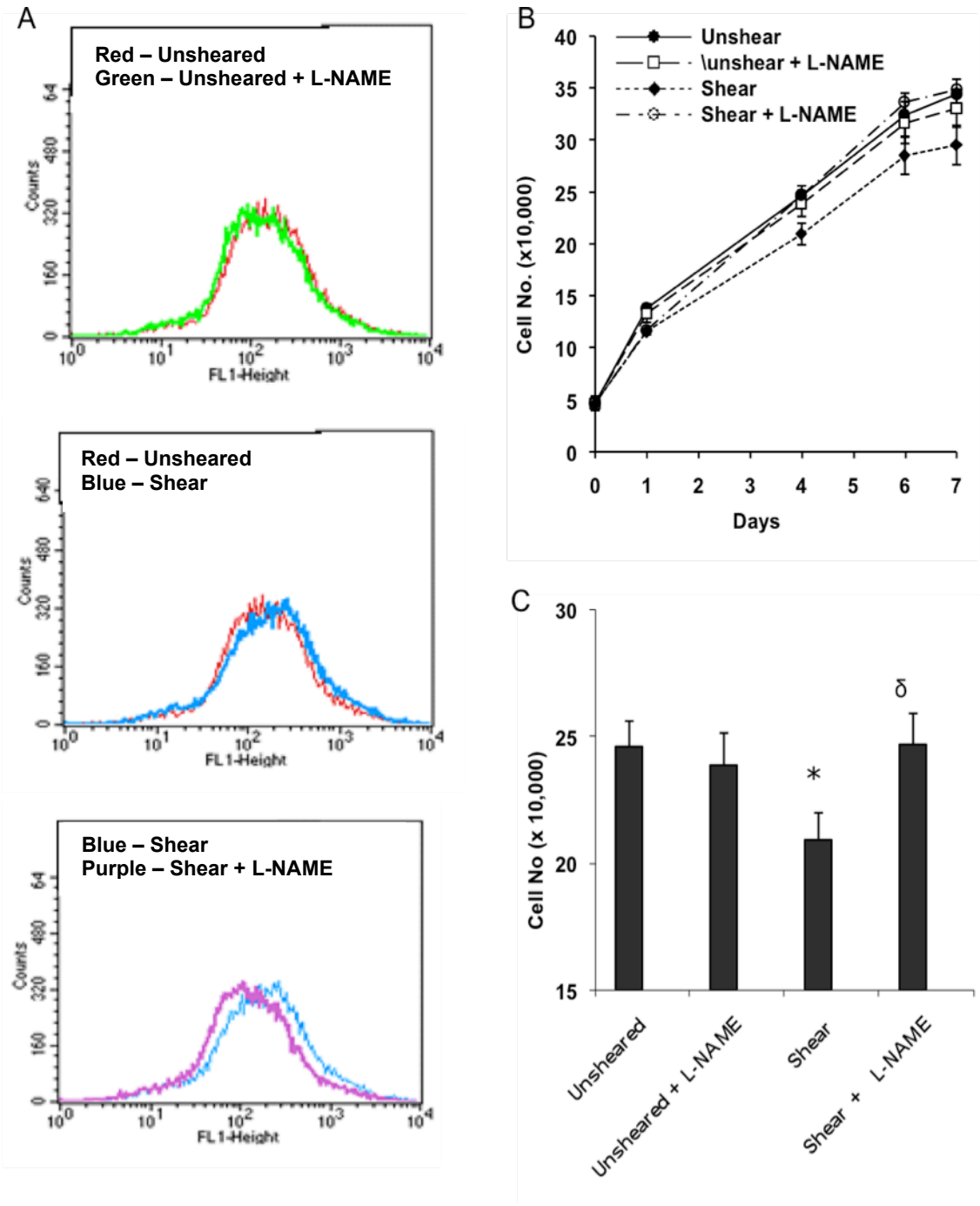


Fig. 5.3: Effect of LSS-derived BCM on BASMC proliferation following BAEC eNOS blockade. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence or presence of L-NAME. The harvested BCM was incubated with static quiescent BASMCs and proliferation was monitored by; (A) FACS analysis at day 4 (Proliferation decreases as the peaks shift to the right) and (B) hemocytometer cell counting over 7 days. Histogram in (C) shows cell counts specifically for day 4 (12±1.0%). Histograms were averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared controls. δP≤0.05 versus shear. FACS scans are representative of 3 experiments.

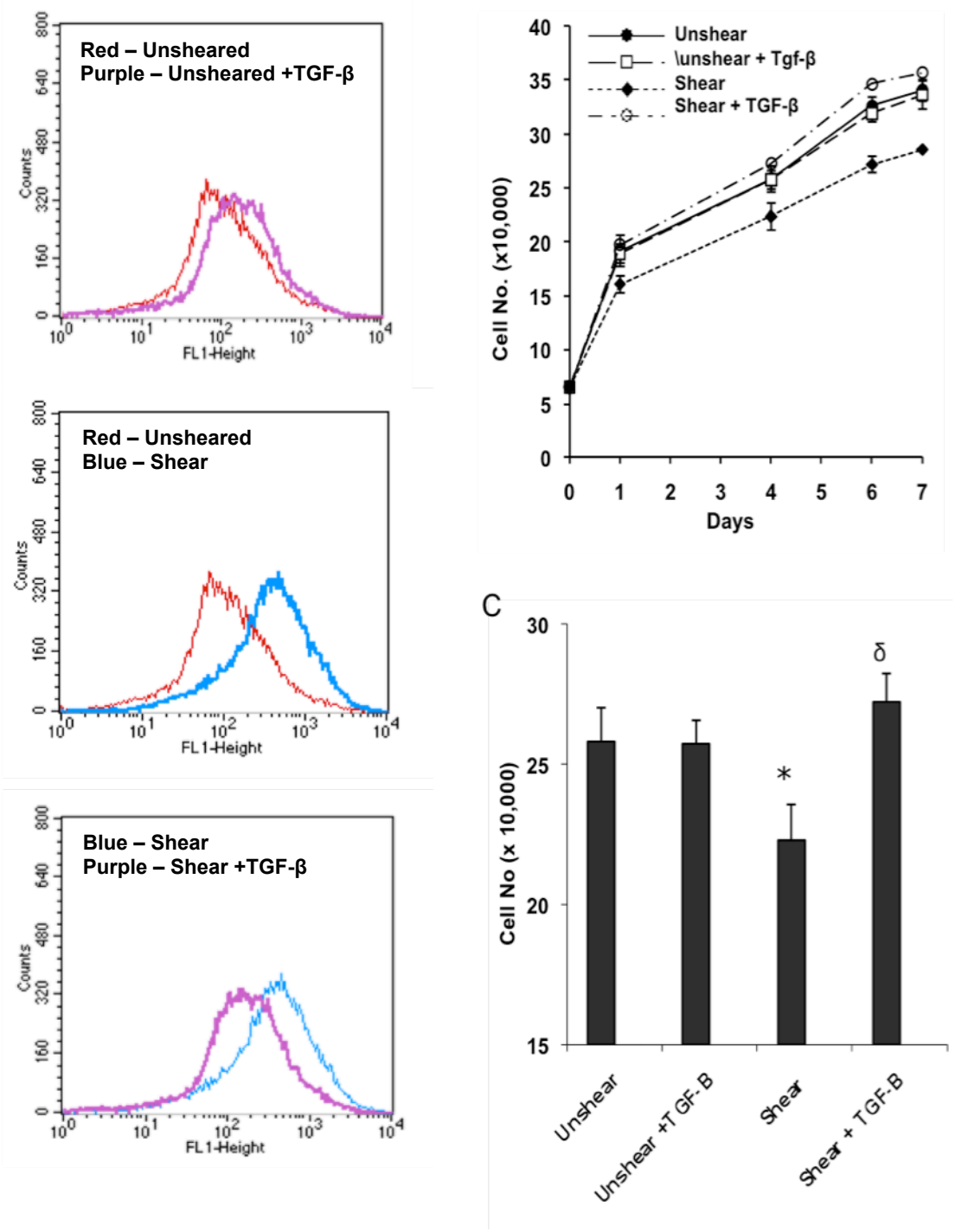


Fig. 5.4: Effect of LSS-derived BCM on BASMC proliferation following BAEC TGF-β blockade. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence or presence of ALK5 R1 Antagonist. The harvested BCM was incubated with static quiescent BASMCs and proliferation was monitored by; (A) FACS analysis at day 4 (proliferation decreases as the peaks shift to the right) and (B) hemocytometer cell counting over 7 days. Histogram in (C) shows cell counts specifically for day 4 (14±1.3%). Histograms were averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared controls. δP≤0.05 versus shear. FACS scans are representative of 3 experiments.

5.2.5 Heterotrimeric G-protein blockade: PTX

To investigate the involvement of Gα-subunits in modulating BCM-dependent effects on BASMC proliferation, pertussis toxin (PTX) (100ng/ml), a specific pharmacological inhibitor for Gα protein subunits was employed (Burns, 1988). BAECs were pre-treated with PTX prior to and for the duration of shear stress (10 dynes/cm^2, 24 h). Static BASMCs were incubated with the harvested BCM and monitored for proliferation by FACS analysis and hemocytometer cell counting. LSS-derived BCM decreased BASMC proliferation by 16±1.2% relative to control (unsheared), an effect that was *not* reversed by BAEC pre-incubation with PTX (20±1.4%) ($P \leq 0.05$) (Fig. 5.5 A-C). A slight baseline inhibitory effect of PTX (3±0.9%) was also observed (albeit statistically insignificant).

5.2.6 ERK1/2 blockade: PD98059

The role of MAPKs were examined using PD98059, a specific inhibitor for ERK1/2 (Alessandrini *et al*., 1999). BAECs were pre-treated with 10 μM PD98059 prior to and for the duration of shear stress (10 dynes/cm^2, 24 h). Static BASMCs were incubated with the harvested BCM and monitored for proliferation by FACS analysis and hemocytometer cell counts. LSS-derived BCM decreased BASMC proliferation by 18±0.7% relative to control (unsheared), an effect exacerbated further by BAEC pre-incubation with PD98059 (27±0.5%) ($P \leq 0.05$) (Fig. 5.6 A-C). A slight baseline inhibitory effect of PD98059 (3±0.5%) was also observed (albeit statistically insignificant).

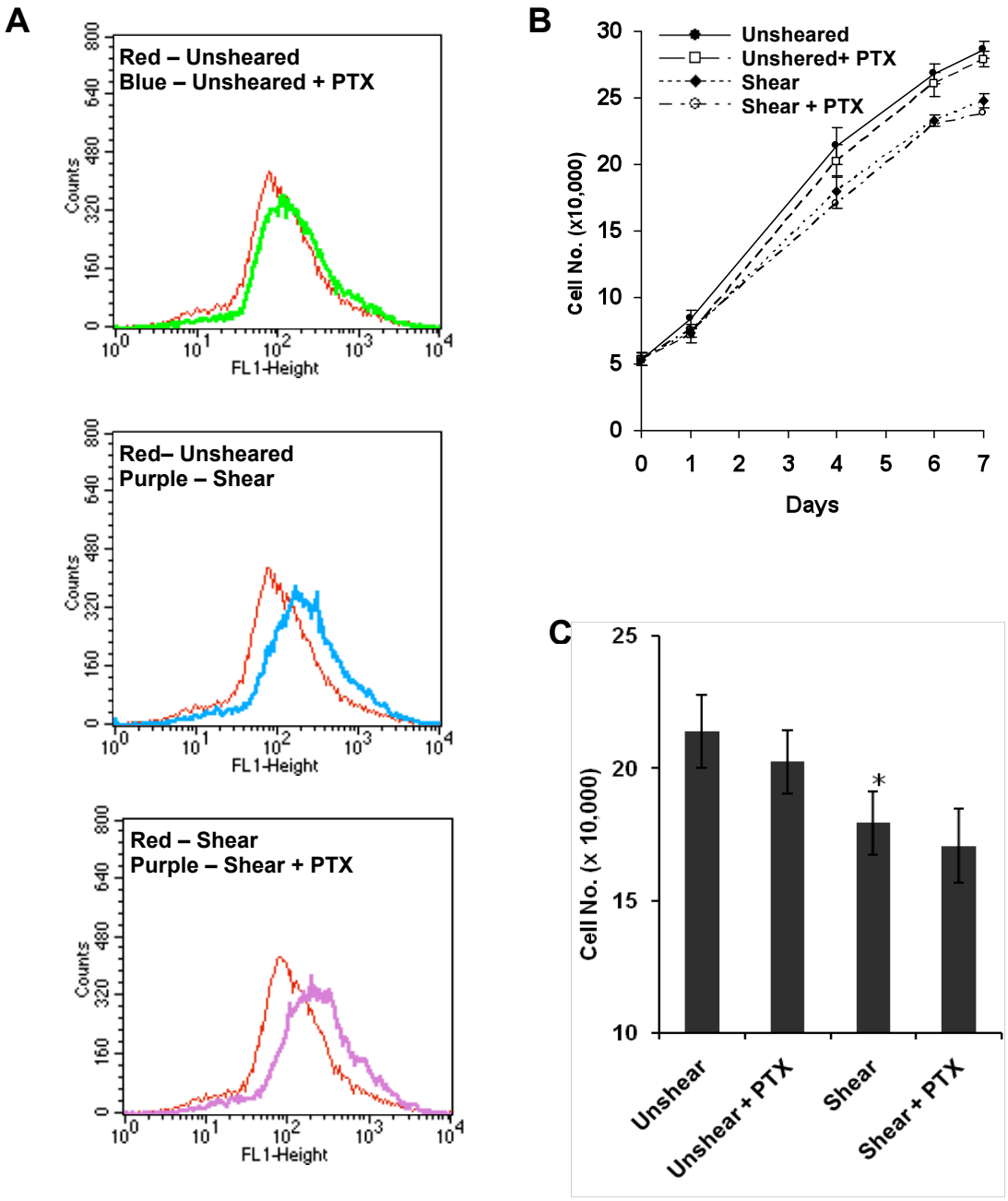


Fig. 5.5: Effect of LSS-derived BCM on BASMC proliferation following BAEC Gα-subunit blockade. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence or presence of PTX. The harvested BCM was incubated with static quiescent BASMCs and proliferation was monitored by; (A) FACS analysis at day 4 (16±1.2% - proliferation decreases as the peaks shift to the right) and (B) hemocytometer cell counting over 7 days. Histogram in (C) shows cell counts specifically for day 4. Histograms were averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared controls. FACS scans are representative of 3 experiments.

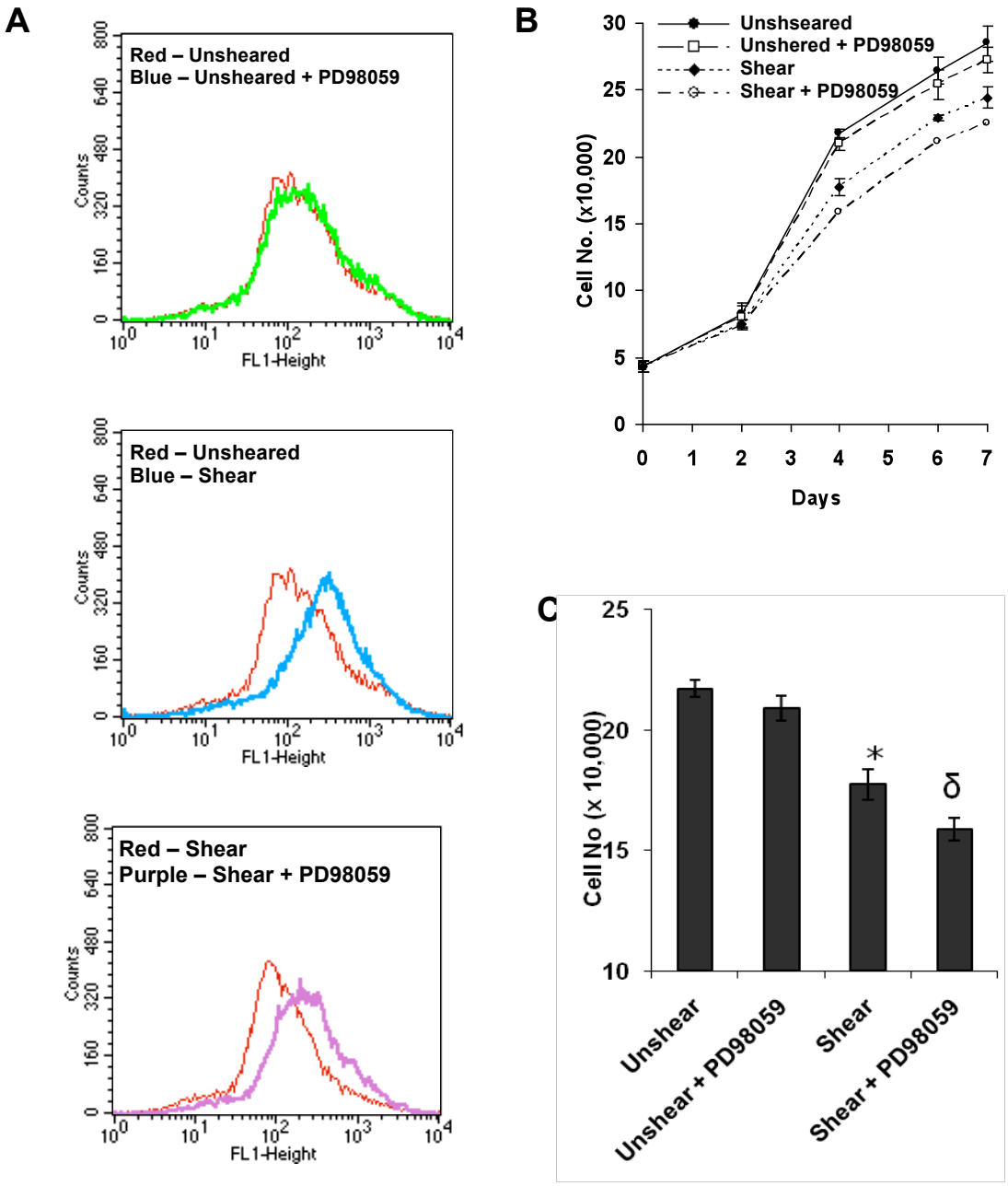


Fig. 5.6: Effect of LSS-derived BCM on BASMC proliferation following BAEC ERK1/2 MAPK blockade. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence or presence of PD98059. The harvested BCM was incubated with static quiescent BASMCs and proliferation was monitored by; (A) FACS analysis at day 4 (18±0.7% - proliferation decreases as the peaks shift to the right) and (B) hemocytometer cell counting over 7 days. Histogram in (C) shows cell counts specifically for day 4. Histograms were averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared controls. δP≤0.05 versus shear. FACS scans are representative of 3 experiments.

5.2.7 NADPH oxidase blockade: Apocynin

To ascertain the role of NADPH oxidase in modulating BCM-dependent effects on BASMC proliferation, BAECs were pre-treated with NADPH oxidase-specific apocynin (Johnson *et al*., 2002) prior to and for the duration of shear stress (10 dynes/cm^2, 24 h). Static BASMC were incubated with the harvested BCM and monitored for proliferation by FACS analysis and cell counts. LSS-derived BCM decreased proliferation by 18±1.0%, an effect exacerbated further by BAEC pre-incubation with 10 μM apocynin ($P \leq 0.05$). A slight baseline inhibitory effect of apocynin was also observed (6.5±0.9%) (Fig. 5.7 A-C).

5.2.8 PTK blockade: Genistein

To investigate the role of PTK's, a specific pharmacological inhibitor was employed. BAECs were pre-treated with (50 μM) Genistein prior to and for the duration of shear stress (10 dynes/cm^2, 24 h). Static BASMCs were incubated with the harvested BCM and monitored for proliferation by FACS analysis and hemocytometer cell counts. LSS-derived BCM decreased BASMC proliferation by 16±0.22% relative to control (unsheared), an effect that was reversed by BAEC pre-incubation with L-NAME (Fig. 5.8 A-C). A slight baseline inhibitory effect of PTX (8±0.63%) was also observed (albeit statistically insignificant).

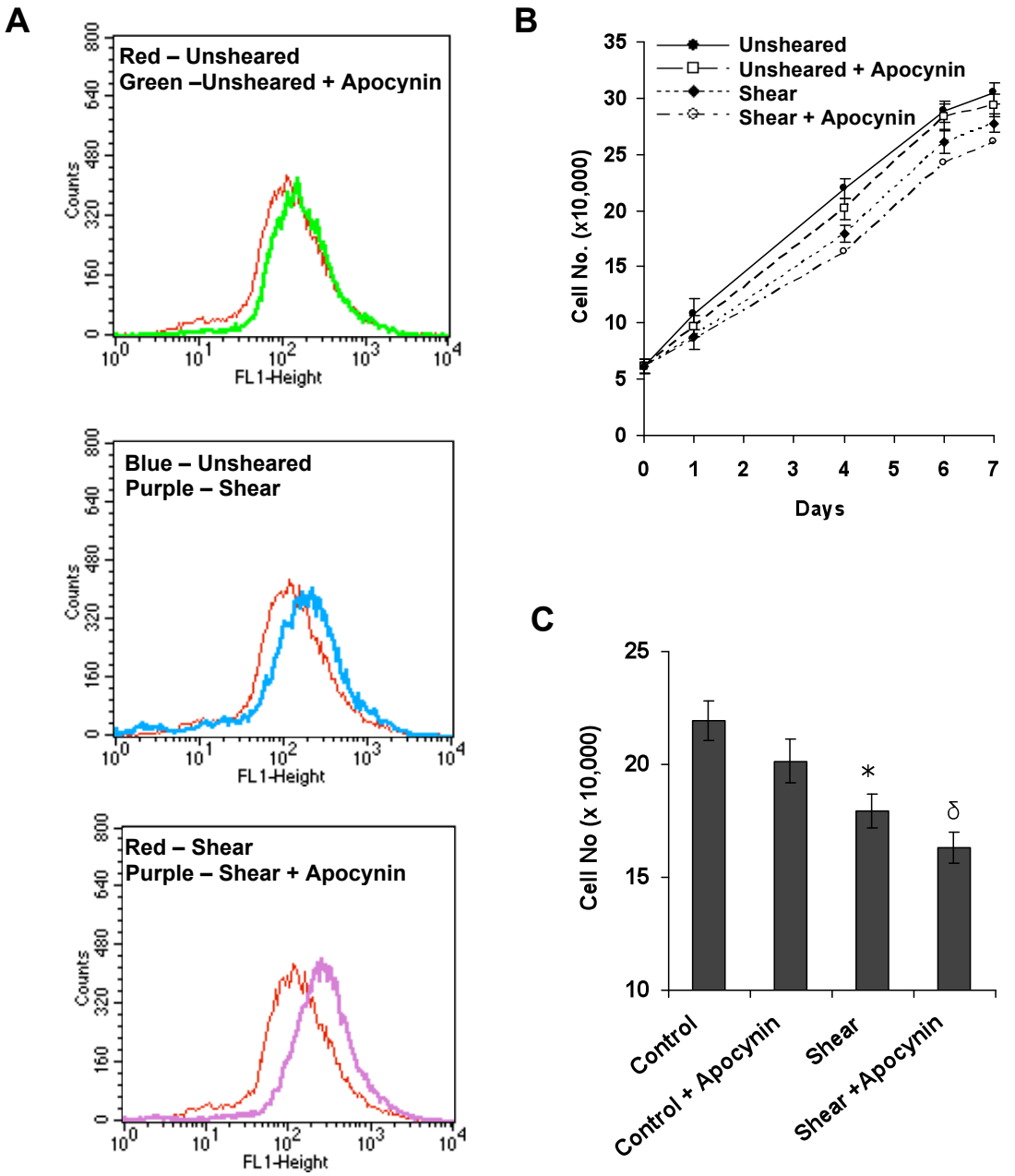


Fig. 5.7 Effect of LSS-derived BCM on BASMC proliferation following BAEC NADPH oxidase blockade. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence or presence of apocynin. The harvested BCM was incubated with static quiescent BASMCs and proliferation was monitored by; (A) FACS analysis at day 4 (18±1.0% - proliferation decreases as the peaks shift to the right) and (B) hemocytometer cell counting over 7 days. Histogram in (C) shows cell counts specifically for day 4. Histograms were averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared controls. FACS scans are representative of 3 experiments.

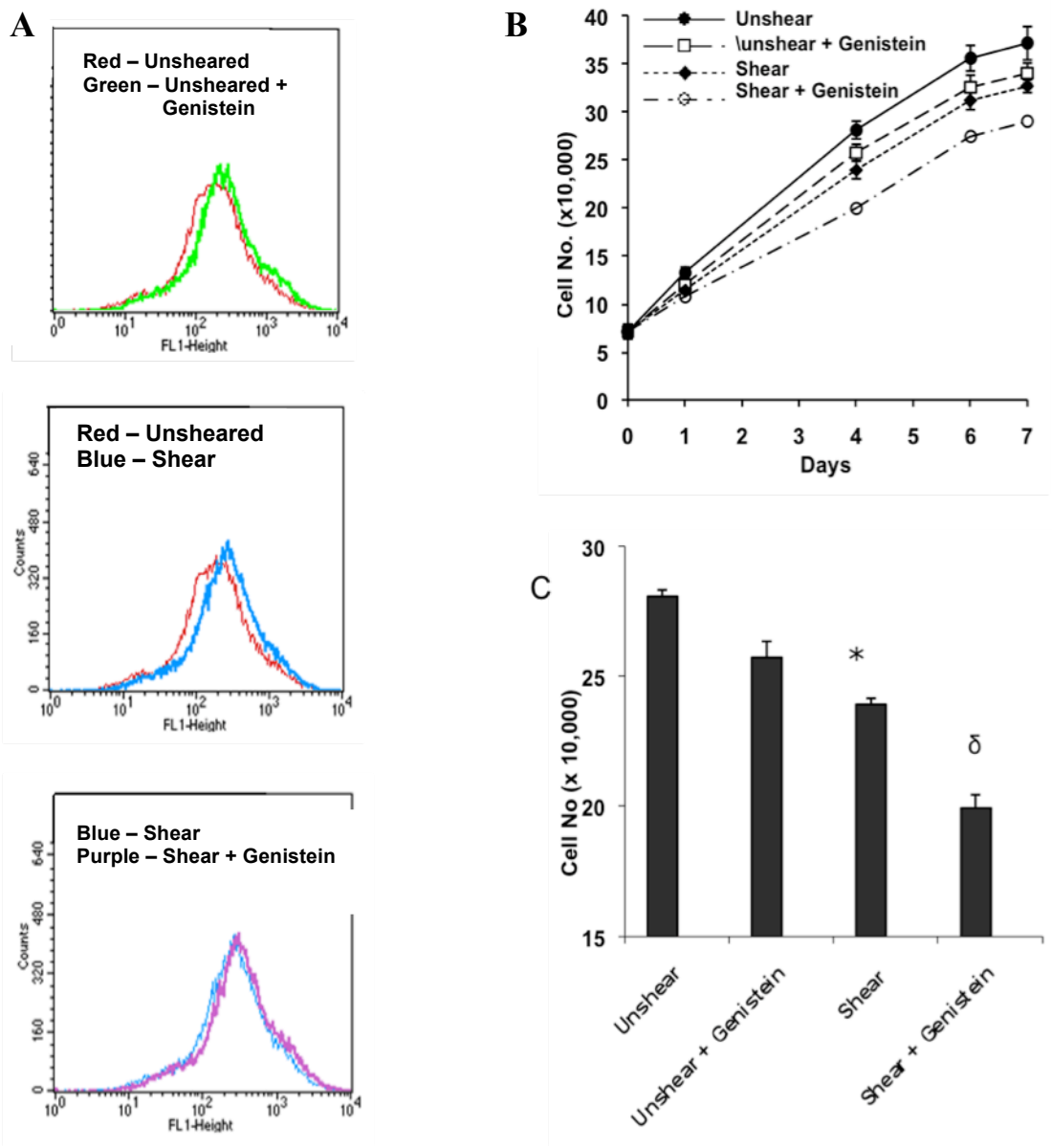


Fig. 5.8: Effect of LSS-derived BCM on BASMC proliferation following BAEC PTK blockade. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence or presence of Genistein. The harvested BCM was incubated with static quiescent BASMCs and proliferation was monitored by; (A) FACS analysis at day (16±0.22% - proliferation decreases as the peaks shift to the right) and (B) hemocytometer cell counting over 7 days. Histogram in (C) shows cell counts specifically for day 4. Histograms were averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared controls. FACS scans are representative of 3 experiments.

5.2.9 MMP blockade: GM6001

To investigate the role of MMP's, a specific pharmacological inhibitor was employed. BAECs were pre-treated with (10 μM) GM6001 prior to and for the duration of shear stress (10 dynes/cm^2, 24 h). Static BASMCs were incubated with the harvested BCM and monitored for proliferation by FACS analysis and hemocytometer cell counts. LSS-derived BCM decreased BASMC proliferation by 15±1.1% relative to control (unsheared), an effect that was reversed by BAEC pre-incubation with GM6001 ($P \leq 0.05$) (Fig. 5.9 A-C). A slight baseline inhibitory effect of GM6001 (1±1.1%) was also observed (albeit statistically insignificant).

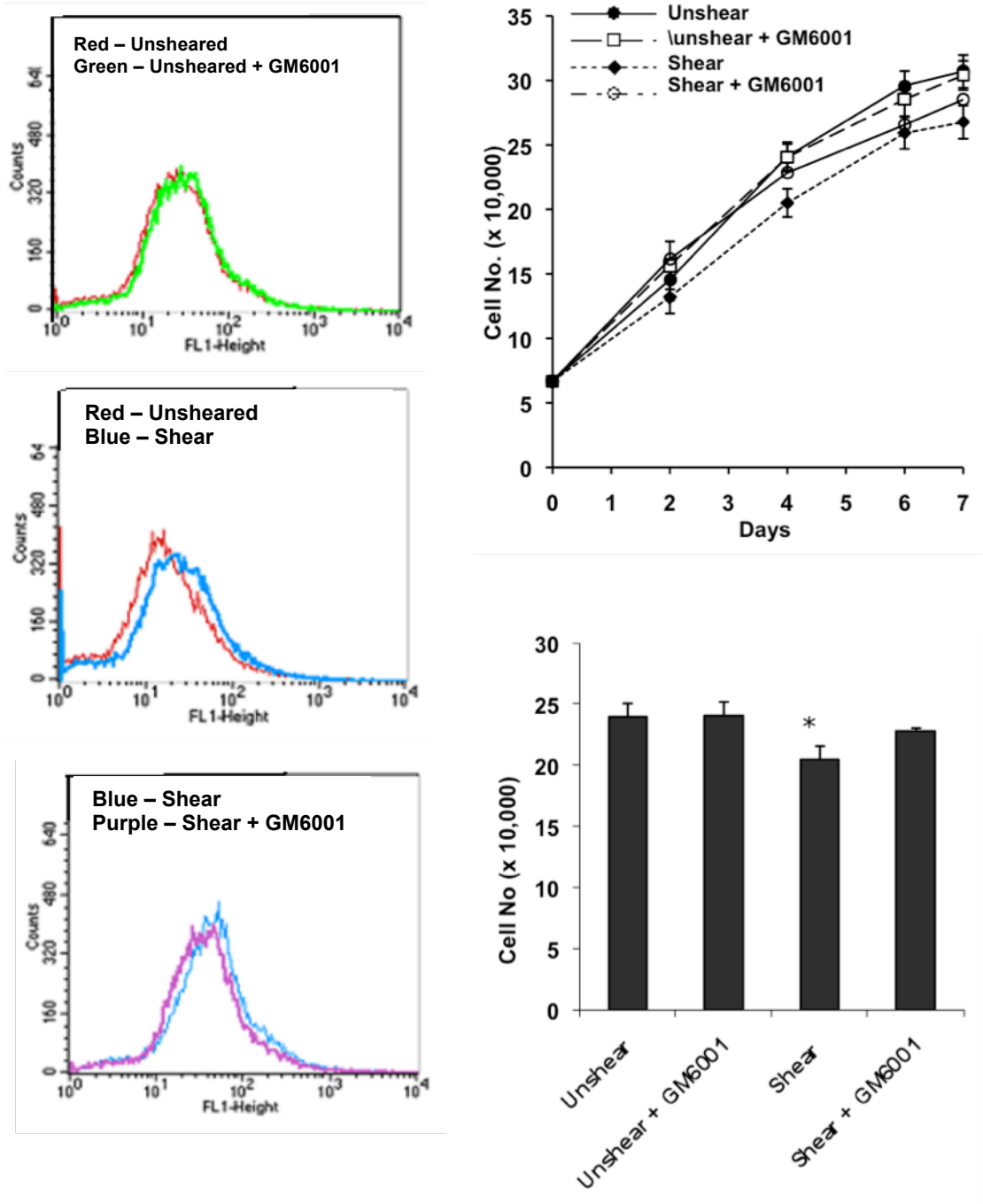


Fig. 5.9: Effect of LSS-derived BCM on BASMC proliferation following BAEC MMP blockade. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence or presence of GM6001. The harvested BCM was incubated with static quiescent BASMCs and proliferation was monitored by; (A) FACS analysis at day 4 (15±1.1%) (Proliferation decreases as the peaks shift to the right) and (B) hemocytometer cell counting over 7 days. Histogram in (C) shows cell counts specifically for day 4. Histograms were averaged from three independent experiments ±SEM. *P≤0.05 versus unsheared controls. FACS scans are representative of 3 experiments.

Signaling Component	Inhibitor	Effect (+/-)
Integrins	cRGD (100 μM)	+
Rac1	NSC23766 (50 μM)	+
G-proteins	PTX (100 ng/ml)	-
PTKs	Genistein (50 μM)	-
ERK1/2	PD98059 (10 μM)	-
NADPH oxidase	Apocynin (10 μM)	-
NO	L-NAME (10 μM)	+
TGF-β	ALK5 R1 Antagonist (20 μM)	+
MMPs	GM6001 (10 μM)	-

Table 5.1: Pharmacological inhibition studies. Table summarises the BAEC signalling components found to putatively mediate the anti-proliferative effects of LSS-derived BCM on BASMCs. (-) inhibitor had no effect, (+) inhibitor reversed anti-proliferative effect.

5.2.10 Proof-of-concept study: HAEC/HASMC model

We next asked if the observations made with the BAEC/BASMC model (with respect to the shear-induced anti-proliferative effects) could be replicated in an equivalent HAEC/HASMC model. Investigating this however presented some methodological hurdles. In the BAEC/BASMC model, both cell types were cultured in the same growth media (RPMI 1640 with FCS and antibiotics), thus ensuring that BASMCs could proliferate in BAEC-conditioned media. However, both HAECs and HASMCs require their own specific media, giving rise to potential problems growing either cell type in a different media. In order to address this, confluent HAECs were exposed to shear stress in Promocell Human Endothelial Cell Basal Media (5% FCS, 5% ECGS/H, 10 ng/ml epidermal growth factor, 1μg/ml hydrocortisone, and antibiotics) and Promocell Human Smooth Muscle Cell Growth Media (5% FCS, 0.5ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, 5 μg/ml insulin, and antibiotics). Both types of HAEC-conditioned media (labelled HCM-EC and HCM-SMC) were harvested and incubated with HASMCs to monitor proliferation over 7 days (FACS analysis and hemocytometer cell counts). Interestingly, HASMCs cultured in the presence of either type of LSS-derived HCM did not show any inhibition in proliferation compared to control (HCM-EC (3±0.7%) and HCM-SMC (0±0.4%)) (Fig. 5.10 A-D).

As LSS-derived HCM had no effect on HASMC proliferation, we decided to examine the possible effects of LSS-derived BCM. Confluent BAECs were incubated in both standard RPMI growth media and Promocell Human Smooth Muscle Cell Growth Media and exposed to shear stress (10 dynes/cm^2, 24 h). Both types of BAEC-conditioned media (labelled BCM-STD and BCM-SMC) were harvested and

incubated with HASMCs to monitor proliferation over 7 days (FACS analysis and hemocytometer cell counts). The results again showed that neither type of BCM appeared to induce an anti-proliferative effect on HASMCs (BCM-STD (3±0.9%) and BCM-SMC (0±0.8%)) (Fig. 5.11 A-D).

Finally, as neither LSS-derived BCM nor -HCM appeared to have an effect on HASMC proliferation, we decided to examine the possible effects of LSS-derived HCM on BASMCs. Confluent HAECs were incubated in both standard RPMI growth media and Promocell Human Endothelial Cell Basal Media and exposed to shear stress (10 dynes/cm^2, 24 h). Both types of HAEC-conditioned media (labelled HCM-EC and HCM-STD) were harvested and incubated with HASMCs to monitor proliferation over 7 days (FACS analysis and hemocytometer cell counts). Both HCM-EC and HCM-STD inhibited BASMC proliferation by 13±0.9% and 11±1.3%, respectively ($P \leq 0.05$) (Fig. 5.12 A-F).

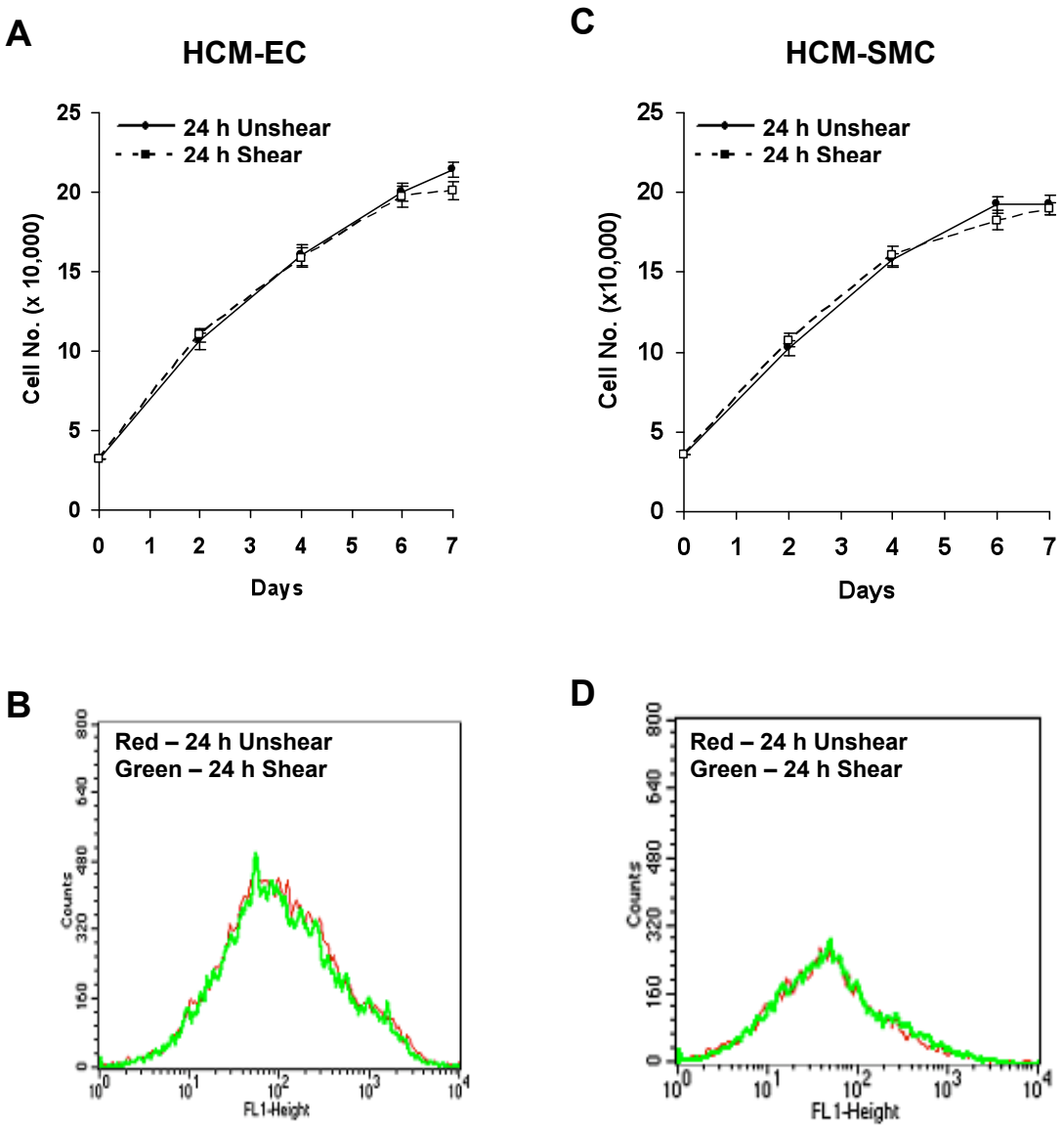


Fig. 5.10 Effect of HAEC-conditioned media on HASMC proliferation. Confluent HAECs were incubated in Promocell Human Endothelial Cell Basal Media or Human Smooth Muscle Cell Growth Media and exposed to shear stress (10 dynes/cm^2, 24 h). The resultant HCMs (termed HCM-EC and HCM-SMC, respectively) were incubated with HASMCs and proliferation was monitored by hemocytometer cell counting and FACS analysis; (A, B) HCM-EC and (C, D) HCM-SMC.No change in proliferation was observed by resultant HCM-EC (3±0.7%) or HCM-SMC (0±0.4%). Proliferation decreases as the peaks shift to the right. FACS scans are representative of 3 experiments.

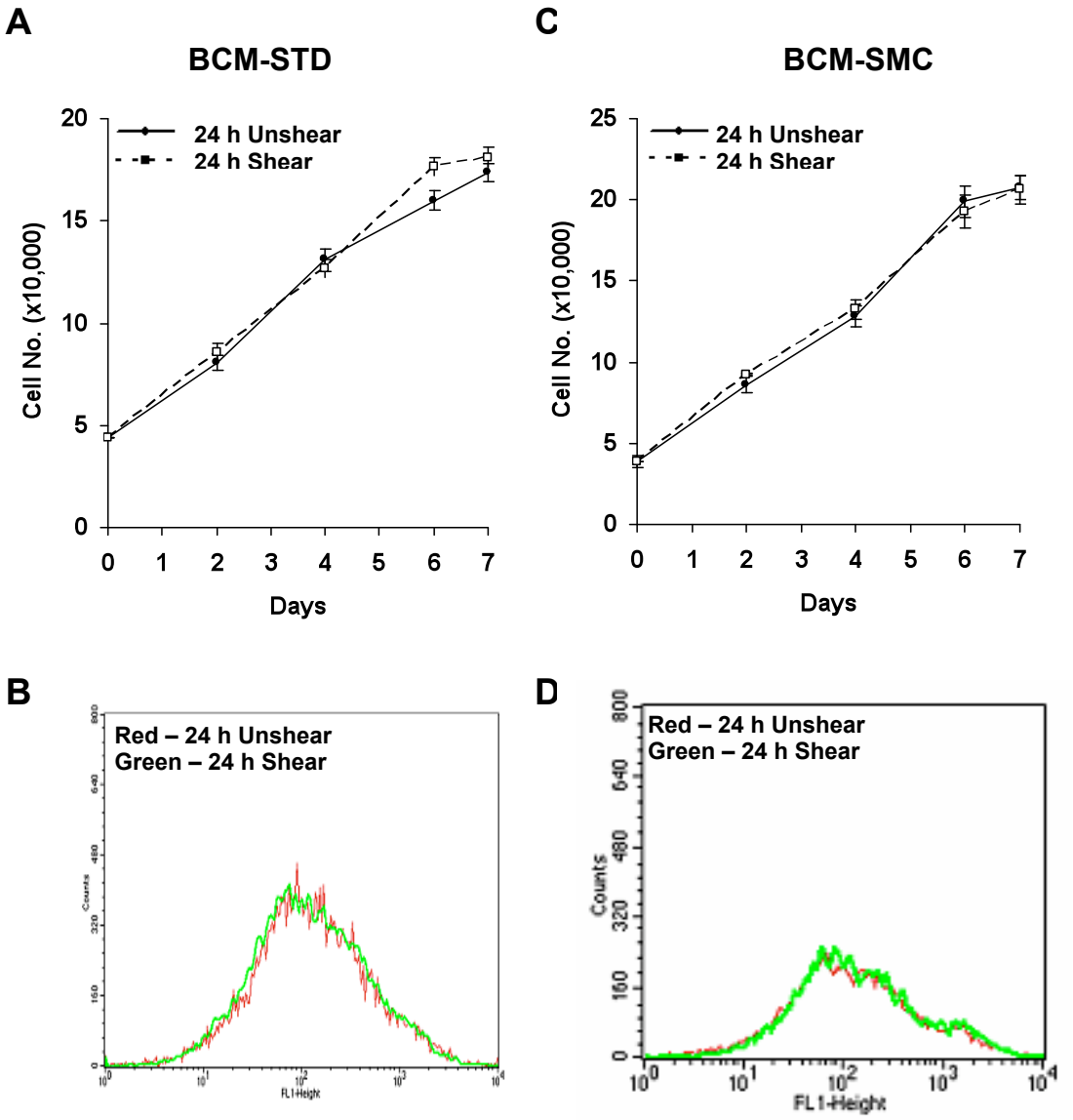


Fig. 5.11: Effect of BAEC-conditioned media on HASMC proliferation. Confluent BAECs were incubated in standard RPMI growth media and Promocell Human Smooth Muscle Cell Growth Media and exposed to shear stress (10 dynes/cm^2, 24 h). The resultant BCMs (termed BCM-STD (3±0.9%) and BCM-SMC (0±0.8%), respectively) were incubated with HASMCs and proliferation was monitored by hemocytometer cell counting and FACS analysis; (A, B) BCM-STD and (C, D) BCM-SMC. Proliferation decreases as the peaks shift to the right. FACS scans are representative of 3 experiments.

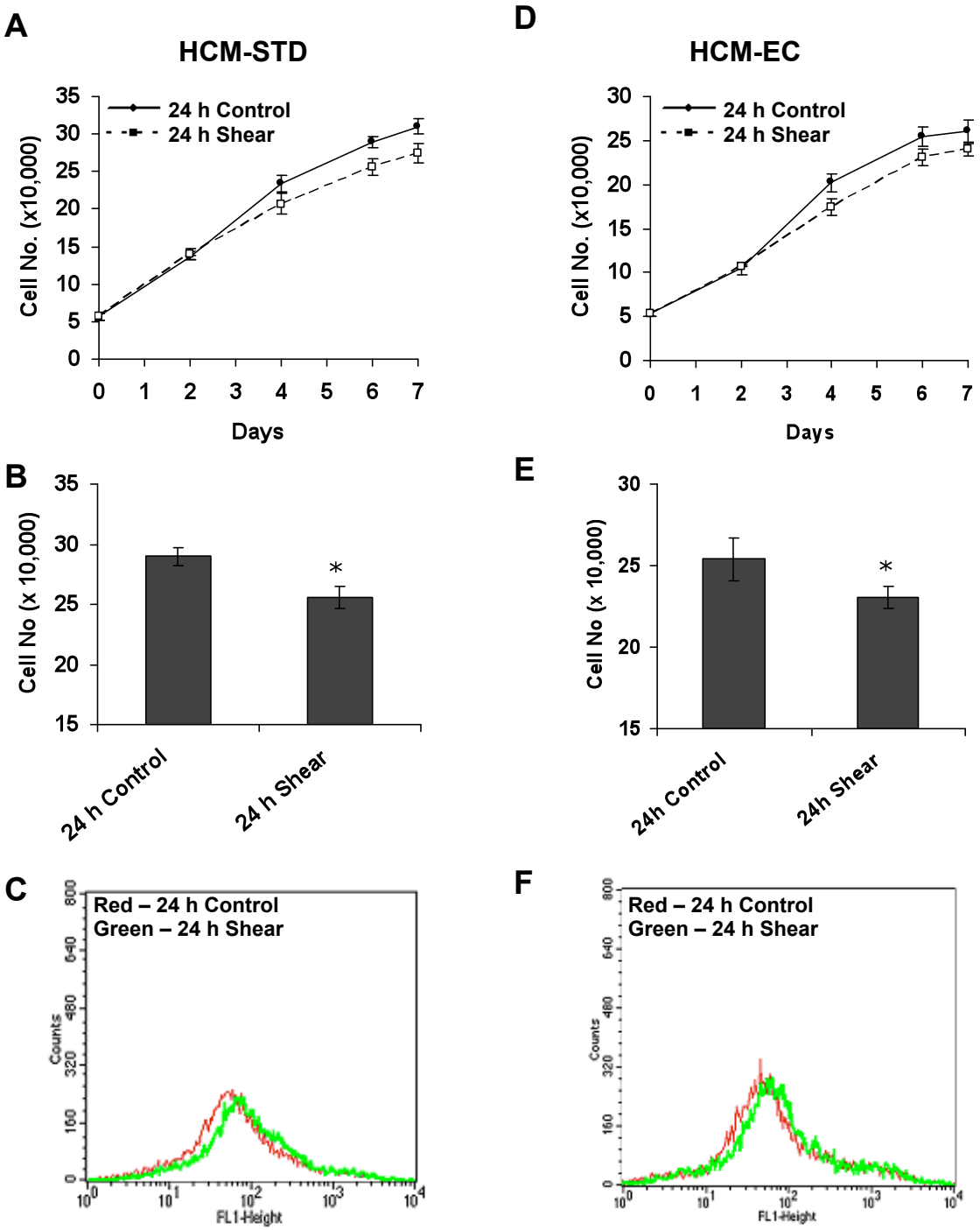


Fig. 5.12: Effect of HAEC-conditioned media on BASMC proliferation. Confluent HAECs were incubated in standard RPMI growth media and Promocell Endothelial Cell Growth Media and exposed to shear stress (10 dynes/cm^2, 24 h). The resultant HCMs (termed HCM-STD and HCM-EC, respectively) were incubated with BASMCs and proliferation was monitored by hemocytometer cell counting and FACS analysis; (A-C) HCM-STD (13±0.9%) and (D-F) HCM-EC (11±1.3%).and (D-F) HCM-EC. Histograms were averaged from three independent experiments ±SEM. *$P \leq 0.05$ versus unsheared controls. Proliferation decreases as the peaks shift to the right. FACS scans are representative of 3 experiments.

5.2.8 Gene Expression Studies

Having initially focussed our attention on the BAEC signalling pathways putatively mediating the anti-proliferative effects of shear on BASMCs, we next decided to extend our model to investigate the direct effects of LSS-derived BCM on cell cycle-associated gene expresion patterns in BASMCs. The cell cycle is regulated by cyclin dependent kinases (CDKs) and cyclins, which when activated, coordinate entry into the successive phases of the cell cycle. Two analytical formats were selected to investigate this; (i) Human Cell Cycle RT² Profiler™ PCR Microarray to monitor the expression of 84 genes key to cell cycle regulation; (ii) Standard RealTime PCR to individually monitor a *selected* number cell cycle-associated genes using gene-specific primers.

5.2.8.1 Impact of EC laminar shear on SMC gene expression: PCR Microarray

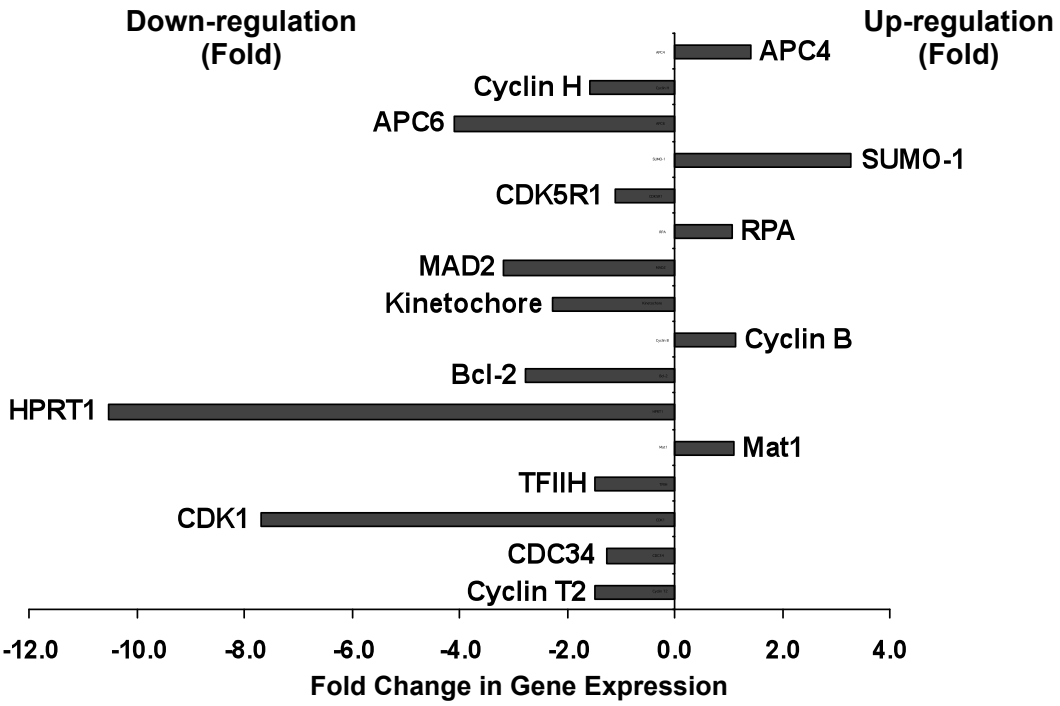
The Human Cell Cycle RT² Profiler™ PCR Microarray (SABiosciences) is a 96-well plate that profiles the expression of 84 genes key to cell cycle regulation. This 96-well array contains pre-designed primers for genes that positively and negatively regulate the cell cycle, DNA replication, checkpoints, and arrest. Five housekeeping genes and three RNA and PCR quality controls are also included in this array. Moreover, in view of the unexpected lack of any anti-proliferative effects on HASMCs following either HCM or BCM treatment (as shown in previous section), we decided to use BASMCs for this study (although mindful of the fact that we may get reduced primer hybridization efficiency on certain target genes using bovine mRNA on a human PCR array). BAECs were exposed to shear stress (0 or 10 dynes/cm^2, 24 h) and the harvested BCM incubated with static BASMCs (4 days).

Total BASMC mRNA was then harvested for PCR microarray analysis (as described in sections 2.4 and 2.4.4, respectively). Results indicated shear-dependent modulation of a number of cell cycle-associated genes in BASMCs (highlighted in Fig. 5.13 A-C).

5.2.8.2 Impact of EC laminar shear on SMC gene expression: RealTime PCR

In addition to PCR microarray, standard RT-PCR was employed to individually monitor a *selected* number cell cycle-associated genes using gene-specific primers. In this regard, we focused on the cyclin/CDK family of genes, a number of which were found to be regulated within the PCR microarray. Specifically, LSS-derived BCM (10 dynes/cm^2, 24 h) was harvested and incubated with BASMCs (4 days). Total BASMC mRNA was harvested and monitored for expression levels of specific genes using RealTime PCR analysis. Our results indicated shear-dependent up-regulation of cyclin D1 (1.44±0.2 fold) and CDK4 (2.67±0.2 fold). By contrast, we observed a down-regulation in CDK1, CDK2 and CDK6 mRNA levels (to 0.25±0.1, 0.51±0.2, and 0.62±0.2 fold of control, respectively) ($P \leq 0.05$). Interestingly, no statistically significant change was observed in p27*Kip*1, cyclin A, or cyclin E levels (Fig 5.14 A,B).

A



B

Position	UniGene	RefSeq	Symbol	Description
B12	Hs.591241	NM_001241	CCNT2	Cyclin T2
C04	Hs.514997	NM_004359	CDC34	Cell division cycle 34
C02	Hs.334562	NM_001786	CDC2	Cell division cycle 2
E04	Hs.577202	NM_005316	GTF2H1	General transcription factor IIH
F05	Hs.509523	NM_002431	MNAT1	Menage a trois homolog 1, cyclin H assembly factor
H02	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1
A09	Hs.150749	NM_000633	BCL2	B-cell CLL/lymphoma 2
B01	Hs.23960	NM_031966	CCNB1	Cyclin B1
E08	Hs.300559	NM_014708	KNTC1	Kinetochore
E10	Hs.591697	NM_002358	MAD2L1	MAD2 mitotic arrest deficient
G05	Hs.487540	NM_002947	RPA3	Replication protein A3
C07	Hs.5000015	NM_003885	CDKR1	Cyclin-dependent kinase 5, regulatory subunit 1
G12	Hs.533273	NM_003334	UBA1	Ubiquitin-like modifier activating enzyme 1
C01	Hs.374127	NM_003903	CDC16	Cell division cycle 16 homolog
B10	Hs.292524	NM_001239	CCNH	Cyclin H
A03	Hs.152173	NM_013367	ANAPC4	Anaphase promoting complex subunit 4

Fig. 5.13: Cell cycle PCR Microarray. Following exposure to LSS-derived BCM, BASMC mRNA was harvested and monitored for cell cycle gene expression using the Human Cell Cycle RT² Profiler™ PCR Array. (A) Fold change in gene expression relative to unsheared controls; (B) Panel of differentially regulated genes identified in the array. Data is the result of one array study (i.e. one control array and one shear array). GAPDH has been used for normalization between control and shear samples.

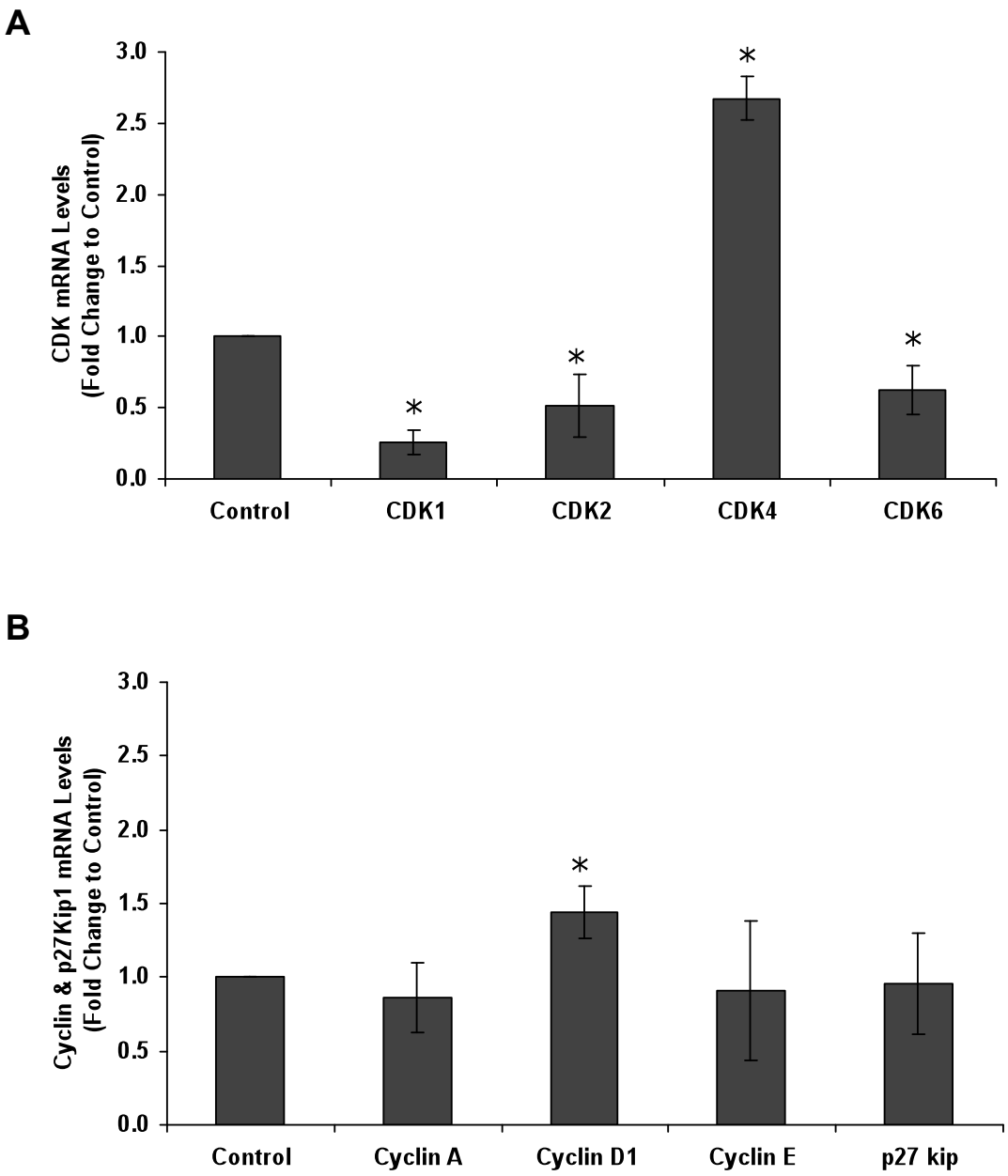


Fig. 5.14: Individual gene expression analysis by RT-PCR. Following exposure to LSS-derived BCM, BASMC mRNA was harvested and monitored for expression of individual cell cycle-associated genes; (A) CDK mRNA levels and (B) Cyclin mRNA levels. Histograms represent shear-dependent fold change in gene expression level relative to the unsheared control normalized to 1.0 for each gene. Up-regulation of cyclin D1 (1.44±0.2 fold) and CDK4 (2.67±0.2 fold). By contrast, we observed a down-regulation in CDK1, CDK2 and CDK6 mRNA levels (to 0.25±0.1, 0.51±0.2, and 0.62±0.2 fold of control). Histograms are averaged from three independent experiments ±SEM. *$P \leq 0.05$ relative to unsheared control.

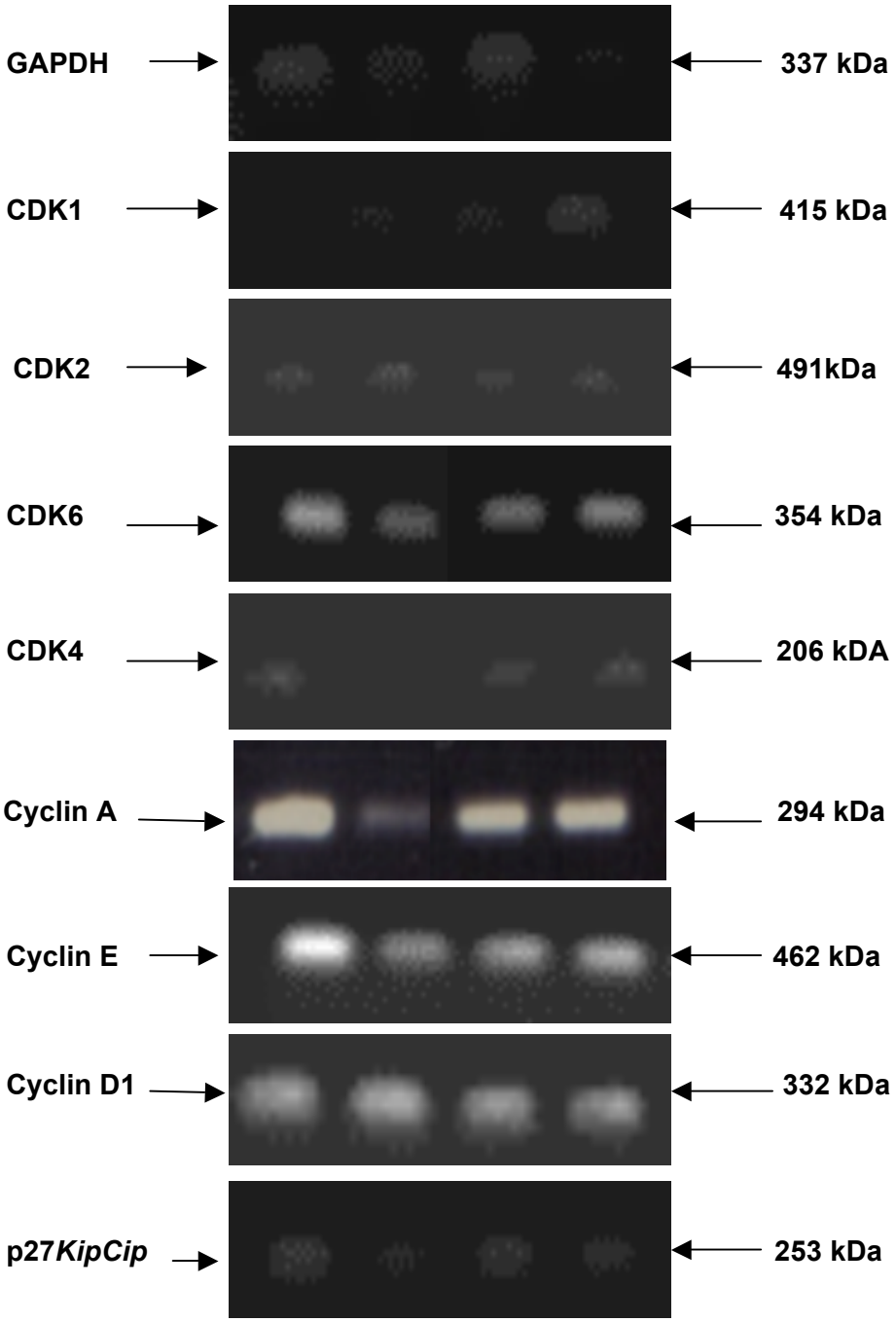


Fig. 5.15: Agarose gels of CDK and cyclin primer sequences. BASMC PCR product for CDKs and cycins were monitored for correct fragment size by agarose gels. Gels are representative.

5.3 Discussion

Due to its unique location between the vessel wall and bloodstream, the endothelium can detect both humoral and biomechanical stimuli and transduce these signals effectively to the underlying smooth muscle cell layer with consequences for smooth muscle cell function and medial integrity. Suitable communication between endothelial and smooth muscle cell is therefore essential for maintaining proper vessel homeostasis. Disruption in blood flow pattern can perturb the normal hemodynamic challenge to the vascular endothelial cell and result in adverse remodelling of the vessel wall. In this regard, endothelial cells can act as intermediates in the mechano-regulation of smooth muscle cell functions through the production of growth factors, cytokines, signalling molecules, and enzymes, which may subsequently alter smooth muscle cell phenotype. With respect to the latter, smooth muscle cell proliferation and apoptosis are particularly important during vascular development. Indeed increased smooth muscle cell proliferation and apoptosis are important contributors to the pathogenesis of several cardiovascular disease states including atherosclerosis, restenosis and hypertension.

The first aim of this study was to investigate the endothelial mechanotransduction systems putatively mediating the effects of hemodynamically-derived BCM on BASMC growth properties. Given the potentially broad nature of this aim, it was decided to focus specifically on the anti-proliferative effects of LSS-derived BCM. For these studies we employed pharmacological inhibitors to selectively block signalling through a number of shear-activated mechanosensory mechanisms in endothelial cells (e.g. G-proteins, integrins, PTKs, NO, NADPH oxidase, MAPKs etc.), and the anti-proliferative effects of the harvested BCM subsequenly assessed by

different methods. Our investigations revealed that the shear-dependent decrease in BASMC proliferation putatively involved integrin-, Rac1- and NO-mediated signalling.

In endothelial cells, integrins are established mechanotransducers known to play a role in various biological processes such as cell migration, proliferation, adhesion and survival (Chen *et al*., 1999). They are heterodimeric ECM-binding receptors that can convert mechanical stimuli into intracellular responses. Studies in endothelial cells have shown that integrin clustering with the adaptor proteins Shc and FAK occurs in response to shear stress, which in turn can activate downstream Ras/MAPK pathways (Shyy *et al*., 2002; Chen *et al*, 1999). Studies have also shown that shear stress in HUVECs up-regulates integrin expression (Urbich *et al*., 2000). In our model, cyclic-RGD, a pharmacological inhibitor of β_1/β_2 integrins was used to ablate integrin activation during BAEC shear (Buckley *et al.,* 1999). Our findings indicated that following integrin blockade with cRGD during the BAEC-shearing process, the decrease in BASMC proliferation by LSS-derived BCM was reversed to baseline proliferation levels (Note: a baseline inhibitory effect was observed for cRGD in unsheared controls). This would suggest that the anti-proliferative impact of BCM on BASMCs is mediated through shear activation of an integrin signalling pathway in endothelial cells.

Rac1 is a member of the Rho GTPase family of proteins known to integrate a number of cellular signalling processes. It regulates a diverse array of cellular events including control of cell growth, cytoskeletal reorganization and activation of protein kinases (Li *et al*., 2000) and is activated by mechanical forces such as shear stress

(Chen *et al*., 1999) and cyclic strain (Birukova *et al*., 2006). Rac1 is also an active component of the NADPH oxidase complex. For these studies we employed NSC23766, a pharmacological inhibitor that specifically inhibits Rac1. When NSC23766 was included in the BAEC-shearing process, the anti-proliferative effects of harvested BCM on BASMCs was completely reversed to baseline proliferation levels (Note: a slight baseline inhibitory effect in unsheared control cells was observed), thus implicating shear activation of endothelial Rac1 in these events. Numerous studies have shown a relationship between integrins and Rac1. For example, previous reports have shown that during endothelial shear, integrins can activate downstream Rac1 to induce flow alignment (Goldfinger *et al.,* 2008). Studies have also shown that Rac1 activition in response to shear stress could be regulated by integrin binding to ECM ligands (Tzima, 2001).

Smooth muscle cell proliferation (and apoptosis) is likely modulated by BAEC-derived paracrine factor(s) that are regulated in turn by endothelial shear stress. Our data so far has implicated potential roles for integrins and Rac1 in the anti-proliferative impact of BCM on BASMCs *in vitro*. Shear stress is one of a number of potent physiological stimuli for eNOS activation and NO production in endothelial cells. NO is a known inhibitor of VSMC proliferation (Buga *et al.*, 1991) and has been shown to inhibit RASMC proliferation via MAPK signalling (Bauer *et al*., 2001). Moreover, Kader *et al*. have shown that eNOS over-expression in endothelial cells can inhibit smooth muscle cell proliferation *in vitro*. In their study, BAECs were transfected with the human eNOS gene and co-incubated with BASMCs *in vitro*. eNOS-transfected BAECs significantly over-expressed eNOS and decreased BASMC proliferation (Kader *et al*., 2000). Our own results revealed that eNOS blockade with

L-NAME during BAEC-shear reversed the anti-proliferative effects of LSS-derived BCM on BASMCs. Whilst this would suggest a direct role for NO within the BASMC phase, it is worth noting that NO is a very short-lived signalling metabolite with half-life of just seconds *in vivo*. Thus, high levels of NO are unlikely to be found in BCM after 24 h shear. Nitrite by-products however, may be present in measurable levels, and may be responsible for the anti-proliferative effects observed on BASMCs. Also relevant, Lee *et al*. have demonstrated NO-dependent regulation of endothelial integrin activation, which may also account for the anti-proliferative effects of eNOS blockade in this shear model (Lee *et al.*, 2000).

TGF-β, which is secreted by endothelial cells in a latent inactive form, has been shown to be involved in the inhibition of SMC proliferation (Ueba *et al*., 1997). Studies have shown that upon exposure to laminar shear stress endothelial cells up-regulate TGF-β production (Ohno *et al*., 1995). In view of these observations, we investigated a potential role for endothelial-derived TGF-β in our BASMC proliferation model. Specifically, we employed an "activin receptor-like kinase 5" (ALK5) antagonist (a blocker of TGF-β receptor 1 activation) to attenuate the autocrine/paracrine action of LSS-induced TGF-β on endothelial cells during shear. Interestingly, the resultant BCM was found to have significantly attenuated anti-proliferative impact on BASMCs, suggesting that shear-induced TGF-β may be acting directly on the endothelial cell with putative consequences for BAEC media conditioning (possibly via modulation of NO production - Schwartz *et al.*, 2005). Conversely, the observations may also arise from residual antagonist present in the BCM following 24 h conditioning, which may be preventing interaction of shear-induced endothelial TGF-β with SMC receptors. A more definitive experiment will be

required to yield more conclusive findings on the role of this effector (e.g. siRNA blockade of TGF-β in endothelial cells prior to shear, treatment of post-shear BCM with a TGF-β neutralizing antisera).

Heterotrimeric G-proteins are composed of α-monomers and βγ-dimers, which transduce signals from activated transmembrane receptors (e.g. G-protein coupled receptors, GPCRs) to intracellular effectors. Several studies have reported the ability of G-proteins to transduce shear stress into intracellular signaling cascades (e.g. Ras activation, ERK-1/2 activation) (Jo *et al*., 1997; Gudi *et al*., 2003). Thus, we hypothesized a putative role for shear-induced endothelial G-protein signalling in the anti-proliferative effects of BCM on BASMCs. The pharmacological inhibitor, PTX, was employed to inhibit heterotrimeric Gα subunits. Results indicated that PTX did not attenuate the decrease in BASMC proliferation by LSS-derived BCM, thus ruling out any hemodynamic involvement of endothelial Gα subunit signalling in these events.

Protein tyrosine kinases (PTKs) play a key role in signal transduction to regulate various cellular responses such as proliferation, migration, differentiation, and survival. Studies have shown that in response to shear stress, an increase in PTK activity was observed in endothelial cells (Ishida *et al*., 1996). PTKs have also been shown to play a role in shear-dependent NO production (Corson *et al.*, 1996). For our studies, genistein was employed to attenuate PTK activation and signalling following shear onset in BAECs. Our results indicated that genistein did not reverse the BCM-induced decrease in BASMC proliferation, likely ruling out a role for PTK signalling in these events.

Our data implicating Rac1 involvement in the anti-proliferative impact of BCM is also worthy of further mention. Rac1 is an activating component of the NADPH oxidase complex (Quinn *et al.*, 1993), suggesting involvement of the latter. Shear stress is also known to modulate NADPH oxidase activation and cellular redox levels (i.e. superoxide) in various instances (Duerreschmidt *et al*., 2006). Pre-treatment of BAECs with apocynin (an NADPH oxidase inhibitor) however, did not recover the anti-proliferative effect of BCM, ruling out Rac1-mediated NADPH oxidase activation in these events.

The MAP kinase family are potential downstream candidates to mediate some of the effects of shear stress on vascular cell function. In response to flow, mechanosensors such as integrins, PTK's, and G-proteins can activate the Ras/MAP kinase signalling cascade, initiating transcription factor activation and protein synthesis and leading to changes in cell growth, transformation, and differentiation (Cowan *et al*., 2003). The MAPK family is comprised of three main and distinct signalling pathways: the extracellular signal-regulated protein kinase (ERK), the c-jun N-terminal kinases or stress-activated protein kinases (JNK/SAPK), and the p38 family of MAP kinases (Cowan *et al*., 2003). Investigations have shown that a shear rate of 12 dynes/cm^2 was found to activate endothelial ERK1/2 and p38, but to reduce activity of JNK (Surapisitchat *et al.,* 2001). Moreover our own group have demonstrated activation of ERK1/2 and p38 in BAECs following a physiological dose of cyclic strain (von Offenberg Sweeney *et al.*, 2005). Shear-dependent ERK1/2 activation also leads to increased expression of c-fos and c-jun to form the activating protein-1 (AP-1) transcription factor which plays a significant role in the expression

of a number of genes (Proud, 1994). For our studies the pharmacological inhibitor, PD98059, was employed to block shear-dependent ERK1/2 activation. Interestingly, our results demonstrated that ERK1/2 blockade had no effect on these events. p38 MAPK was not examined in this instance, and so its putative involvement downstream of integrin/rac1 activation cannot be discounted at this time.

Matrix metalloproteinases (MMPs) are a diverse family of mechanosensitive zinc-dependent proteases which degrade ECM components (e.g. collagen, laminin and fibronectin) and non-matrix substrates (e.g. growth factors and cell surface receptors). Previous reports have confirmed that MMPs play a significant role in regulating SMC proliferation, as MMP inhibitor (BB94) was shown to inhibit intimal thickening after arterial injury *in vivo* by decreasing SMC proliferation (Zempo *et al*., 1996). Extracellular proteases, including MMPs, have also been shown to activate TGF-β, a known inhibitor of smooth muscle cell proliferation (Annes *et al*., 2003; Mc Caffrey *et al*., 1995). Moreover, several studies have shown that hemodynamic challenge can differentially modulate MMP expression and function in different vascular cell types, with consequences for vascular cell fates. Previous studies in our lab have demonstrated that cyclic strain of endothelial cells up-regulates MMP-2 and MMP-9 expression, activity and secretion, implicating a pivotal role for these collagenases in hemodynamically-mediated vascular remodelling. Particularly noteworthy, studies by von Offenberg Sweeney and co-workers have shown that strain-induced endothelial MMP-2 significantly reduced smooth muscle cell migration in a BCM model of paracrine regulation (von Offenberg Sweeney *et al.,* 2004), a study which prompted us to consider a putative role for MMP-2 in our SMC proliferation model. Of relevance, shear stress has been shown to regulate MMP's, both in cultured

endothelial cells and in animal models, albeit in an apparently opposite manner to cyclic strain. Studies have shown for example, that MMP-2 production is actually reduced (via NO) in microvascular endothelial cells in response to laminar shear stress (Milkiewicz *et al*., 2006). Bassiouny *et al*. and Palumbo *et al*. also report decreased endothelial MMP-2 production and release in response to high laminar shear (Bassiouny *et al*. 1998; Palumbo *et al*., 2002), whilst oscillatory (but not laminar) shear increased MMP-9 in murine lymphoid endothelial cells (Magid *et al*., 2003). Consistent with the reported down-regulatory effect of shear on gelatinase production and release, our own studies confirm (by gelatin zymmography – data not shown) a shear-dependent increase in intracellular MMP-2, likely due to decreased secretion and enhanced intracellular accumulation. Moreover, blockade of MMP activity in LSS-derived BCM had no statistically significant effect on its anti-proliferative effects on BASMCs.

We have implicated Rac1, integrins and NO in mediating this anti-prolfierative effect. However, their linear progression or indeed other potential downstream components involved are still unknown. Interestingly, our results have ruled out a possible role for both NADPH oxidase and ERK1/2. As previously mentioned, Rac1 is an activating component of the NADPH oxidase complex (Quinn *et al.*, 1993) and shear stress has been shown to modulate NADPH oxidase activation and cellular redox levels (i.e. superoxide) in various instances (Duerreschmidt *et al*., 2006). Interestingly, pre-treatment of BAECs with apocynin (an NADPH oxidase inhibitor) did not recover the anti-proliferative effect of BCM, ruling out Rac1-mediated NADPH oxidase activation in these events. This suggests that Rac1 is mediating this effect via other signalling components such as integrins and NO. Also, several studies

have implicated Rac1, integrins and NO in the regulation of MAPK pathways (ERK1/2, JNK1/2, and p38 MAPK) (Nosaka et al., 2001; Murga et al., 2002). Indeed MAPK have been shown to be activated in response to shear stress. Given that our results demonstrated no role for ERK1/2 in mediating this anti-proliferative effect suggests a possible role for JNK1/2 or p38, however further studies are needed to address this.

We next examined whether the anti-proliferative effects observed in the BAEC/BASMC laminar shear model could also be replicated in an equivalent HAEC/HASMC model. Interestingly, our results revealed that shear-conditioned media from neither HAECs nor BAECs attenuated HASMC proliferation. In a parallel control study, HAEC-conditioned media however, was found to induce an anti-proliferative effect on BASMCs. This latter finding suggests that the lack of observable effect on HASMC proliferation in these studies cannot simply be ascribed to a "species difference" issue (as HAECs can induce an anti-proliferative effect in response to shear). We suspect however, that the extremely rapid doubling time of the commercially available HASMCs (≤24 h) may account somewhat for this phenomenon, possibly in conjunction with a need for significantly higher HAEC shear levels (>20 dynes/cm^2).

In a final series of experiments, we endeavoured to investigate the impact of LSS-derived BCM on the regulation of smooth muscle cell cycle-associated gene expression. Our approach to this study took two formats; (i) Human Cell Cycle RT^2 Profiler™ PCR Microarray to monitor the expression of 84 genes key to cell cycle regulation; (ii) Standard RealTime PCR to individually monitor a *selected* number

cell cycle-associated genes using gene-specific primers. Using the PCR microarray approach, we observed differential regulation of up to 16 genes. Moreover, 8 of these genes displayed a change in expression in excess of 1.5 fold. Notable examples included up-regulation of UBA1 (ubiquitin-like modifier activating enzyme 1) and down-regulation of CDC2 (cell cycle division 2 or CDK1), CCNH (cyclin H), CDC16 (cell division cycle 16 homolog), GTF2H1 (general transcription factor 2H), BCL2 (Bcl-2), KNTC1 (kinetichore associated 1), and MAD2L1 (MAD2 mitotic arrest deficient-like 1).

These changes are basically consistent with a cell cycle inhibitory effect (and thus, anti-proliferative effect). CDK1 (CDC2) for example, is one of the components of the maturation promoting factor (MPF) controling cell division cycle. When CDK1 binds to cyclin B it allows the cell to progress through mitosis (Sherr, 1993). Significant down-regulation of CDK1, as seen in our PCR microarray study, would therefore be expected to block mitotic progression by reducing cyclin B-CDK1 complex formation. GTF2H1 (TFIIH), a multi-subunit complex composed of 9 polypeptides, is a general transcription factor for RNA polymerase, which also plays a role in nucleotide/DNA excision repair (Zawel and Reinberg 1995; Friedberg, 1996). GTF2H1 can be separated into 2 stable sub-complexes; the core GTF2H1 complex and the CDK-activating kinase (CAK) complex. The CAK complex consists of CDK27 (kinase catalytic complex), cyclin H (regulatory subunit), and Mat1 (assembly factor) (Nigg, 1996; Johnson and Walker, 1999). CDK27 has recently been shown to phosphorylate the carboxyl-terminal domain of polymerase II (Roy *et al.*, 1994; Feaver *et al.*, 1994). This induces the RNA polymerase to start producing RNA, marking the end of initiation and the start of elongation (Maldonado and Reinberg,

1995). The kinase activity of GTF2H1 also appears to play a role in promoter clearance and/or elongation of polymerase II transcription (Friedberg, 1996). Our PCR microarray study demonstrated down-regulation of both GTF2H1 and CCNH (cyclin H), again consistent with anti-proliferative events. It is tempting to speculate a possible role for p53, a growth suppressor and a regulator of cell cycle progression, in this context. *In vitro* and *in vivo* studies have shown that wild-type p53 can bind to cyclin H, leading to a down-regulation of the CAK complex activity (Schneider *et al.*, 1998). This may involve p53-mediated transactivation of p21*Cip*1/*Waf*1, an efficient inhibitor of cyclin dependent kinases (Eldeiry *et al.*, 1993). Further studies examining the effects of BCM on p53 and p21*Cip*1/*Waf*1 expression and activation in BASMCs are needed to address this question.

Down-regulation of KNTC1 (Kinetochore associated 1) was also observed in our study. This is the protein structure on chromosomes where the spindle fibers attach during division to pull the chromosomes apart. Thus, down-regulation of KNTC1 prevents chromosomal segregation normally associated with mitosis and meiosis. Interestingly, BCL2 (Bcl-2), an anti-apoptotic protein was also down-regulated, likely pointing to the pro-apoptotic effects observed in BASMCs following BAEC shear.

Finally, the up-regulation of UBA1 (Small Ubiquitin-like MOdifier activating enzyme-1, SUMO-1) deserves some mention. Ubiquitin-like proteins affect the ability of the modified target protein to interact with other cellular factors (unlike ubiquitination, which generally targets proteins for degradation) (Vertegal *et al.*, 2006). Reports on SUMO-1 have shown that it can conjugate to growth suppressor p53 to enhance its transactivation (Gostissa *et al.*, 1999). Thus, up-regulation of

SUMO-1 may increase transactivation of p53 leading to a cell cycle arrest and elevated apoptosis, consistent with the findings of our study. In further support of an underlying role for p53 in these events, previous studies have also shown that p53 interacts with and inhibits the ability of RPA3 (Replication protein A3) to bind to ssDNA (Dutta, 1993; Dutta *et al.*, 1993), thereby preventing the onset of S-phase. RPA3 is a hetero-trimeric single-stranded DNA-binding protein that is highly conserved in eukaryotes. It plays essential roles in many aspects of nucleic acid metabolism including DNA replication (Wobbe *et al*., 1987; Wold and Kelly, 1988), DNA excision repair (Coverley *et al*., 1992; Coverley *et al*., 1991), and recombination (Heyer *et al*., 1990; Moore *et al*., 1991).

Preliminary results from the Human Cell Cycle RT² Profiler™ PCR microarray therefore confirm our hypothesis that the anti-proliferative (and pro-apoptotic) effects of LSS-derived BCM on BASMCs may be reflected in changes in the expression patterns of key cell cycle-regulating genes. In view of the large change observed in CDK1 expression, and of the pivotal role played by cyclin-CDK dynamics in cell cycle progression, it was decided to examine more closely the impact of our shearing paradigm on the expression of a range of cyclins and CDKs in BASMCs using standard RT-PCR in conjunction with pre-designed gene-specific primers.

Following treatment of BASMCs with LSS-derived BCM as described in Methods, our results indicated that cyclin D1 and CDK4 are signficantly up-regulated, whilst CDK1, CDK2, and CDK6 are down-regulated. Induction of cyclin D1 and CDK4 likely initiates G_1 phase progression. Cyclin D is the first cyclin to be produced and assembles with its catalytic partners, CDK4 and CDK6, to progress through the G_1

phase (Sherr *et al*., 1993). Entry into the next phase of the cycle typically relies on CDK2 activation, as CDK2 is sequentially activated by cyclin A and E (Girard *et al.,* 1991; Ohtsubo *et al*., 1995). However, down-regulation of CDK2 likely reduced formation of an active cyclin E-CDK2 complex, preventing G_1/S phase transition. CDK2 down-regulation would also reduce cyclin A-CDK2 complex formation and thus prevent the G_2/M phase transition. Also noteworthy, significant down-regulation of CDK1 would be expected to reduce cyclin B-CDK1 complex formation, which is necessary for the progression of mitosis (King *et al*., 1994; Arellano and Moreno, 1997). Collectively, these expression changes indicate that the anti-proliferative effect of LSS-derived BCM on BASMC putatively involves blockade of G_1/S and G_2/M phase transitions, as well as mitotic progression. These findings also correlate with other studies. Several reports suggest that inhibition of CDK4 and CDK6 may not be necessary to arrest cell cycle progression and that inhibition of CDK2 alone may be sufficient to achieve cell cycle arrest (Brooks *et al*., 1997; Ishida *et al*., 1997; Sasaguri, 1996).

As previously mentioned, cyclin-CDK complexes are regulated by a variety of specific CDK inhibitor proteins that bind to and inactivate CDKs. Members of the KIP/Cip family (p27*Kip*1 and p21*Cip*1/*Waf*1) have an inhibitory effect on cyclin E-CDK2 complex formation (Sherr and Roberts, 1999), thus halting the cell cycle in the G_1 phase. Studies have shown that p27*Kip*1 over-expression completely inhibits CDK2 activity and cell growth (Polyak *et al*., 1994; Toyoshimap *et al*., 1994). Similarly, recombinant p21*Cip*1/*Waf*1 inhibits CDK2, but not CDK4, activity (Poon *et al*., 1996), and p21*Cip*1/*Waf*1-deficient mouse embryo fibroblasts showed an increase in CDK2, but not CDK4, activity compared to wild type animals (Brugarolas

et al., 1998). For our PCR study, we prepared gene-specific primers to examine the expression of p27*Kip1*. Interestingly, our results revealed that endothelial shear did not appear to affect p27*Kip1* expression levels in SMCs. Whilst this suggests that this inhibitor is not involved in our specific shear model, post-translational modification and activation of cellular pools of p27*Kip1* cannot be ruled out at this point.

On a related note, endothelial-derived NO within the vasculature stimulates generation of cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP) in both the endothelium and the underlying smooth muscle cell layer. cAMP and cGMP are second messengers involved in the intracellular signal transduction of a wide variety of extracellular stimuli. These signals regulate many biological processes including cell proliferation, differentiation, migration, and apoptosis (Koyama *et al*., 2001). Studies have shown that cAMP can inhibit smooth muscle cell proliferation *in vitro* (Southgate and Newby, 1990; Assender *et al*., 1992) and reduce formation of neointimal lesions after arterial injury *in vivo* (Indolfi *et al*., 1997; Wang *et al*., 2000). Interestingly, cAMP suppresses expression of both cyclin D and A, and stimulates up-regulation of p27*Kip*1 in smooth muscle cells, thus preventing G_1/S transition (Vadiveloo *et al*., 1997; Fukumoto *et al*., 1999). cAMP induction of p27*Kip*1 in-turn has also been shown to suppress CDK2 and CDK4 activities in human smooth muscle cells (Koyama *et al*., 2001; Fukumoto *et al*., 1999). Recent reports also show a potential role for cGMP in regulation of cell cycle progression. Specifically, cGMP has been reported to up-regulate p21*Cip*1/*Waf*1, which associates with and suppresses CDK2 activity (Ishida *et al*., 1997; Tanner *et al*., 2000). Whilst our data implicates a role for NO (and/or NO-derived nitrite by-products) in the anti-proliferative effect of LSS-derived BCM on

BASMCs, the lack of either p27*Kip*1 up-regulation or CDK4 suppression suggests NO may be working via a cAMP-independent pathway. In this regard, the observed down-regulation of CDK2 may possibly be attributable to cGMP-dependent up-regulation of p21*Cip*1/*Waf*1 (not examined in our investigations).

In conclusion therefore, we have shown that laminar shear stress of BAECs *in vitro* decreases BASMC proliferation. Within the endothelium these events are mediated through integrin-, Rac1-, and NO-dependent signalling pathways. Furthermore, our studies implicate endothelial shear in the regulation of cell cycle-associated gene expression in SMCs, with consequences for cell cycle progression at the G_1 phase.

CHAPTER 6

Final Summary

6.1 Final Summary

The vascular endothelium is strategically located between the bloodstream and the vessel wall, where it plays a pivotal role in vascular homeostasis (Sagripanti *et al*., 2000). Due to its unique location, it regulates the impact of physiological stimuli (mechanical and humoral) on endothelial dependent vessel tone and remodelling events. Under normal conditions it imparts an atheroprotective effect displaying anti-platelet, anti-coagulant, and fibrinolytic properties. Thus, a critical balance between the endothelium-derived relaxing and contracting factors is necessary to maintain vascular homeostasis. Of particular importance are the mechanical or hemodynamic forces associated with blood flow, which impact upon the endothelium. These include cyclic circumferential strain, the transmural force acting perpendicularly to the vessel wall, and fluid shear stress, the frictional force generated by blood flow. Both forces can profoundly impact the endothelium and can regulate vascular cell functions including cell morphology, cell function/fate and the synthesis/secretion of various macromolecules essential for the regulation of vessel remodelling processes (Traub *et al*., 1998). Moreover disruption of normal hemodynamic forces can be either causative of, or contributory to vascular diseases such as atherosclerosis and stroke (Esper *et al*., 2006).

In view of the importance of hemodynamic forces to endothelium function and vessel health, the aim of this study was to investigate the putative impact(s) of hemodynamic challenge to the endothelium on the properties of the underlying medial layer SMCs. In this regard, four experimental models were employed, namely; (i) laminar shear stress; (ii) turbulent shear stress; (iii) pulsatile laminar shear stress with

co-culture; and (iv) equibiaxial cyclic strain. For laminar shear stress studies, BAECs were exposed to shear in 6-well plates via rotation on an orbital shaker (0-10 dynes/cm^2, 0-48 h) as previously described (Hendrickson *et al*., 1999; Colgan *et al*., 2007). BAEC-conditioned media (BCM) was subsequently harvested and incubated with quiescent BASMCs, after which, BASMC proliferation and apoptosis were monitored. For turbulent shear stress, an improvised model (back-forth shaking) was employed to generate TSS-derived BCM for studies with BASMCs. For pulsatile shear studies, a CELLMAX® Artificial Capillary System (a perfused transcapillary co-culture system) was employed. This is a far more accurate mimic of both the three-dimensional hemodynamic environment of a blood vessel and the spatio-temporal dynamics of endothelial-smooth muscle cell communication. For cyclic strain studies, BAECs were into custom 6-well Bioflex® plates comprising a flexible pronectin-bonded growth surface and exposed to equibiaxial cyclic strain (0-10% strain, 60 cycles/min, 24 h, cardiac waveform) using a Flexercell® Tension Plus™ FX-4000T™ system (Flexcell International Corp.- Hillsborough, NC).

We commenced our studies by examining the basic characteristics of commercially available cell lines (bovine and human endothelial/smooth muscle cells) under *static* conditions in complete growth media. These initial studies confirmed normal endothelial and smooth muscle cell morphology ("cobble-stone" versus "spindle-shaped"), as well as expression of cell-specific markers (ECs: von Willebrand Factor, SMCs: SMC-specific α-actin). Bovine endothelial cells were also examined under conditions of *hemodynamic* challenge to ensure normal hemodynamic responsiveness. Specifically, we examined the effect of laminar shear stress and cyclic strain on BAEC morphology, F-actin dynamics, and tight junction-associated zonula

occludens-1 (ZO-1) localisation. Exposure of BAECs to chronic shear stress (10 dynes/cm^2, 24 h) resulted in distinct morphological realignment of cells in the direction of the shear vector, along with a clear realignment of the actin cytoskeleton in the direction of flow. Moreover, Rhodamine-Phalloidin staining demonstrated that cortical actin formation (consistent with elevated barrier function) was clearly up-regulated and visible along the cell periphery in response to shear stress. Cell-cell border localisation of ZO-1 was also increased as it appeared more continuous and evenly distributed along the cell-cell border under shear stress. With respect to tight junction ZO-1, the regulatory association between hemodynamic forces and endothelial tight junction assembly and barrier function has been well established (Conklin *et al*., 2002; Collins *et al*., 2006; Colgan *et al.*, 2007). Regulation of barrier integrity is crucial for normal endothelial-mediated vascular homeostasis and is a central pathophysiological mechanism of many vascular remodelling-associated processes (Balda and Matter 1998; Harhaj and Antonetti, 2004). Indeed, vascular pathologies exhibiting altered vessel hemodynamic loading with associated remodelling (e.g. atherosclerosis and restenosis) frequently correlate with compromised endothelial barrier integrity (Harhaj and Antonetti 2004; van Nieuw *et al*., 2002). Also of relevance, up-regulation of eNOS mRNA and protein levels were also observed in BAECs after exposure to shear stress. In response to cyclic strain (10% equibiaxial strain, 24 h cardiac waveform), enhanced ZO-1 cell-cell border localisation was also observed in parallel with an up-regulation of MMP-2 mRNA, observations again consistent with previous publications (Collins *et al*., 2006; von Offenberg Sweeney *et al*., 2004a).

Vascular remodelling in response to mechanical stimuli requires careful regulation of smooth muscle cell properties such as proliferation and apoptosis (Galis *et al.,* 2002; Clarke and Bennett 2006). A number of endothelial-derived biomolecules released in response to mechanical forces putatively impact vascular smooth muscle cell fates (Wang *et al.,* 2003; Sumpio *et al.,* 1998; Cucina *et al.,* 2003; Powell *et al.,* 1998; Fillinger *et al.,* 1997). Interactions between ECs and SMCs in the vessel wall are considered to be an important factor in the control of blood vessel growth and function (Spagnoli *et al*., 1982). Previous studies in animal models have demonstrated that SMC proliferation occurs quickly after endothelial injury and may account for early lesion formation (Clowes and Schwartz, 1985), with many advanced plaques consisting of a SMC-rich fibrous cap overlying a necrotic core. Until recently, SMCs have been viewed as being directly responsible for initiating the atherosclerotic plaque, through elevated proliferation, migration from the medial layer, and synthesis of matrix proteins (Ross, 1993). By contrast, recent work has presented evidence showing a beneficial/protective role of SMC proliferation in atherosclerosis (Weissber, 1996). These studies indicate a paucity of SMCs in plaques that have undergone plaque rupture compared with stable lesions (Davies, 1993). Moreover, arteries prone to develop atherosclerosis, as well as unstable plaques typically have "fewer" SMCs (Davies *et al*., 1993; Tracy, 1995). It is now generally accepted that SMCs can protect the integrity of the *developed* plaque by promoting stability through elevated proliferation. Indeed, a lack of SMC proliferation in plaques, due in part to cellular senescence, and even SMC death via apoptosis or other mechanisms, is associated with the late consequential events in the atherosclerotic plaque progression and destabilisation (Newby and Zaltsman, 1999). Thus, whilst elevated SMC proliferation may be a contributory feature of endothelial dysfunction and early

plaque development, it also paradoxically serves as a protective feature wrt plaque nature, stabilization, and rupture.

SMC apoptosis has been implicated in a number of deleterious aspects of atherosclerosis, including plaque rupture, vessel remodelling, coagulation, inflammation, and calcification. Apoptosis of SMCs within atherosclerotic plaques has been shown to induce multiple features of plaque vulnerability (to rupture), including fibrous cap thinning, increased necrotic cores, and inflammation. The local inflammation may further weaken the fibrous cap by secretion of matrix metalloproteinases, leaving the "thinned" cap prone to rupture (Clarke and Bennett 2006). In early lesions, apoptotic frequencies are minimal (relative to elevated proliferative state) but peak in advanced plaques with both SMCs and macrophages showing features of elevated apoptosis (Lutgens *et al*., 1999). Indeed, plaques from patients with unstable symptoms show higher levels of apoptosis than those with stable lesions (Geng *et al*., 1995). This is consistent with the reports of reduced SMC proliferation in unstable plaques, and points to the reciprocal coupling of proliferative and apoptotic status in SMCs during plaque initiation and progression to maturity.

Mindful of these facts, we began to address our experimental objective; To investigate the impact of hemodynamic challenge to BAECs on BASMC proliferation and apoptosis using various hemodynamic modeling paradigms (described above). Initial investigations using the orbital rotation model of shear demonstrated that LSS-derived BCM (0-10 dynes/cm^2, 0-48 h) decreased BASMC proliferation whilst increasing apoptosis. The force-and time-dependency of these effects was also confirmed. As the two vascular cell types were not in physical contact during shear,

this indicated that the effects observed could be attributed to a BAEC-secreted factor(s) acting on BASMCs. As a control experiment, we also examined the effect of TSS of BAECs (using a highly improvised model of turbulent shear stress) on BASMC proliferation and apoptosis. In vessel areas typically exposed to TSS, vessel homeostasis becomes imbalanced leading to endothelial dysfunction and atherogenesis (Esper *et al*., 2006). Our results showed that TSS-derived BCM slightly increased BASMC proliferation (without effecting BASMC apoptosis), essentially in contrast to the anti-proliferative effect induced by LSS-derived BCM.

A more superior pulsatile laminar shear model, the CELLMAX® Artificial Capillary System, was next used to investigate the impact of pulsatile laminar shear on BAEC-dependent regulation of BASMC proliferation and apoptosis in a perfused *co-culture* format (0.3 -vs- 20 dynes/cm^2, 5 days). Our initial results indicated that BASMC mono-cultures (i.e. no intra-lumenal BAECs) increased their proliferation slightly under high pulsatile shear stress, likely due to elevated hydrostatic pressure (Birney *et al*., 2004). In the presence of intra-lumenal BAECs however, significant anti-proliferative effects on co-cultured BASMCs were clearly observed at high shear. This is consistent with our earlier observations of anti-proliferative effects with LSS-derived BCM (i.e. from the orbital rotation shear system) and also builds very significantly on the earlier, rudimentary findings of Nackman and co-workers (Nackman *et al.*, 1998). Using this pulsatile co-culture model, we also noted with interest that high shear enhanced BASMC apoptosis to a similar extent in both the absence and presence of intra-lumenal BAECs. Whilst this is consistent with the earlier findings of Birney *et al*., in which high pulse-pressure was found to elevate BASMC apoptosis (Birney *et al*., 2004), it is in contrast with the BAEC-dependent

pro-apoptotic effects observed using LSS-derived BCM. We concluded that this finding may point to an (undefined) apoptotic pathway that can be induced by either hydrostatic pulse pressure *or* BCM in a non-additive manner.

Consistent with the above findings following BAEC shear stress, BCM harvested from BAECs exposed to cyclic strain (0-10% strain, 60 cycles/min, 24 h, cardiac waveform) also demonstrated an anti-proliferative and pro-apoptotic impact on BASMCs. This finding is in full agreement with an earlier study from our laboratory in which treatment of BAECs with an identical cyclic strain regimen significantly down-regulated BASMC migration via an MMP-2-dependent mechanism (von Offenberg Sweeney *et al*., 2004b).

For the final chapter of the thesis, we decided to specifically focus on *how* laminar shear stress of BAECs impacts BASMC proliferation, with initial emphasis on the endothelial mechanosensor and effector components putatively mediating the anti-proliferative effects of LSS-derived BCM. Pharmacological inhibitors were employed to selectively block endothelial signalling mechanisms during shear-dependent BCM generation. In this regard, our investigations point to the importance of endothelial integrin-, Rac1- and NO-dependent mechanisms in the shear-dependent conditioning of BCM and subsequent anti-proliferative effects on BASMCs (Fig. 6.1).

Integrins were initially examined as previous studies report their frequent role in transduction of mechanical forces in endothelial cells (Shyy *et al*., 2002; Chen *et al.,* 1999). They are also involved in activating downstream pathways which modulate cellular responses. Our results revealed that incubation of BAECs with cRGD during

BCM generation could reverse the anti-proliferative effects of LSS-derived BCM on BASMCs. Interestingly, the cRGD-dependent reversal appeared more significant at day 6, as opposed to day 4 (and vice-versa for Rac1). This most likely stems from counting error in analysis of the BASMC growth curves over 7 days, or may possibly reflect an undefined temporal factor(s) in these experiments. Rac1 has been implicated in a diverse array of cellular events such as control of cell growth and cytoskeletal reorganisation, and therefore plays a pivotal role in the responsiveness of endothelial cells to shear stress (Li *et al.,* 2000; Ridley *et al*., 1992; Nobes and Hall 1995; Tzima *et al*., 2002). Not surprisingly, incubation of BAECs with NSC23766, a potent Rac1 inhibitor, reversed the anti-proliferative action of LSS-derived BCM. Of relevance, studies have shown that in endothelial cells under shear, integrins may activate downstream Rac1 to induce flow alignment of endothelial cell morphology (Goldfinger *et al.,* 2008). The integrin-dependent regulation of Rac1 activity in endothelial cells is likely due to integrin binding of ECM ligands (Tzima, 2001). Conversely, studies have also demonstrated that Rac1 may activate integrins downstream in respone to shear stress in endothelial cells. Fujiwara *et al*. have shown that Rac1 activation is pivotal in endothelial cells for integrin-mediated cell spreading and migration (Fujiwara *et al*., 2004). Mohri *et al*., have also shown that Rac1 can up-regulate integrin expression and induce cell adhesion (Mohri et al., 2002). The present study therefore suggests that shear activation of the integrin-Rac1 signalling axis not only have consequences for endothelial properties, but also for endothelial-dependent regulation of underlying SMCs.

Nitric oxide (NO) is produced in endothelial cells in response to normal levels of shear stress and is known to have numerous effects on the vessel wall, including anti-

proliferative effects on SMCs (Buga *et al*., 1998). Indeed, areas of low shear manifest reduced NO production (Buga *et al*., 1991), a potential contributor to proliferation of smooth muscle cells and lesion initiation *in vivo*. Incubation of BAECs with L-NAME (blockade of eNOS) appeared to recover the anti-proliferative action of LSS-derived BCM, suggesting NO involvement in this model. As NO has a relatively short (seconds) physiological half-life (Hakim *et al*., 1996), one is unlikely to find considerable levels of NO in BCM after 24 h shear. Rather, nitrite by-products are likely to be present in measurable levels and may possibly contribute to the anti-proliferative effects seen on BASMCs. NO-dependent regulation of endothelial integrin activation as previously described (Lee et *al*., 2000) may also possibly account for the apparent anti-proliferative effects of L-NAME in this regulatory model.

Interestingly, selective blockade of heterotrimeric Gα-subunits, protein tyrosine kinases (PTK), and ERK-1/2 signalling in endothelial cells had *no* significant effect on the anti-proliferative effects of BCM. Shear-dependent activation of these signalling components has previously been reported in endothelial cell studies. Heterotrimeric G-proteins for example, have been shown to activate ERK1/2 and Ras GTPase activity in response to shear stress (Jo *et al*., 1997; Gudi *et al*., 2003), and have also been implicated in flow-induced angiogenesis (Cullen *et al*., 2002). Shear-dependent activation of PTK has also been demonstrated in endothelial cells with consequences for downstream events including NO production (Corson *et al*., 1996), MAPK activation (Ishida *et al.*, 1996; Takahashi *et al.*, 1996). Moreover, ERK1/2 has also been shown to be activated by shear stress leading to changes in endothelial growth properties (Surapisitchat *et al*., 2001).

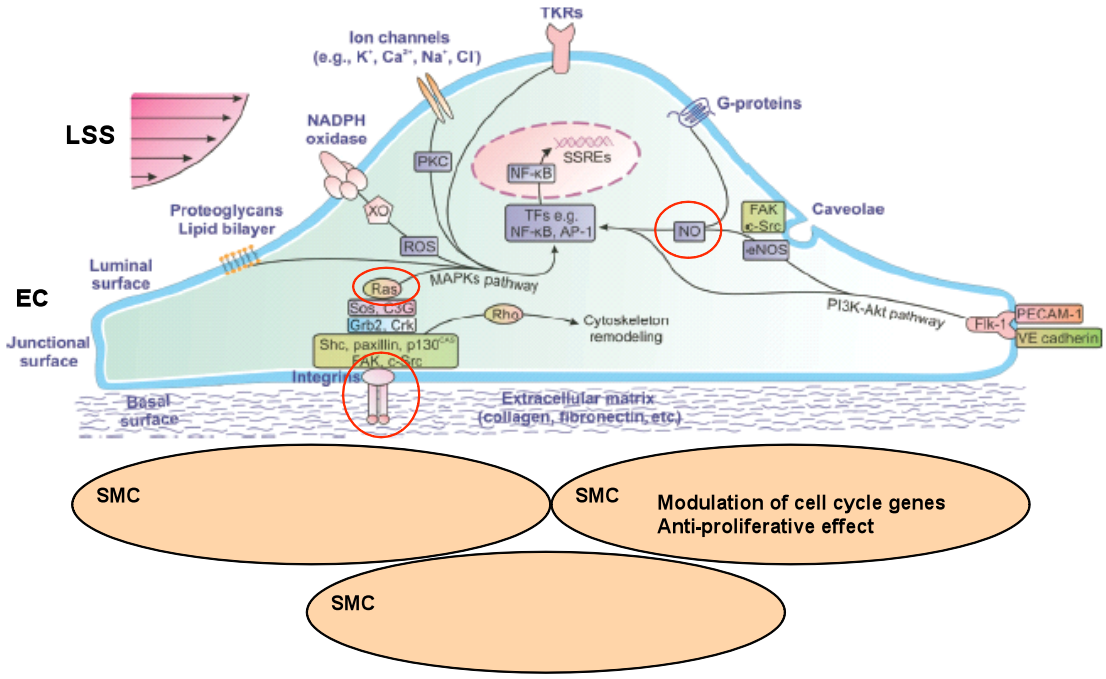


Fig. 6.1: Proposed Signaling model: The above schematic depicts the endothelial signalling mechanism proposed to transduce endothelial shear into reduced SMC proliferation. LSS-dependent activation of an Integrin-Rac1 signaling a likely upstream event, with possible consequences for NO production downstream. NO production can give rise to intracellular and extracellular cAMP, cGMP and nitrite by-products. Both transcrptional and translational events are proposed to contribute to media conditioning during hemodynamic challenge (Chatzizisis et al.,2007)

In a separate series of experiments, we examined whether the anti-proliferative effects observed in the BAEC/BASMC laminar shear model could also be replicated in an equivalent HAEC/HASMC model. Interestingly, our results revealed that shear-conditioned media from neither HAECs nor BAECs attenuated HASMC proliferation. In a parallel control study, HAEC-conditioned media however, was found to induce an anti-proliferative effect on BASMCs. This latter finding suggests that the lack of observable effect on HASMC proliferation in these studies cannot simply be ascribed to a "species difference" issue (as HAECs can induce an anti-proliferative effect in response to shear). We suspect however, that the extremely rapid doubling time of the commercially available HASMCs (≤24 h) may account somewhat for this

phenomenon, possibly in conjunction with a need for significantly higher HAEC shear rates than the 10 dynes/cm^2 employed here.

In a final series of experiments, we used RealTime PCR (microarray and individual primers) to investigate the impact of LSS-derived BCM on the regulation of smooth muscle cell cycle-associated gene expression. Using the PCR microarray approach, we observed differential regulation (i.e. ≥1.5 fold) of up to 8 genes. Notable examples included up-regulation of UBA1 (ubiquitin-like modifier activating enzyme 1) and down-regulation of CDC2 (cell cycle division 2 or CDK1), CCNH (cyclin H), CDC16 (cell division cycle 16 homolog), GTF2H1 (general transcription factor 2H), BCL2 (Bcl-2), KNTC1 (kinetichore associated 1), and MAD2L1 (MAD2 mitotic arrest deficient-like 1). These changes are basically consistent with a cell cycle *inhibitory effect* (and thus, *anti-proliferative effect*) and appear to confirm our hypothesis that the anti-proliferative (and pro-apoptotic) effects of LSS-derived BCM on BASMCs may be reflected in changes in the expression patterns of key cell cycle-regulating genes. CDK1 (CDC2) for example, is one of the components of the maturation promoting factor (MPF) controling cell cycle division. When CDK1 binds to cyclin B, it allows the cell to progress through mitosis (Sherr, 1993). Significant down-regulation of CDK1 (over 7 fold), as seen in our PCR microarray study, would therefore be expected to block mitotic progression by reducing cyclin B-CDK1 complex formation. Down-regulation of KNTC1 (Kinetochore associated 1), the protein regulating chromosmal segregation during mitosis/meiosis, was also observed in our study. BCL2 (Bcl-2), an anti-apoptotic protein was also down-regulated, likely pointing to the pro-apoptotic effects observed in BASMCs following BAEC shear.

Similarly, the up-regulation of UBA1 (SUMO-1) observed in our PCR microarray study is also highly relevant. Reports on SUMO-1 have shown that it can conjugate to growth suppressor p53 to enhance its transactivation (Gostissa *et al*., 1999). Thus, up-regulation of SUMO-1 may increase transactivation of p53 leading to a cell cycle arrest and elevated apoptosis, consistent with the findings of our study. In further support of an underlying role for p53 in these events, previous studies have also shown that p53 interacts with and inhibits the ability of RPA3 (Replication protein A3) to bind to ssDNA (Dutta, 1993; Dutta *et al.*, 1993), thereby preventing the onset of S-phase.

In view of the considerable fold down-regulation observed in CDK1 expression using the Human Cell Cycle RT² Profiler™ PCR microarray, and in view of the pivotal role played by cyclin-CDK dynamics in cell cycle progression, we decided to examine more closely the impact of our shearing paradigm on the expression of a range of cyclins and CDKs in BASMCs using standard RT-PCR in conjunction with pre-designed gene-specific primers. Following treatment of BASMCs with LSS-derived BCM as described in Methods, our results indicated that cyclin D1 and CDK4 are signficantly up-regulated, whilst CDK1, CDK2, and CDK6 are down-regulated. The down-regulation of CDK2 is particularly significant as CDK2 up-regulation and activation is normally required for G_1/S phase transition within the cell cycle. CDK2 is sequentially activated by cyclin A and E (Girard *et al.,* 1991; Ohtsubo *et al*., 1995). Thus, down-regulation of CDK2 would likely reduce formation of an active cyclin E-CDK2 complex, preventing G_1/S phase transition. CDK2 down-regulation would also reduce cyclin A-CDK2 complex formation, thus preventing the G_2/M phase transition. These findings also correlate with other studies. Several reports suggest that inhibition

of CDK4 and CDK6 may not be necessary to arrest cell cycle progression and that inhibition of CDK2 alone may be sufficient to achieve cell cycle arrest (Brooks *et al*., 1997; Ishida *et al*., 1997; Sasaguri, 1996). Also noteworthy, significant down-regulation of CDK1 would be expected to reduce cyclin B-CDK1 complex formation, which is necessary for the progression of mitosis (King *et al*., 1994; Arellano and Moreno, 1997). In conclusion therefore, these expression profiles indicate that the anti-proliferative effects of LSS-derived BCM on BASMCs putatively involve blockade of G_1/S and G_2/M phase transitions, as well as mitotic progression (Fig. 6.2).

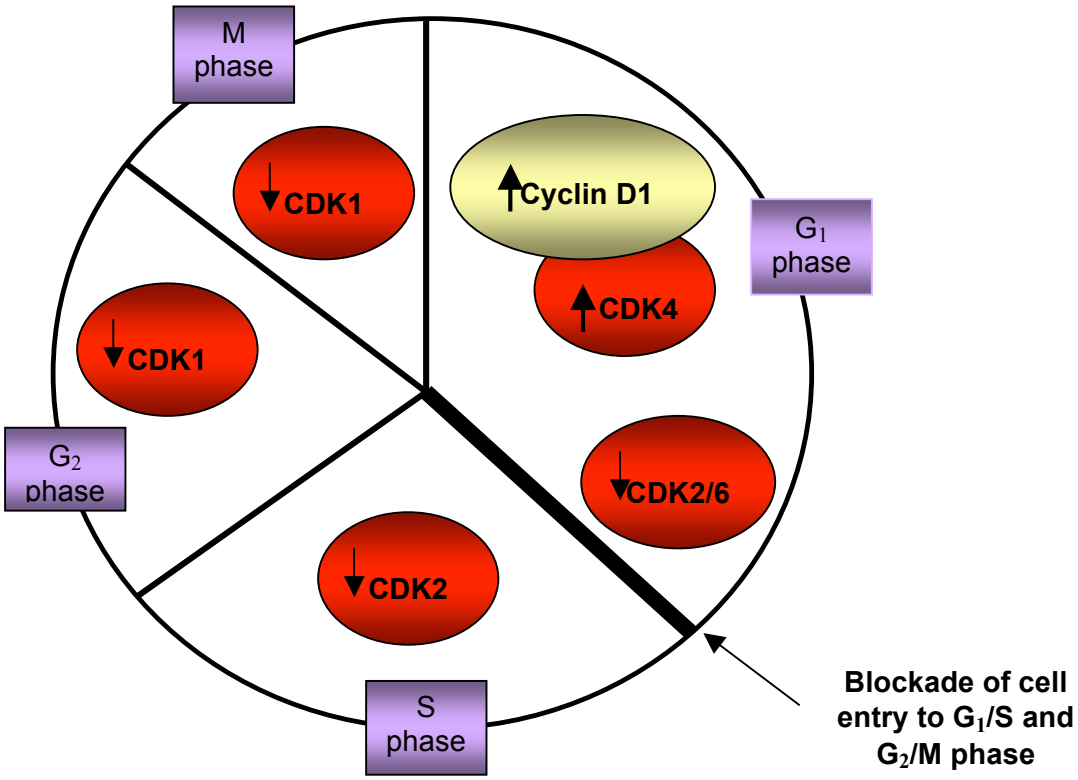


Fig. 6.2: Cell cycle regulation: The above schematic depicts the impact of LSS-derived BCM on regulation of cell cycle-associated cyclin-CDK gene expression. Arrows indicate up- or down-regulation of gene expression.

In conclusion, the aim of this thesis was to model the regulatory impact of the vascular endothelium on smooth muscle cell function(s) as a consequence of the blood flow-associated hemodynamic forces, shear stress and cyclic strain. To this end, we can conclude that defined hemodynamic challenge to BAECs can induce release of paracrine signals leading to anti-proliferative and pro-apoptotic effects on BASMCs (at physiological hemodynamic loadings). We speculate that a Rac1-NO signalling pathway, possibly upstream of integrin production, may be mediating this endothelial regulatory response under laminar shear stress. With respect to the anti-proliferative effects of LSS, BCM-induced blockade of the smooth muscle cell cycle at the G_1/S and G_2/M phase transitions, as well as blockade of mitotic progression, are proposed.

This study has highlighted the importance of hemodynamic forces to proper vessel homeostasis and endothelial-dependent remodelling, with normal hemodynamic stimulation clearly prompting the endothelium to suppress SMC growth and migration. Indeed, hemodynamically-sensitive areas of the vasculature manifesting pathological blood flow patterns can be transduced by ECs into paracrine signalling events with consequences for dysregulation of SMC gene expression, unchecked growth/migration and ultimately initiation of atherosclerotic plaque formation.

Finally, over the course of these studies we have addressed the question of hemodynamic EC-SMC regulation using a broad range of modelling approaches, in conjunction with multiple analytical read-outs. To our knowledge, this is the first time this question has been addressed to this extent and it builds on other directly related studies in this field published through this laboratory (von Offenberg Sweeney *et al.*,

2004b). Moreover, several endothelial and smooth muscle cell-associated signalling components have been identified as being relevant to this process. We anticipate that these findings will contribute to a better overall understanding of the mechanoregulatory dynamic that exists between these two cell types, with future benefits for the development of strategies to treat atherosclerosis and other vascular diseases.

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