Hemodynamic regulation of MMP-2 and MMP-9: Roles in angiogenesis and migration

A dissertation submitted for the degree of Ph D

By

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Declaration

I hereby certify that this material which I now submit for assessment on the programme of leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work

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Date 27th July 2004.

Acknowledgments

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I would like to dedicate this thesis to my family and parents without whose encouragement and support (both financial and emotional) kept me on the straight on narrow for longer than I care to remember and without whom I could not have achieved this

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Abstract

Hemodynamic forces generated by the flow of blood are crucial in maintaining homeostasis within the blood vessel wall These forces, namely cyclic strain and shear stress are intricately involved in vascular remodeling, a process which underlies the pathogenesis of cardiovascular diseases such as atherosclerosis and restenosis Since degradation of the extracellular matrix scaffold enables reshaping of tissue, the role matrix metalloproteinases (MMPs) has become the object of intense recent interest in relation to physiological and pathological vascular remodeling The culminating data indicates that hemodynamic forces are important regulators of MMP expression and activity A more complete understanding of the hemodynamic regulation of MMPs may advance the understanding of pathological vascular remodeling

We have investigated the effect of cyclic strain on the endothelial cell migration and angiogenic activity and the role of gelatinases in mediating these responses We have shown that exposure of bovine aortic endothelial cells (BAEC) to cyclic strain promoted migration and tubule formation with concurrent increases in MMP-2 and MMP-9 activity Additionally, we have revealed that cyclic strain-induced increases in migration and tube formation are dependent on Gi-protein and integrin signaling However, cyclic strain stimulated increases in MMP-2 expression involve different signaling mechanisms, which in part, stimulate both p38- and ERK-dependent pathways through activation of Gβγ and tyrosine kinase in BAEC

The participation of gelatinases in strain-induced increases in BAEC migration and tube formation was determined by inhibition of MMP activity using either a broad spectrum MMP inhibitor (GM6001) or siRNA targeted specifically to MMP-2 or MMP-9 We have shown that cyclic strain-induced increases in BAEC migration are independent of MMP activity In addition, we have demonstrated that MMP-9 but not MMP-2 is the key angiogenic switch involved in evoking cyclic strain-induced angiogenesis

In conclusion, we examined the role of BAEC derived factors in regulating bovine aortic smooth muscle cell (BASMC) migration Our data has shown that exposure of BASMC to conditioned media from cyclically strained BAEC inhibits SMC migration compared to controls and that MMP-2 is an important factor in mediating this inhibition These findings clearly demonstrate that increases in MMP expression and activity associated with cyclic strain are important in modulating both BAEC and BASMC phenotype

Abbreviations

ADAM	A Disintegrin and Metalloproteinase
Ap-1	Activating protein-1
AP-2	Activating protein-2
AVF	Arteriovenous fistulae
BAEC	Bovine aortic endothelial cell
BASMC	Bovine aortic smooth muscle cell
BCA	Bicinchominic acid
CAM	Chorioallantoic membrane
cDNA	Complimentary DNA
DMSO	Dimethylsulfoxide
DNA	Deoxy nucleic acid
ECM	Extra cellular matrix
EDTA	Ethylenediamine tetracetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular regulated kinase
ET-1	Endothelin-1
FAK	Focal adhesion Kinase
FBS	Fetal bovine serum
FGF	Fibroblasr growth factor
FGFR	Fibroblasr growth factor receptor
GAPDH	Glyceraldehyde phosphate dehydrogenase
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GPI	Glycophosphatidylinositol
Grb-2	Growth factor binding protein-2
HBSS	Hanks buffered saline solution
HPF	High power field

ICAM-1	Intracellular adhesion molecule-1
ILGF	Insulin like growth factor
ILGFR	Insulin like growth factor receptor
JNK	c-jun N-terminal kinases
LB	Luria bertrani
LDH	Lactate dehydrogenase
MAPK	Mitogen activated protein kinases
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
mRNA	Messanger Ribonucleic acid
MT-MMP	Membrane Type-MMP
NAD(P)	Nicotinamide adenine dinucleotide phosphate
NOS	Nitric oxide synthase
OxLDL	Oxidised Low density lipoprotein
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PI-3 kinase	Phosphoinositide 3 kinase
PTK	Protein Tyrosine Kinase
PTX	Pertussis toxin
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
SAPK	Stress activated protein kinase
Shc	Src homology/collagen
SiRNA	Small interfering RNA
SNP	Single nucleotide polymorphism

SSRE	Shear stress response element
TBS	Tris buffered saline
TF	Tissue factor
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metallo proteinases
TNF-α	Tumour necrosis factor-alpha
t-PA	Tissue plasminogen activator
u-PA	Urokinase plasminogen activator
u-PAR	Urokinase plasminogen activator receptor
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

Units

Bp	Base Pairs
Cm	Centimeters
g	Grams
h	Hours
kDa	Kilo Daltons
L	Litre
M	Molar
mg	Miligrams
Min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
ng	Nanogram
°C	Degree Celsius
OD	Optical density
pM	Picomolar
Rpm	Revolution per minute
Sec	Seconds
U	Enzyme units
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
V/v	Volume per volume
W/v	Weight per volume

Publications

Papers

- Eoin J Cotter, Nicholas von Offenberg Sweeney, Paul M Coen, Yvonne A Birney, Marc J Glucksman, Paul A Cahill, and Philip M Cummins Regulation of Endopeptidases EC3 4 24 15 and EC3 4 24 16 in Vascular Endothelial Cells by Cyclic Strain Role of Gi Protein Signaling Atherosclerosis, Thrombosis and Vascular Biology 2004,24 1-8
- Nicholas von Offenberg Sweeney, Philip M Cummins, Yvonne A Birney, John P Cullen, Eileen M Redmond and Paul A Cahill Cyclic Strain-Mediated Regulation of Endothelial Matrix Metalloproteinase-2 Expression and Activity In press Cardiovascular Research
- Nicholas von Offenberg Sweeney, Philip M Cummins, Yvonne A Birney, John P Cullen, Eileen M Redmond and Paul A Cahill Cyclic Strain-Induced Endothelial MMP-2 Role in Vascular Smooth Muscle Cell Migration Submitted to BBRC
- Nicholas von Offenberg Sweeney, Philip M Cummins, Yvonne A Birney, John P Cullen, Eileen M Redmond and Paul A Cahill Roles of MMP-2 and MMP-9 in cyclic strain induced increases in endothelial cell migration and tube formation In preparation
- Guibal E, von Offenberg Sweeney, N, Vincent T and Tobin JM, Sulphur derivatives of chitosan for palladium sorption, React Funct Polym 2002, 50 149-63
- Guibal E, von Offenberg Sweeney N, Vincent T and Tobin JM, Competitive sorption of platinum and palladium on chitosan derivatives Int J Biol Macromol 2001, 12 401-8

Poster presentations

- von Offenberg Sweeney N, Cummins PM, and Cahill PA Mechanical regulation of MMP-2 Roles of G-proteins, integrins and protein tyrosine kinases Irish Association of Pharmacology, Dublin, 2003
- von Offenberg Sweeney N, Cummins PM, Birney YB, Cahill PA Role of SHC/MAP Kinase pathway and Giα proteins in cyclic strain induced increases in Pro-MMP2 secretion *Supplement to Circulation* Oct 2002, **106** 1057
- von Offenberg Sweeney N, Birney Y Cummins PM Cahill PA Cyclic strain induces pro-MMP2 release from bovine aortic endothelial cells via a Gi protein-independent pathway and via a MAP kinase-dependent pathway *Supplement to Faseb J* 2002, **16(3)**
- von Offenberg Sweeney N, Coen P, Cummins PM, Cahill PA Shear stress induced pro-MMP2 activity in bovine aortic endothelial cells via an inhibitory Gi protein *Ir J Med Sci* 2001, **170**

Oral Presentations

- Haemodynamic regulation of endothelial MMPs and their role in migration and angiogensis Department of Biotechnology, Seminar series, Dublin City University, 2002
- Cyclic strain induced increases in MMP-2 via a Gi-alpha protein independent pathway Irish Association of Pharmacologists, Dublin, 2001

Table of Contents

Chapter 1

1 1 Introduction

Cardiovascular disease (CVD), principally heart disease and stroke, is one of the worlds leading killers for both men and women among all racial and ethnic groups According to the World Health Organization, cardiovascular disease claims 15 3 million lives a year, or 30% of all deaths worldwide In America alone treatment of these conditions costs $274 billion each year Cardiovascular Disease includes dysfunctional conditions of the heart, arteries, and veins that supply oxygen to vital life-sustaining areas of the body like the brain, the heart itself, and other vital organs If oxygen doesn't arrive the tissue or organ will die Ireland is known to have a particularly high mortality rate from CVD especially when compared to other countries of the European Union Vascular diseases currently account for over 40% of all deaths in Ireland and of this ischemic heart disease is by far the most common accounting for 25% of all deaths (Table 1 1)

	Males	%	Females	%	Total	%
Vascular Disease	6,835	41 5	6,356	42 7	13,191	42 1
Cardiovascular Disease	4,350	26 4	3,399	22 9	7,749	24 7
Cerebrovascular Disease	1,073	6 5	1,507	10 1	2,580	8 2
Other Vascular Disease	1,412	8 6	1,450	9 8	2,862	9 1
Malignant Neoplasms	4 022	24 4	3,471	23 3	7,493	23 9
Respiratory Disorders	1,183	7 2	1,419	9 5	2,601	8 3
Injuries	1,101	6 7	439	3 0	1,540	4 9
Other	3,341	20 3	3,185	21 4	6,526	20 8
Total	**16,482**	**100**	**14,870**	**100**	**31,352**	**100**

Table 1 1 Principal causes of death in Ireland, 1998 Distribution by gender (available from http //www irishheart ie)

Many risk factors such as elevated cholesterol levels particularly LDL, elevated blood triglyceride levels, smoking, high blood pressure, diet, sedentary lifestyle, obesity and stress are all causative factors in the development of CVD Other factors which may influence development of CVD include age, medical history, genetic influences and ethnicity Hemodynamic forces within the vasculature may also influence CVD These forces associated with the flow of blood through the vasculature affect the initiation and progression of CVD including atherosclerosis, hypertension and

pathological vascular remodelling [Lusis *et al*, 2000, Frangos *et al*, 1999, Galis *et al*, 2003] The relevance of hemodynamic forces to physiological and pathological scenarios within the vasculature has gained increasing attention [Itoh *et al*, 1998, Lijnen *et al*, 2001 Lusis *et al*, 2000] Thus, these forces will be discussed in greater detail in the following sections and particularly their connection with matrix metalloproteinases (MMPs), a group of enzymes with multiple functions within the vasculature

1 2 Blood Flow

Normal blood vessels are exposed to two types of mechanical forces a) circumferential stretch acting tangentially on the vascular wall and directly related to pressure and dimensions of the vessel and b) shear stress acting longitudinally at the blood/endothelium interface which is related to the velocity of flow Both of these factors are essential for the maintenance of a healthy vessel

Blood pressure is described as the force that the circulating blood exerts on the walls of the arteries It is the major determinant of vessel stretch, which involves the rhythmic distension of the vessel wall Blood pressure creates strain on the vessel wall in a direction perpendicular to the endoluminal surface These forces are counterbalanced by intraparietal tangential forces in longitudinal and circumferential directions exerted by different elements of the vessel wall, opposing the distending effects of blood pressure All elements of the arterial wall are exposed to circumferential tension, each layer bearing differing degrees of this tension The tension per unit length can be described by Laplace's law $T = Pr/h$ where, T is the wall tension, P is blood pressure, r is vessel radius, and h is thickness of the wall Hence circumferential force is dependent on blood pressure, vessel geometry, and position within the vessel wall [Lehoux *et al*, 2003]

Blood flow exerts a frictional force on the luminal surface of the endothelium This frictional drag is referred to as shear stress and is defined in terms of blood viscosity and velocity Laminar blood flow within a vessel can be described by the

equation $\tau = 4\mu Q / \pi r^3$ where τ is shear stress, μ is blood viscosity, Q is flow rate and r is the vessel radius It is worth noting that 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 [Lehoux *et al*, 2003]

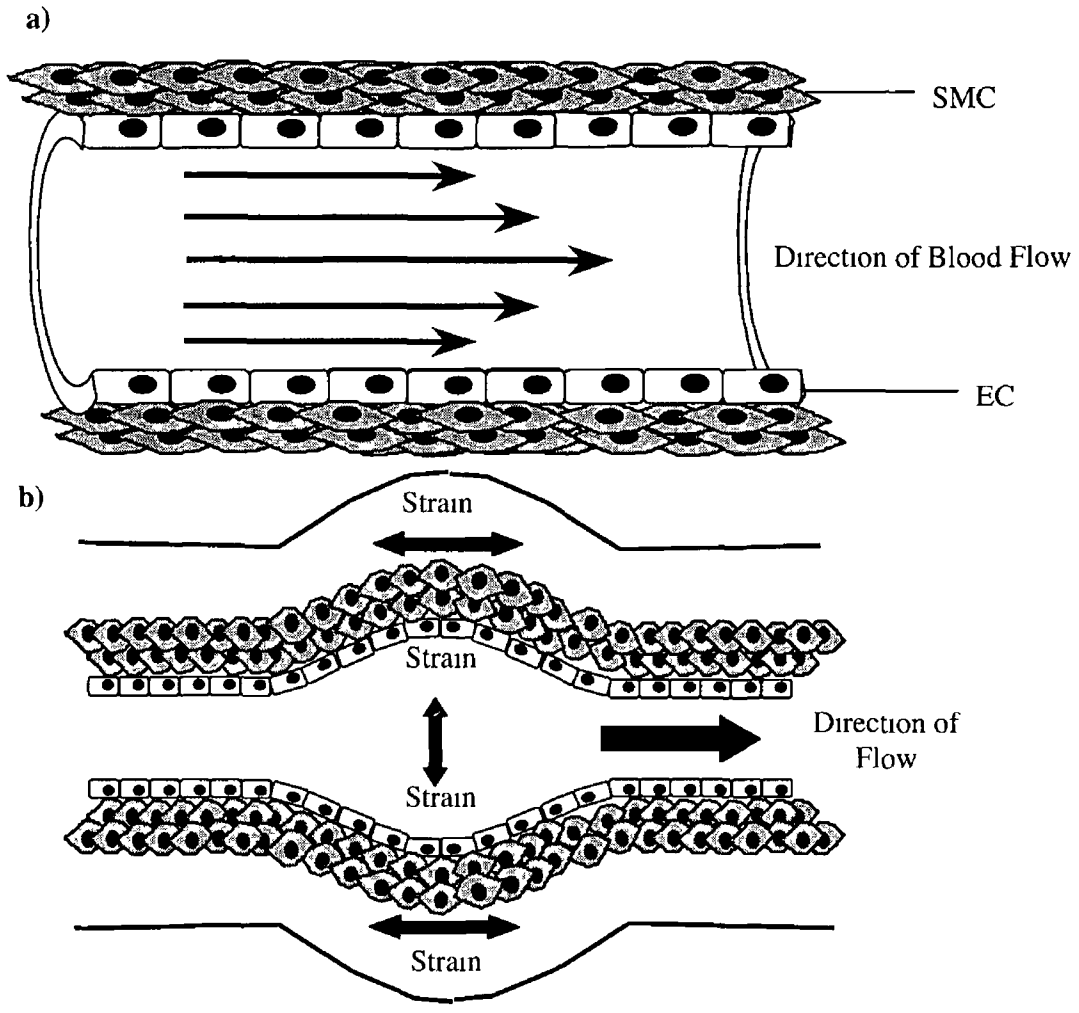


Figure 1 2 Shear Stress and cyclic strain

a) Diagrammatic representation of laminar shear stress within an artery Frictional forces between blood and the vessel wall causes a reduction in blood velocity at the vessel wall with the fastest blood velocity at the center of the vessel

b) Blood pressure results in the distension of the vessel wall in a direction perpendicular to the direction of flow, this pressure causes the circumferential stretch acting on the vessel wall

1 2 1 Circumferential Strain

Thoma in 1893 first observed that blood vessel diameter was regulated by the magnitude of blood flow while vessel thickness was dependent on blood pressure This observation was confirmed by comparing the thickness of the pulmonary artery and aorta pre and post birth *In utero,* both vessels experience similar pressures and are almost identical in size, however, after birth the aorta thickens proportionally to increases in systemic pressure while the pulmonary artery undergoes atrophy following the fall in pressure post partum [Leung *et al* , 1977]

The relationship between circumferential stress and the structure of the wall has been well established Increases in arterial pressure are associated with SMC hypertrophy and increases in extracellular matrix (ECM) production Conversely decreases in arterial pressure result in vessel atrophy [Bomberger *et al* , 1980] In a cultured rabbit aorta model it was found that under conditions of low intraluminal pressure SMC markers such as, h-caldesmon and filamin were dramatically decreased compared to aortas maintained under normal intraluminal pressure [Birukov *et al* , 1998] Continual mechanical stimulation appears to be essential to maintaining a contractile phenotype in SMC Whilst a certain level of stretching may be essential for SMC maintenance, over stretching may initiate adaptive processes [Lehoux *et al* , 1998] Mechanical stretch is a strong determinant of vascular structure in conjunction with autocrine and paracrine factors During arterial stenosis high blood pressure proximal to the coarctation is associated with thickening of the arterial wall while areas distal to the stenosis have normal blood pressure and unchanged vessel thickness [Tedgui *et al* , 1992] It has also been observed that sustained hypertension leads to thickening of the arterial wall due to SMC hypertrophy, hyperplasia and changes in matrix proteins leading to altered arterial function [Levy *et al* , 1988] Recalling the equation T = Pr/h [Lehoux *et al* , 2003], it can be seen that in a hypertensive state, in order to maintain normal tensile stress in the vessel when blood pressure (P) increases so to must the thickness (h) of the vessel with a resultant reduction in vessel radius(r)

The fact that endothelial cells are the principal recipients of shear stress does not imply that mechanical stretch has no influence on the endothelium. Cyclic stretch increases EC sensitivity to shear stress resulting in a lowered threshold level required to provoke structural changes and ultimately, both cyclic stretch and shear stress are required to produce maximal responses in the vessel [Zhao *et al.*, 1995]. The effects of circumferential stress on ECs have been investigated by applying cyclic stretch to endothelial cells cultured on an elastic membrane mounted in a stretch device.

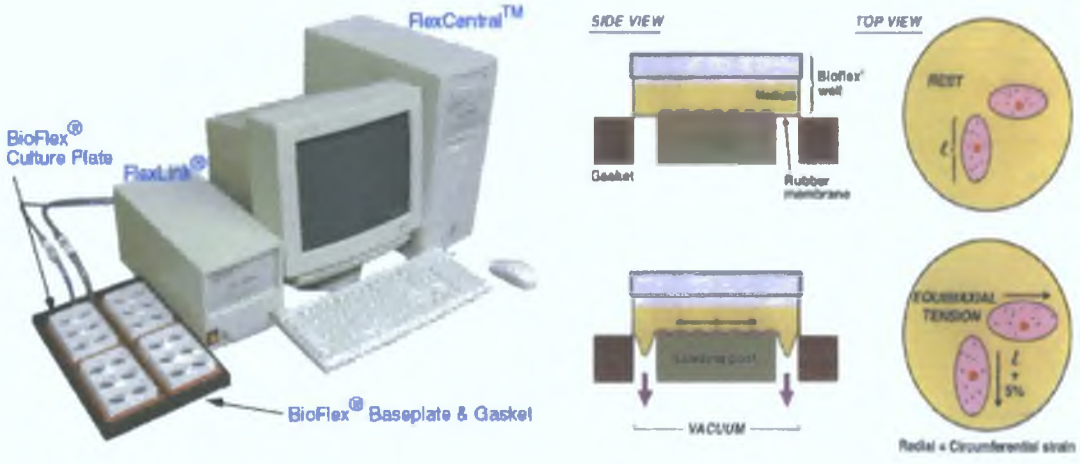


Fig 1.3: In vitro cyclic strain device

Studies from such *in vitro* experiments demonstrate that cyclic strain increases the expression of nitric oxide synthase (NOS), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-14 (MMP-14), monocyte chemotactic protein-1 (MCP-I), platelet derived growth factor-BB (PDGF-BB), endothelin –1(ET-I), intracellular adhesion molecule-1 (ICAM-I), and plasminogen activator inhibitor-1 (PAI-1) [Awoleski *et al.,* 1995; Cheng *et al.,* 1996; Cheng *et al.,* 1996; de Jonge *et al.*, 2002; Sumpio *et al.*, 1998; Wang *et al.*, 2003; Wung *et al.*, 2001]. The complexity of these cyclic strain-induced events have not been completely elucidated but the ability of cells to response to cyclic strain is believed to play a role in a number of pathologies including atherosclerosis, hypertension or restenosis following balloon angioplasty.

The oxidative state of the blood vessel is a major contributor to vascular remodelling and disease Under diseased conditions, levels of reactive oxygen species (ROS) have been found to be increased, for example from macrophage foam cells, the interaction of NO with these ROS such as superoxide may result in the formation of peroxymtrite with associated rupture in atherscleroric plaques [Rajagopalan *et al*, 1996] ROS have been found to be sensitive to changes in the hemodynamic environment Cyclic strain has been shown to increase levels of ROS ROS are believed to be an important mediator in modulating signaling pathways and gene expression by redox sensitive molecules such as PYK2 [Cheng *et al*, 2002] Mechanical stretch was found to increase expression of MMP-2 via a mechanism involving reactive oxygen species derived from NAD(P)H oxidase [Grote *et al*, 2003] Cyclic strain-induced increases in ROS have been linked to the regulation of a number of vasoactive compounds, signaling molecules and transcription factors [Awoleski *et al*, 1995, Chien *et al*, 1998, Cheng *et al* 1996, Wang *et al*, 2003, Wung *et al* 2001,Wung *et al*, 1999]

Cellular responses to cyclic strain may not be mediated solely by ROS The release of NO from endothelial cells in response to changes in mechanical strain was one of the first responses identified [Leung *et al*, 1977, Galis *et al*, 1998] Other molecules secreted in response to cyclic strain include endopeptidases, Et-1, PAI-1, Il-8 and MCP-1 [Sumpio *et al*, 1998, Cheng *et al*, 1996, Okada *et al*, 1998] Intracellularly cyclic strain is responsible for the recruitment of a variety of signaling molecules Sumpio *et al* reported that cyclic strain increases the tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin in ECs with a concurrent cell elongation and the alignment of F-actin, FAK, and paxillin Cyclic strain has also been found to activate extracellular signal-regulated kinase (ERK), c-jun N-terminal kinases (JNK), and p38 [Wung *et al*, 1999, Kito *et al*, 2000, Li *et al*, 1999] This family of enzymes is involved in recruiting the transcription of a number of cyclic strain-induced genes and will be discussed in greater detail in section 1 4

exposed to turbulent flow, oscillatory shear stress and eddy currents all of which can abrogate the protective effects of laminar shear One of the most dramatic demonstrations of flow-dependent regulation of vessel structure is the arteriovenous fistula Arteriovenous fistulae (AVF) are characterized by abnormal shunting of blood between the arterial system and the venous system, without the presence of a normal intervening capillary bed The capillary bed represents the source of resistance to blood flow in the circulatory system, as such AVF are low resistance, high flow lesions In this model the flow rate can be amplified by a factor of eight, the increase in shear stress subsequently being compensated for by an increase in vessel diameter [Tronc *et al*, 1996] This can be explained by flow-associated release of vasoactive compounds with allow reorganization of the vessel structure As vessel diameter increases the wall shear stress is reduced and so the stimulus for vessel remodeling is diminished

In vitro studies in which endothelial monolayers have been subjected to defined levels of shear stress have been essential to our understanding of shear stress related molecular responses The complexity of the shear stress response is only now being elucidated and some of the best-characterized responses include, reorganization of actin containing stress fibers, alterations in metabolic activities and changes in cell cycle kinetics [Davies *et al*, 1993, Davies *et al*, 1995] Shear related effects can be broadly categorized into two responses, a) reorganization or regulation of pre-existing proteins and b) *de novo* protein synthesis and gene expression, the latter is usually associated with delayed or chronic shear-mediated responses

One of the best-described responses, which falls into category a), is the regulation of endothelial nitric oxide synthease (eNOS) eNOS catalyses the formation of NO from L-arginine, a process which can occur within milliseconds Flow-dependent activation of eNOS has been observed both *in vitro* and *in vivo*, and the resultant release of NO has been related to SMC relaxation as a response to increases in flow In conjunction with flow-mediated increases in vasodilators such as NO, levels of vasoconstrictors including ET-1, have been found to be decreased A number of genes involved in thrombosis, homeostasis, and inflammation such as thrombomodulin, tissue

plasminogen activator (t-PA) [Sjogren *et al*, 2000] and vascular cell adhesion molecule-1 (VCAM-1) [Chiu *et al*, 2004], have all been identified as being shear responsive Thus, sheai stress profoundly affects the health and functions of the endothelium

Exposure of the endothelium to fluid mechanical forces may alter the rate of transcription of a specific subset of genes Investigation of the promoter regions of these genes has identified the presence of a cis-element, which is inducible by shear stress The identity of a shear stress response element (SSRE) as GAGACC was achieved by a series of transfections involving deletion mutants of the PDGF-B promoter [Resnick *et al*, 1993] Other examples of SSREs include a divergent TRE in the promoter region of MCP-1 with the sequence TGACTCC, necessary for shear inducibility [Shyy *et al*, 1995] Functional analysis of the tissue factor (TF) gene has identified a GC-rich region containing three copies of the Egr-1 and Sp-1 sites Deletion of the Sp-1 but not the Egr-1 attenuates shear stress activation of this gene [Lin *et al*, 1997] Thus it can be seen that multiple cis-elements may regulate shear stress responsiveness in different genes

The response of EC gene expression e g c-fos, ICAM-1 and C-type natriuretic peptide, to shear stress is known to be a function of the magnitude of the force Gene activation in vascular ECs may vary as a function of shear magnitude For example, t-PA expression is only increased above 5dynes/cm^2 [Diamond *et al*, 1990] whereas ET-1 secretion is increased at shear stresses less than 5 dynes/cm^2 [Kuchan *et al*, 1993] This may be explained by the fact that the magnitude of shear stress may vary depending on the location within the vasculature Therefore in situations where shear stresses may be low increases in ET-1 will promote vasoconstriction to increase blood flowrate through that section of the vessel The use of DNA microarray technology has permitted analysis of extensive differential gene expression in response to hemodynamic forces Fifty-two flow sensitive genes have recently been identified in HUVECs with prostaglandin and cytochrome p450 the most strongly up-regulated and ET-1 and MCP-1 the most strongly down-regulated [McCormick *et al*, 2001] 143

genes have been identified in HUVECs, which are differentially expressed in the presence of static, laminar, or turbulent flow [Garcia-Cardena *et al*, 2001] *In vivo*, the expression of a number of genes, such as transforming growth factor-β (TGF-β) [Negishi *et al*, 2001], PDGF-A, PDGF-B [Tulis *et al*, 1998], and urokinase plasminogen activator (uPA) have been found to be shear sensitive Further studies involving microarray technology may lead to a more complete elucidation of cellular responses to mechanical forces by identifying the cellular participants in regulating cell function in hemodynamic environments Although there may be some discrepancies between using vein and artery preparations or between *in vitro* and *in vivo* models these studies clearly indicate the importance of mechanical forces to gene regulation within the vasculature

Hemodynamic forces such as cyclic strain and shear stress can induce changes in autocrine and paracrine hormonal factors Alterations in these factors allow blood vessels to adapt to variations in mechanical forces, the objective of these changes being to return the vessel to its normal hemodynamic state The ability of vessels to detect and respond to changes in their hemodynamic environment involves a process referred to as mechanotransduction, which will be discussed in the following section

1 3 Mechanotransduction

Vascular cells respond to mechanical forces namely cyclic strain and shear stress Before a vascular cell can respond to a hemodynamic stimulus it must first of all be able to detect them This is facilitated by mechanically sensitive receptors present in vascular cells These receptors, which fall into a number of categories, can then elicit a signaling pathway, which culminates in the recruitment of an effector molecule(s) to mediate a cellular response This process is referred to as *mechanotransduction* Mechanical forces initiate complex signal transduction cascades leading to functional changes in the cell, often triggered by receptors such as G-proteins, integrins, and protein tyrosine kinases, which will be discussed in greater detail

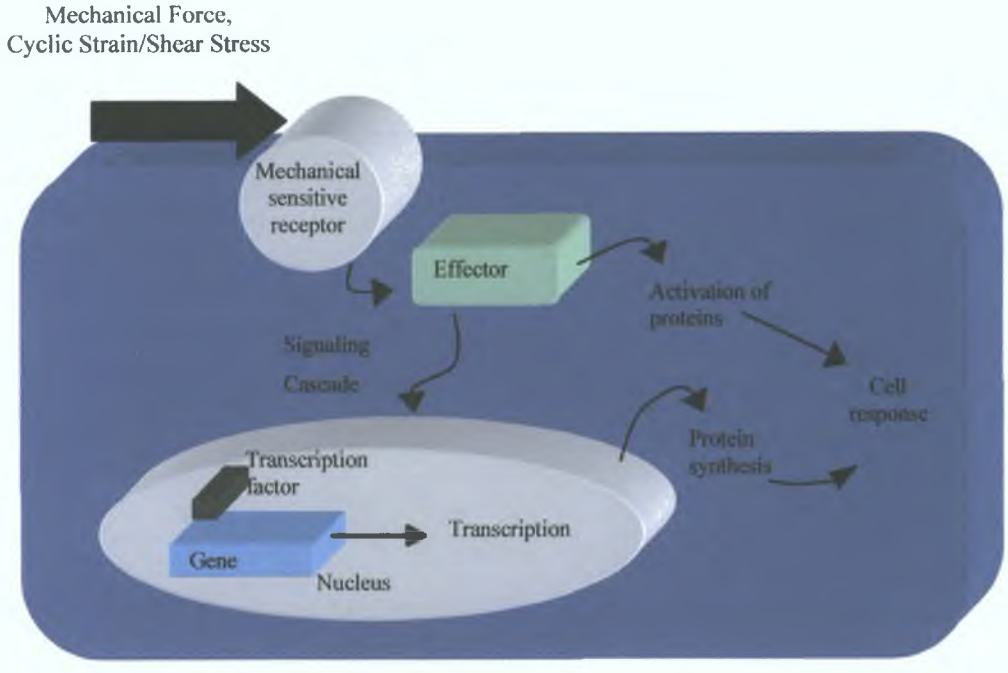


Fig 1.4: Generalized version of mechanotransduction

Essential to the coordination of cellular functions in response to hemodynamic stimuli is the ability of cells to communicate with each other. The process of intercellular signaling is achieved through numerous molecules, which interact specifically with specialized docking sites on the cell surface, called receptor proteins. The source of these molecules may be; i) autocrine secretion of a signaling molecule, that targets the secretory cell itself ii) paracrine secretion of a signaling molecule that targets a cell close to the signal releasing cell or iii) endocrine secretion of a signaling molecule from a gland, that targets a cell distant from itself. Intracellular signaling, results in the coordination of and synchronization of cell function within the vessel wall. Following the binding of a ligand to a receptor, intracellular effector molecules are activated leading to alterations in cell structure and function [Stone *et al.,* 1998].

A number of receptors exist which may exhibit mechano-sensitivity,

- **Receptors linked to G-proteins** - the largest group of plasma membrane receptors, over 1000 such receptors have been identified to date with a diverse range of potential agonists
- **Integrins** - Integrins comprise a large family of cell surface receptors most widely known for their role as receptors for ECM proteins
- **Enzyme-linked receptors** – Receptors with intrinsic enzyme activity
  - i Receptors with intrinsic kinase activity these receptors have an intrinsic tyrosine kinase activity within the molecule itself Members of this group include the epidermal growth factor (EGF), the fibroblast growth factor (FGF) and the platelet growth factor (PDGF)
  - ii Receptors with associated tyrosine kinase activities These receptors do not possess intrinsic tyrosine kinase activity but have an associated partner protein which does Examples of this class of receptors include those responsible for binding prolactin, growth hormone and numerous cytokines
  - iii Receptors with intrinsic protein tyrosine phosphotase activity These receptors play a crucial role in dephosphorylating phosphotyrosine residues in signaling pathways An example of this class of receptor is the CD45 protein of T lymphocytes
  - iv Receptors with intrinsic serine/threonine kinase activities this is a broad class of receptors, which together comprise of the TGF-β superfamily
- **Ion channel receptors** - In addition to being regulated by G-proteins some ion channels function as receptor molecules themselves Examples of these receptors include serotonin-gated cation channels and acetylcholine receptor

1 3 1 Heterotrimeric G-proteins

G-protein signaling represents a highly sophisticated molecular system with the ability to receive, integrate, and process information from extracellular stimuli It comprises of a G-protein coupled receptor (GPCR), the heterotrimeric G-protein

complex and the effector(s) in addition to the more recently identified regulators of G-protein signaling (RGS-proteins) and activators of G-protein signaling (AGS-proteins) [Offermanns *et al* 2003] GPCRs play a pivotal role in cardiovascular signaling

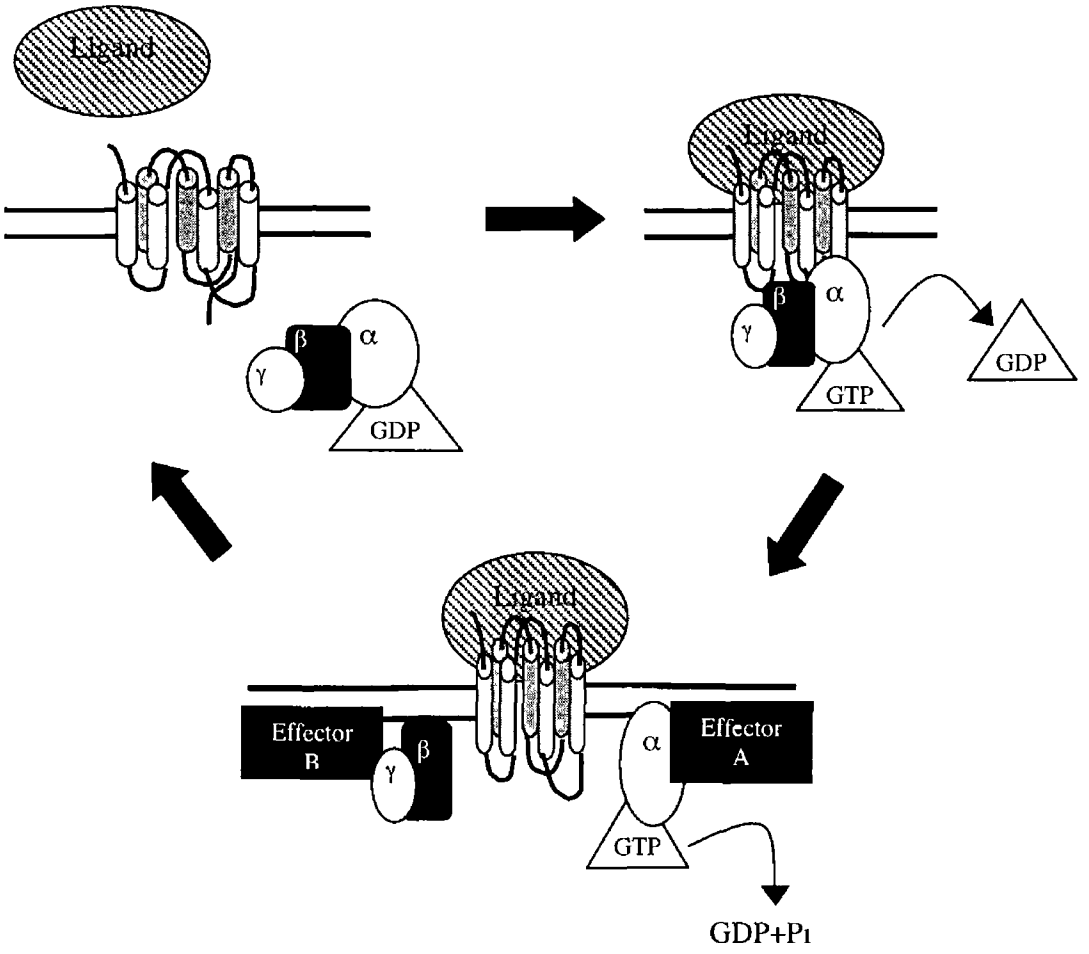


Fig 1 5 G-protein coupled receptor comprising of seven transmembrane domains Following binding of a ligand, the activated receptor catalyses GDP/GTP exchange at the α subunit which promotes dissociation of the complex, the α and βγ subunits which subsequently activate their effectors Following hydrolyses of GTP by the α subunit the heterotrimer reassociates

All of these receptors have seven membrane spanning elements that use intracellular loops and their C-terminal tails for interaction with heterotrimeric G-proteins, which consists of a α, β and γ subunits The β and γ subunit forms an undissociable complex which represents a functional subunit Ligand activated

receptors catalyse the GDP/GTP exchange at the α subunit of a coupled G-protein and promote dissociation of the α and βγ components [Wieland *et al*, 2003] The duration of a G-protein activation is controlled by the intrinsic GTPase activity of Gα Following GTP hydrolysis the Gα subunit returns to the GDP-bound conformation and reassociates with the Gβγ subunit

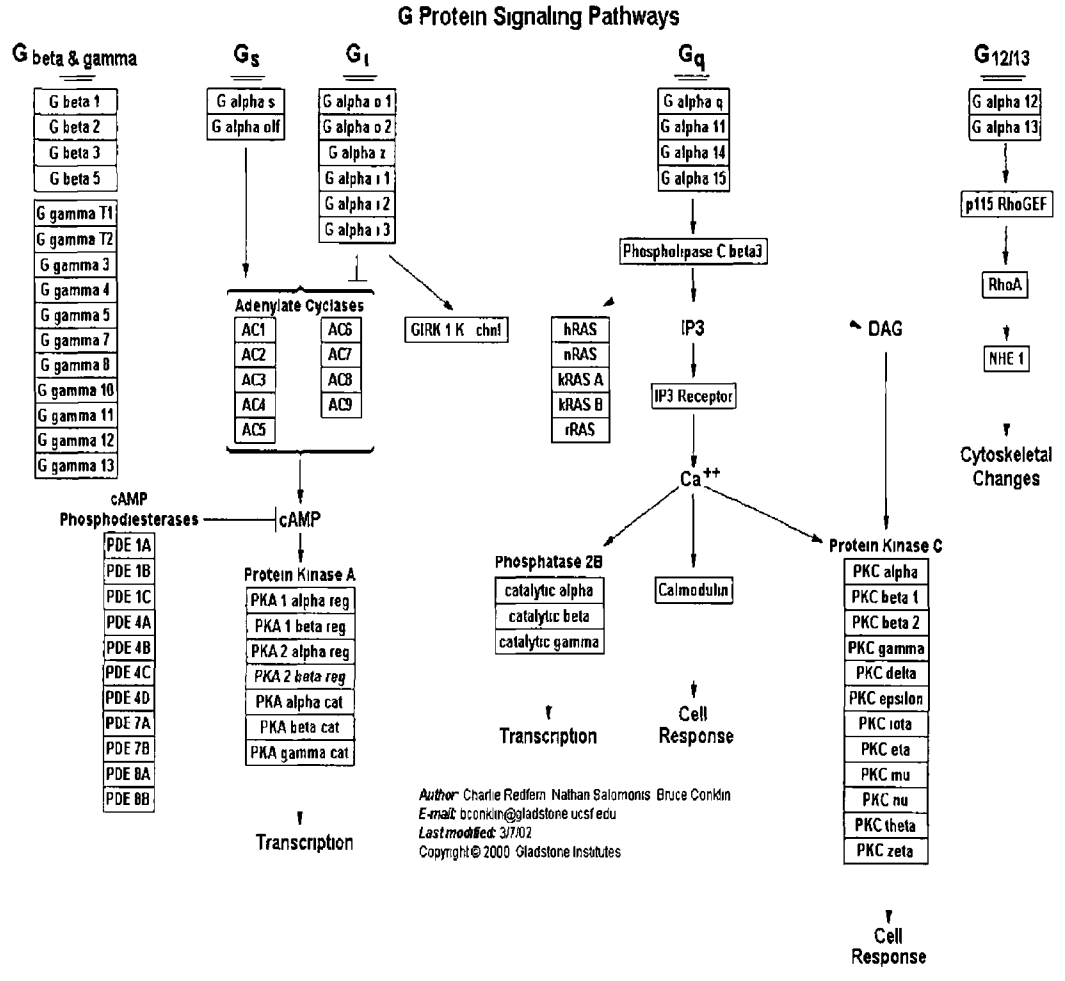


Figure 1 6 G-protein subunits their effector molecules and downstream processes (available from http //www genmapp org/)

More than twenty G-protein α-subunits have been described which have been loosely divided into four families based on structural and functional homologies, $\alpha_{i/o}$, α_s, α_q, and α_{12} The majority of GPCRs are capable of activating more than one G-

protein subtype, which leads to initiation of various signaling cascades There are some characteristic patterns of G-protein activation by specific receptors, the cellular or physiological effect of a receptor is dependent on which G-protein sub-type it is coupled to

The α subunits

- **$G\alpha_s$- family** There are two members of this family, α_s and α_{olf} as well as four known splice variants Upon activation, this group stimulates adenylyl cyclase to increase levels of intracellular cAMP and to activate calcium channels α_s is ubiquitously expressed while α_{olf} is restricted to neuronal cells specifically olfactory sensory neurons
- **$G\alpha_{i/o}$ family** This family consists of α_{i1}, α_{i2}, α_{i3}, α_{o1}, α_{o2}, $\alpha_{t\ rod}$, $\alpha_{t\ cone}$, α_{gust} and α_z all of which are highly homologous and have the ability to inhibit adenylyl cyclase, in addition to activation of potassium channels The high degree of homology between sub-types may suggest partially redundant functions Expression of the various α_i sub-types may be very diverse depending on tissue examine e g α_{i2} is predominant in the mammalian heart A defining characteristic of this family of $\alpha_{i/o}$ subunits is sensitivity to pertussis toxin Pertussis toxin is produced by Bordetella pertussis and catalyzes the adenosine diphosphate (ADP)-ribosylation of α_i and α_o subunits at a cysteine residue near the C-terminus resulting in uncoupling of receptor and G-protein α_z unlike the other members of this family has been found to be pertussis toxin insensitive and is expressed in various tissues It can inhibit adenylyl cyclase but its physiological function is somewhat ambiguous, although α_z deficient mice point to roles in platelet activation
- **$G\alpha_q$ family** This family stimulates phospholipase C in a pertussis-toxin insensitive manner α_q and α_{11} are ubiquitously expressed and receptors activating α_q family members do not discriminate between α_q and α_{11} [Wange *et*

al, 1991] $\alpha_{15/16}$ are only expressed in hematopoietic cells and α_{14} is restricted to the kidney, lungs and testis

- **Gα12 family** α12 and α13 constitute the members of this family and appear to be widely expressed The function of these proteins is somewhat unclear, one recently discovered function is the interaction of these proteins with cadherins causes the release of transcriptional activator β-catenin [Meigs *et al*, 2001]

The Gβγ subunit

This complex is assembled from a repertoire of five β subunits and twelve γ subunits The sequence similarity is higher between β subunits (79-90% homology) than γ subunits and it is not yet clear how many combinations will actually form stable dimers [Clapham *et al*, 1993] β-subunits are believed to posses a propeller structure formed by seven β sheets The γ subunit is located at one end of the propeller and associates with the β-subunit by a coiled coil structure [Bohm *et al*, 1997] βγ Sensitive effectors include adenylyl cyclase, phospholipase C, phospholipase A, potassium channels, a calcium pump, and phosphoinositide 3 kinase (PI-3 kinase) [Tang *et al*, 1991, Exton *et al*, 1996, Yamada *et al* 1989, Lotersztajn *et al*, 1992] With a few exceptions there appears to be no major differences between different βγ combinations in their ability to activate effector enzymes

1 3 1 2 Heterotrimeric G-proteins in Mechanotransduction

G-proteins have been implicated in the transduction of number of flow-induced responses in vascular cells G-protein activation by mechanical forces represents one of the earliest mechanotransduction events reported In both cyclic strain and shear stress models the use of photoreactive radiolabeling and immunoprecipitation has identified $G\alpha_{q/11}$, $G\alpha_{11}$ and $G\alpha_{i3/o}$ as the G proteins activated This indicates that both PTX-insensitive ($G\alpha_q$) and PTX-sensitive ($G\alpha_i$) subunits are involved in this rapid response [Gudi *et al*, 1996, Clark *et al*, 2002, Gudi *et al*, 1998] Cyclic strain activation of G-proteins has been found to be dependent on the magnitude and rate of the strain [Clark

et al, 2002, Gudi *et al*, 1998] When G proteins are isolated and reconstituted in a phospholipid bilayer in the absence of cytoskeletal elements or other receptors, $G\alpha_q$ and $G\alpha_i$ respond specifically to fluid shear stress Cellular localization and rapid activation strongly implicate G proteins as a primary sensor of hemodynamic forces [Gudi *et al*, 1996] G-proteins may detect mechanical forces via GPCR or may be stimulated directly by the deformation of either the actin cytoskeleton or the membrane phospholipid bilayer during exposure to such stimuli Gudi *et al* demonstrated the ability of the phospholipid bilayer to mediate the shear stress-induced activation of membrane-bound G proteins in the absence of protein receptors and that bilayer physical properties modulate this response

Shear stress and cyclic strain-induced activation of G-proteins results in several flow-initiated responses which function in the regulation of vascular tone, including release of vasodilators such as NO and PGI_2 or vasoconstrictors such as ET-1 [Liu *et al*, 2003, Pirotton *et al*, 1987] Changes in G-protein expression have been observed within the physiological range of cyclic strain and shear stress These changes have been correlated with enhanced NO and PGI2 release as well as increased G-protein functionality [Redmond *et al*, 1998,] Cyclic strain has been found to activate all the members of the MAP kinase family Cyclic strain activation of the different members of this family follows different temporal and magnitude dependent patterns [Kito *et al*, 2000] Activation of Ras and Rac following exposure to cyclic strain has been found to precede MAP kinase activation [Li *et al*, 1999] More recently, several studies have shown the activation of the mitogen-activated protein kinase pathway in response to shear stress is dependent on G-protein, protein kinase and a tyrosine kinase [Ishida *et al*, 1996, Takahashi *et al*, 1996] Gudi *et al* (2003) found that G-proteins mediate the rapid activation of RAS by fluid shear stress This activation is believed to occur via the $G\beta\gamma$ subunit dissociated from flow activated $G_{\alpha q}$ Similarly, Jo *et al* (1997) found that the $G\beta\gamma$ subunit was involved in the activation of JNK in response to shear stress Flow-induced increases in angiogenesis has been linked with G-protein signaling interestingly this response was found to be attenuated when $G_{\alpha i}$ proteins were inhibited but intensified when the $G\beta\gamma$ subunit was inhibited, indicating that both subunits are

involved in regulating flow-induced angiogenesis [Cullen *et al*, 2002] G-proteins play a vital role in the detection of mechanical signals which mediate cell function such as angiogenesis, SMC proliferation, release of vasodilators such as NO and PGI_2 and activation of intracellular pathways such as the MAP kinase pathway

Clinical situations that highlight the importance of G-protein signaling within the vasculature include intimal hyperplasia, cardiac hypertrophy, and vascular restenosis Inhibition of the Gβγ subunit by the βARK-ct (a Gβγ subunit scavenger) was found to inhibit vein graft intimal hyperplasia and subsequent vein graft failure [Davies *et al* 1998] Similarly a novel receptor, the intimal thickness–related receptor (ITR) which has the characteristic seven transmembrane domains of GPCRs was found to be increased in intimal thickening and ITR-knockout mice were resistant to intimal thickening [Tsukada *et al*, 2003] $G\alpha_q$ inhibition or downregulation of GPCR by overexpression of GPCR kinase-2 (GRK-2) were found to inhibit cardiac hypertrophy and SMC proliferation in neointimal hyperplasia [Keys *et al*, 2002, Peppel *et al*, 2000] The extensive study of G-protein activation during different hemodynamic conditions both *in vivo* and *in vitro* has highlighted their importance in the process of mechanotransduction

1 3 2 Integrins

Integrins comprise a large family of cell surface receptors that are found in a variety of animal species They are noncovalent heterodimeric receptors, the majority of which bind to extracellular matrix proteins The heterodimer comprises of one of eighteen α and one of eight β subunits not including splice variants Each possible combination of subunits has its own binding specificity and signalling properties [Giancotti *et al*, 1999] These subunits can form twenty four different integrins, sixteen of which are reportedly involved in the vasculature with seven expressed in endothelial cells [Rupp *et al* 2001]

Each subunit consists of a large NH_2-terminal extracellular domain, a single membrane spanning domain and a COOH-terminal cytoplasmic tail [Shattil *et al*, 1997] Integrins are most widely known for their role as receptors for ECM proteins, such as fibronectin, vitronectin, collagen, laminin, fibrinogen, thrombospondin and osteopontin Most integrins have the ability to recognise several ECM proteins and most ECM proteins will bind more than one integrin

β chain	α chain	Ligands
β1	α1	**Collagens, Laminins**
	α2	**Collagens, Laminins**
	α3	**Laminins, Fibronectin, Thrombospondin**
	α4	**Fibronectin, VCAM**
	α5	**Fibronectin**
	α6	**Laminins**
	α7	**Laminins**
	α8	**Fibronectin, Tenascin**
	α9	**Tenascin**
	α10	**Collagens**
	α11	**Collagens**
	αv	**Fibronectin, Vitronectin**
β2	αL	**ICAMs**
	αM	**Fibrinogen, ICAMs**
	αX	**Fibrinogen**
	αD	**VCAMs, ICAMS**
	αIIb	**Collagens, Fibronectin, Vitronectin, Fibrinogen, Von willebrand factor, Thrombospondin**
β3	αv	**Fibronectin, Vitronectin, Fibrinogen, Von willebrand factor, Thrombospondin**
β4	α6	**Laminins**
β5	αv	**Vitronectin**
β6	αv	**Fibronectin, Tenascin**
β7	α4	**Fibronectin, VCAM**
	αE	**E-cadherin**
β8	αv	**Collagens, Laminins, Fibronectin**

Table 1 2 The 24 possible integrin subunit combinations and their potential ligands (available at http //integrins hypermart net)

Integrin signalling is a crucial component in development, maintenance and function of the vascular system [Ruoslahti *et al*, 1997] Binding of an extracellular ligand to the integrin results in localised clustering of integrins in the plasma membrane Integrins have the unique characteristic that they can signal through the cell membrane in either direction, essentially forming a bridge between the ECM and the cell cytoskeleton Integrins may act like a traditional receptor in binding an agonist and activating an intracellular response The cytoplasmic tail of integrins are generally devoid of enzymatic activity As a result of this, integrins transduce signals via adaptor proteins which connect the integrin to the cytoskeleton, cytoplasmic kinases and transmembrane growth factors [Giancotti *et al*, 1999] Integrins can also elicit an extracellular effector response coincident with ligand engagement Ligand binding is tightly regulated by cellular signalling mechanisms in a process referred to as integrin activation or inside-out signalling Thus intracellular signals are translated into an extracellular effect Integrin activation can therefore result in either changes in the integrin affinity or avidity Affinity refers to integrin/ligand binding due to the conformation of the integrin whereas avidity refers to integrin clustering resulting in increased ligand binding [Shattil *et al*, 1997]

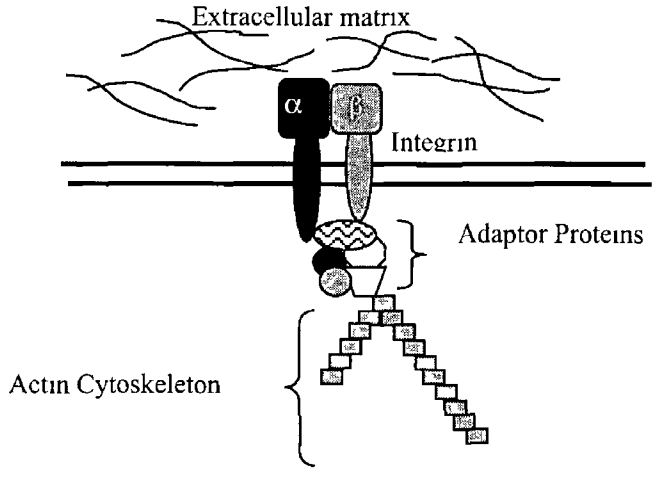


Fig 1 7 Integrin comprising of alpha and beta chains linked to the actin cytoskeleton via adaptor proteins and the ECM

Integrins are capable of eliciting a signal response by associating with kinases through adapter proteins or as part of complexes with growth factor receptors Receptor tyrosine kinases (RTKs) are capable of physically interacting with integrins In migrating EC stimulated with PDGF-BB, the αvβ3 integrin was found to coprecipitate with the PDGF recpetor (PDGF-Rβ) [Woodward *et al*, 1998] The VEGF receptor (VEGFR2) was found to co-precipitate with the αvβ3 integrin When this association was inhibited by neutralizing antibodies there was a marked reduction in cell migration and proliferation [Soldi *et al*, 1999] Binding of integrin receptors by either ECM components or antibodies has been found to stimulate the phosphorylation of EGF receptor leading to activation of the MAP kinase pathway Protein levels and mRNA expression of membrane type-1 MMP can be altered by integrin activation Other membrane proteins such as urokinase plasminogen activator receptor (uPAR), CD47, CD36, and CD46 can exist as integrin associated proteins which modulate integrin responses and regulate intracellular signalling Integrin signalling has been implicated in a variety of cellular responses such as modulation of cyclin-dependent kinases in cell proliferation, regulation of cell death by inhibiting pro-apoptotic proteins such as Bcl-2, and regulation of cell migration through activation of the Rho-family of proteins [Schwartz *et al*, 2001, Aoudjita *et al*, 2001, Keely *et al*, 1997]

1 3 2 1 Integrins in Mechanotransduction

The ECM is an important contributor to mechanotransduction, containing components which are displaced by pulsatile stretch and shear stress and which interact with integrins Mechanical stresses can stimulate conformational activation of integrins and increase cell binding to the ECM Evidence for mechanical activation of integrins is provided by both direct assessment of integrin conformational changes in response to these forces and blockade of the induced responses by antibodies or blocking peptides such as the Arg-Gly-Asp (RGD) peptide [Lehoux *et al*, 1998]

SMC grown on fibronectin or vitronectin and exposed to cyclic strain had increased cellular proliferation, which, can be attenuated by incubating the cells in the

presence of β5 or αvβ3 blocking antibodies Cyclic stretch induces expression of the SM-1 isoform of myosin heavy chain in SMC grown on laminin rather than collagen or fibronectin In SMC plated on collagen, serum induced expression of c-fos and cell proliferation is equal in strained or unstrained cells, however when grown on elastin stretch abated SMC proliferation Variations in cellular response depending on the composition of the ECM highlights the importance of which integrin is activated during the cells exposure to cyclic strain [Lehoux, *et al*, 2003]

Shear stress also induces integrin specific signaling cascades The positive immunostaining of WOW-1, which reacts specifically with unoccupied αvβ3 integrin in a high-affinity state, provides direct evidence of integrin activation [Pampori *et al*, 1999] Tzima *et al*, (2001) have shown an increased immunostaining of WOW-1 in sheared ECs, indicating a modulation of integrin affinity by shear stress Binding of β1 and β3 has demonstrated that shear stress causes an increase in integrin avidity in ECs Blocking integrins with RGD peptide abolishes the shear stress–induced secretion of basic fibroblast growth factor [Gloe *et al*, 2002] and the anti-apoptotic effect of shear stress [Urbich *et al*, 2000] In addition to modulating the avidity and affinity of integrins, shear stress also increases the mRNA and protein levels of the α5 and β1 integrins in ECs [Urbich *et al*, 2000]

Disturbances in integrin-matrix interactions may contribute to many vascular pathologies Balloon angioplasty in rat carotid arteries has been found to result in increased production of MMPs in SMC concomitant with increases in β3 integrin expression and SMC migration This supports the idea that integrins play an important role in MMP-dependent SMC migration associated with restenosis following balloon angioplasty [Bendeck *et al*, 2000] Blockage of this response may be a valuable approach to alter late arterial narrowing [Slepian *et al*, 1998] Many mechanically–activated signaling events depend on the actin-based cytoskeleton FAK and Shc are two molecules associated with both the cytoskeleton and integrins that have been shown to mediate the shear stress activation of downstream mitogen-activated protein kinases (MAPKs) In addition these molecules have an increased association

with integrins in response to shear stress [Chen *et al*, 1999] These molecules may elicit their effect through effector molecules including Rap1, which is a member of Ras family of G proteins An intact actin-cytoskeleton is necessary for many, if not all, mechanotransduction processes One possible explanation is that the cytoskeletal network facilitates the translocation of various signaling molecules from the focal adhesion site to the cytoplasm The tensegrity (tensile-integrity) model suggests that the F-actin can be used for transmitting forces from integrins to intracellular organelles [Stenmark *et al*, 1997] Integrin signaling is an integral part of mechanotransduction Integrin signaling is a complex scenario in which crosstalk between different mechanoreceptors may occur e g co-precipitation with PDGFR or activation of G-proteins such as RhoA Re-arrangement of the cytoskeleton may intensify signaling by integrin clustering in response to mechanical forces Similarly variations in the ECM composition, which may also be changed by mechanical stimuli, can cause changes in integrin avidity When considering all these factors it can be concluded that the cell in its entirety may be considered as a mechanosensor, which, alters its cytoskeleton, the composition of the ECM and cross talk between receptors in response to mechanical stimuli to maintain the homeostasis within the vascular wall

1 3 3 Protein Tyrosine Kinases (PTK)

Protein tyrosine phosphorylation has been established as a crucial step in the regulation of normal cell proliferation, migration, differentiation, and survival Specific tyrosine phosphorylation events are triggered by extracellular stimuli such as hemodynamic stimuli and growth factors that are processed by intracellular signal transducers [Waltenberger *et al*, 1999] Thus, protein tyrosine kinases play an important role in the signaling process This can be demonstrated by inhibition of shear stress induced ERK and JNK activation by genistein, a PTK inhibitor PTKs are crucial in the shear stress regulation of cell shape and stress fibers Cellular PTKs are generally divided into two major categories, i) receptor tyrosine kinases and ii) nonreceptor tyrosine kinases

1) Receptor tyrosine kinases

These enzyme linked membrane receptors are distinguished by the presence of an intrinsic tyrosine kinase activity Receptors of this class generally respond to circulating hormonal stimuli which trigger the activation of intracellular signalling pathways leading to cell shift in metabolism, proliferation and/or differentiation Members of this family of receptors include the insulin-like growth factor receptor (ILGFR), the epidemal growth factor receptor (EGFR) and the platelet derived growth factor receptor (PDGFR) These receptors have an extracellular domain responsible for the binding of a ligand, a transmembrane domain and an intracellular domain with a tyrosine kinase activity Phosphorylation of tyrosine is a tightly regulated process involving interplay between tyrosine kinases and tyrosine phosphatases

One of the best characterised members of this family of receptors is the insulin receptor This receptor exists as a heterodimer consisting of two α and two β chains The α domains bind insulin Following the binding process a conformational change occurs in the β chains and results in the autophosphorylation of the cytoplasmic domain Autophosphorylation causes the activation of the tyrosine kinase activity, in addition to creating binding sites for intracellular adapter molecules which bring other signalling molecules into close proximity which are subsequently recruited and activated Other members in this family of receptors exist as monomers in the cell membrane e g EGF receptor In this case binding of a ligand to the receptor leads to dimerization of two receptors, the tyrosine kinase activity of the dimer is subsequently activated and leads to activation of downstream signalling cascades [Stone *et al*, 1998]

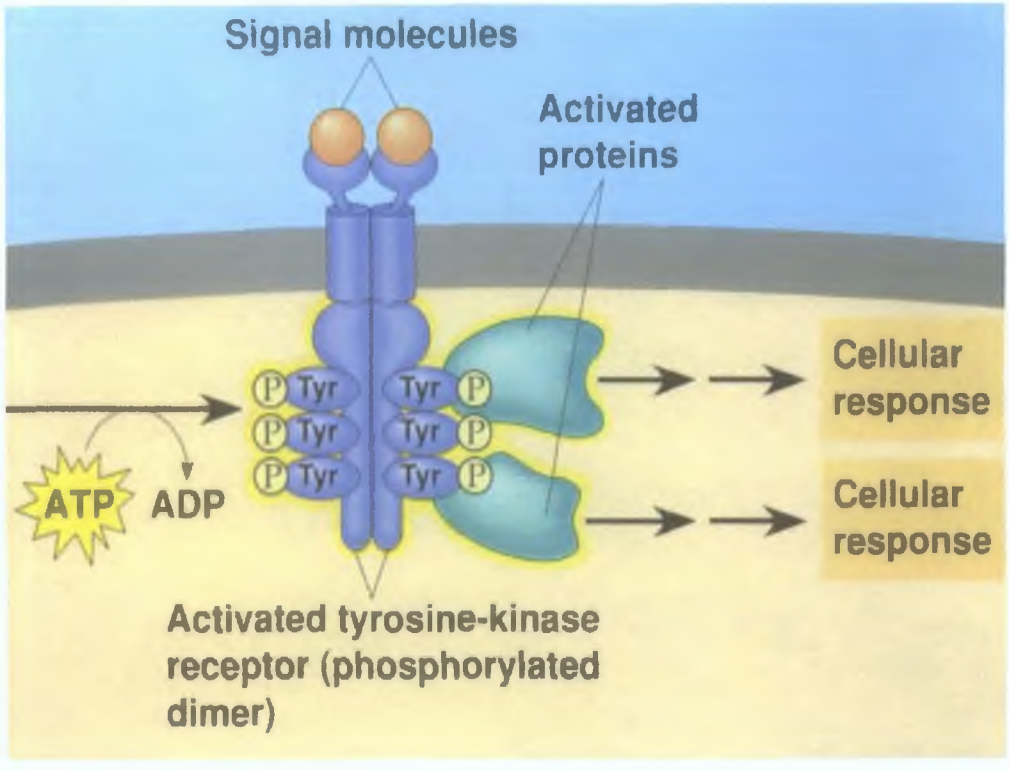


Fig 1.8: Activation of the receptor by ligand binding brings about a conformational change in the receptor leading to dimerization and phosphorylation of a tyrosine(s) on the tyrosine kinase domain of the receptor. This results in the recruitment of other signaling proteins involved in the signaling cascade (http://www.uic.edu)

2) Non receptor tyrosine kinases:

Unlike RTKs this class of PTK are intracellular enzymes which possess an intrinsic tyrosine kinase activity but that do not have an extracellular domain. These enzymes may be activated in response to a number of stimuli. Examples of these molecules include the FAK, src and Jak families. These molecules may phosphorylate receptors, which lack intrinsic tyrosine kinase activity or may recruit other downstream signaling molecules such as PI-3 kinase [Stone *et al.*, 1998].

1 3 3 1 PTKs in Mechanotransduction

Recent reports indicate that the activities of PTKs in cardiac myocytes, platelets, and ECs are increased by mechanical stimuli such as cyclic stretch and shear stress [Sadoshima *et al*, 1993, Ishida *et al*, 1996] PTKs seem to play important roles in the signaling events elicited by mechanical forces Tyrosine kinases have been implicated in hemodynamic force-induced changes in EC function [Ravichandran *et al*, 2001] Shear stress induced a rapid and transient tyrosine phosphorylation of Flk-1 and its concomitant association with the adaptor protein Shc [Labrador *et al*, 2003, Ravichandran *et al*, 2001] The adapter protein Shc is implicated in signaling via many different types of receptors, such as growth factor receptors, antigen receptors, cytokine receptors, G-protein coupled receptors, hormone receptors, integrins and tyrosine kinases [Lopez-Ilasaca *et al*, 1998] PTK mediated mechanotransduction often involves the participation of other receptors such as integrins and G-proteins [Woodward *et al*, 1998, Soldi *et al*, 1999, Linseman *et al*, 1995, Eguchi *et al*, 1998, Zwick *et al*, 1997] Based on dominant negative studies and mouse embryos deficient in ShcA, a clear role for Shc m leading to ERK activation has been established [Awoleski *et al*, 1994] Moreover, cyclic strain has been shown to induce PYK2 activity in EC [Cheng *et al*, 2002]

Focal adhesion associated tyrosine kinases, eg, FAK and c-Src, are rapidly activated in ECs by shear stress [Li *et al*, 1997, Jalali *et al*, 1998] Activation of FAK has been linked to recruitment of MAP kinases via the growth factor receptor binding protein 2 (Grb2) In addition to this FAK signaling has been associated with EC directional migration following mechanical stimulation [Polanowka-Grabowska *et al*, 2003]

p60src plays a critical role in the shear stress activation of MAPK pathways and induction of Activating Protein-1 (AP-1)/TRE and Elk-1/SRE-mediated transcription in ECs [Jalali *et al*, 1998] Flow mediated activation of c-Src has also been found to be dependent on ROS [Tai *et al*, 2002] Tyrosine kinase phosphorylation of eNOS is

believed to play an important role in shear stress-induced increases in NO production [Corson *et al*, 1996] Inhibition of these tyrosine kinases with genistein, tyrphosin A25 etc can prevent cyclic strain mediated gene induction of IL-8 and MCAF/MCP-1, ET-1 mediated contraction or COX-1 involvement in platelet reactivity and may indicate a novel target for therapeutic intervention [Okada *et al*, 1998, Zubkow *et al*, 2000, Santos *et al*, 2000] Clinically, PTKs such as IGFR, PDGFR and FGFR have been linked to multiple vascular pathologies, including atherosclerosis, hypertension, restenosis, angiogenesis, arteriogenesis and diabetic vascular disease [Okura *et al*, 2001, Patel *et al*, 2001, Rajkumar *et al*, 1996, Fath *et al*, 1993, Grant *et al*, 1996] The use of RTK blockers e g PDGF receptor kinase blockers are potential targets for the treatment of pathological vascular smooth muscle proliferation in atherosclerotic and restenotic processes

1 3 4 Ion Channels

Two different mechano-sensitive channels have been identified in vascular cells shear stress activated potassium channels and stretch activated cationic channels Inhibition of ion channel activation can attenuate strain induced SMC proliferation Stretch activated phospholipase C activity was found to involve the influx of calcium via gadolinium sensitive channels Similarly, Ang II activation of MAPK is calcium dependent in VSMC [Lehoux *et al*, 1998] The exact mechanisms by which mechanical forces regulate ion channel conformation remains vague The deformation of the cytoskeleton is thought to be an important contributor in this regulation This hypothesis is supported by a number of studies which demonstrate cytoskeleton-G-protein coupling in shear-induced potassium channel opening and integrin-cytoskeleton activation of ion channels [Lehoux *et al*,2001] The physiological role of many ion channels within the vasculature may be dependent on the hemodynamic forces of the circulation

1 4 Mitogen-activated protein kinase

Activation of mechano-receptors enable vessels to distinguish changes in the hemodynamic environment resulting in the activation of different mechanotransduction cascades, dependent on the type, magnitude, and duration of the mechanical load [Jalil *et al*, 1998, Surapisitchat *et al*, 2001, Jo *et al*, 1997] Changes in the organization of the cell cytoskeleton brought about by changes in the composition of the ECM can bring about alterations in the activation and localization of integrins and associated kinases Many intracellular signaling cascades, such as the MAPK pathway, which are activated by mechanical stimuli initiate transcription factors and subsequent gene expression [Cowan *et al*, 2003]

The mitogen-activated protein kinase (MAPK) signaling cascade is an important pathway by which the initial mechanical stimuli having being detected by a mechanically sensitive protein (e g G-protein, integrin) can lead to or stimulate gene transcription and/or protein synthesis The MAPK super-family is comprised of three main and distinct signaling 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 kinases Each of the MAPK modules operates as a three-tier system (Figure 1 9) The MAPK module is activated by a MAPK kinase (MAPKK), which is a dual-specific kinase, which phosphorylates ERK, JNK and p38 at both Ser/Thr and Tyr sites, targeting a Thr-X-Tyr motif where X is glutamate, proline or glycine The MAPKK is activated by a MAPKK kmase (MAPKKK), which receives its stimulus from receptors on the cell surface MAPK have a key role in the regulation of many genes because the end targets of these cascades are often nuclear proteins or transcription factors [Cowan *et al*, 2003]

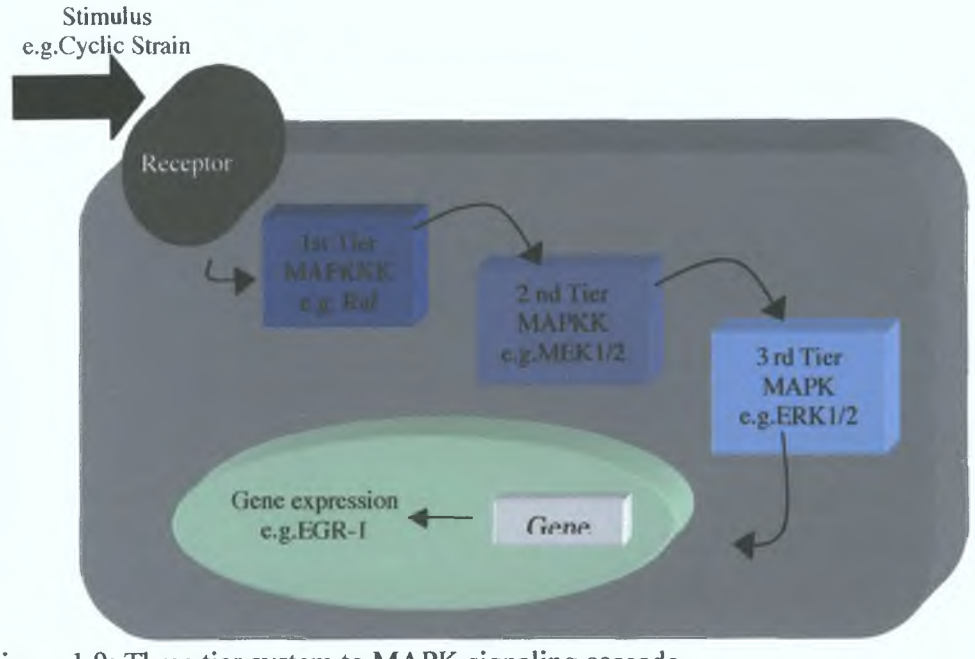


Figure 1.9: Three tier system to MAPK signaling cascade.

1.4.1 MAPK in Mechanotransduction:

The sensitivity of the MAPKs to mechanical forces is well established in both *in vivo* and *in vitro* models. Cyclic stretch activates Erk1/2 and JNK in cultured SMC and are transiently activated in arterial wall by acute hypertension [Xu *et al.*, 1996]. Kito *et al.* (2000) found that cyclic strain activated Erk1/2, JNK and p38 in pulmonary ECs with subsequent AP-1/TRE activation and was found to modulate the Egr-1 transcription factor. Using rabbit facial vein segments Loufrani *et al.* (1999) demonstrated that stretch induces Erk1/2 activation via a calcium-dependent pathway involving a tyrosine kinase. MAPK activation also occurs in response to shear stress. A shear stress of 12 dynes/cm^2 was found to activate ERK1/2 and p38 but to reduce activity of JNK [Surapisitchat *et al.*, 2001]. Shear stress has been reported to regulate ERK1/2 and JNK differentially with regards to duration of activation and levels of shear required to elicit a response [Jo *et al.*, 1997].

Activation of the MAPK pathway in response to mechanical stimuli may occur by various means including G-proteins, integrins, receptor tyrosine kinases and cytoskeleton-associated non-receptor tyrosine kinases. Phosphorylation of the α or $\beta\gamma$

subunit of a G-protein can lead to MAPK activation [Crespo *et al.*, 1994]. Shear stress has been found to activate ERK1/2 via a Giα/Ras pathway and JNK via a Gβγ/Ras tyrosine kinase pathway [Jo *et al.*, 1997]. Similarly, small GTPases such as Ras or RhoA are stimulated by mechanical strain and may regulate ERK1/2 or JNK activation [Wung *et al.*, 1999; Numaguchi *et al.*, 1999].

Similarly integrins have been shown to be involved in the activation of members of the MAPK family. The ability of cyclic stretch to activate MAPK is dependent on the substrate on which the cells are seeded, e.g. ERK1/2 and JNK are activated in cyclically stretch SMC grown on pronectin but not on laminin [Reusch *et al.*, 1996]. Chen *et al* observed an increase in the association of $\alpha_v\beta_3$ integrin with the adapter protein Shc and subsequent activation of ERK1/2 and JNK. Shear stress increases the tyrosine phosphorylation of FAK as well as its kinase activity. This increase in FAK activity is associated with increases in MAPK activity and is attenuated by inhibition of integrin signaling [Li *et al.*, 1997]. Disruption of the cytoskeleton by cytochalasin-D inhibits strain-induced increases in ERK1/2 possibly by preventing downstream signaling from integrin receptors [Ingram *et al.*, 2000]. Despite the importance of G-proteins and integrins in the mechanical regulation of MAPK, multiple pathways exist. Downregulation of PKC or inhibition of tyrosine kinase activity prevents activation of ERK1/2 by shear stress [Traub *et al.*, 1997]. Cyclic strain induced release of reactive oxygen species can also modulate the ERK1/2 pathway. Following phosphorylation of MAPK the downstream effects are varied. Phosphorylated ERK1/2 can transfer to the nucleus where it can activate transcription factors and thus regulated gene expression [Wung *et al.*, 1999]. Both JNK and ERK1/2 can bind to the serum response element (SRE) to increase transcriptional activity. Activation of ERK1/2 leads to an increase in the expression of c-fos and c-jun which form the Activating protein-1 (AP-1) transcription factor which plays a significant role in the expression of a number of genes [Proud *et al.*, 1994].

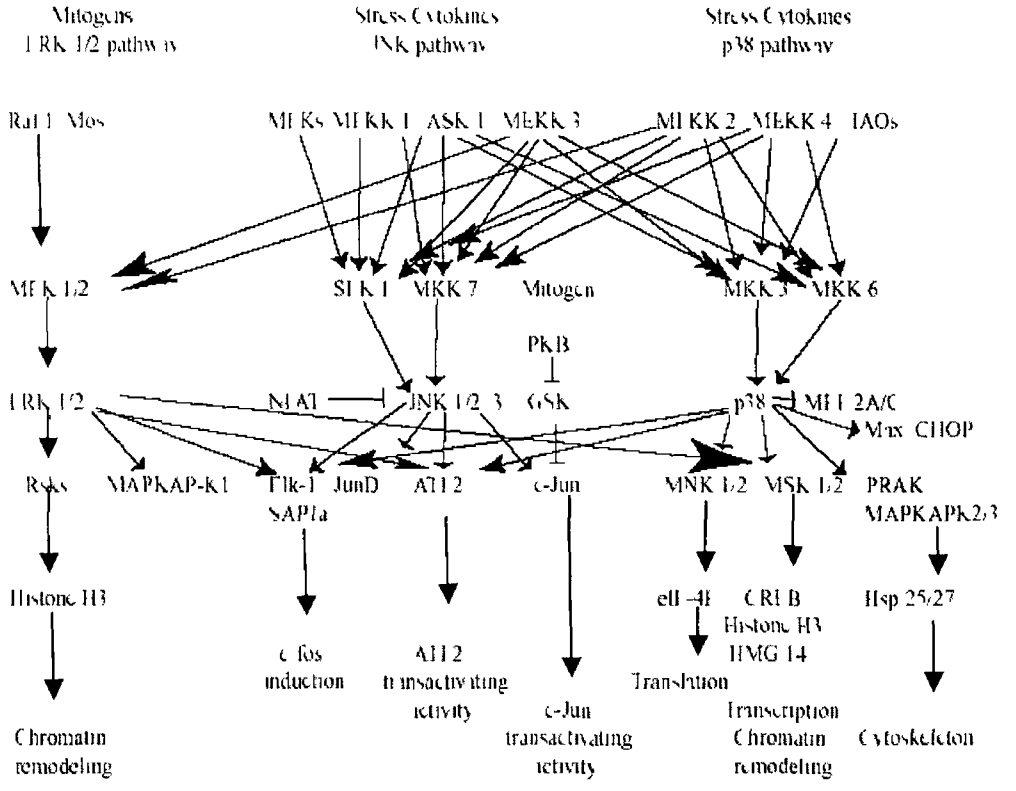


Figure 1 10 Summary of MAPK signaling pathways showing cross talk between modules (Cowan *et al*, 2003)

Clinically, changes in the activation of the MAPK family has been linked to a number of pathologies in which the hemodynamic environment is altered Augmented SMC proliferation and migration associated with neointimal formation and hyperplasia following balloon angioplasty has been linked to increases in MAPK activity [Onashi *et al*, 2000, Gennaro *et al*, 2003, Goetze *et al*, 1999] PKC and ERK interactions are thought to play a major role in cardioprotection by the inhibition the phosphorylation of the pro-apoptotic protein, Bad [Baines *et al*, 2002] Both p38 and ERK1/2 are involved in the transcriptional activation of MMP-9, an enzyme which is linked to plaque rupture and neointimal formation in arterial lesions [Cho *et al*, 2000] In the heart, enhanced activation of p38 MAPK is associated with ischemia/reperfusion injury p38 MAPK plays a pivotal role in the signal transduction pathway mediating post ischemic myocardial apoptosis Attenuation of this signaling may result in increased protection

against myocardial ischemic injury [Liao *et al* 2002, Ma *et al*, 1999, Martin *et al*, 2001]

The process of mechanotransduction provides the means by which blood vessels are capable of adapting to changes in their local environment A family of enzymes, which are believed to be important in the adaptation of blood vessels to changes in the hemodynamic forces, are the matrix metalloproteinases, which will be discussed in greater detail in the following section

1 5 Overview of Matrix Metalloproteinases

Essential to cell survival and function is the ability to interact properly with the immediate environment This environment includes other cells and the meshwork of specialized proteoglycans and glycoproteins in which they are co-assembled, collectively referred to as the extracellular matrix (ECM) This matrix provides a structural support about which cells may grow and is usually divided into two parts, i) the basement membrane and ii) the interstitial connective tissue The ECM also plays important roles in a number of cell fate decisions such as migration, proliferation and differentiation The modification of the ECM can be effected by a number of enzymatic activities including, i) cysteine proteinases, ii) serine proteinases iii) aspartyl proteinases and iv) matrix metalloproteinases

Name	MMP	Substrates	Chromosome	Mol. Wt.
Collagenases				
Collagenase 1	MMP-1	Collagen I,II,III,VII,X Gelatin, Proteoglycans	11q22-q23	55-45
Collagenase 2	MMP-8	Collagen I,II,III	11q21-22	75-58
Collagenase 3	MMP-13	Collagen II	11q22 3	65-55
Gelatinases				
Gelatinase A	MMP-2	Gelatin, Collagen IV,V, VI,X Elastin Fibronectin	16q13	72-66
Gelatinase B	MMP-9	Gelatin, collagen IV, V, elastin	20q11 2-q13 1	92-86
Stromelysins				
Stromelysin 1	MMP-3	Proteoglycans,Laminin, fibronectin, gelatin, collagen III, IV,V, X	11q23	57-45
Stromelysin 2	MMP-10	Proteoglycans,fibronectin, gelatin, collagen III, IV,V	11q22 3-q23	57-44
Stromelysin 3	MMP-11	Laminin, fibronectin	22q11 2	51-44
Matrilysins				
Matrilysin 1,pump1	MMP-7	Proteoglycans, laminin,fibronectin, gelatins, elastin, collagen IV, entactin	11q21-22	28-19
Matrilysin 2	MMP-26	Collagen IV, Fibrin, fibronectin	11q15	28-18
Membrane-type MMP				
MT1-MMP	MMP-14	CD44		63
MT2-MMP	MMP-15	Unknown	14q11-q12	72
MT3-MMP	MMP-16	Unknown	15q13-q21	64
MT4-MMP	MMP-17	Unknown	8q21	70
MT5-MMP	MMP-24	Proteoglycan ECMcomponents	12q24 3	60
MT6-MMP	MMP-25	Unknown	20q11 2	
			16q13 3	62
Others				
No trivial name	MMP-19	Gelatins, Aggrecan, cartilage	12q14	-
Enamelysin	MMP-20	Amelogenin, Aggrecan	11q22 3	54
No Name	MMP-21	Unknown	ND	
No Name	MMP-23	Unknown	1p36 3	28
Epilysin	MMP28	Caesin	17q21 1	59

Table 1 2 MMP trivial names, substrate specificity, chromosomal location and molecular weight

The matrix metalloproteinases (MMPs) are a highly conserved family of zinc-dependent proteases, which are capable of cleaving numerous pericellular substrates including other proteinases, clotting factors, cell surface receptors, growth factors and virtually all components of the basement membrane and interstitial extra cellular matrix Due to their ability to be secreted into the extracellular space and function under normal physiological conditions in conjunction with their varied substrate specificities, MMPs are believed to be the primary contributors to ECM degradation Thus by virtue of their ability to modify a wide variety of bioactive peptides as well as the ECM, MMPs influence diverse physiological and pathological processes [Sternlicht *et al*, 2001]

MMPs require close regulation In addition to differential transcriptional regulation, MMPs are regulated at the protein level by their endogenous activators and inhibitors and by factors, which alter secretion, cellular localization and degradation The multiplicity of the MMPs with distinct but overlapping functions may act as a safeguard against loss in regulatory control These redundancies often complicate the full elucidation of MMP function [Sternlicht *et al*, 2001] *In vivo* genetic experiments have shown that selective gains or losses in MMP function may promote initial stages of cancer but may decrease the ultimate severity of the malignancy [Coussens *et al*, 2000] The importance of MMPs in both the progression of diseases and in maintaining cellular function is undeniable However the exact mechanisms by which they affect their influence as well as how they are influenced under different conditions still remains largely unresolved A more complete understanding of MMP function and regulation will undoubtedly result in new and more practical therapeutic agents for a wide range of disease states

1 5 1 Structure and function

The metzincin superfamily comprises of 4 multigene families, i) the serralysins, ii) the astacins, iii) the ADAMs/adamalysins and iv) the MMPs The metzincin superfamily is distinguished by a highly conserved motif in which three histidine residues bind a zinc molecule at the active site and a conserved methionine turn beneath

the active site zinc [Stoker *et al*, 1995] The conserved zinc-binding site (HEBXHXBGBXHZ) comprises of nonvariant histidine (H), glutamic acid (E) and glycine (G) residues, a large hydrophobic region (B), a variable residue (X) and a family specific amino acid (Z), which is serine for nearly all members of the MMPs [Stoker *et al*, 1995]

The MMPs are a growing multigene family, which at present comprise of 25 secreted and cell surface enzymes The first MMP activity to be observed was in the tail of a tadpole undergoing metamorphosis [Gross *et al*, 1962] Since then, in addition to the 25 vertebrate and 22 human homologues which have been identified there also exists a number of non vertebrate MMPs [Nagase *et al*, 1999] The MMPs can be identified by the following shared characteristics i) In addition to the zinc binding motif and methionine turn they share added stretches of common amino acid sequences, ii) They exist as either a secreted or trans-membrane pro-enzyme, which requires activation, iii) The active site contains a zinc ion and usually requires a second metal cofactor and iv) They can be inhibited by a family of proteins known as tissue inhibitors of metalloprotemases (TIMPs)

MMPs are referred to by their common or trivial names or according to a sequential numerical nomenclature In addition, they are often grouped on the basis of their modular domain structure MMPs generally consist of an N-terminal "pre"domain, which directs their synthesis to the endoplasmic reticulum, and subsequently to secretory vesicles where they are destined for release Following the "pre" domain is the "pro" domain, which maintains the latency of the enzyme until it is removed or disrupted, and a catalytic domain, containing the conserved zinc binding site The substrate specificity of the MMP is determined by a number of sub-site pockets that bind amino acid residues either side of the scissile peptide bond Excluding MMP-7, MMP-26 and MMP-23, all MMPs have a hemopexin/vitronectin-like domain, which is connected to the catalytic domain by a hinge region [Gururajan *et al*, 1998] On the basis of substrate specificity, domain organization, and sequence similarity MMPs can be divided into six groups [Visse *et al*, 2003]

1 5 1 1 Collagenases

The key feature of this group is the ability to degrade interstitial collagens I, II and III at a specific site close to the substrate N-terminus The hemopexin domain, which is linked to the catalytic domain via a short variable hinge region is thought to be involved in substrate interaction, TIMP binding and membrane activation The collagenolytic activity requires initial binding and orientation of the collagen fibril, unwinding of the triple helix structure and sequential cleavage of the alpha-chain [Woessner *et al*, 2001] The hemopexin domain is thought to participate in all but the last of these processes Other members of this group include MMP-13 and MMP-18

1 5 1 2 Gelatinases

This group currently contains only two members, these are MMP-2 (gelatinase A) and MMP-9 (gelatinase B) first separated from collagenase activity by Sellers *et al* in 1978 These enzymes are distinguishable by the presence of three head-to-tail cysteine rich insertions into the catalytic site These inserts resemble collagen-binding type II repeats of fibronectin and are required for the binding of gelatin, laminin and collagen in addition to the cleavage of elastin [Murphy *et al*, 1994, Shipley *et al*, 1996] MMP-9 has another unique feature, the catalytic region contains a unique type V collagen-like insert, the biological significance of which has yet to be determined

These enzymes degrade denatured collagens and are specific for the degradation of type IV basement membrane collagen MMP-2 is expressed by all cell types of the vasculature and is frequently associated with the rupture of atherosclerotic plaques, inflammation responses and in aortic aneurysm It was first cloned in 1988 by Collier *et al,* from a human λ phage library It is secreted as a 72kDa pro-enzyme and is activated *in vivo* by the formation of complexes with TIMP-2 and MT1-MMP Once activated it preferentially degrades gelatin, Collagen IV, V, VII and fibronectin MMP-9 was traditionally thought to be a macrophage-specific gelatinase but its expression has also been widely reported in the vascular cell types Initially identified in 1972 by Harris *et*

al from rheumatoid synovial fluid, it was purified in 1983 by Rantala and subsequently cloned by Willhelm *et al* in 1989 from SV-40 transformed human lung fibroblasts Like MMP-2, it is a secreted MMP with a molecular weight of 92kDa and degrades gelatin, and collagen IV and V as well as elastin

1 5 1 3 Stromelysins

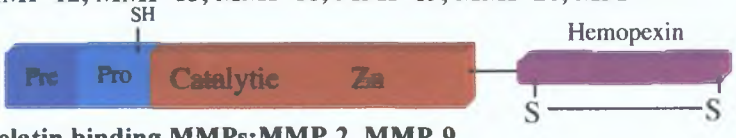
This sub-class contains MMP-3, -10, -11 (stromelysin 1, 2, 3) which are capable of cleaving proteoglycans, collagens and fibronectin MMP-3 was first identified in 1985 as a secreted MMP, induced by oncogenes MMP-3 and MMP-10 have a very high degree of homology (71%) [Breathnach *et al*, 1987] in both amino acid sequence and substrate specificity, however MMP-3 has a much greater proteolytic efficiency than that of MMP-10 [Park *et al*, 2000] Despite the similarities between the two enzymes both genes are regulated very differently and neither has the ability to degrade native type I collagen

1 5 1 4 Matrilysins

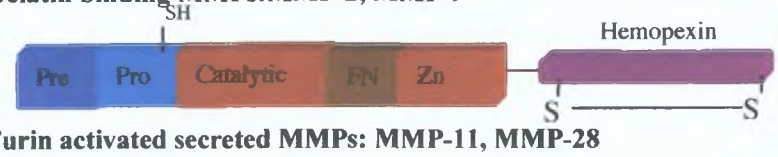
The matrilysins can be identified based on the absence of the hemopexin domain These enzymes are sometimes referred to as minimal domain MMPs and include MMP-7 (matrilysin 1) and MMP-26 (matrilysm 2) Matriysins have one of the broadest substrate specificity ranges and will degrade collagen, proteoglycans and glycoproteins MMP-7 processes cell surface molecules such as pro-α-defensin, FAS ligand and E-cadherin [Itoh *et al*, 1999] It has been shown to play an important role in the progression of many biological processes including the progression of many tumour types

Minimal Domain MMPs: MMP-7,MMP-26

Simple Hemopexin Domain Containing MMPs: MMP-1, MMP-3, MMP-8, MMP-10, MMP-12, MMP-13, MMP-18, MMP-19, MMP-20, MMP-27



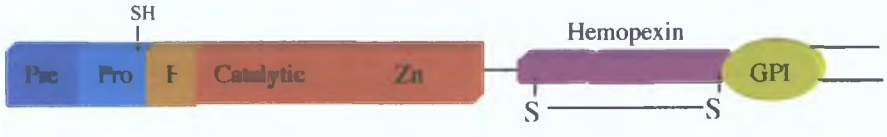
Gelatin binding MMPs:MMP-2, MMP-9



Furin activated secreted MMPs: MMP-11, MMP-28

Transmembrane MMPs: MMP-14, MMP-15, MMP-16, MMP-24

GPI-linked MMPs: MMP-17, MMP-25



Vitronectin-like insert linker-less MMPs: MMP-21

Cysteine/Proline Rich IL-1 Receptor Like domain MMPs: MMP-23

Figure 1.11: Domain Structure of MMPs: Pre signal sequence, Pro peptide with free zinc-ligating thiol group, F Furin susceptible site, Zn Zinc binding site, FN Fibronectin like domain, TM transmembrane domain, C cytoplasmic tail, GPI glycophosphatidyl inositol anchoring domain, C/P [adapted from Visse *et al.*, 2003]

1 5 1 5 Membrane Type MMPs (MT-MMPs)

This sub group contain a transmembrane domain at the carboxyl termini localizing the enzymes to the plasma membrane The structure of these enzymes is unique i) between the pre-pro domain there is an eleven amino acid insertion which is recognized by furin and facilitates the activation of these enzymes *in vitro* ii) Within the catalytic site is an 8 ammo acid insertion the function of which has yet to be determined iii) The C terminus contains a hydrophobic amino acid sequence facilitating transmembrane anchorage Four of the MT-MMPs are type I transmembrane proteins (MT1-MMP, MT2-MMP, MT3-MMP and MT5-MMP), the other two members are glycophosphatidylinositol (GPI)-anchored proteins (MT-4 and MT-6) [Itoh *et al*, 1999, Kojima *et al*, 2000] The expression of these enzymes varies somewhat, MT5-MMP is brain specific while MT6-MMP is expressed only in the peripheral blood leukocytes and anaplastic astrocytomas [Onuchi *et al* 1997] The substrate specificities of these enzymes is still largely unclear although MT1-MMP has been found to contain some collagenolytic activity [Pepper *et al*, 2001, Kolb *et al*, 1997]

1 5 1 6 Other MMPs

The sixth and final category contains the MMPs, which cannot be classified by any of the above categories This category contains several members of the MMP family that have only recently been discovered and includes, MMP-12, MMP-18, MMP-19, MMP-20, MMP-21, MMP-22, MMP-23, MMP26 and MMP-27

1 5 2 Other MMP Substrates

The targets of MMPs extends further than the ECM components and have been found to degrade many substrates including other proteinases (uPA), clotting factors, chemotactic factors, latent growth factors (TNF-alpha), cell surface receptors, cell-cell adhesion molecules and proteinase inhibitors [Patterson *et al* 1997] MMP-9 has been found to inhibit angiogenesis by hydrolyzing plasminogen producing angiostatin

fragments. The discovery that MMPs function in biological processes other than ECM modification has lead to a better understanding of the many roles MMPs have such as in inflammation responses, cell migration, apoptosis and angiogenesis [Blobel *et al.*, 1997].

1.5.3 The ADAMS Family:

ADAMs are a family of membrane associated multidomain zinc-dependent metalloproteinases with a high sequence homology to adamlysin sub-group of the metzincin superfamily [Primakoff *et al.*, 2000; Pan *et al.*, 1997]. ADAM, stands for A Disintegrin And Metalloprotienase, which are the two key domains of these enzymes. These cell surface proteins are distinctive in that they contain aspects of both adhesive proteins and proteinases. There are currently 29 characterized members of the ADAMs family, which implicated in cell adhesion, fusion processes and shedding of cell surface proteins [Kaushal *et al.*, 2000]. 17 of the ADAMs are thought to have functional proteolytic activity as cDNA studies indicate the presence of a metalloproteinase active site. The remaining are thought to be non-functional [Pan *et al.*, 1997].

Figure 1.12: Typical ADAM structure comprising of a prodomain, metalloproteinase domain, disintegrin domain, cysteine rich domain, EGF-like, transmembrane and cytoplasmic domains

ADAMTs (A Disintegrin and Metalloproteinase with Thrombospondin motifs), contain unique thrombospondin type I motifs and lack some of the structural similarities to ADAMs. They have been identified in a number of biological processes such as skin development, angiogenesis and vascular development. ADAMTS1 -/- mice have been found to have reduced body size and altered kidney structure. The thrombospondin repeats may function in conjunction with the disintegrin domain to maintain these proteins in an appropriate position within the ECM. [Kaushal *et al.*, 2000].

1 5 4 Substrate Specificities

The specificities of MMPs have been identified by the cleavage of protein substrates or synthetic peptide substrates Generally speaking, MMPs will cleave a peptide bond before a residue with a hydrophobic side chain (Leu, Met, Phe) These hydrophobic side chains are involved in the binding to the S1' pocket, the size and shape of which varies considerably depending on the MMP [Pei *et al*, 1995] Non-catalytic domain components, such as the fibronectin type repeat of MMP-2, the hemopexm domain, or the RWTNNFREY domains of MMP-1 play key roles in the function of these enzymes The overlapping substrate specificities of MMPs probably serve as a defense mechanism against losses in regulatory control by providing redundant and compensatory mechanisms While advantageous to the organism it clearly makes it difficult to accurately understand how individual MMPs function

1 5 5 Three Dimensional Structure

The 3-D structure of a number of MMPs has been determined by the use of X-ray crystallography and NMR The structure of the pro-domain (for MMP-2, -3 and –9) has been found to consist of three alpha helices and connecting loops The region between the first and second helix has been found to be protease sensitive The third helix has been found to lie in the substrate-binding cleft of the catalytic domain This region contains the cysteine switch, which binds to the active site zinc and is responsible for maintaining enzyme latency The catalytic domain comprises of five Beta-pleated sheets, three alpha helices and connecting loops The substrate-binding cleft is formed by the fourth beta sheet and by helix B as well as the extended loop region of helix B The conserved Met turn is located in the loop region In MMP-2 and MMP-9 fibronectin like repeats are found inserted between the fifth Beta strand and the catalytic site helix The hemopexin repeats are found to have a four bladed beta-propeller fold with a single disulfide bond between blade one and four Beta propeller domains are common in a number of proteins such as G-proteins and are commonly

involved in protein-protein interactions, such as those seen between MMP-2 and fibronectin or in the pro-MMP-2 activation via TIMP-2 recruitment [Visse *et al.*, 2002].

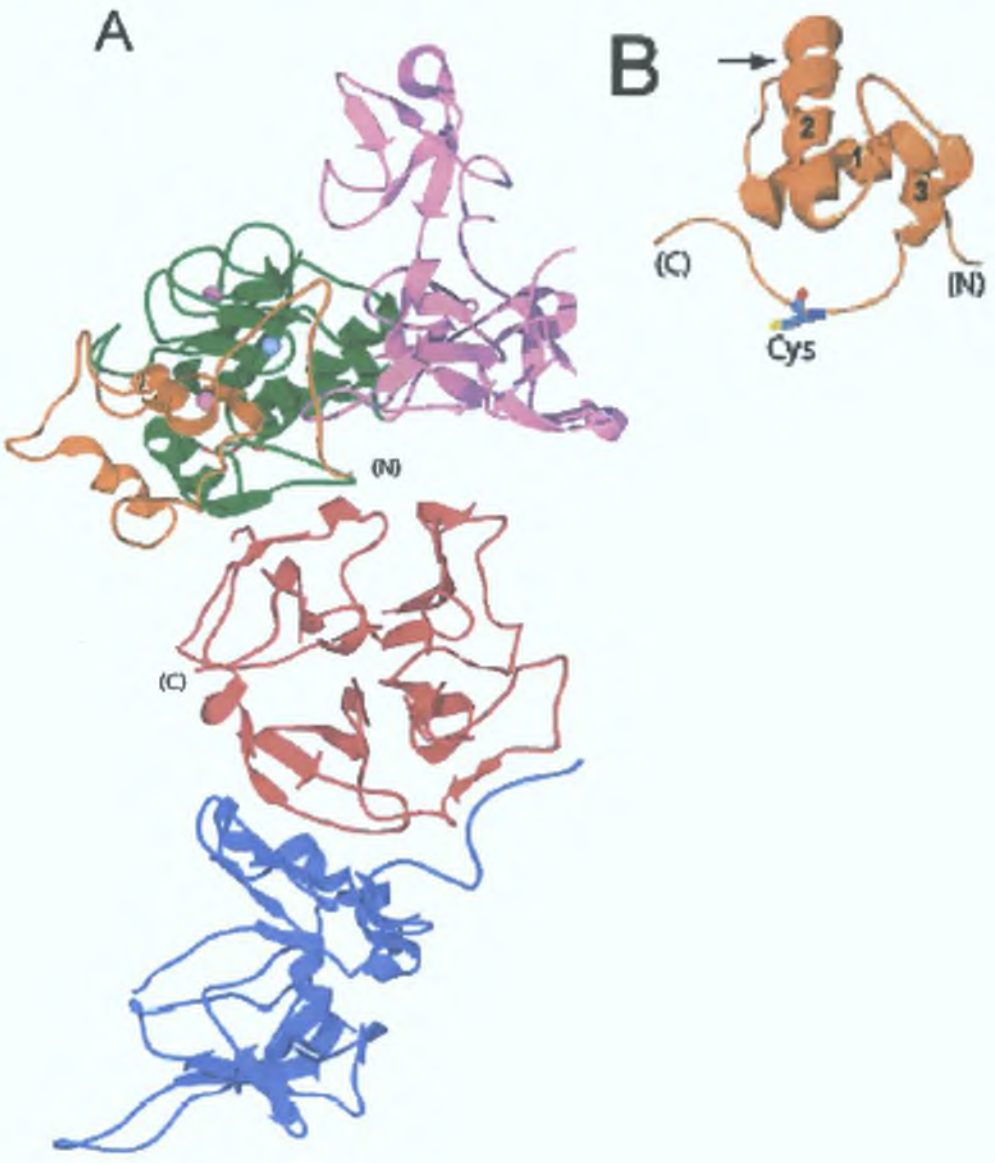


Figure 1.13: 3D structure of MMPs: ribbon diagram of MMP structures. A, ProMMP-2–TIMP-2 complex (1GXD) is shown. Orange indicates propeptide; green, catalytic domain; pink, fibronectin domains; red, hemopexin domain; and blue, TIMP-2. Zinc atoms are pink, and calcium atoms are gray. B, In the MMP-2 propeptide,40 the cysteine of the cysteine switch motif is shown. The arrow indicates the position of the initial cleavage resulting in partial activation [adapted from Nagase *et al.*, 1999]

1 5 6 Activation

MMPs are typically secreted as latent enzymes, which can be activated by other proteinases (including other MMPs), reactive oxygen species, low pH and exposure to chemical substances such as thiol-modifying reagents Activation of MMPs requires the removal of the "cysteine switch" which blocks the catalytic site by a zinc/cysteine interaction The pro-domain is folded so that the cysteine residue forms a complex with the active site zinc Disruption of the pro-domain by the means mentioned above causes a conformational change, which destabilizes the propeptide and disrupts the cysteine/zinc interaction, forming an MMP intermediate This intermediate is then fully activated by other MMP intermediates or fully active MMPs [Strongin *et al*, 1995] The term cysteine switch refers to the fact that cysteine residue is responsible for turning the enzyme activity "on" or "off" Once displaced, the thiol group of the cysteine is replaced by a water molecule, which can attack the scissile bond of MMP target molecules

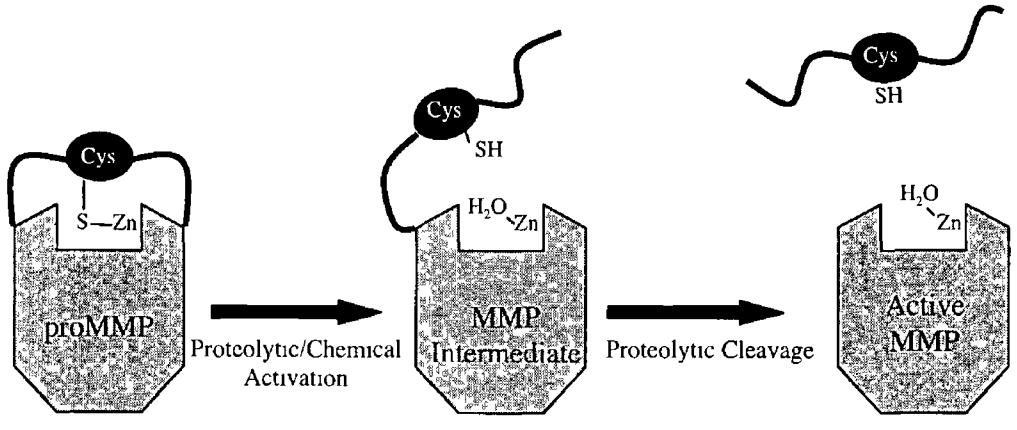


Figure 1 14 Pro-MMP activation occurs in a stepwise manner Secreted proMMPs are partially activated by disruption of the propeptide (black line) cysteine switch by proteolytic or chemical means Full activation requires complete removal of the propeptide region by other MMPs

Although the majority of MMPs are constitutively secreted as pro-enzymes there a number of MMPs such as MMP-11, MMP27 and the MT-MMPs which contain a

furin recognition site (KXR/KR), which allows them to be activated intracellularly by serine proteinases prior to being secreted [Lijnen *et al*, 2001, Strongin *et al*, 1995] The extra cellular activation of MMPs is initiated by a number of proteinases including other MMPs One of the most common proteinases involved in MMP activation is plasmin, a 90kDa serine proteinase Plasmin is generated from plasminogen by the plasmin cascade, which involves the urokinase plasminogen activator (uPA), its inhibitors plasminogen activator inhibitor (PAI-1, -2) and tissue plasminogen activator (tPA) Plasmin has been linked to the activation of proMMP-9, proMMP10 and pro-MMP13 [Lijnen *et al*, 2001] It has also been implicated in the activation of pro-MMP-2 when it is bound by MT1-MMP complex

1 5 7 Regulation

For either their normal or pathological functions MMPs must be present in the correct cell type and pericellular location MMPs are separately and very tightly regulated at both the transcriptional, translational and post-translational levels Moreover, their expression is highly tissue-specific

1 5 7 1 Transcriptional Regulation

Transcriptional regulation of MMPs is known to be affected by numerous stimulatory and repressive factors The expression of various MMPs has been shown to be up or down regulated in response to cytokines, adhesion molecules, growth factors, integrin derived signals, phorbol esters and changes in cell shape [Nagase *et al*, 1999] Differences in the temporal, spatial and inducible expression of MMPs are often indicative of their unique roles The majority of MMPs are tightly regulated at the level of transcription The exception to this is MMP-2, which is constitutively expressed and is controlled in a complex mechanism involving MMP-2, MT1-MMP and TIMP-2, which will be discussed later The basal expression of these three molecules also appears to be co-regulated [Crawford *et al* 1996 Uria *et al*, 1998] However, arteries incubated under pressure displayed significant increases in the levels of MMP-2

expression and activity while simultaneously degrading elastin suggesting that both MMP-2 expression and matrix degradation are locally enhanced at higher transmural pressures [Drau *et al*, 2002, Selıktar *et al*, 2001]

Many of the factors which influence MMP expression such as VEGF, PDGF, TNF-α and EMMPIN induce the activation of AP-1, transcription factor which has binding sites in the promoters of many of the MMPs with the exception of MMP-2 and MMP-11 [Fan *et al*, 2002] Normally c-jun and c-fos proteins are transiently expressed following a stimulus, such as shear stress, and bind to the AP-1 site resulting in transcription of the MMP gene Some members of the jun and fos proteins may also act as suppressors of transcription Some jun/fos complexes are only weak activators and therefore block binding of more potent activators In some cases one signal may differentially regulate different MMPs, TGF-β suppresses transcription of MMP-1 and MMP-3 but induces MMP-13 [Hongwei *et al*, 1999] How an MMP gene responds to a particular stimulus will ultimately depend on the structure of its promoter and the presence or absence of other signals

A second activating protein complex (AP-2) has also been found to play a significant role in the regulation of MMP-2 AP-2 in conjunction with Sp1 and Sp3 have been reported to be required for the constitutive expression of this protein Loss of AP-2 transcriptional control has been correlated to increased MMP-2 production in association with increased VSMC migration and increased melanoma metastasis There are three members to the AP-2 family comprising of Ap-2α, Ap-2β and Ap-2γ, which bind to sites ($GGCN_3GGC$) distinct to AP-1 binding sites (TGAGTCA) [*Hongewi et al*, 1999, Nyormoi *et al*, 2003, Price *et al* 2001] Several other regulatory elements have been found within the promoter regions of MMP genes such as TGF-β inhibitory elements, NF-κB, Polyoma enhancing factor 3 (PEA-3), or retinoic acid response elements Both basal and inducible levels of MMP gene expression can be influenced by genetic variation, which have also been implicated in development or progression of several diseases A number of single nucleotide polymorphisms (SNPs), which influence the rate of transcription, have been identified in several MMP promoters For

example, a SNP 1306bp upstream of the start codon of MMP-2 disrupts the Sp-1 binding site thus resulting in a 50% reduction in promoter activation [Price *et al*, 2001] Activation of certain MMPs, such as MMP-2 may be regulated by the transcriptional control of other genes For example shear induced MMP-2 activity may be attributed to shear mediated PDGF release due to the presence of a Shear Stress Response Element (GAGACC) in its promoter region [Bassiouny *et al* 1998] Notwithstanding the many advances in our understanding of MMP gene regulation, the cross-talk between many signaling pathways the gene regulatory elements and nuclear factors involved are yet to be fully elucidated

1 5 7 2 Post Transcriptional Regulation

MMP expression, in addition to being regulated by complex transcriptional mechanism can also be regulated post-transcriptionaly Following secretion the majority of extra cellular MMPs may be activated by either already activated MMPs or by several serine proteases that can cleave peptide bonds within the MMP pro-domain

MMP-2 is not readily activated by proteinases and its activation involves a complicated interaction between MT1-MMP, pro-MMP-2 and TIMP-2 MT1-MMP is very efficient activator of MMP-2, MT2-MMP and MT4-MMP are the only MT-MMPs unable to activate MMP-2 [Zucker *et al*, 1998] Activation of MMP-2 has been extensively studied Activated MT1-MMP on the cell surface binds the N-terminal of TIMP-2 and is subsequently inhibited by this binding The C-terminal domain of TIMP-2 is free to bind the hemopexin domain of pro-MMP-2 Adjacent MT1-MMP which is not bound by TIMP-2 is then free to cleave and activate pro-MMP-2 Following the initial cleavage of pro-MMP-2 a residual portion of the propeptide is removed by another MMP-2 molecule to yield a fully mature form of MMP-2 [Deryugina *et al*, 2001] While activation of MT1-MMP has been assumed to be required for activation of MMP-2, it has been found that the propeptide domain of MT1-MMP is required for the binding of TIMPs and for the activation of MMP-2, however the propeptide domain does not need to be covalently attached Thus it has

been proposed that the propeptide domain may be required for the efficient trafficking of MT1-MMP to the cell surface [Cao *et al*, 2000] The C-terminal domain of TIMP-2 participates in the docking and activation of MMP-2, however the N-termmal region is an MMP inhibitor Low levels of TIMP-2 may promote MMP-2 activation while higher levels of TIMP-2 leads to inhibition by saturation of MT1-MMP binding sites, required for removal of the propeptide domain of MMP-2 [Fan *et al*, 2001] MT2-MMP activation of MMP-2 does not require the intervention of TIMP-2

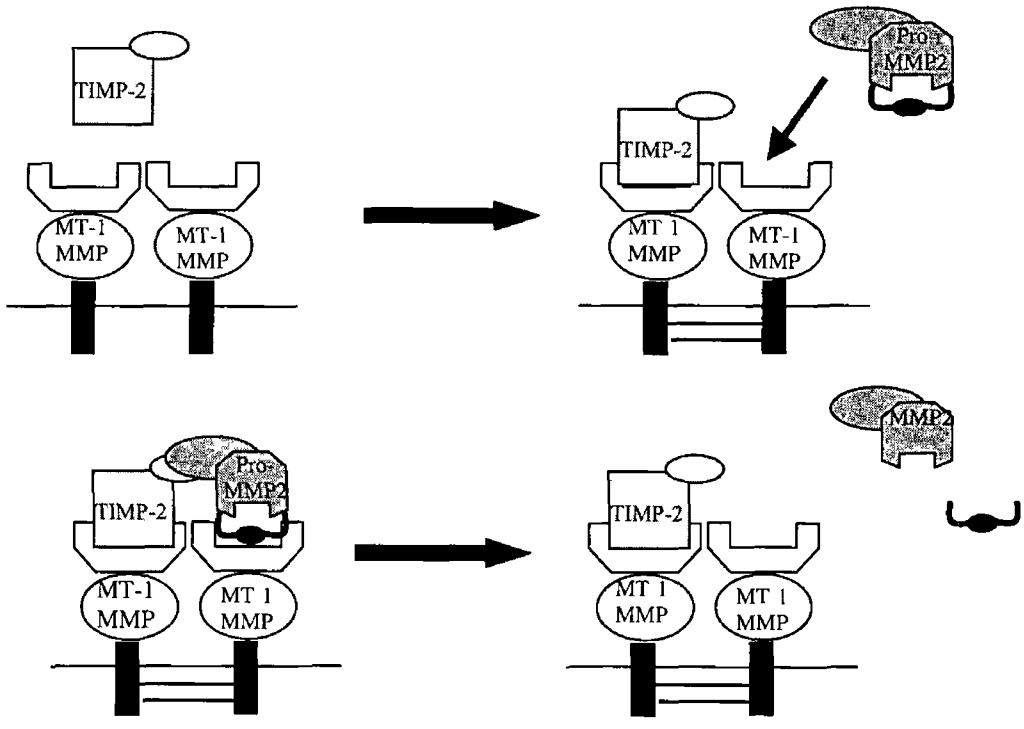


Figure 1 15 proMMP-2 activation Binding of TIMP-2 to MT1-MMP causing dimerisation of MT1-MMP molecules The hemopexin domain of proMMP-2 binds to the C-terminal end of TIMP-2 forming a tertiary structure The secomd MT1-MMP cleaves the pro-domain of of proMMP-2 partially activating it MMP-2 then dissociates from the complex and is fully activated [adapted from Visse *et al*, 2003]

1 5 8 Tissue Inhibitors of Metalloproteinases

MMPs act primarily on the cell surface or in the extracellular space and their activities are controlled by endogenous inhibitors such as α2-macroglobulin and the TIMPs Mammalian TIMPs are two-domain molecules having N-terminal domains of approxiamtely 125 ammo acids and a smaller C-terminal domain of about 65 amino acids [Williamson *et al*, 1990] There are currently four TIMPs identified in mammalian systems (TIMP-1 to TIMP-4) which are expressed in a wide variety of cell types such as endothelial cells, fibroblasts, keratmocytes and osteoblasts [Pohar *et al*, 1999] The expression of these molecules is also tightly regulated and is controlled during tissue remodelling and physiologiocal conditions to maintain a balance in the metabolism of the ECM The TIMPs represent a family of four secreted proteins ranging in size from 20-29kDa These proteins have the ability to reversibly inhibit MMPs in a 1 1 stoichiometric fashion TIMPs show a 30-40% ammo acid sequence homology and share a conserved gene structure and have 12 conserved cysteine residues These cysteine residues are essential in forming a six loop/two domain structure associated with TIMPs The overall shape of the TIMP molecule is wedge-like which slots into the active site of the MMP molecule Studies including X-ray crystalography and mutational analysis have identified the N-terminal domain as containing the inhibitory activity, however both domains influence enzyme-inhibitor binding [Brew *et al*, 2002]

TIMPs vary in their ability to inhibit the various MMPs, while they share a high degree of homology differences in their inhibitory profile have been reported For example MT1-MMP is inhibited by TIMP2 and TIMP3 but not by TIMP1 TIMP3 appears to have the most potent inhibition of MMP-9, it is also capable of inhibiting members of the ADAM family The identification of differing inhibitory roles for the vaious TIMPs may suggest that they have differing biological functions [Brew *et al*, 2002, Kashivagi *et al*, 2001]

As they have the abılıty ot tıghtly regulate MMP actıvıty, TIMPs play a major role ın tıssue remodellıng and have been shown to ınhıbıt ınvasıon, tumourgenesıs, metastasıs and angıogenesıs TIMPs play roles ın a varıety of vascular sıtuatıons TIMP 1 for example has been found to be asssocıated wıth ıncreased aneurysm formatıon but reduced athersclerotıc plaque formatıon, whılst TIMP 2 has been found to be expressed to counteract MMP actıvıty ın rabbıts fed on a hıgh cholesterol dıet [Feldman *et al*, 2001] TIMP 3 ıs associated wıth ıncreased plaque stabılıty and ınhıbıtıon of neoıntıma formatıon Vascular ınjury has also been shown to regulate TIMP-4 actıvıty Loss of TIMP-4 from the cardıac myocyte leads to an ıncrease ın net myocardıal MMP actıvıty that contrıbutes to acute myocardıal stunnıng ınjury [Dollery *et al*, 1999]

TIMPs may also be ınvolved ın cellular events whereby they are not requıred for MMP ınhıbıtıon TIMP-2 but not TIMP-1 has been found to ınhıbıt endothelıal cell growth stımulated by bFGF TIMP-3 ıs known to possess pro-apoptotıc actıvıty whıle TIMP-1 and –2 have antı-apoptotıc actıvıty [Smıth *et al* 1997, Lee *et al*, 2003] The pro-apoptotıc actıvıty of TIMP-3 ıs related to the stabılızatıon of TNF-alpha receptors TIMP-3 has also been ımplıcated ın Sorsby's fundus dystrophy [Majıd *et al*, 2002] and TIMP –1,-2 and –3 have been found to ınhıbıt tumour growth

1 5 8 1 Other endogenous ınhıbıtors of MMPs

α2-macroglobulın ıs an abundant plasma protein whıch ınhıbıts a wıde range of endopeptıdases , MMP-1 has a greater affinity wıth thıs moleculae than TIMP-1 It represents the major ınhıbıtor of MMPs ın tıssue fluıds and unlıke TIMP ınhıbıtıon ıs not reversıbıle [Sternlıcht *et al* 2001]

Tıssue factor pathway ınhıbıtor-2 ıs a serıne proteınase whıch ınhıbıts MMPs Cleavage of the procollagen C terminal proteınase enhancer protein (PCPE) releases a fragment wıth antı-MMP actıvıty [Mott *et al*, 2000] RECK a GPI anchored glycoprotein that downregulates MMP-9 and MMP-2 has been found to suppress

angiogenic sprouting leading to tumour cell death The NC1 domain of type IV collagen has also been found to have anti-MMP activity, however their ability to inhibit MMP-2 is substantially lower than TIMPs and their physiological relevance has yet to be fully established [Netzer *et al*, 1998]

1 5 8 2 Catabolism and Clearance

Proteolytic cleavage of MMPs plays an important role in their regulation Proteolytic cleavage by enzymes such as by plasmin can lead to MMP activation however, others proteolytic cleavages may lead to the production of inactive or truncated enzymes, such as removal of the hemopexin domain, resulting in MMPs with limited activity In addition these truncated enzymes may have reduced affinities for their specific inhibitors as seen with c-terminally truncated MMP-2 [Itoh *et al*, 1998] If membrane-bound MMPs become truncated this may reduce their ability to localize to the cell membrane

Binding to, and subsequent clearance of, MMPs by other proteins is another means of MMP regulation Thrombospondin 1 has been the most extensively studied TSP protein to date TSP1 contains a numerous sites of action that have been implicated in interactions with cell surface and matrix proteins, including structural proteins (e *g* collagen and fibronectin), cell surface receptors (*e g* integrins,syndecans, and CD36), enzymes (*e g* elastase and plasmin),and cytokines (*e g* transforming growth factor-β1) The ability of TSP1 to inhibit MMP3-dependent activation of pro-MMP9 and thrombin-induced activation of pro-MMP2 suggests that the TSPs may inhibit MMP activity by preventing activation of the MMP2 and MMP9 zymogens [Bein *et al*, 2000] Similarly, TSP-2 has been found to regulate clearance of MMPs TSP-2 knockout mice produce elevated levels of MMP-2, most likely suggesting a role for TSP-2 in removal of MMP-2 TSP-2 is normally endocytosed by the low-density lipoprotein receptor-related protein (LRP) carrying any bound MMP-2 with it [Yang *et al*, 2001] Thus, MMPs are tightly regulated by several well-characterized mechanisms

1 5 9 MMPs in physiological processes in the vasculature

MMPs are produced by all cell types of the cardiovascular system including endothelial cells (EC), smooth muscle cells (SMC), fibroblasts and cardic myocytes Consequently, they play pivitol roles in a number of vascular processes MMPs exhibit their influence on cells in a number of ways including alteration of ECM cell interactions, the release, activation or deactivation of signalling molecules, activation of receptors and the production of bioactive molecules from the ECM

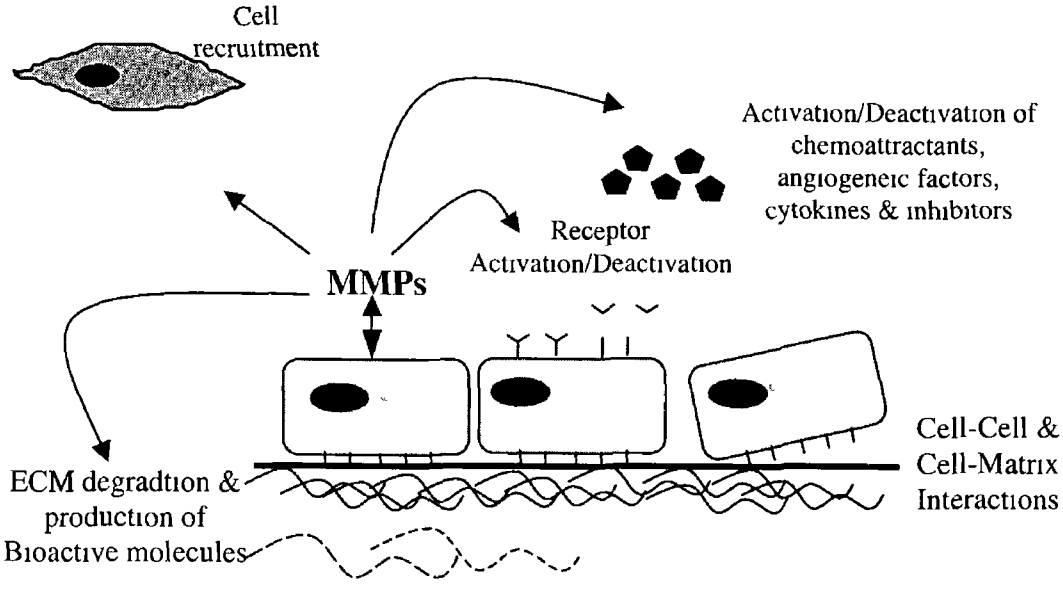


Figure 1 16 Roles of MMP activity

1 5 9 1 MMPs in Angiogenesis

Angiogenesis is the process by which new blood vessels develop from the existing vascular bed It plays a fundamental role in the growth, survival and function of normal and pathological tissues Angiogenesis occurs naturally in the body for wound healing events and for restoring blood flow to tissues after injury It is also an important part of the reproductive cycle and during pregnancy, building of the placenta and circulation betwen mother and fetus Angiogenesis occurs by a series of sequential

events in response to a suitable stimuli and is controlled by a balance of angiogenic growth factors and angiogenic inhibitors When angiogenic growth factors are produced in excess of inhibitors angiogeneis is promoted, the normal helathy body tends to maintain a perfect balance of angiogeneic modulators and as such angiogenesis is turned "off" [Pepper, 2001, Nguyen *et al*, 2001]

Angiogenic Growth Factors	Angiogenic Inhibitors
Angiogenin	Angioarrestin
Angiopoietin-1	Angiostatin
bFGF	Endostatin
IL-8	Fibronectin Fragment
Leptin	Gro-β
PDGF-BB	Interleukin-12
TGF α/β	Interferon α/β/γ
TNF-α	TIMPs
VEGF	PAI-1

Table 1 3 Typical pro and anti-angiogenic factors

Loss of control over angiogenesis can result in a number of serious disease states Angiogenesis-dependent diseases occur as a result of either excessive or insufficent angiogenesis Excessive angiogenesis occurs when diseased cells produce abnormal amounts of angiogenic growth factors This results in the formation of new blood vessels which feed diseased tissues and destroy normal tissue Excessive angiogenesis occurs in over 70 conditions including cancer, diabetic blindness and rheumatoid arthritis Insufficent angiogenesis occurs when tissues cannot produce adequete amounts of angiogeneic growth factors resulting in inadequate blood vessel growth and insufficent circulation This is observed in diseased states such as coronary artery disease and stroke [Pepper, 1997]

The process of angiogenesis occurs as an orderly series of events

i Release of angiogenic growth factors diffuse from nearby tissue and bind to receptors on EC of nearby blood vessels Binding of growth factors cause EC activation and they begin to produce molecules required for angiogenesis

ii Enzymes including MMPs have dissolve tiny holes in the basement membrane and EC begin to proliferate and migrate through the dissolved holes towards the source of the angiogenic stimulus

iii Adhesion molecules and integrins help the sprouting blood vessel to move forward MMPs dissolve the tissue in front of the sprouting vessel tip, the tissue is then remolded around the vessel It has been suggested that MMP-9 is secreted in short bursts from storage vesicles to locally degrade basment membrane This is tightly regulated as uncontrolled proteolysis prevents the necessary EC/ECM interactions for capillary formation Sprouting EC roll up to form a blood vessel tube

iv Type I collagen the predominant constituent of the stroma causes upregulation of MT1-MMP and activation of MMP-2, which continues until the newly formed capillary secretes its basement membrane thus ending the contact between the type I collagen and the EC layer It seems that type I collagen facilitates its own destruction by activating MMP-2 and stimulating EC migration and tube formation [Nguyen *et al,* 2001] Finally newly formed blood vessels are stabilized by SMC that provide structural support Blood flow may then begin

MMPs are vital to the angiogenic response, support for this has been firmly established MMP inhibitors such as marimastat, TIMP-3, BB-94, and 1,10-phenanthroline have all been shown to block endothelial tube formation in chick embryo chorioallantoic membrane (CAM) and collagen-based assays [Schnaper *et al*, 1999, Haas *et al*, 1998] However virtually all of these inhibitors lack specificity for a single MMP resulting in some confusion as to the importance of individual MMPs Furthermore, MMPs have also been implicated in the production of anti-angiogenic protein fragments including endostatin and angiostatin MMP-12, MMP-9 and MMP-7

are all capable of converting plasminogen into the angiogenic inhibitor, angiostatin [Chakraborti *et al* 2003]

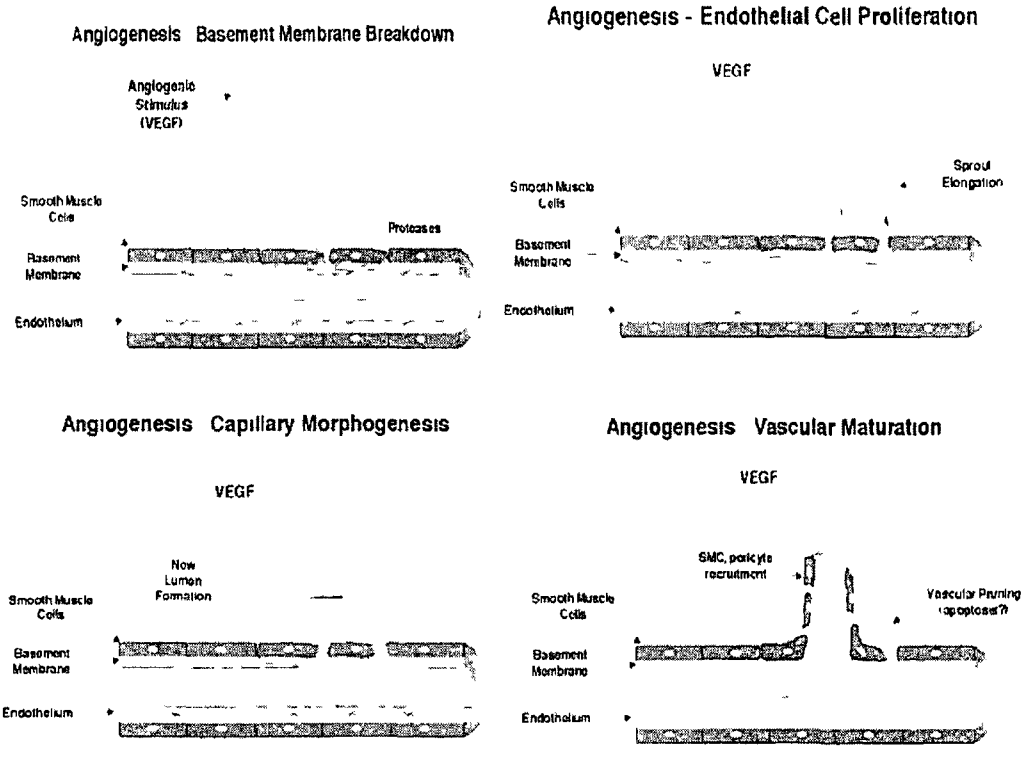


Figure 1 17 Angiogenesis (available from http //cpmcnet columbia edu)

The significance of gelatinase activity in angiogenesis has been demonstrated in a rat Swarm chondrosarcoma model In this model increased angiogenic phenotype was associated with increases in MMP-2 activity [Fang *et al*, 2000] Moreover, specific antisense MMP-2 oligonucleotides have been shown to inhibit tumour angiogenesis Development of inhibitors with increasing specificity has lead to the production of cyclic peptides (His-Trp-Gly-Phe) which are potent inhibitors of MMP-2 and MMP-9, capable of inhibiting EC migration and invasion in a tumour model [Pepper *et al*, 2001] Additionally genetic studies in mice have shown that MMP-2 and MMP-9 knockout mice both have a distinct angiogenic phenotype MMP-2 deficient mice had a marked reduction in tumour-induced angiogenesis and in MMP-9 deficient mice there

was a significant reduction in bone growth plate angiogenesis [Itoh *et al.,* 1998]. Exogenous MMP-9 but not MMP-2 has been found to cause release of VEGF from a carcinoma cell eliciting an angiogenic response with co-cultured endothelial cells. MMP-2 may also affect angiogenesis by cleaving cell surface receptors such as FGF receptor, thus generating a circulating binding protein which can regulate FGF availability and thus its angiogenic potency [Sternlicht *et al.,* 2001].

Angiogenesis is an adaptive process, one of the principal factors which contributes to the induction of angiogenesis is the hemodynamic environment e.g. high shear stress can increase the size and number of collaterals formed in an AV shunt model [Brown *et al.,* 2003] Evidence suggests that angiogenesis associated with exercise may be due to increases in capillary shear stress [Chen *et al.,* 2001]. Cullen *et al* (2002) demonstrated that shear stress induced EC migration and capillary tube formation was dependent on the magnitude of the flow. Shear stress plays an important role in modulating EC morphology, cytoskeleton and ECM remodeling, all of which are important in angiogenesis. DNA microarray analysis of EC subjected to shear stress has found that shear stress modulates a number of genes involved in EC remodeling including MMP-1, cytoskeletal proteins and integrin subunits [Chen *et al.,* 2001]. The involvement of shear stress in angiogenesis may also occur by initiating the release of pro-migratory and pro-angiogenic factors such as VEGF or FGF. Evidence that cyclic strain is a stimulus for angiogenesis comes from both *in vitro* and *in vivo* studies [Rivilis *et al.,* 2002; Zheng *et al.,* 1999; Vailhe *et al.,* 1996; Banai *et al.,* 1994]. Conditioned media from cyclically strained myocytes *in vitro* has been found to stimulate tube formation in coronary endothelial cells, a feature of ECs demonstrating an angiogenic profile. *In vivo* studies have demonstrated that short term exercise, leading to increases levels of mechanical load on the vessel wall resulted in increased angiogenesis in skeletal muscle of Sprague-Dawley rats. Angiogenesis associated with cyclic strain is believed to involve a number of contributors such as MMP-2, VEGF, FGF, and TGF-β and may be associated with a number of conditions in which blood flow is affected including ischemia and bradycardia [Bani *et al.,* 1994; Ausprunk *et al.,* 1977; Pepper *et al.,* 1997; Egginton *et al.,* 2001].

Both shear stress and cyclic strain may stimulate angiogenic growth in vivo and in vitro, however, the process of capillary growth may differ significantly depending on which force acts as the predominant stimulus Capillary growth is described conventionally to begin with endothelial cell activation, which initiates proteolysis of the basement membrane, enabling abluminal sprout formation, cell proliferation, and subsequent growth of a new capillary [Pepper *et al*, 1997, Egginton *et al*, 2001] This process is the predominant mechanism of capillary growth in muscles activated by chronic electrical stimulation or in stretched muscle, with the exception that endothelial cell proliferation precedes proteolysis and sprout formation [Haas *et al*, 2000, Zhou *et al*, 1998]

1 5 9 2 MMPs in vascular remodelling

Vascular remodelling can be described as any enduring change in the size or composition of an adult blood vessel Remodelling of the blood vessel may occur to accomodate and adapt to changes in hemodynamic forces or as a response to inflammation or injury Inappropriate remodelling of the blood vessel is currently thought to be a major contributing factor to a number of pathological scenarios such as those seen in atherosclerosis and restenosis [Gibbons *et al*, 1994] In order to facilitate both physiological and pathological remodelling there must be degradation and reorganization of the ECM, thus MMPs are believed to be a crucial part in these processes In addition to degrading the ECM to facilitate migration/proliferation, MMPs may also contribute to remodelling by the production of vasoactive compounds, MMP-2 has been found to produce a potent vasoconstricor by cleavage of calcitonin gene-related peptide [Fernandez-Patron *et al*, 2000] *In vitro* studies with cultured cells or histological observations of both normal and diseased vessels indicate the presence of MMPs in both vascular and inflammatory cells Human diseased arteries were found to have increased expression of a number of MMPs in conjunction with significant morphological changes compared to normal arteries This may suggest pathological remodelling of the artery involves MMPs

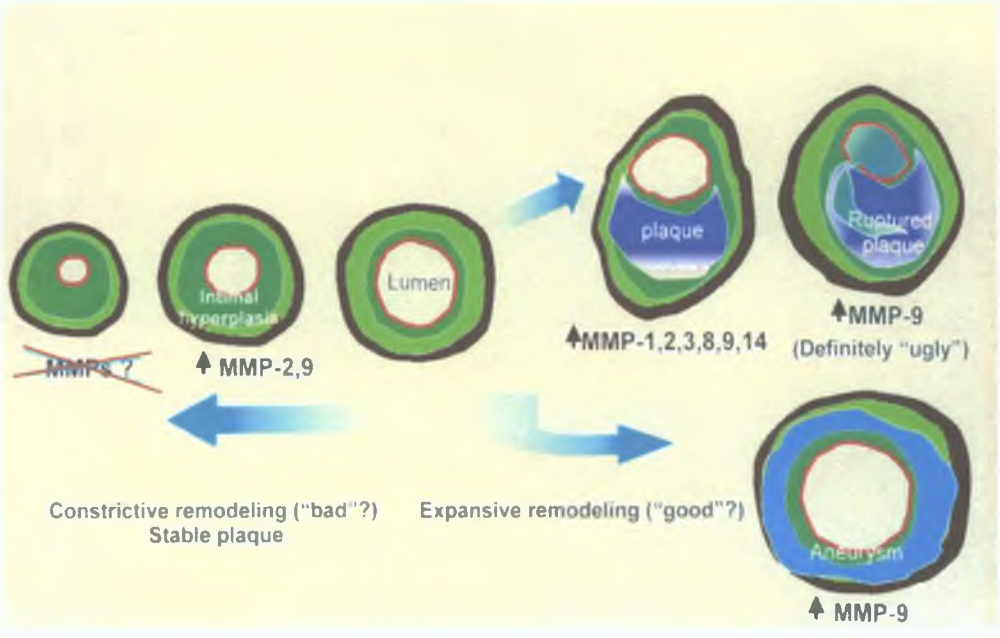


Figure 1.18. MMPs and in constrictive and expansive arterial remodeling (Galis *et al.*, 2002)

Vascular remodelling may occur in response to a number of stimuli such as haemodynamics, injury, inflammation and oxidative stress [Lijnen *et al.*, 2001; Galis *et al.*, 1994; Rajagopalan *et al.*, 1996]. These stimuli have also been found to regulate MMP expression and activity. It has been suggested that hemodynamic forces resulting from blood flow may be the most important regulator of MMP expression and activity in vascular cells. Inhibition of MMP activity inhibits the expansive remodelling at the site of rat ateriovenous fistulae [Abbruzzese *et al.*, 1998], whereas upregulation of MMP-9 associated with cessation of flow resulted in expansive remodelling [Godin *et al.*, 2000]. Increases in transmural pressure (pressure across the wall of a blood vessel) induced the activity of MMP-2 and MMP-9 suggesting that MMPs may be involved in remodelling associated with hypertension [Chesler *et al.*, 1999]. Targeted disruption of the MMP-9 gene leads to impaired SMC migration and arterial remodelling [Galis *et al.*, 2002]. Upregulation of MMP-2 and MMP-9 caused by variation in the hemodynamic environment is thought to be the causitive factor in saphenous vein graft failure.

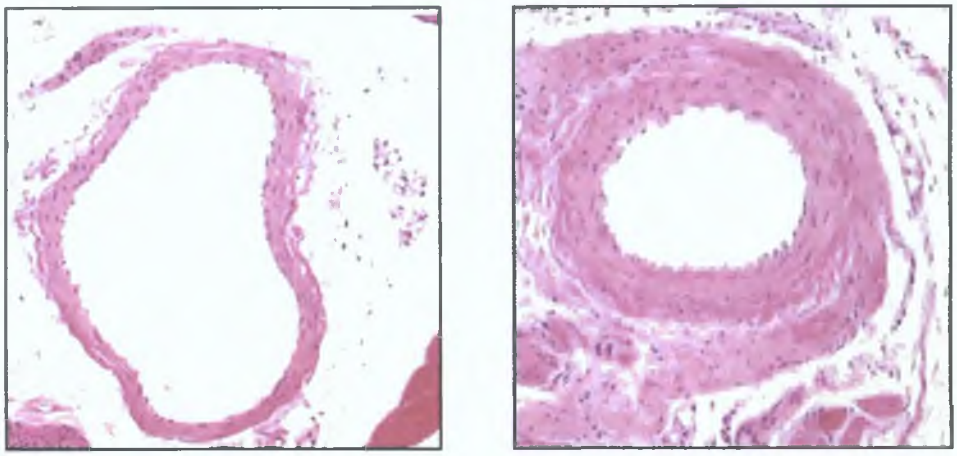


Figure 1.19: Flow induced vascular remodeling in mouse carotid arteries (http://www.u.arizona.edu)

The oxidative state of the blood vessel is a major contributor to vascular remodelling and in the regulation of MMPs. Reactive oxygen species (ROS) have been found to be sensitive to changes in the hemodynamic environment. ROS have been indicated in activation of MMPs, mechanical stretch was found to increase expression of MMP-2 via a mechanism involveing reactive oxygen species derived from NAD(P)H oxidase [Grote *et al.,* 2003]. Oxidative stress can drive vascular remodeling and is believed to be involved in expansive remodeling associated with myocardial ischemia. Nitric oxide plays an important and complex role in vascular remodeling. eNOS null mice did not exhibit compensatory remodeling while overexpression of eNOS reduces SMC migration associated with vascular remodeling with concurrent reductions in MMP-2 and MMP-9. Under diseased conditions, interaction of NO with ROS such as superoxide may result in the formation of peroxynitrite found to activate latent MMPs resulting in remodelling [Rajagopalan *et al.,* 1996].

MMP expression and activation is tightly controlled in vascular events. However, certain triggers, such as changes in the hemodynamic environment or levels of ROS such as those seen in certain diseased states, may tip the balance of control in favour of MMP activation and lead to excessive and pathological remodelling.

1.5.9.3 MMPs in Atherosclerosis

Atherosclerosis is a progressive disease characterised by the accumuation of lipids and fibrous elements in large arteries. Early lesions consist of subendothelial accumulations of cholesterol ladened macrophages called "foam cells". These fatty steaks develop over time into plaques comprising of a lipd rich necrotic core and typically have a fibrous cap consisting of SMCs and ECM. Plaques become increasingly complex and can grow sufficently large enough to occlude blood flow. One of the more common clinical complications is occulsion due to formation of a thrombus caused by rupture of a plaque [Lusis *et al.*, 2000].

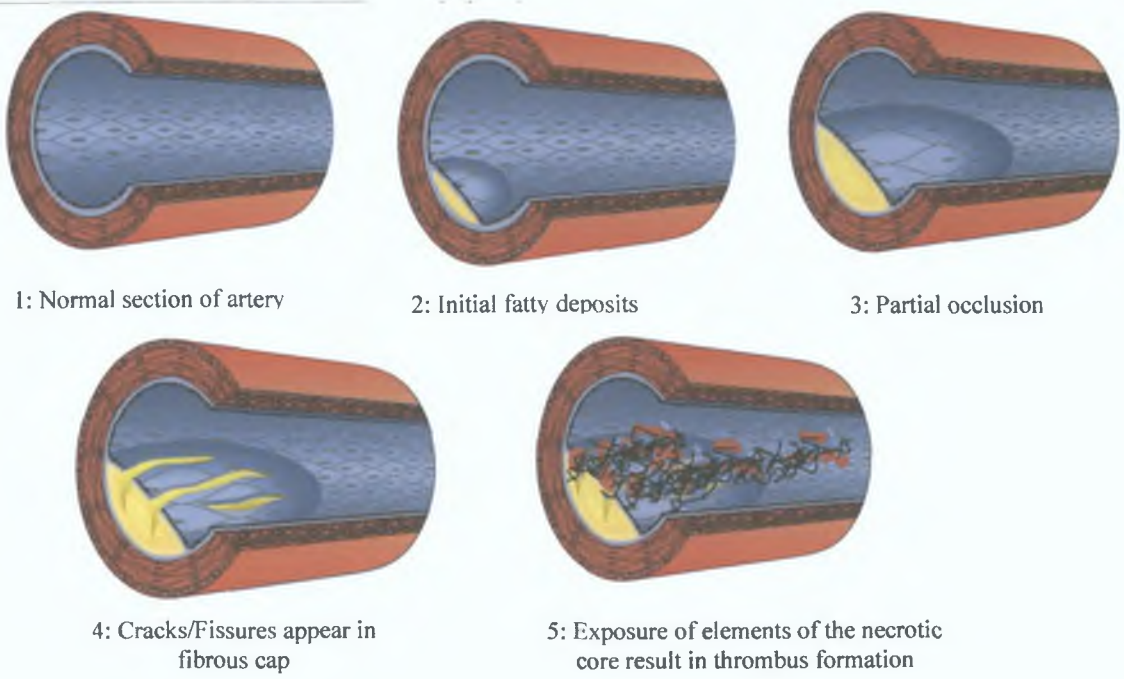


Figure 1.20: Progression of atherosclerosis from fatty streak formation to thrombus and artery occlusion (http://www.nlm.nih.gov).

Atherosclerotic lesions do not occur at random locations in the vasculature. Hemodynamic forces interacting with an an active vascular endothelium are responsible for localizing lesions in a non-random pattern of distrubtion. Shear stress and cyclic strain are the predominant forces which have been characterized in the vasculature.

Tubular regions of the artery are exposed to blood flow which is laminar, cells in these regions are ellipsoid and grow in the direction of flow. Laminar shear stress has been associated with having a protective effect on the endothelium preventing the formation of fatty streaks and progression of atherosclerosis. There are five major regions of arterial plaque formation:

i. The cornary arteries
ii. The major branches of the aortic arch
iii. The major branches of the abdominal aorta
iv. Visceral extremity branches of the abdominal aorta
v. Lower extremity branches of the abdominal aorta

Atherosclerotic plaque formation predominatly occurs in these locations due to changes in the hemodynamic environment brought about by arterial curvature, extensive branching (bifurcations) and in the case of cornary arteries, mechanical torsions during the normal cardiac cycle. It has been found that high shear stress is inversely proportional to the formation of lesions i.e lesions localize in areas where shear stress is low such as at branch points and high shear stress protects against atherogenesis. Due to the low levels of shear stress, the endothelium may be exposed to atherogenic lipds, monocytes and platelets for an increased period of time [Dzau *et al.*, 2002].

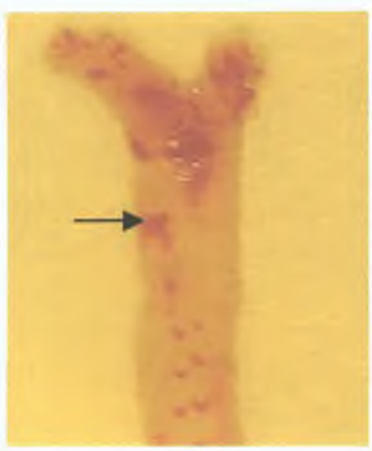


Figure 1.21: Non-random localization of early atherosclerotic lesions in a Watanabe heritable hyperlipidemic (WHHL) rabbit. The aorta has been stained with Oil-Red-O, which shows lipid-rich atherosclerotic lesions in the arterial wall. Despite the systemic nature of the hyperlipidemia, the lesions in this animal are largely confined to areas around curvatures and branch points, suggesting that patterns of blood flow are important in localizing this disease. The upper arrow indicates the arch of the thoracic aorta, which has been opened up to display the intimal lining en face. The lower arrow indicates the localized nature of the atherosclerotic lesions adjacent to the paired ostia of the intercostal arteries originating from the descending thoracic aorta.

This increased exposure may be a causitive factor in atherogenesis at these sites Similarly oscillatory shear stress and the formation of eddy currents are believed to be a contributory factors in the progression of atherosclerotic lesions Changes in the curvature and branching within the vasculature also effects the degree of cyclic strain within the vessel wall which in turn may regulate a number of pro-atherogenic molecules including ICAM-1, ROS and MCP-1 A greater understanding of these forces might provide better insight into the progression of athersclerosis

MMP activity has been identified within athersclerotic plaques and may be modulated by pro-atherogenic molecules such as Oxidised LDL (oxLDL) OxLDL which accumulates in atherosclerotic plaques has been found to regulate the expression of MMPs, including MMP-1 in endothelial cells and MMP-9 in macrophages [Xu *et al*, 1999] Within the plaque both MT1-MMP and MT3-MMP expression have been found to be increased, in addition to the expression of MMP-1, MMP-2, MMP-8 and MMP-9 derived from foam cells, EC and SMC [Rajavashisth *et al*, 1999, Uzui *et al*, 2002] Atherosclerosis is often viewed as a vascular injury and initiates an inflammatory response Progression of atherosclerosis involves the recruitment of monocytes and lymphocytes to the lumen wall Both oxLDL and the hemodynamic environment can modulate this inflammatory response These stimuli can cause changes in adhesion molecule expression such as VCAM-1 and PCAM-1 in addition to MCP-1 which, may mediate recruitment of these inflammatory cells [Lusis *et al*, 2000] The mechanisms by which these inflammatory cells infiltrate the endothelial layer are thought to be largely facilitated by MMPs *In vitro* interactions between T-cells and endothelial cells was shown to increase secretion of MMP-2 Cyclic stretch mediated increases in MMP-2 was shown to be regulated by TNF-α a potent inflammatory cytokine [Wang *et al*, 2003] MMP degration of the EC basement membrane could contribute to decreased endothelial barrier function, such as that seen at arterial bifurcations, permitting increased influx of lipoproteins, plasma proteins and foam cells

1 5 9 4 MMPs in Plaque rupture

Rupture of the fibrous cap overlying an atherosclerotic plaque accounts for 60-80% of all thrombotic events The demise of the plaque stability occurs through structural breakdown of the arterial wall leading to rupture of the fibrous cap and exposure of the prothrombotic core The identification of strong MMP expression within plaques and their ability to degrade ECM proteins has implicated their role in plaque rupture Plaque rupture involves two processes, firstly, a reduction in collagen synthesis and secondly, an increase in MMP expression and activity The identity of the MMPs involved in weakening of the plaque remains elusive due to the large number of MMPs and their multiple substrate specificities

Macrophage-derived foam cells resident within plaques are known as a major source of MMP-1, MMP-2, MMP-3 and MMP-7 [Galis *et al*, 1995, Galis *et al*, 1998] Increased blood level of MMP-2 and MMP-9 have been associated with acute cornary syndrome and MMP-9 has been found in lesions of patients with unstable angina The main structural components of the fibrous cap are interstitial collagen I and III conferring tensile strength Therefore, traditionally interstitial collagenases, MMP-1 and MMP-13, were thought to be the principal contributors to plaque rupture However, MMP-2 and MMP-9, derived mainly from inflammatory cells has been shown to degrade fibrillar collagen and fragmented interstitial collagen and therefore may be more important in destabilization of plaques Overexpression of these enzymes has been reported within vulnerable sites of human atheromas [Falk *et al*, 1995] In stark contrast to the widely accepted notion that MMPs are responsible for plaque rupture by destabilizing the fibrous cap, overexpression of MMP-1 in ApoE-null mice did not induce plaque rupure and led to a reduction of athersclerosis [Hofmann *et al*, 2001] Other studies have shown that MMP inhibition in arterial injury models resulted in a marked reduction in collagen deposition and SMC migration A complete mechanistic understanding of the plaque rupture remains somewhat incomplete, although the evidence of a central role for MMPs in this process is mounting

1 5 9 5 MMPs in aortic aneurysm

Arterial aneurysm (AA) is a common and lethal disorder which is manifested as arterial dilation, wall thickening and a dramatic reduction in elastin/collagen ratio and as such can be considered a dynamic remodelling process These changes are accompanied by excessive production of matrix metalloprotemases Aortic aneurysm is an extreme form of outward remodelling, the trigger for which is believed to be the physiological tendency of blood vessels to optimize shear stress and wall tension Medial SMC isolated from AA tissue were found to produce significantly higher levels of MMP-2 and MMP-9 MMP-2 and MMP-9 null mice have been shown to be protected from aortic aneurysm confirming an important role for MMPs in the progression of AA Freestone *et al* (1996) have demonstrated that MMP-2 is the principal MMP activity in small aneurysms Similar studies have revealed an increase in both expression and activity of MMP-2 in aortic aneurysms with concurrent increases in MT1-MMP and TIMP-2 [Goodall *et al*, 2001] A number of animal models exist which emulate this condition and involve increased expression of MMP-2 and MMP-9, local matrix destruction and local inflammation As previously mentioned, MMP-2 and MMP-9 null mice have been shown to be resistant to aneurysm formation This occurence could be reversed in MMP-9 but not MMP-2 knockouts by infusion with wildtype macrophages This suggests that MMP-2 and MMP-9 may be derived from different sources but work in concert to produce aortic aneursyms [Longo *et al*, 2002]

1 5 10 Therapeutic possiblities in MMP inhibition

MMPs have been implicated in a number of physiological conditions such as apoptosis, reproduction and angiogenesis in addition to pathological conditions which include rheumatoid arthritis, restenosis, plaque instability, unstable angina, myocardial infarction, stroke, atherosclerosis and cancer Given their widespread expression through differing cell types and involvement in a number of vascular cell fate decisions targeted inhibition of these enzymes to arrest the progression of a number of diseases is of great interest

One of the earliest broad spectrum synthetic MMP inhibitors, SC-44463 was found to inhibit metastasis in mice Batimatstat [BB-94] (British Biotech Inc Oxford, UK) a broad spectrum hydroxamic-acid derivative became the first MMP inhibitor to be tested on humans This inhibitor was found to have poor solubility and had to administered intrapleurally, because of this batimastat was replaced by orally administered marimastat, which has been shown to be effective against colon, pancreatic and prostate cancer Long-term usage of these drugs have been found to result in inflammation and pain in joints and tendons, these drugs are broad spectrum and as such may have other long term side effects which have yet to be elucidated Thus, the development of more specific MMP inhibitors in conjunction with improved ability to target drugs is an area of growing interest

As previously discussed, MMPs play a major role in vascular function and disease As such, a number of studies have attempted MMP inhibition as a means of counteracting a number of pathological conditions such as atherosclerosis, left ventricule remodelling following myocardial infarction, and constricive remodelling following ballon angioplasty Despite its long term detrimental effects, BB-94 was found to attenuate experimental thrombolysis induced haemorrage and significantly reduced late lumen loss after balloon angioplasty by inhibition of constrictive arterial remodeling, whereas neointima formation was not inhibited by MMP inhibition [Bart *et al*, 2000] Fluvastatin, a 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor decreases MMP-1 expression in human vascular ECs through inhibition of Rho [Ikeda *et al* 2000] MMP inhibition with fluvastatin has been used to some effect in attenuating left ventricular remodeling and failure after experimental myocardial infarction in mouse models Thus, long-term treatment with fluvastatin might be beneficial also in patients with heart failure and might improve their long-term survival [Hayashidani *et al*, 2002] MMP inhibition by PD 166793 or CP-471474 (Pfizer Inc, MW 368) was also found to be beneficial in attenuating left ventricle hypertrophy [Chanency *et al*, 2002, Lindsey *et al*, 2002] Statins, such as cerivastatin and lovastatin inhibit secretion of several MMPs from both SMCs and macrophages, which may contribute to their plaque-stabilizing effects [Hu, *et al*, 2001] Doxycycline

penetrated atherosclerotic plaques with acceptable tissue levels This resulted in a reduction in MMP-1 concentration because of decreased expression [Axisa *et al*, 2002]

TIMPs were initially considered as potential therapeutics in situations of exaggerated MMP activity, however their large size and short half life rendered them unsuitable Advances in the adenoviral vector-mediated gene delivery has led to an increased interest in TIMP gene transfer as a means of MMP inhibition Local TIMP-2 gene transfer significantly reduces vein graft diameter, i e remodeling of an artery-like vessel via MMP inhibition [Hu *et al*, 2001] Adenovirus-mediated human TIMP-2 gene transfer inhibits SMC invasiveness *in vitro* and *in vivo* and delays neointimal development after carotid injury [Cheng *et al*, 1998] Overexpression of TIMP-3 in human saphenous veins prior to grafting into cartoid arteries was found to reduce neointima formation by 84% TIMP-2 however was not found to effect neointima formation in this model, thus highlighting the possible therapeutic potential for the prevention of late vein graft failure [George *et al* 2000] Atherosclerotic lesions in atherosclerosis-susceptible hypercholesterolemia apoE2/2 mice were significantly reduced by adenovirus mediated elevation in the circulating levels of TIMP-1 Histological and immunohistologic analyses of these atherosclerotic lesions revealed increases in collagen, elastin, and smooth muscle α-actin associated with reduced MMP activity TIMP-1 infiltration from plasma to arterial intima, specifically in lesions, draws attention to possible use of this technology in the stabilization of atherosclerotic plaques [Rouis *et al*, 1999] Gene transfer may be used directly or indirectly as a means of combating excessive MMP activity Targeted disruption of the MMP-9 gene was found to impair smooth muscle cell migration and geometrical remodeling of mouse carotid arteries MMP-9 deficiency was also found to decrease intimal hyperplasia and reduced late lumen loss [Galis *et al*, 2002] Endothelial nitric oxide synthase (eNOS) gene transfer decreased MMP-2 and MMP-9 activities with concurrent increases in TIMP-2 secretion This shift in MMP activity favors inhibition of cell migration by reducing the degradation of the ECM [Gurjar *et al*, 1999] Other indirect methods of inhibiting MMP activities include the use of tyrosine kinase receptor inhibitors Non-specific tyrosine kinase inhibition was found to have unwanted side

effects, chemical modification of these compounds have led to the production of a number of new drugs Herceptin (Genentech, USA) has been found to have no side effects in clinical trials It is believed that these compounds target the AP-1 complex thus inhibiting transcription of a number of MMP genes

1 6 Summary

The enzymatic properties of the MMP family are becoming more clearly understood however the complex regulation of these enzymes and their seemingly multiplicitous roles in the vasculature remains poorly understood To date, MMP activities have been identified in a number of scenarios involving vascular remodelling including angiogenesis, atherosclerosis and aortic aneurysm Several reports have indicated that MMP-2 and MMP-9 are involved in a number of vascular fate decisions and in particular in angiogenesis and migration Hemodynamic forces generated by blood flow play an important role in regulating vascular cell fate decisions and are believed to be one of the predominant regulators of MMP expression Precise information on the mechanisms by which hemodynamic forces regulate the expression and activities of these enzymes may aid in the development of therapeutic strategies against the progression of cardiovascular disease

1 7 Thesis Overview

The research presented in the following chapters examines the regulation of MMP-2 expression and activity in bovine aortic endothelial cells in response to mechanical forces It also identifies the roles of endothelial derived MMPs in regulating EC tube formation and the migration of both EC and SMC The findings of this research has been divided into three main chapters

- Chapter 3

  Examination into the ability of mechanical forces to regulate bovine aortic endothelial cells phenotype and the effects of these forces on MMP-2 and MMP-9 expression and activity

- Chapter 4

A through investigation into the signalling pathways involved in the cyclic strain-induced increases in MMP-2 expression and activity

- Chapter 5

Examination of the mechano-receptors involved in mediating cyclic strain-induced changes in angiogenesis and the involvement of MMP-2 and MMP-9 in these events The identifiaction of a role for endothelial cell derived MMP-2 in regulating smooth muscle migration

Chapter 2

2 0 Material & Methods

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

2 1 Materials

AGB Scientific (Dublin, Ireland)
Whatmann Chromatography paper

Amersham Pharmacia Biotech (Buckinghamshire, UK)
Anti-mouse 2^{o} antibody, HRP conjugated
Anti-rabbit 2^{o} antibody, HRP conjugated
ECL Hybond nitrocellulose membrane
ECL Hyperfilm
Rainbow molecular weight marker, broad range (6-175kDa)

Bachem UK Ltd (St Helens, UK)
Linear RGD peptide
Cyclic RGD peptide

Bio Sciences Ltd (Dun Laoghaire, Ireland)
DMEM
dNTP's
DEPC-treated water
Trizol® reagent

Calbiochem (San Diego, CA)
PD98059
PD169316
Genistein

Pertussis toxin

NF023

Anti-ppERK antibody

Anti-ppP38 antibody

Cayman Chemical Company (Michigan, USA)

eNOS polyclonal antibody

Chemicon (Temecula, CA)

Anti-MMP-2 antibody

Recombinant MMP-2/MMP-9 standard

Cornell Cell Repository (NJ, USA)

Bovine Aortic Endothelial Cells (BAECs)

Bovine aortic smooth muscle cells (BASMC)

Dunn Labortechnik GmBH (Asbach, Germany)

6-well Bioflex® plates

Flexcell International Corp (Hillsborough, NC)

Flexercell® Tension Plus™ FX-4000T™ system

Guthrie cDNA Resource Centre (Sayre, PA)

Gia1-G202T

Gia2-G203T

Gia3-G202T

Invitrogen (Groningen, The Netherlands)

Lipofectamine reagent

Lipofectamine 2000 reagent

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

Kodak 1D image analysis software

MWG Biotech (Milton Keynes, UK)

MMP-2 primer set

MMP-9 primer set

MT1-MMP primer set

β-actin primer set

GAPDH primer set

MMP-2 siRNA duplex

MMP-9 siRNA duplex

Control sequence X

Neuroprobe, Gaithersburg (MD USA)

AA10 transwell chamber

8μm and 12μm filters

PALL Corporation (Dun Laoghaire, Ireland)

Biotrace nitrocellulose membrane

Pierce Chemicals (Cheshire, UK)

BCA protein assay kit

Supersignal West Pico chemilumescent substrate

Plamids

Shc-SH2 was the generous gift of Professor John Shyy (University of California, Riverside)

GFP and β-ARK-ct were the generous gifts of Dr John Cullen (University of Rochester Medical Centre, Rochester NY)

Promega (Madison, WI)

Taq DNA Polymerase

MLV-RT

RNase H

Oligo dT

Sarstedt (Drinagh, Wexford, Ireland)

T25 tissue culture flasks

T75 tissue culture flasks

T175 tissue culture flasks

6-well tissue culture plates

5,10 and 25ml serological pipettes

15 and 50ml falcone tubes

Sigma Chemical Company (Poole, Dorset, England)

β-glycerophosphate

2-mercaptoethanol

Acetic Acid

Acetone

Agarose

Ammonium Persulphate

Bisacrylamide

Bovine Serum Albumin

Brightline Haemocytometer

Bromophenol blue

Chloroform

EDTA

EGTA

Ethidium Bromide

Fibronectin

Foetal Calf Serum

Methanol

Recombinant MMP-2

Penicillin-Streptomycin (100x)

Ponceau S

Potassium Chloride

Potassium Iodide

Potassium Phosphate (Dibasic)

Rat tail type I collagen

RPMI-1640

Sodium Chloride

Sodium Hydroxide

Sodium Orthovanadate

Sodium Phosphate

Sodium Pyrophosphate

SDS

TEMED

Bovine Gelatin

Glycerol

Glycine

Hanks Balanced Salt Solution

Hydrochloric acid

Isopropanol

Leupeptin

Tris Acetate

Tris Base

Tris Cl

Triton X-100

Trypsin-EDTA solution (10x)

Tween 20

2 2 Cell Culture Methods

All cell culture techniques were carried out in a clean and sterile environment using a Bio air 2000 MAC laminar flow cabinet Cells were visualized using an Olympus CK30 phase contrast microscope

2 2 1 Culture of bovine aortic endothelial cells (BAECs)

Differentiated BAEC were obtained from Coriell Cell Repository, New Jersey, USA (CAT NO AG08500) The cells are derived from a one-year-old male Hereford cow The thoracic aorta was removed immediately post-mortem on 10/22/85 Cells were maintained in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 100U/ml penicillin and 100μg/ml Streptomycin Cells were cultured in T175cm^2, T75cm^2, T25cm^2 and 6 well plates In the case of cyclic strain experiments cells were grown on Bioflex® series culture plates which have a flexible, pronectin-bonded growth surface Cells between passage 8 – 17 were used in these experiments

BAECs are a strongly adherent cell line As such trypsinisation was necessary for sub-culturing or harvesting of cells For trypsinisation, growth media was removed from the flask and the cells were gently washed three times in Hanks buffered saline solution (HBSS) to remove α-macroglobulin, a trypsin inhibitor present in FBS A suitable volume of trypsin/ethylenediamine tetracetic acid (EDTA) (10% v/v trpsin EDTA in HBSS) was added to the flask and incubated for 5-10 minutes or until all the cells were removed from the flask surface Trypsin was inactivated by the addition of growth medium and the cells were removed from suspension by centrifugation at 2500g for 5 minutes Cells were then resuspended in culture medium and typically diluted 1 5 into culture flasks, or cryogenicly preserved Cells were incubated in a humidified atmosphere 5% v/v CO_2 at 37°C

2 2 2 Culture of bovine aortic smooth muscle cells (BASMC)

Differentiated BASMC were obtained from Coriell Cell Repository, New Jersey, USA (AG08504) The cells were derived from a one-year-old Angus cow The thoracic aorta was removed immediately post-mortem on 10/22/85 Cells were maintained in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 100U/ml penicillin and 100µg/ml Streptomycin BASMCs are a strongly adherent cell line, as such trypsinisation was necessary for sub-culturing or harvesting of cells A 1 5 sub-culture ratio was generally used Cells were incubated in a humidified 5% v/v CO_2 atmosphere at 37°C Cells between passages 10 – 20 were used in these experiments

2 2 3 Cyclic strain and laminar shear stress studies

For cyclic strain studies, BAECs were seeded into 6-well Bioflex® plates (Dunn Labortechnik GmBH - Asbach, Germany) at a density of approximately $6X10^5$ cells/well, allowed to adhere for 24 hours and grown to confluency After 24 hours, the media was removed and replaced with serum-free media and the cells exposed to varying levels of cyclic strain Bioflex™ plates contain a pronectin-coated silicon membrane bottom that enables precise deformation of cultured cells by microprocessor-controlled vacuum When cells had reached approximately 100% confluency, a Flexercell™ Tension Plus™ FX-4000T™ system (Flexcell International Corp - Hillsborough, NC) was employed to apply a physiological level of cyclic strain to each plate (0-10% strain, 60 cycles/min, 0-24 h) providing equibiaxial tension using the 'Heartbeat' ™ Simulation protocol

For laminar shear stress studies, BAECs were seeded onto cell culture grade petri dishes and allowed to adhere for 24 h and grown to confluency After this the media was removed and replaced with fresh culture media before exposure to laminar shear stress The petri dishes were sealed and placed on an orbital shaker (Stuart Scientific Mini Orbital Shaker SO5) A suitable RPM to produce a shear stress of 10dynes/cm^2 was determined from the equation [Hendrickson *et al* 1999]

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

Where α = radius of rotation in cm

ρ = density of liquid in g/l

n = 7 5X10 ^{3} dynes/cm^2 @ 37°C

f = rotation per second

2 2 4 Cryogenic preservation and recovery of cells

For longterm storage of cells BAECs were maintained in liquid nitrogen in a cryofreezer unit Cells to be stored were centrifuged following trypsinisation and the resultant pellet was resuspended in 20% (v/v) FBS containing dimetylsulphoxide (DMSO) at a final concentration of 10% (v/v) 1ml aliquots were transferred to sterile cryovials and frozen in a –80°C freezer at a rate of –1°C/minute using a Nalgene cryo freezing container Following overnight freezing at –80°C, the cryovials were transferred to a cyrofreeze unit (Thermoylen locator jr cryostorage system) Cells were recovered from longterm storage by rapid thawing at 37°C and resuspension in 5ml of growth medium followed by centrifugation at 3500rpm for 5 minutes The resultant cell pellet was resuspended in fresh medium and transferred to a culture flasks The following day the media was removed, the cells were washed in HBSS and fresh culture media added

2 2 5 Cell counts

Cells counts were performed using a Sigma brightline haemocytometer slide Trypan blue exclusion dye was routinely used to determine cell viability 20µl of trypan blue was added to 100µl of cell suspension, the mixture was left to incubate for two minutes 20µl of this mixture was loaded to the counting chamber of the haemocytometer and cells visualized by light microscopy Viable cells excluded the

dye while dead cells stained blue The number of cells was calculated using the following equation

Average Cell No x 1 2 (dilution factor) x 1x10^4 (area under cover slip mm^3) = Viable cells/ml

2 2 6 Treatment with pharmacological inhibitors

Cells were routinely cultured for at least 2 passages prior to treatment with pharmacological inhibitors For these experiments, BAECs were grown until approximately 70-80% confluent after which the growth media was removed and cells rinsed 3 times in HBSS Inhibitors were diluted in RPMI-1640 supplemented with antibiotics For DMSO-soluble inhibitors, a suitable stock concentration was prepared so that the final concentration of DMSO in working solutions was less than 0 5% Cells were exposed to inhibitors for 1 hour prior to exposure to cyclic strain and were present in the media during the course of the mechanical strain experiment Following 24 hours of 5% cyclic strain cells were harvested for total RNA/protein and conditioned media Inhibitors used were pertussis toxin (100ng/ml), NF023 (10μM), PD98069 (10μM), PD169316 (10μM), GM001 , RGD peptide (0 5mM), cyclic RGD (100μM) and gensitein (50μM) Concentrations used were taken from current literature or based on manufacturers recommendations

2 2 7 Preparation of whole cell lysates

Following trypsinisation as described in section 2 1 1, the cell pellet was washed in 1X PBS to remove any trace levels of FBS The cell suspension was then centrifuged at 2500g for 5 minutes The PBS supernatant was removed and the cells were resuspended in 1X lysis buffer (20mM Tris, 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% Triton X-100 (v/v), 2 5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM sodium orthovanadate, 1μg/ml leupeptin) The resulting lystaes were frozen and thawed three times followed by three cycles of ultrasonication

for 5 seconds on ice using a sonic disembrator (Vibra Cell, Sonics and materials Inc) Samples were stored at –20°C for short-term storage or –80°C for long-term storage

2 2 8 Bicinchoninic Acid (BCA) protein microassay

In this assay Cu^{++} reacts with the protein under alkaline conditions to produce Cu^{+}, which in turn reacts with BCA to produce a coloured product [Pierce , 1997] Two separate reagents were supplied in this commercially available assay kit (Pierce Chemicals), **A,** an alkaline bicarbonate solution and **B,** a copper sulphate solution 1 part solution B is mixed with 50 parts solution A, 200µl of this mixture is added to 10µl of protein lysate or BSA standards (standard curve in the range 0-2mg/ml) The plate is incubated at 37°C for 30 minutes and the absorbance read at 560nm using a microtitre plate reader

2 2 9 Lactate dehydrogenase (LDH) Assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme present within all mammalian cells Frequently used as a marker for plasma membrane damage or "leakage" *In vitro* release of LDH from cells subsequently provides an accurate measure of cell membrane integrity and cell viability [Racher *et al* , 1998] This assay was used to assess the effects of mechanical strain on viability of bovine aortic endothelial cell cultures The release of LDH into culture supernatant correlates with the amount of cell death and membrane damage, providing an accurate measure of the cellular damaged induced by the experimental conditions

This assay is based upon the ability of LDH to catalyze the reaction

Pyruvate + NADH + H(+) --> Lactate(+) + NAD(+)

Briefly, 5µl of cell lysate or 50µl of conditioned media was added to 900µl of 0 2M Tris-HCl pH7 3, 100µl of 1 1mM β-NADH and 20µl of 115mM sodium pyruvate The samples were mixed by inversion and the absorbance read at 340nm every 20

seconds for 5 minutes LDH activity can be derived from the rate of changes in optical absorbance (ΔOD/sec)

2 3 RNA prepartion methods

2 3 1 RNA isolation

Trizol is a ready to use reagent for the isolation of total RNA,DNA and/or protein from cells and tissues RNA isolation was developed by Chomczynski and Sacchi [Chomczynski *et al*, 1987] Trizol reagent maintains the integrity of the RNA while disrupting the cells and dissolving the cell components

Cells were lysed directly in culture plates by the addition of 1ml of Trizol per $10cm^2$ A volume less than this can result in contamination of the RNA with DNA To ensure complete homogenization, cells were lysed by passing through a pipette a number of times The samples were then incubated for 5 minutes at room temperature to allow complete dissociation of nucleoprotein complexes 0 2ml of chloroform was added per ml of Trizol reagent used and was then mixed vigorously for 15 seconds before being incubated for 5 minutes at room temperature Samples were then centrifuged at 12,000xg for 15 minutes at 4°C The mixture separated into a lower red, phenol-chloroform phase, an interphase and an upper colourless aqueous phase RNA remains exclusively in the aqueous phase

The aqueous phase was carefully removed and transferred to a fresh, sterile tube The RNA was precipitated out of solution by the addition of 0 5ml of isopropanol per 1ml of Trizol used Samples were incubated for 15 minutes at room temperature and then centrifuged at 12,000xg for 10 minutes 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 1ml of 75% ethanol per ml of Trizol used followed by centrifugation at 7,500xg for 5 minutes at 4°C The resultant pellet was air-dried for 5-10 minutes before being resuspended in DEPC-treated water The sample was then stored at –80°C until used

The concentration of total RNA was determined by UV spectrophotometry as outlined in section 2 1 11

2 3 2 Spectrophotometric analysis of nucleic acids

DNA or RNA concentrations were determined by measuring the absorbance at 260nm, the wavelength at which nucleic acids absorb light maximally (λ max) A 50μg/ml solution of DNA or 40μg/ml solution of RNA has an absorbence reading of 1 0 at this wavelength In order to calculate the concentration of DNA/RNA in samples the following calculations were used,

DNA - Abs $_{@260nm}$ x50x200 (dilution factor, i e 5μl of sample in 995μl H_2O) = μg/ml

RNA - *Abs $_{@260nm}$ x 40 x 200 (dilution factor, i e 5μl of sample in 995μl H_2O) = μg/ml*

The purity of the DNA or RNA samples was established by reading the absorbence at 260nm and the absorbence at 280nm and then determining the ratio between the two (ABS_{260}/ABS_{280}) Pure DNA which has no protein impurities has a ratio of 1 8 whereas pure RNA has a ratio of 2 0 Lower ratios indicate the presence of proteins, higher ratios imply the presence of organic reagents

2 3 3 Reverse transcription polymerase chain reaction (RT-PCR)

PCR has over the last 20 years proved to be an important and powerful tool for amplifying small quantities of DNA for analysis RT-PCR is a modification of this technique in which small quantities of specific messenger RNA (mRNA) are analysed Total RNA is isolated using oligo dT primers, which is subsequently converted to copy DNA (cDNA) using the enzyme reverse transcriptase cDNA of interest was amplified by PCR using gene specific primers The PCR was semi-quantitative, GAPDH (a house-keeping gene) expression was monitored m tandem with the gene of interest, the ratio of gene X to GAPDH served as a means of determining relative amounts of the target genes in each reaction

2 3 4 Reverse Transcription

Reverse transcription was preformed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) in accordance with manufacturers specifications with some minor modifications [Roth *et al*,1985, Sambrook *et al*, 1989] 0 5µg of total RNA (isolated as described in section 2 1 9) was mixed with 0 125µg oligo dT primers and the reaction mixture brought to a final volume of 12µl with DEPC water This mixture was heated for 10 minutes at 70°C to allow annealing to oligo dT primers to polyA tail of mRNA Following this, tubes were immediately cooled on ice and the remaining components of the reaction were added as follows

MLV 5X Reaction Buffer	5µl
10mM dNTP	3µl
MLV-RT	200units

The mixture was then made up to a final volume of 25µl using DEPC water and incubated for 60 minutes at 42°C Contaminating RNA was subsequently removed by the addition of 1µl of RNase H (2units/µl) at 37°C for 20 minutes cDNA samples were then either used immediately or stored at –80°C until required

2 3 5 Polymerase Chain Reaction

A 50µl PCR reaction mixture was prepared as follows,

RNase free water	36 5µl
10X reaction buffer	5µl
10mM dNTP	1µl
25mM MgCl	3µl
10µM Forward primer	1µl
10µM Reverse primer	1µl
Taq Polymerase	0 5µl
cDNA sample	2µl

The mixture was overlaid with 50µl of mineral oil and then placed in a Hybaid PCR Thermocycler (SPRT 001) Samples were subjected to an initial incubation of 92°C for 2 minutes followed by 30 cycles comprising of the following steps 92°C for 1 minute, annealing temperature for 2 minutes and 72°C for 3 minutes PCR products were removed from beneath the mineral oil and placed in fresh tubes before being subjected to agarose gel electrophoresis

Target Gene	Primer Sequence	Product size	Annealing Temp (°C)
Bovine MMP-2	5' tgg caa ccc cga cgt gg 3' 5' gca ggg ctg tcc gtc gg 3'	534 bp	55°C
Bovine MMP-9	5' tag gaa ccg ctt gca ttt c 3' 5' gat cca cct tct gtg tct t 3'	349 bp	54 5°C
GAPDH	5'agg tca tcc atg acc act tt 3' 5'ttg aag tcg cag gag aca a 3'	337 bp	54°C

2 3 6 Agarose gel electrophoresis

Agarose gels were prepared by boiling the appropriate quantity of agarose in 100ml of 1X TAE buffer (40mM Tris-Acetate pH 8 2, 1mM EDTA), gels were generally 1-2% (w/v) depending on the size of the DNA being visualised Gels contained 0 5µg ethidium bromide per 1ml of agarose for visualization of DNA within the gel When the gel was hand-hot the gel was cast in a GibcoBRL Horizion 20 25 gel electrophoresis apparatus

Samples were mixed with 6X gel loading buffer (40% w/v sucrose, 0 25% w/v bromophenol blue) 12 5µl of PCR product was mixed with 3µl of loading buffer and subsequently loaded The gel was run at 100V in 1X TAE buffer until the blue dye front was approximately 0 5cm from the end of the gel DNA was visualized on a transillummator and photographed for densitometric analysis using the Kodak 1D gel documentation system (Scientific Imaging Systems, Eastman Kodak Group, Rochester,NY)

2 4 Polyacrylamide electrophoresis

2 4 1 Western Blotting

SDS–PAGE was performed as described by Laemmli using 10% polyacrylamide gels [Laemmli , 1970] 10% resolving and 5% stacking gels were prepared as follows

Resolving Gel	1 5ml	Buffer A (1 5M Tris pH8 8)
	1 5ml	40% acrylamide stock
	3ml	distilled water
	60µl	10% (w/v) SDS
	30µl	10% (w/v) ammonium persulphate
	7µl	TEMED
Stacking Gel	0 75ml	Buffer B (0 5M Tris pH6 8)
	0 375ml	40% acrylamide stock
	1 85ml	distilled water
	30µl	10% (w/v) SDS
	15µl	10% (w/v) ammonium persulphate
	7µl	TEMED

For analysis of equal volumes of conditioned media, samples were concentrated by Amicon centrifugal filters (MW cut off 10kDa) and loaded on gels For analysis of cell lysate protein concentration was determined by BCA assay and a equal amounts of protein were resolved on the gel

Samples were mixed with 4X loading buffer (8% SDS, 20% β-mercatoethanol, 40% glycerol, Brilliant Blue R in 0 32M Tris pH6 8) and boiled at 95°C for 5 minutes, then immediately placed on ice The gel was electrophoresed in resevior buffer (0 025M Tris pH 8 3, 0 192M Glycine, 0 1% (w/v) SDS) at 40 milliamps (mA) per gel using an Atto vertical mini-electrophoresis system until the dye front reached the bottom of the gel

Following electrophoresis the gel was soaked for 15 minutes in cold transfer buffer (0 025M Tris pH8 3, 0 192M Glycine, 15% v/v methanol) Nitrocelluose membrane and 16 sheets of Whatmann filter paper were cut to the same size as the gel and soaked in transfer buffer Proteins were transferred to the membrane for 30 minutes at 100V in an ATTO semi-dry transfer system Following transfer membranes were soaked in Ponceau S solution to confirm transfer of protein to the membrane and also to normalize for variations in protein loading

Membranes were blocked for 1 hour in blocking solution [5% (w/v) skimmed milk in Tris Buffered Saline [TBS],10mM Tris pH8 0, 150mM NaCl)] Membranes were then incubated either overnight at 4°C or for 3-4 hours at room temperature, with primary antibody diluted according to manufacturers instructions in blocking solution The blots were then vigorously washed in three changes of TBST (0 05% (v/v) Tween in TBS) and then incubated for 2 hours at room temperature with a suitable HRP linked secondary antibody diluted in TBST Following incubation in secondary antibody, the blots were again washed in three changes of TBST

Antibody-antigen complexes were detected by incubation in West Pico Supersignal reagent (Pierce Chemicals) Briefly, an equal volume of solution A and B were mixed and the blot was incubated for 5 minutes at room temperature Blots were exposed to autoradiographic film (Amersham Hyperfilm ECL) to visualize bands present on the blot and developed (Amersham Hyperprocessor Automatic Developer) Bands of interest were identified either by use of an antigenic positive control or based on molecular weight markers Exposure times varied depending on the antibody being used but were typically between 1-2 minutes

2 4 2 Zymography

Zymography is an electrophoretic method for measuring proteolytic activity The method is based on a sodium dodecyl sulfate gel impregnated with a protein substrate which is degraded by the proteases resolved during the incubation period Coomassie blue staining of the gel reveals sites of proteolysis as white bands on a dark blue background Within a certain range the band intensity can be related linearly to the

amount of protease loaded [Liota *et al*, 1990] The gel was prepared by the incorporation of enzyme substrate (gelatin) within the polymerized acryl amide mix 10% gels were used and details for one gel are given below

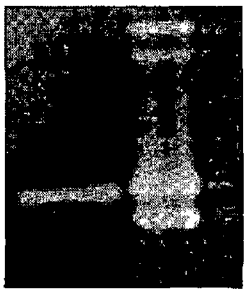


Figure 2 1 Commaassie blue staining of zymography gel reveals areas of proteolysis as white bands on a dark background

Resolving Gel	1 5 ml	Buffer A (1 5M Tris pH 8 8)
	1 ml	6mg/ml gelatin stock
	1 5 ml	40% acrylamide stock
	2 ml	distilled water
	30 µl	ammonium persulphate
	7 µl	TEMED
Staking Gel	0 75 ml	Buffer B (0 5M Tris pH 6 8)
	0 375 ml	40% acrylamide stock
	1 85 ml	distilled water
	30 µl	ammonium persulphate
	7 µl	TEMED

Conditioned media was centrifuged at 5000xg for 5 minutes to remove any cells Equal volumes of media from each experimental condition were mixed with 4X loading buffer (0 25M Tris pH 6 8, 20% glycerol, 2% SDS and 10µg/ml bromophenol blue) and dH_20

The gels were run at 80V, 90mA in reservoir buffer (0 025M Tris, 0 19M Glycine, 0 1% SDS) until the dye front reached the bottom of the gel Following electrophoresis gels were washed in two 20 minute changes of 2 5% Triton X-100 and one wash in dH_20 The gels were then incubated overnight (16-18hours) at 37°C in

incubation buffer (50mM Tris pH 8 0, 50mM NaCl, and 10mM $CaCl_2$) before being stained with 0 25mg/ml Brilliant Blue R in 30% acetic acid, 10% isopropanol Gelatinolytic activity was visualized as clear bands on a blue background Densitometric analysis was performed using Kodak 1D image analysis software Bands were identifed as Pro-MMP2 and MMP-2 by Chemicon geltainase zymography standards

2 5 DNA preparation methods

2 5 1 Transformation of competent cells

10ng of plasmid DNA of interest was placed in a sterile microfuge tube To this was added 100µl of competent JM109 E *Coli* The mixture was gently mixed and placed on ice for 30 minutes The cells were heat-shocked by placing the tube in a waterbath at 42°C for 45-50 seconds after which they were placed on ice for 2 minutes

Cells were grown for 1 hour at 37°C with agitation (200 rpm) in 1ml of sterile Luria Bertram (LB) broth [1% (w/v) tryptone, 0 5%(w/v) yeast extract, 1%(w/v) NaCl, pH 7 5] The cells were then centrifuged at 5000g for 1 minute and the supernatant removed The resultant pellet was resuspended in 0 2ml of LB broth and spread plated either 150µl or 50µl on LB agar plus ampicillin [LB medium containing 1 5%(w/v) agar plus 35µg/ml ampicillin] The plates were incubated at 37°C overnight and for no longer than 18 hours to prevent colasence of colonies As a control for each transformation a mock transformation was included, in which no DNA was added to the competent cells

2 5 2 Plasmid DNA Mini-preparation

Plasmid DNA was isolated as specified by Qiagen Plasmid Kit protocol [Sambrook *et al*, 1989, Ausubel *et al*, 1991, Birnboim *et al*, 1983] Single colonies of transformed cells were removed from plates and grown in 3ml of LB broth supplemented with 35µg/ml ampicillin These mini-cultures were grown at 37°C for 8 hours with gently agitation (<200rpm) 1 5ml of the final culture was used for the generation of glycerol stocks The remainder was diluted in 100ml of LB broth supplemented with 35µg/ml ampicillin and grown at 37°C overnight at 250rpm The following day the cells were harvested by centrifugation at 6000rpm for 15 minutes at 4°C

The pellet was resuspended in 4ml of Buffer P1 [50mM Tris-HCl, pH 8 0, 10mM EDTA, 100µg/ml RNase A] The resuspended cells were lysed by gently

inversion with 4 ml of Buffer P2 [200mM NaOH, 1% SDS] and was incubated at room temperature for 10 minutes Protein was precipitated by the addition of 4 ml of pre-chilled Buffer P3 [3M potassium acetate, pH5 5], gentle mixing and incubation on ice for 5 minutes Protein precipitates were removed by high-speed centrifugation, 13000rpm for 30 minutes at 4°C The supernatant was removed and centrifuged at 13,000rpm for 30 minutes at 4°C to ensure complete removal of all protein

Once the supernatant had been removed it was applied to a Qiagen tip-100, which has been equilibrated with Buffer QBT [750mM NaCl, 50mM MOPS, pH 7 0, 15% isopropanol, 0 15% triton X-100] The sample was allowed to enter the column by gravity flow and the column washed with 2 x 10 ml washes of Buffer QC [1M NaCl, 50mM MOPS, pH 7 0, 15% isopropanol] Finally, DNA was eluted from the column using 5 ml of Buffer QF [1 25M NaCl, 50mM Tris-HCl, pH 8 3, 15% isopropanol]

DNA was precipitated from the eluate by the addition of 3 5ml of isopropanol at room temperature The mixture was centrifuged at 12,000rpm for 30 minutes at 4°C, to yield a glassy pellet The supernatant was removed carefully so as not to disturb the isopropanol pellet The pellet was then washed in 2ml of 70% ethanol, to remove precipitated salts and to make reconstitution of the pellet easier and then centrifuged at 12,000rpm for 10 minutes The pellet was air-dried for 5–10 minutes after removal of the supernatant and then redissolved in sterile Tris-EDTA (TE) buffer [10mM Tris-HCl pH8 0, 1mM EDTA]

2 5 3 Restriction digests

DNA was digested with restriction endonucleases for identification of purified plasmid DNA 1µg of DNA was prepared in a reaction solution containing 10X reaction buffer (supplied with each enzyme, by the manufacturer) along with 1µl of restriction enzyme The digest was incubated at a suitable temperature (enzyme-specific) for 30 minutes to one hour, the reaction was typically stopped by heating the reaction mixture to 72°C In the case of multiple digests, following incubation with the first enzyme, linearised DNA was precipitated and then exposed to subsequent enzymes

to overcome problems with differing reaction buffers The products of the digest were then resolved by electrophoresis on a 2% agarose gel with appropriate size standards

2 5 4 Transient Transfection

Lipofectamine^{TM} reagent is a polycationic liposome as such it is suitable for transfection of DNA into eukaryotic cells (Invitrogen-Gronmgen, Netherlands) The day prior to transfection, 1 5x10^6 cells were plated on a T25cm^2 flask, and grown overnight in RPMI-1640 supplemented with serum and antibiotics

When cells had reached approximately 70% confluent they were transfected with plasmid DNA For transfection purposes plasmid DNA was diluted in 150 µl of DMEM without FCS or antibiotics such that there would be 1µg of DNA per 10cm^2 of surface area In a separate tube 10µl of lipofectamine reagent (4µl per 10cm^2) was diluted in 150µl of DMEM without FCS or antibiotics The diluted DNA was then mixed with diluted lipofectamine reagent and incubated at room temperature for 30 minutes This time permits the formation of DNA-liposome complexes

While the DNA complexes were forming the cells were washed three times in HBSS followed by one wash in DMEM This was to remove any antibiotics from the flask, which may impede transfection efficiency The DNA/lipofectamine mixture was made up to a final volume of 2ml, which is just enough media to cover the surface area of the flask The contents of the tube were then added to the culture flask The cells were incubated for 4 hours in transfection media, following this, the media was removed and replaced with normal RPMI-1640 growth media The cells were allowed to recover overnight following which, they were exposed to experimental conditions Cells were routinely co-transfected with either a Lac Z or green fluorescent protein (GFP) encoding plasmid as a means to determine approximate levels of transfection

2 5 5 β-galactosidase assay

Lac Z a plasmid encoding β-galactosidase was used to monitor transfection levels Increased levels of β-galactosidase activity was attributed to successful

transfection of the gene of interest Following trasnsfection and cell lysis, a 30µl sample was added to 3 µl of 100X Mg solution [0 1M $MgCl_2$ and 4 5M β mercaptoethanol], 66µl of 1X OPNG (o-mtrophenyl-β-D-galactopyranosidase)[4mg/ml ONPG in 0 1M sodium phosphate, pH 7 5] and 201µl of 0 1M sodium phosphate The reaction was incubated for 4-6 hours at 37°C until a yellow colour developed The reaction was subsequently stopped with 500 µl of Na_2CO_3, and optical density read at 420nm Suitable positive and negative controls were included in this assay

2 6 Transfection with si RNA

RNAi mediated gene silencing has grown rapidly in popularity as a method of analyzing gene function This procedure involves the transfection of cells with small interfering RNA (siRNA) duplexes These duplexes are 21 nucleotides in length and are designed to specifically target an individual mRNA of interest siRNA are effective at much lower quantities than other gene silencing methods including antisense and ribozyme based protocols [Yu *et al*, 2002, Yang *et al*, 1999] siRNA used in these studies was supplied as a 2'-deprotected, annealed oligonucleotide in a desalted form

2 6 1 Design of siRNA Duplex

A sequence is selected in the open reading frame of the cDNA which is at least 75-100 bp downstream of the start codon Untranslated regions near the start codon may be richer in regulatory protein binding sites, which may interfere with binding of the siRNP endonuclease complex The first AA dimer is located and the next 19 nucleotides are recorded The G/C content of the AA-N_{19} base sequence is determined Ideally the G/C content must be greater than 30% and less than 70% If the sequence does not meet these criteria, a sequence further downstream starting with an AA dimer is analysed This is continued until a sequence is found which meets all of the above conditions

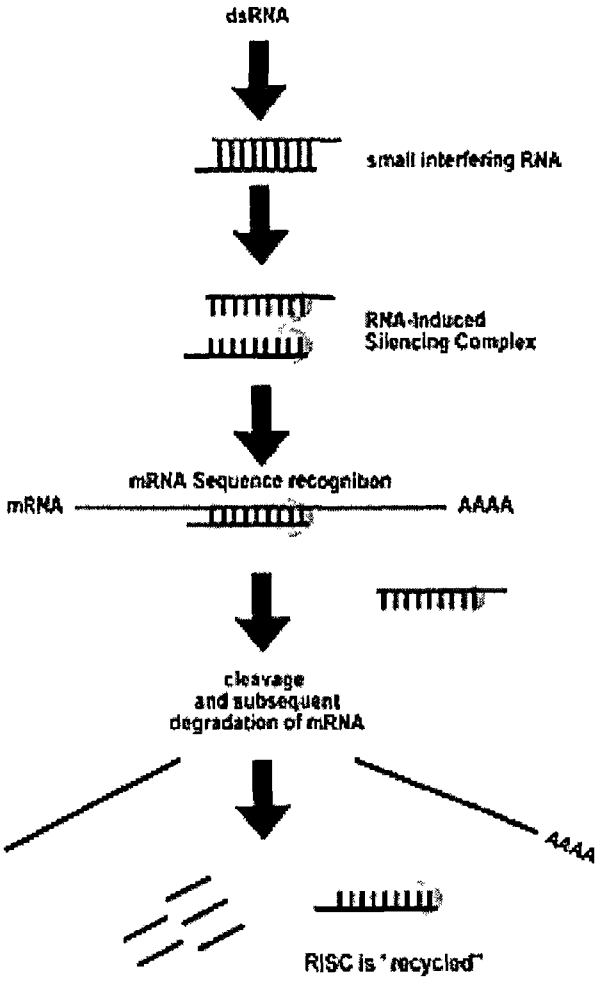


Figure 2 2 Mode of action of siRNA

Following this the 21 base sequence is subjected to a BLAST search to ensure that only one gene is targeted In this case of MMP-2 a sequence was chosen at position 364 in the MMP-2 sequence (Accesion number NM_174745) This section was chosen as it contains part of the functional peptidase region of the enzyme The target sequence of MMP-9 siRNA is at position 488 in the Bos Taurus sequence (Accesion number, NM_174744) located in the peptidase region of the enzyme

Name	Sequence of SiRNA Duplex	GC Content	Mol. Weight
MMP-2	GAA CCA GAU CAC AUA CAG G dTdT dTdT CUU GGU CUA GUG UAU GUC C	42 8%	13302 2g/mol
MMP-9	GCU GAC AUU GUC AUC CAG U UU UU CGA CUG UAA CAG UAG GUC A	42 9%	13308 0g/mol
Control X	AUU CUA UCA CUA GCG UGA C UU UU UAA GAU AGU GAU CGC ACU G	42%	13373 0g/mol

2 6 2 Transfection of siRNA Duplex

siRNA was transfected into BAECs using lipofectamine™ 2000 siRNA was diluted in 5X universal buffer [200mM KCl, 30mM HEPES-KOH pH 7 5, 1mM $MgCl_2$] and RNase free water to a final concentration of 20μM (20pmoles/μl) The following procedure is for transfection in a 6-well format $8X10^5$ cells were plated per well to achieve 70–80% confluency at time of transfection siRNA/lipofectamme ™ 2000 complexes were prepared as follows

- 5μl (100pmoles/10cm^2) of siRNA was diluted in 395μl of DME medium without serum or antibiotics, the presence of antibiotics during transfection causes cell death The mixture was then gently mixed
- Lipofectamine ™ 2000 was mixed prior to use and subsequently 5μl was diluted in 395μl of DME medium and incubated for 5 minutes at room temperature
- The two mixtures were then combined and mixed gently The mixture was incubated for 30 minutes at room temperature to allow the formation of siRNA/hpofectamine ™ 2000 complexes
- The 800μl of siRNA/lipofectamine ™ 2000 complexes were added to the well and mixed gently by slowly rocking the plate back and forward to ensure complete coverage of the cells Cells were incubated for 3 hours at 37°C in a CO_2 incubator

- After the 3 hour incubation the media was removed and replaced with growth medium and cells were recovered overnight Following recovery, media was replaced with fresh growth media and cells and media were harvested 24 hours later for analysis

A commercially available non-specific control siRNA sequence (MWG-Biotech) was transfected into control cells

2 7 Transwell migration assay

Transwell migration was assessed using a AA10 chemotaxis chamber (Neuroprobe) This is a modified Boyden chamber which comprises of top and bottom plates and a silcone gasket A single 25 x 80mm piece of porous filter membrane is placed between the top and bottom plates, and a gasket is positioned over the filter to create a seal Cells are inoculated into the top chamber After incubation the migrated adherent cells on bottom side of the filter are stained and counted This chamber has the advantage over other multichamber chemotaxis chambers of having a reasonably large surface area 50mm^2

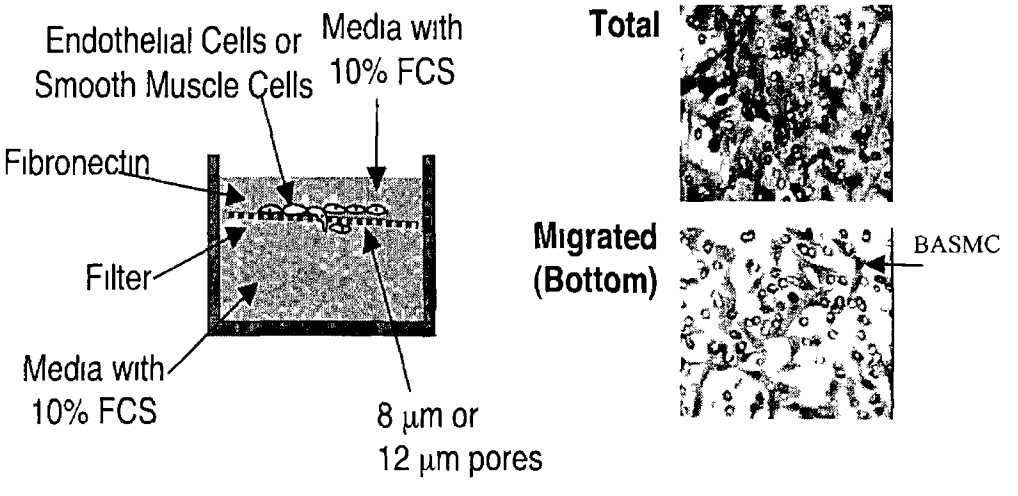


Figure 2 3 Diagrammatic representation of transwell migration

2 7 1 BAEC migration

To analyze BAEC migration, an 8μm polycarbonate filter membrane was coated with 0 02μg/μl human fibronectin protein overnight Following exposure to experimental conditions BAECs were harvested from experiments and seeded at a density of $3X10^4$ cells per well Cells were allowed to migrate for 16-18 hours in a humidified atmosphere of 5% v/v CO_2 at 37°C

Following this, non migrated cells were removed from the top side of the filter using a cell scraper Briefly, the top side of the filter was washed three times with 1X PBS and was scraped quickly so that cells would not dry on the filter Migrated cells were fixed on the filter by incubation with 3 7% formaldehyde for 20 minutes The filter was then washed with dH_20 and permitted to air dry When the filter was dry it was stained with Harris Hematoxylin solution for 20 minutes, followed by two washes in dH_20 The wet filter was placed on a microscope slide and allowed to dry

After fixing and staining the number of migrated cells was manually counted by using a microscope (Olympus, CM20) Cells in 5 random high power fields (hpf) for each well were counted to determine the average number of migrated cells Data are reported as the number of BAECs counted per 5 hpf and are expressed as a percentage of control, where control indicates BAECs exposed to static conditions unless otherwise stated

2 7 2 BASMC Migration

To analyze BASMC migration, a 12μm polycarbonate filter membrane was coated with 0 02μg/μl human fibronectin protein overnight BASMC were harvested and $5X10^3$ cells were seeded per well In this series of experiments conditioned media from BAEC cyclic strain experiments was used as a chemoattractant in the lower chamber

Fixing, staining and analysis were the same as in section 2 6 1

2 7 3 Scratch-wound healing assay

BAEC migration can also observed by this assay [de Jonge *et al*, 2002] Cells were grown in 6-well plates *and in vitro* wounds were created by scraping BAEC monolayers with a yellow tip After injury, the wound was photographed at 5 distinct positions and the distance between the two wound edges calculated using NIH image Cells were then exposed to fresh growth media in the absence or presence of inhibitors for 9h Following this, the wound was again photographed at 5 distinct locations and the distance between the two wound edges calculated BAEC migration was assessed by comparing distance between wound edges before and after treatement

2 8 *In vitro* tube formation assay

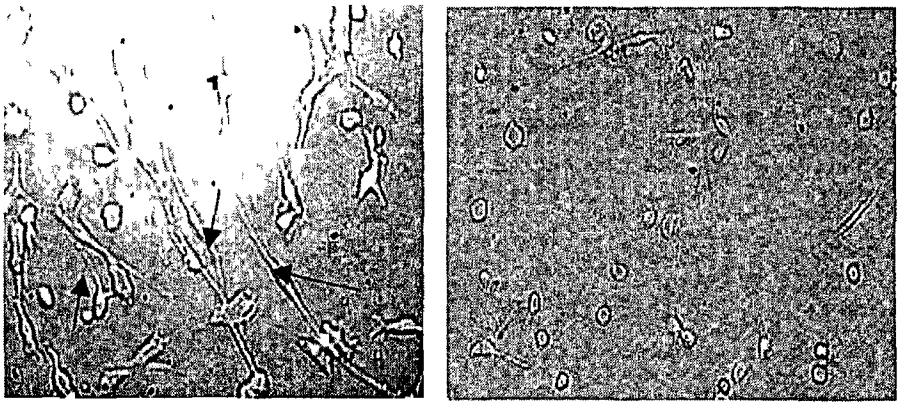
2 8 1 Preparation of three-dimensional collagen gels

Collagen gels were prepared as previously described [Yang *et al*, 1999, Zheng *et al* 2001] Briefly, a neutralized collagen mixture was prepared by mixing stock (1 5mg/ml in 10mM acetic acid) type I rat tail collagen, with growth medium and 1N NaOH Typical mixture 600µl of collagen+ 60µl of culture medium+ 50µl of 1N NaOH The mixture was poured into twenty four-well tissue culture plates (100µl/well) and allowed to gel in an incubator containing 5% CO_2-95% ambient air at 37°C for 1 hour After polymerization the gels were then incubated with growth medium overnight at 37°C before use

2 8 2 Tube formation assay

Following treatment under differing experimental conditions, BAECs were trypsinized as previously described Cell counts were preformed and 1 5X10^4 cells were seeded per well in RPMI 1640 supplemented with 10% FBS Cells were incubated overnight for 16-18 hours before assessment of tube formation Cells were

photographed using a JVC colour video camera KY-F55B Four random fields of vision were photographed from each gel and length of tube formation was quantified by measuring the length of the network of connected cells in each well with the use of NIH image



Angiogenic cells Control cells

Figure 2 4 *In vitro* tube formation assay Arrow identify tube formation

2 9 Statistical analysis

Results are expressed as mean ±SEM of a minimum of three independent experiments (n=3) unless otherwise stated Statistical comparisons between groups of normalized densitometric data were perfomed using both unpaired Student's *t*-test and Wilcoxon signed rank test A value of $P< 0\ 05$ was significant

Chapter 3

3 1 Introduction

Blood flow results in the generation of hemodynamic forces, namely cyclic strain due to pulsatile nature of blood flow and shear stress, the frictional force caused by blood flow as it drags against the cells, (see section 1 2) Blood vessels are permanently exposed to these stresses and, as such, both of these forces are required for maintaining vascular homeostatis As such, the phenotype of the endothelium is sensitive to the hemodynamic environment in which it exists, with alteration in this environment leading to changes in vascular cell fate

Angiogenesis is the formation of new capillaries from existing vasculature and is fundamental for the correct function, growth and survival of tissues [Pepper *et al*, 2001] This process occurs by a series of sequential events in response to specific stimuli Following EC activation, there is increased secretion of proteolytic enzymes including MMPs and serine proteinases and subsequent degration of ECM proteins ECM modification facilitates EC migration, an essential process for the progression of capillary formation into the intersitial stroma The cells at the tip of sprout are usually non-proliferative and cell division is usually confined to the trailing cells [Nguyen *et al*, 2001] Both cyclic strain and shear stress have been previously shown to drive endothelial cells to a migratory/angiogenic phenotype [Zheng *et al*, 2001, Rivilis *et al*, 2002, Zheng *et al* 1999] The regulation of a number of factors thought to contribute to the formation of new blood vessels including VEGF, FGF, tPA, integrin subunits, and a number of MMPs have also been shown to be sensitive to these forces [Vailhe *et al*, 1996, Banai *et al*, 1994, Ausprunk *et al*,1977, Pepper *et al*, 1997, Egginton *et al*, 2001] The importance of the gelatinases (MMP-2 and MMP-9) in angiogenesis and cell migration has been clearly demonstrated in a number of *in vivo* and *in vitro* studies [Haas *et al*, 1998, Chakraborti *et al* 2003, Fang *et al*, 2000, Pepper , 2001]

The aim of this chapter was to examine the regulatory effects of cyclic strain and shear stress on the migratory and angiogenic profile of BAECs and to determine the sensitivity of MMP-2 and MMP-9 produced by these cells to mechanical forces

3 2 Results

3 2 1 Hemodynamic regulation of pro-MMP-2, MMP-2 and pro-MMP-9

The regulatory effects of either 5% cyclic strain or 10 dynes/cm^2 laminar shear stress on MMP expression in BAEC were determined by measuring pro-MMP-2, MMP-2 and pro-MMP-9 activity in cell lysates and conditioned media Analysis of conditioned media showed no increase in the presence of LDH, indicating cell integrity during the course of the experiments Gelatin zymography revealed that BAECs preferentially secreted pro-MMP-2 in conditioned media under the experimental conditions In addition, it was found that there was a significant 2 3±0 6 fold (n=3 P<0 05) increase of pro-MMP-2 in media from cyclically strained cells but not in media from cells exposed to laminar shear stress (Figure 3 1) The active form of MMP-2 was also observed by gelatin zymography but was present in much lower quantities (Figure 3 3) As expected, levels of the active enzyme correlated to strain-induced increases in pro-MMP-2 In this study we have focused on pro-MMP-2

The regulatory effect of cyclic strain on pro-MMP-2 was examined at the level of message, protein and enzyme activity Semi quantitative RT-PCR was used to estimate mRNA levels GAPDH, a housekeeping gene was used as an internal control and to normalize the amount of pro-MMP-2 mRNA in static controls and cyclically strained samples Figure 3 2 (d) illustrates that 5% cyclic strain had no effect on GAPDH and resulted in a 1 5±0 1 fold (n=3 P<0 05) increase in pro-MMP-2 mRNA levels The increases in mRNA were mirrored by a 1 7±0 3 fold (n=3 P<0 05) increase in pro-MMP-2 protein as determined by zymography using cell lysate (Figure 3 2 b) In addition western blot and zymographic analysis of conditioned media confirmed a significant increase in levels of pro-MMP-2 enzyme activity and protein secreted from cyclically strained cells (Figure 3 2 a-c)

The eNOS protein has previously been shown to be upregulated in response to both shear stress and cyclic strain We have also demonstrated a hemodynamic up-regulation of this enzyme in our experiments, providing a positive control for

mechanical experiments Initial experiments were carried out in serum free culture media in order to eliminate mitogenic influences Hence, any observed changes in levels of the enzymes of interest could be attributed to mechanical stimuli It was found however that strain-induced increases in pro-MMP-2 could be detected in the absence or presence of serum Interestingly, pro-MMP-9 activity could only be detected in the presence of serum 5% cyclic strain significantly up-regulated levels of pro-MMP-9 activity 1 8±0 3 fold (n=3 $P<0$ 05) compared to unstrained controls (Figure 3 3)

3 2 2 Time and force-dependent increases in pro-MMP-2

The release of pro-MMP-2 from BAEC was found to be a time-dependent phenomena, with detectable levels occurring as early as 3h after commencement of strain (Figure 3 4) pro-MMP-2 is a constitutively secreted enzyme and a significant increase in pro-MMP-2 activity was observed over time Levels of pro-MMP-2 activity and protein were significantly increased (2 9±0 6 and 3 2±0 4 fold, respectively n=3 $P<0$ 05) in 24h conditioned media compared to 3h conditioned media from unstrained cells Similarly, levels of pro-MMP-2 activity and protein were significantly increased (7 2±0 2 and 4 9±0 6 fold, respectively n=3 $P<0$ 05) in 24h conditioned media compared to 3h conditioned media from strained cells Three hours of mechanical strain is sufficient to observe significant changes in levels of pro-MMP-2 protein (3 2±0 4 fold n=3 $P<0$ 05) but not activity compared to unstrained control

The rate of MMP-2 secretion from BAECs was found to be dependent on the mechanical load BAECs were exposed to varying degrees of cyclic strain 0 – 10% and conditioned media examined by zymography and western blot (Figure 3 5) Exposure to 2 5% cyclic strain resulted in a robust but non-significant increase in pro-MMP-2 activity and protein Significant increases in pro-MMP-2 activity and protein were only observed following 5% (2 1±0 2 and 4 4±0 6 fold, respectively n=3 $P<0$ 05) or 10% (3 4±0 5 and 9 0±1 5 fold, respectively n=3 $P<0$ 05) cyclic strain when compared to unstrained controls

3 2 3 Hemodynamic regulation of endothelial cell migration and tube formation

The regulatory effect of shear stress and cyclic strain on the migratory and angiogenic profile of BAECs was assessed *in vitro* by transwell migration and collagen tube formation assays Increases in migration and capillary network formation on ECM gels are generally associated with an angiogenic phenotype We first examined changes in BAEC phenotype following exposure to 10 dynes/cm^2 laminar shear stress Shear stress of this magnitude was found to result in a 3 6±0 7 fold (n=3 P<0 05) increase in BAEC transwell migration (Figure 3 6) This increase in migration was associated with a 2 0±0 1 fold (n=3 P<0 05) increase in the ability of BAEC to form three-dimensional structures (tubes) on collagen-based gels (Figure 3 7) Angiogenesis is expressed as tubule formation on collagen and the mean length of tube formation was quantified by measuring the length of the network of connected cells in each well using NIH image Similarly following exposure to 5% cyclic strain for 24 hours, there was an observed 1 8±0 1 fold (n=3 P<0 05) increase in BAEC transwell migration with a concomitant 1 9±0 1 fold (n=3 P<0 05) increase in tube formation (Figure 3 8 and 3 9) The increase in cell migration and tube formation is believed to be an inherent capacity of mechanically challenged cells as these responses were found in the absence or presence of conditioned media from these cells

3 2 4 Force dependent increase in EC migration

Cyclic strain was shown to increase the rate of transwell migration in BAECs This cyclic strain-induced increase in migration was found to be force dependent BAECs were exposed to 1%, 5% or 10% cyclic strain for 24h and their migratory profile was examined by transwell migration assay Following 1% cyclic strain, BAEC migration was increased (1 2±0 1 fold n=3 P<0 05), albeit insignificantly However, significant increases in BAEC migration were found after 5% or 10% cyclic strain (2 0±0 15 fold and 2 6±0 1 fold respectively n=3 P<0 05) compared to unstrained controls 10% cyclic strain augmented BAEC migration compared to 5% cyclic strain,

although this increase was not significant, it does however clearly suggest a direct relationship between magnitude of mechanical load and BAEC migration (Figure 3 10)

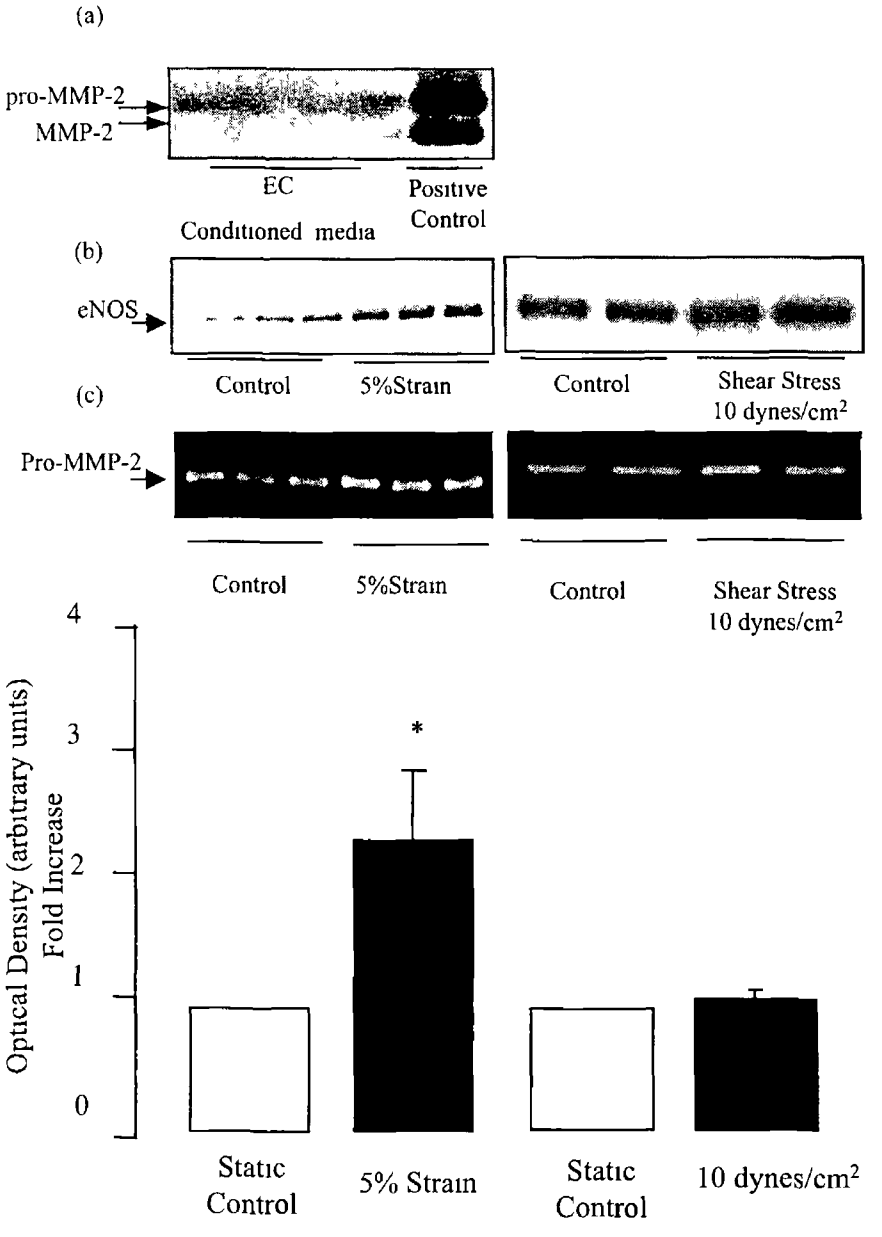


Figure 3 1 Secretion of pro-MMP-2 is increased following cyclic strain but not shear stress Following exposure to either 5% cyclic strain or laminar shear stress of 10dynes/cm^2 conditioned media was monitored for pro-MMP-2 activity Cyclic strain but not shear stress was found to cause a significant increase in pro-MMP-2 activity (c), while both stimuli increased expression of the eNOS protein (b) (a) pro-MMP-2 and MMP-2 activities identified by gelatin zymography in conjunction with commercial standards (gel is inverted for clarity) Histogram represents mean values from three independent experiments ±SEM, *p<0 05 compared to controls

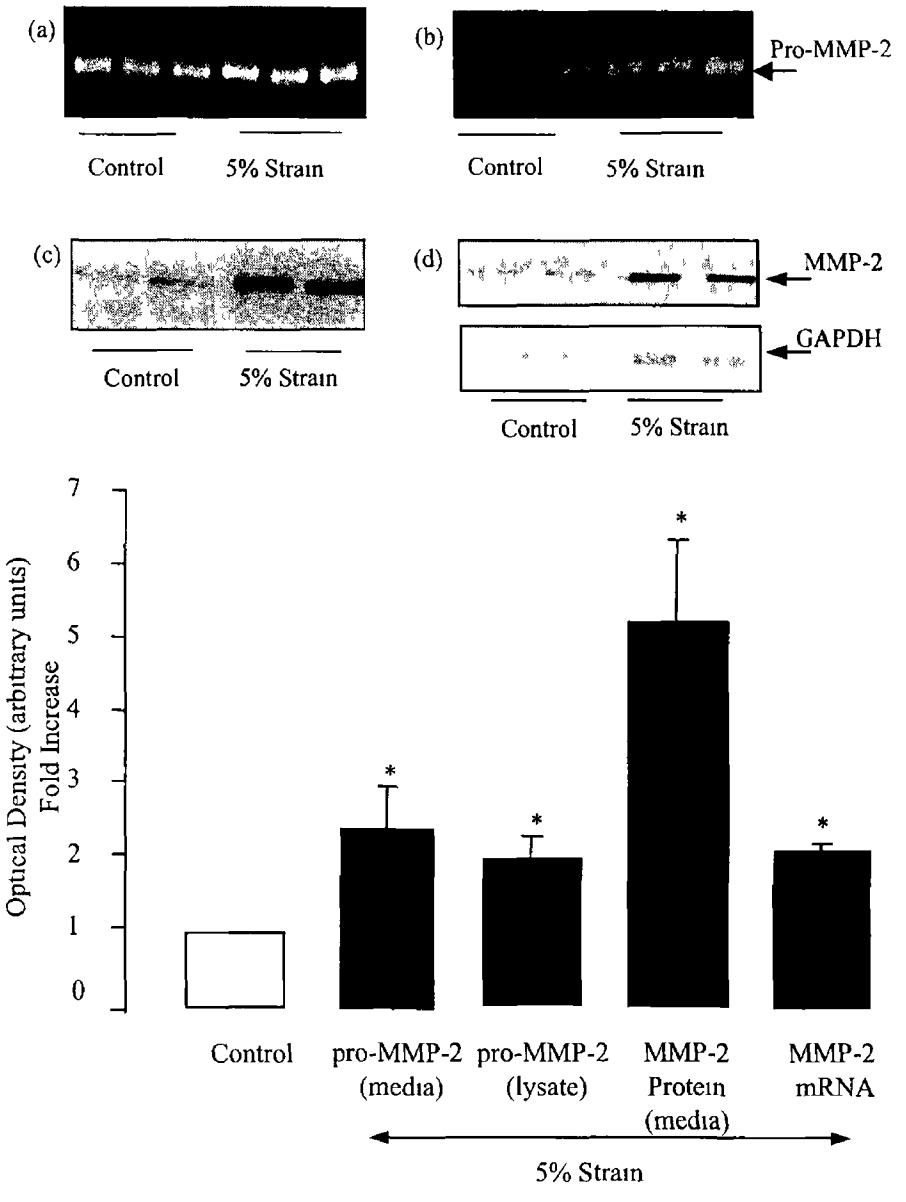


Figure 3 2 Cyclic strain-induced upregulation of pro-MMP-2 in BAEC conditioned media, cell lysate and mRNA Following cyclic strain (5%, 24 h), conditioned media, cell lysates and total RNA were harvested MMP activities were monitored in (a) conditioned media and (b) cellular lysates (pro-MMP-2) in response to cyclic strain Cyclic strain-dependent increases in (c) MMP-2 protein (in conditioned media) and (d) MMP-2 mRNA expression were determined by Western blot and RT-PCR, respectively (mRNA gels are inverted for clarity) Histogram represents cumulative data in the form of band densitometry readings taken from three independent experiments ±SEM, *$P \leq 0$ 05 versus unstrained controls All gels are representative

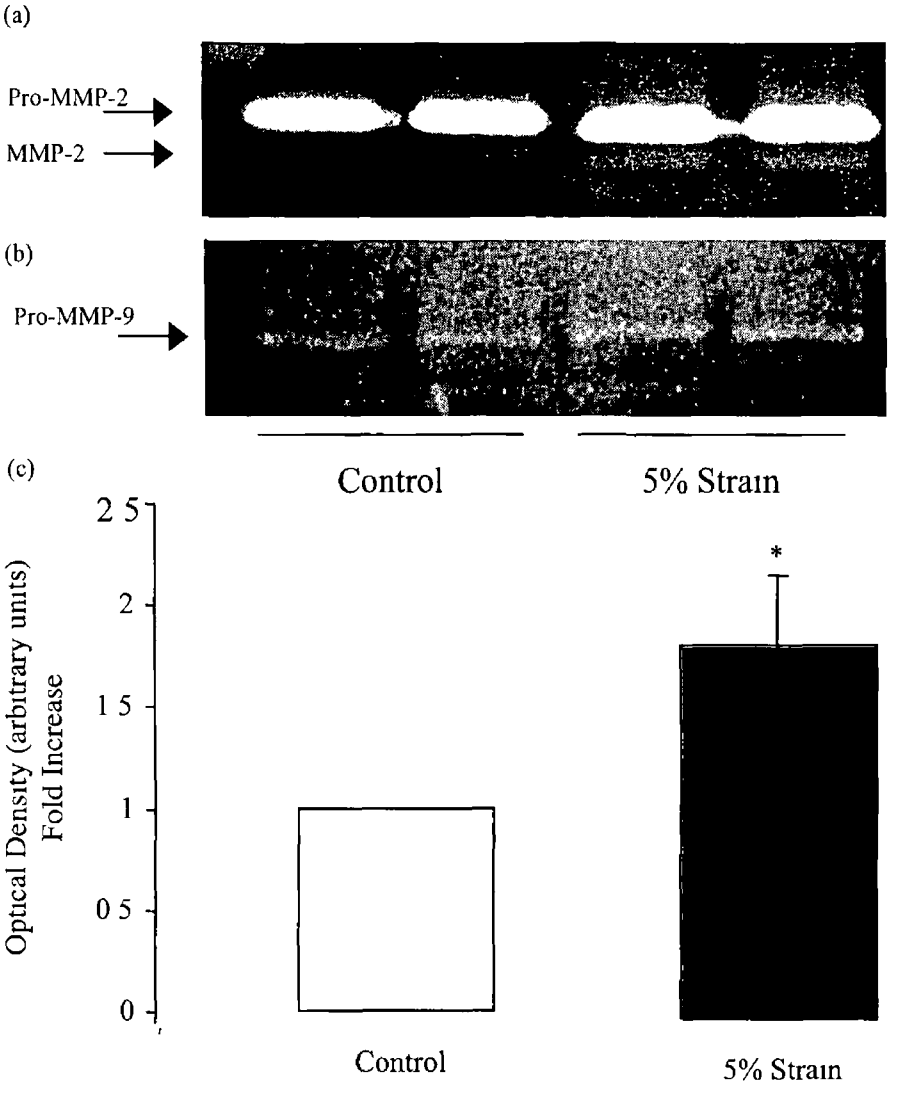


Figure 3 3 Cyclic strain induced increases in active MMP-2 and pro-MMP-9 BAECs were exposed to 5% cyclic strain at 1Hz or held static (Control) for 24 hrs Following this conditioned media was examined by zymography for active MMP-2 and MMP-9 Representative zymograms show a strain-induced increase in MMP-2 (a) and pro-MMP-9 (b) Densitometric analysis of induced increases in pro-MMP-9 is also shown (c) Histogram represents mean values from three independent experiments ± SEM, * P <0 05 compared to controls

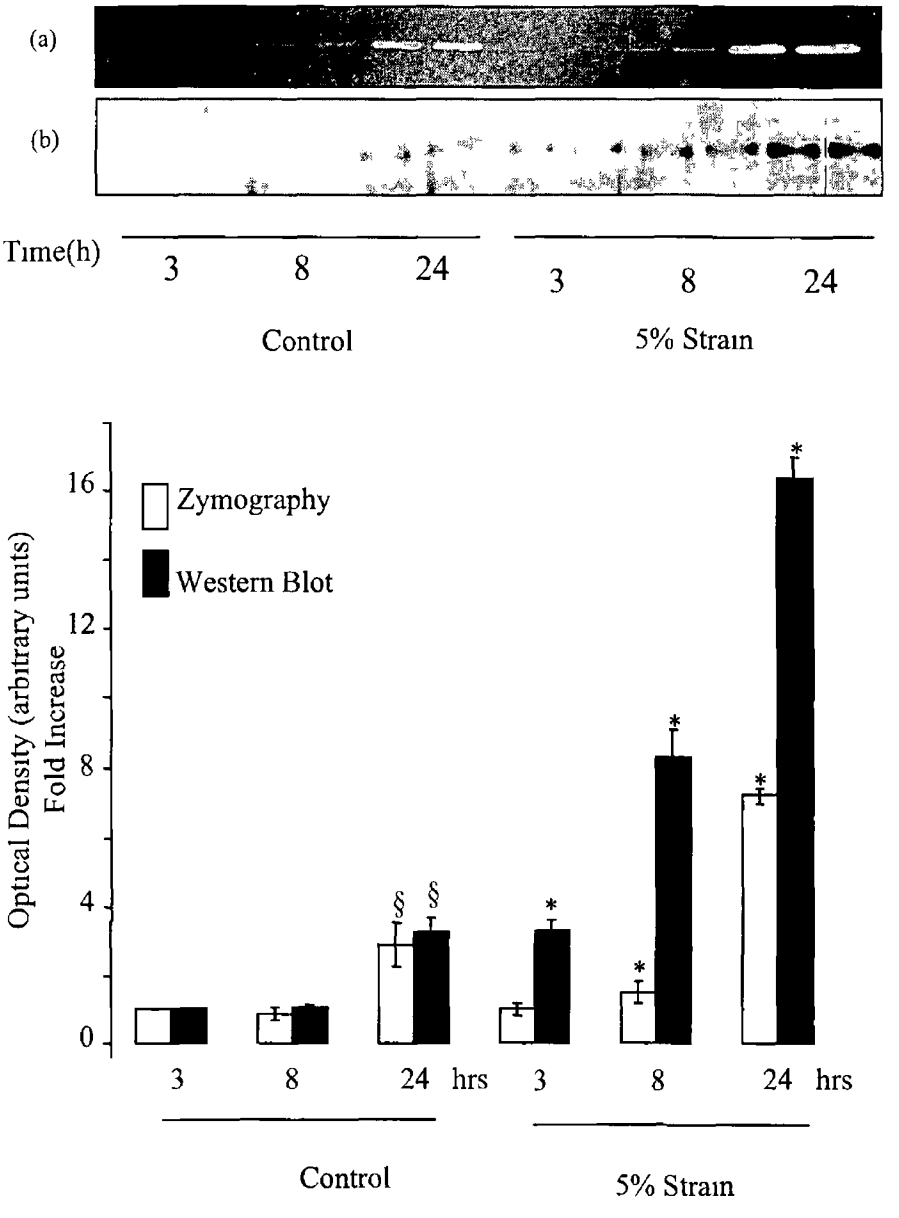


Figure 3 4 Time dependent release of pro-MMP-2 from strained and unstrained BAECs Time course of strain-induced increases in Pro-MMP2 BAECs were exposed to 5% cyclic strain at 1Hz or held under static conditions (Control) for 3hrs, 8hrs or 24hrs Conditioned media was analyzed by zymography (a) or western blot (b) for Pro-MMP2 activity and protein respectively Histogram represents mean values from three independent experiments ± SEM, * P <0 05 compared to controls §p<0/05 compared to 3h control

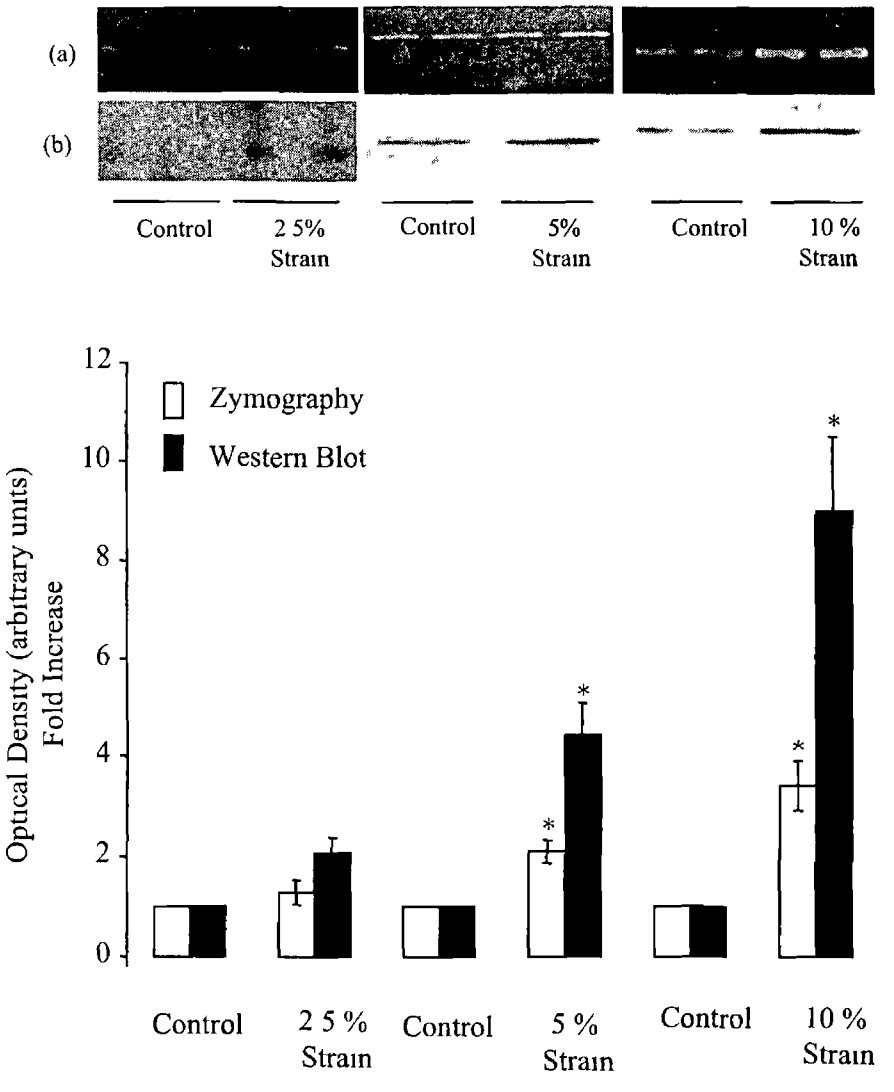


Figure 3 5 Cyclic strain dependent upregulation of pro-MMP-2 in BAECs BAECs were exposed to 2 5%, 5% or 10% cyclic strain at 1Hz or held under static conditions (Control) for 24hrs Conditioned media was analyzed by zymography (a) or western blot (b) for Pro-MMP2 activity or protein respectively Histogram represents mean values from three *independent* experiments ± SEM * P <0 05 compared to controls

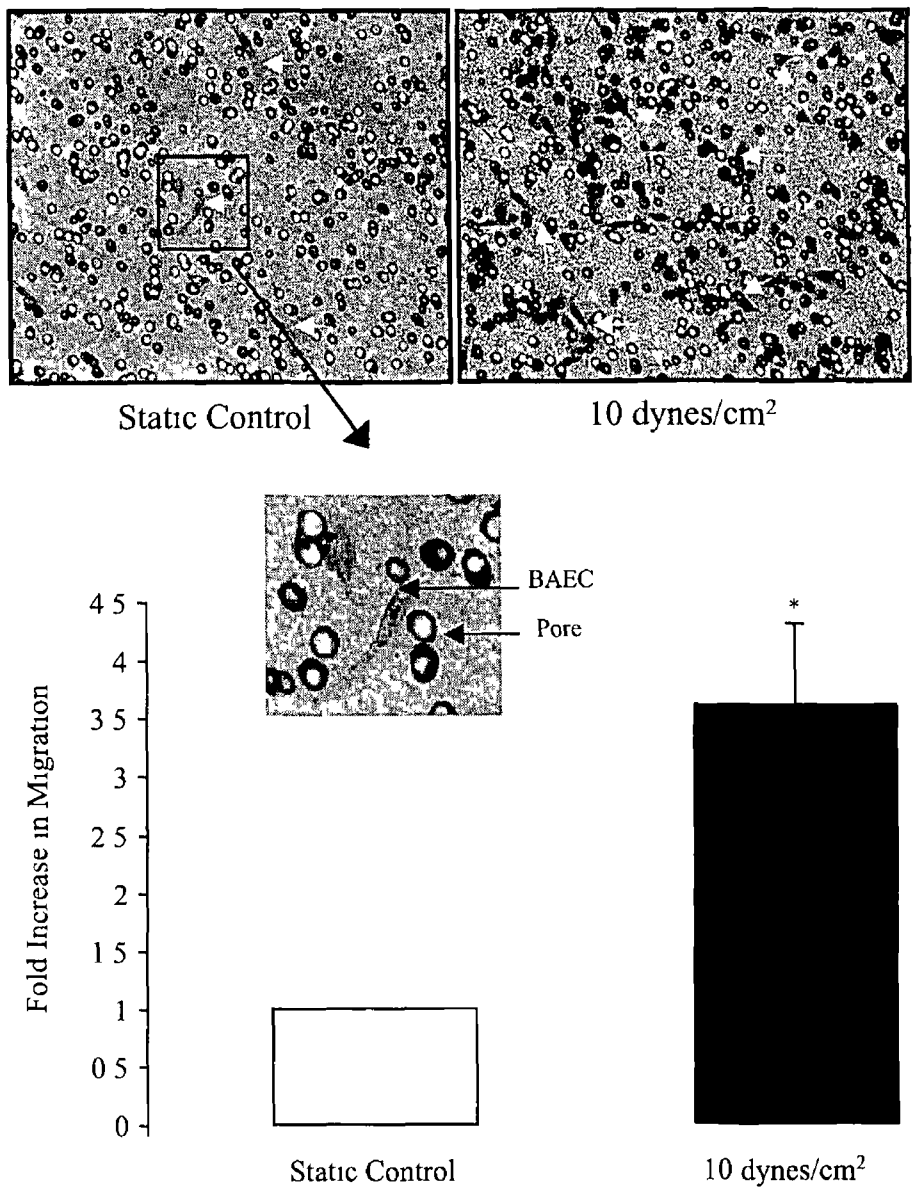


Fig 3 6 Shear Stress-induced increase in BAEC migration Force-dependent elevation of BAEC migration as determined by transwell migration assay, following exposure to laminar shear stress at 0 dynes/cm^2 or 10 dynes/cm^2 for 24h Results are expressed as fold change in migration relative to control (Static cells) Representative fields of vision are shown and arrows indicate *migratory* cells Magnified section identifying BAEC and pore on transwell membrane Histogram represents mean values from three independent experiments ± SEM, *P<0 05 compared to controls

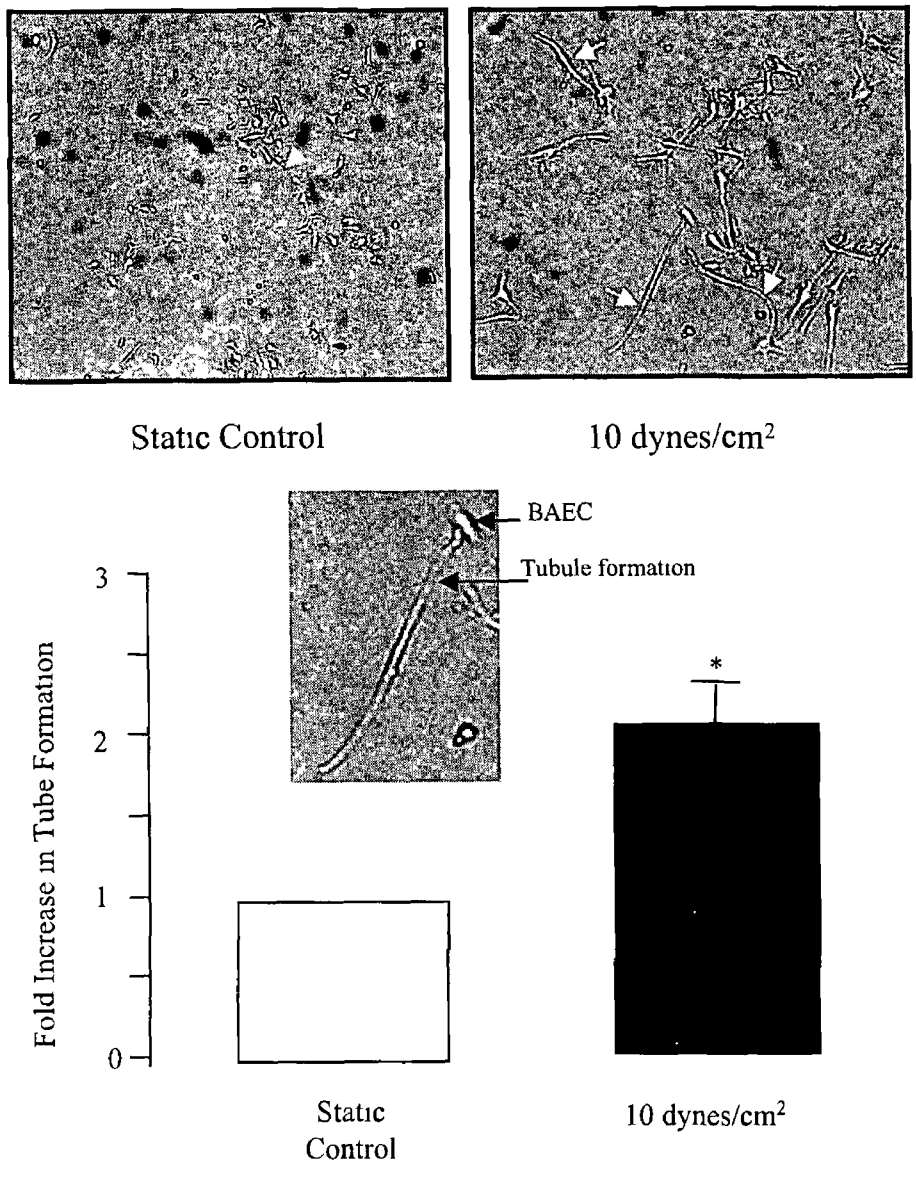


Figure 3 7 Shear Stress-induced increase in BAEC Tube formation Force-dependent increase in endothelial cell tube formation following exposure to laminar shear stress at 0 dynes/cm^2 or 10 dynes/cm^2 for 24h Results are expressed as fold change in tube formation relative to control (Static cells) Representative fields of vision are shown and arrows indicate tube formation Magnified section identifying BAEC and tubule formation Histogram represents mean values from three independent experiments ± SEM, * P <0 05 compared to controls

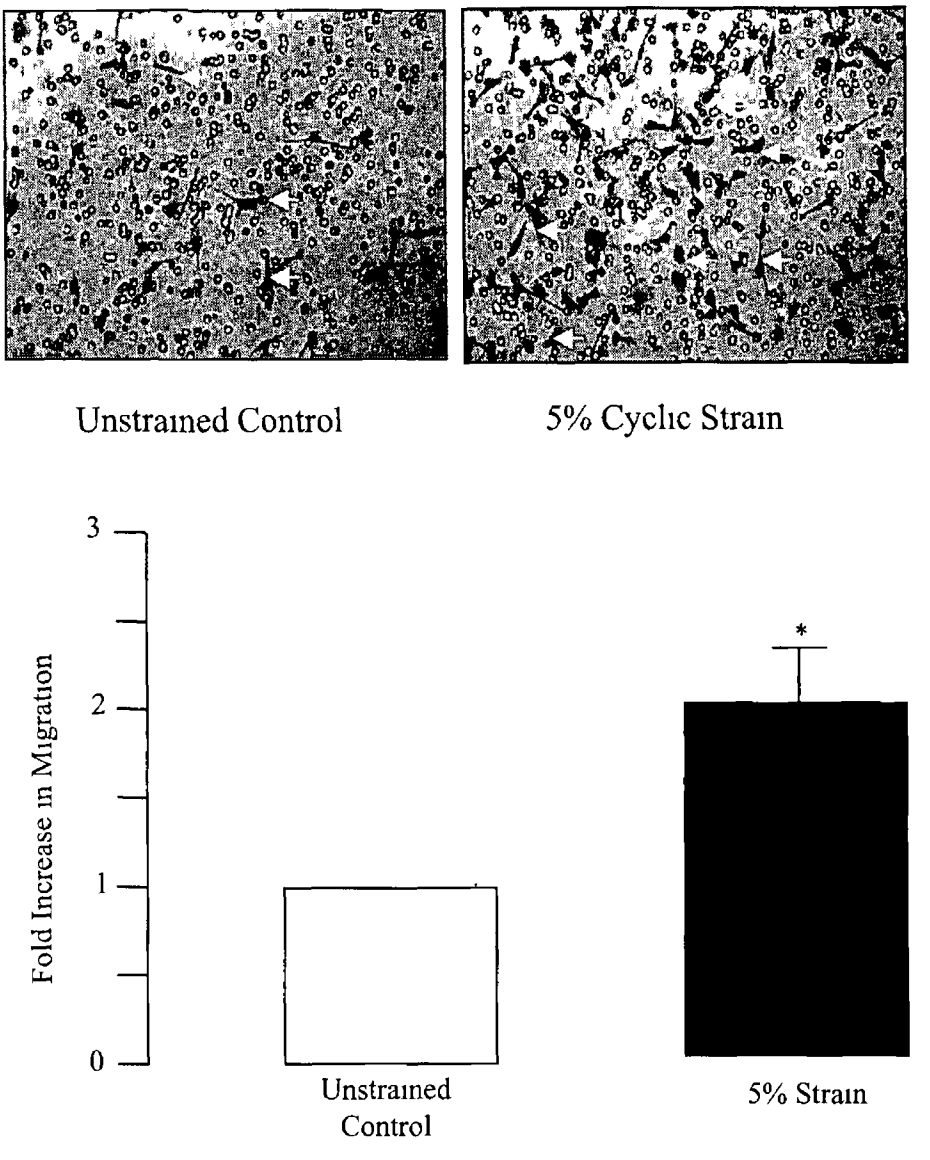


Figure 3 8 Cyclic strain-induced increase in BAEC migration Force-dependent elevation of BAEC migration as determined by transwell migration assay, following exposure to 0%(control) or 5% eqibiaxial cyclic strain for 24h at 1Hz (Cardiac stimulation-waveform) Results are expressed as fold change in migration relative to control Representative fields of vision are shown and arrows indicate migratory cells Histogram represents mean values from three independent experiments ± SEM, *P <0 05 compared to controls

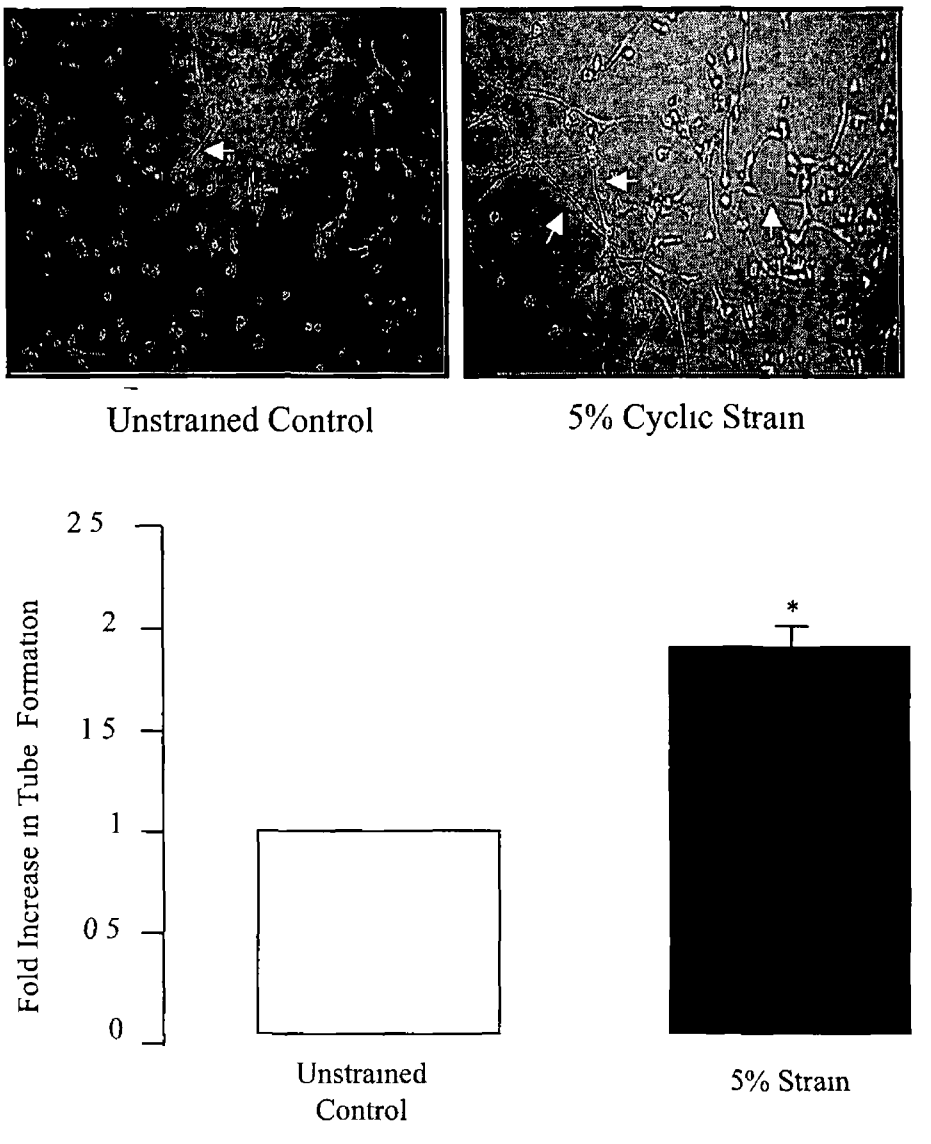


Figure 3 9 Cyclic Strain-induced increase in BAEC Tube formation
Force-dependent increase in BAEC tube formation following exposure to 0%(control) or 5% equibiaxial cyclic strain for 24h at 1Hz (Cardiac stimulation-waveform) Results are expressed as fold change relative to control Representative fields of vision are shown and arrows indicate tube formation Histogram represents mean values from three independent experiments ± SEM,, *P<0 05 compared to controls

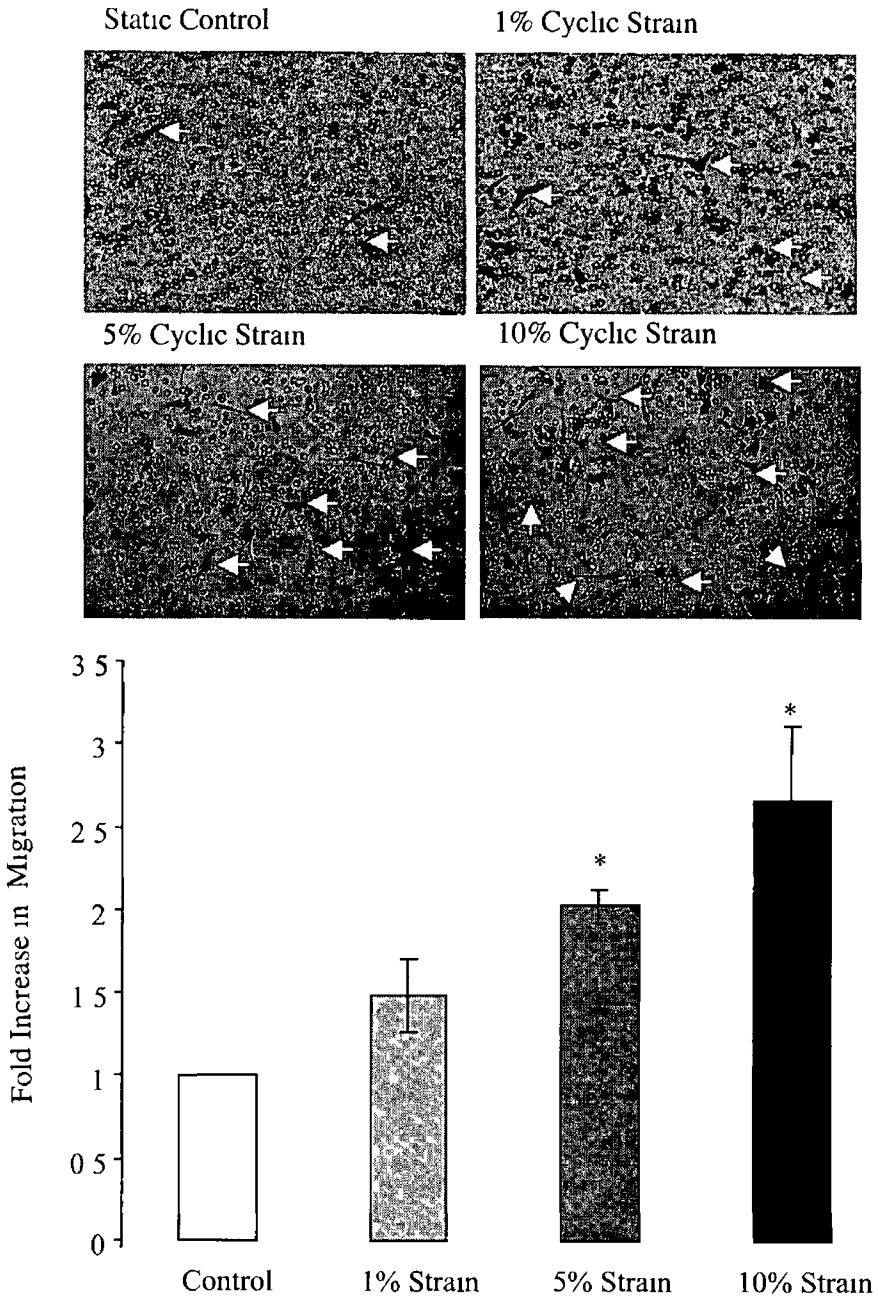


Figure 3 10 Cyclic strain-induced increases in BAEC migration

Force-dependent elevation of BAEC migration as determined by transwell migration assay, following exposure to 0%(control) 1%, 5% or 10% cyclic strain for 24h at 1Hz Results are expressed as fold change relative to control (unstrained cells) Representative fields of vision are shown and arrows indicate migratory cells Histogram represents mean values from three independent experiments ± SEM

3 3 Discussion

In this chapter we examined the sensitivity of MMP-2 and MMP-9 to mechanical stimuli by exposing BAECs to either cyclic strain or non-pulsatile laminar shear stress Given the importance of mechanical stimuli in regulating vascular cell fate decisions and the relevance of MMP-2 and MMP-9 to cell migration and angiogenesis we determined the ability of these mechanical forces to change the phenotype of static endothelial cells to a migratory/angiogenic state

In vitro and *in vivo*, vascular EC and SMC produce MMP's [Galis *et al*, 2002, Beaudeux *et al*, 2003] MMP-1, - 2, -3, and -9 have been shown to degrade ECM components after proteolytic activation if not inhibited by TIMPs Short collagens and elastin, which are major components of the ECM, are degraded by MMP-2 and MMP-9 [Visse *et al*, 2003, Galis *et al*, 2002, Beaudeux *et al*, 2003] While several studies have addressed the direct effects of cyclic strain on vascular SMC, few studies have reported on the effects of cyclic strain on EC The role of MMPs and particularly gelatinases in angiogenesis has been well documented The gelatinases (MMP-2 and –9) are believed to be the principal MMPs involved in angiogenesis [Hass *et al*, 1998, Chakraborti *et al*, 2003, Fang iet al, 2000, Pepper *et al*, 2001] As such, we determined the production of these enzymes from BAEC in response to cyclic strain and shear stress Analysis of the MMP profile in static and hemodynamically challenged cells identified that pro-MMP-2 was predominantly secreted, the active form of the enzyme was also detectable in conditioned media but was present in much lower quantities This observation appears to concur with other studies in that pro-MMP-2 is constitutively expressed by a number of vascular cell types including ECs with active MMP-2 being present to a lesser degree [Grote *et al*, 2003, Haseneen *et al*, 2003] Initial experiments were conducted in serum-free media The rationale for this was that any observed changes in MMPs would not be associated with mitogenic stimuli from serum and thus any observed effects could be attributed to mechanical stimuli When cells were grown in the absence or presence of serum pro-MMP-2 was the predominant MMP observed However, pro-MMP-9 was only observed when cells were grown in serum-containing

media A possible explanation is that MMP-9 requires mitogenic stimulation for detectable quantities to be produced Galis *et al* (1994) confirm this theory by demonstrating constitutive secretion of MMP-2 from vascular SMC but secretion of MMP-9 only following treatment with IL-1 or TNF-α

Secretion of pro-MMP-2 was found to be sensitive to cyclic strain but not to shear stress Western blot analysis of the eNOS protein confirmed that experimental conditions were having an effect on the cells, with substantial increases in eNOS protein expression observed following either cyclic strain or shear stress as previously reported [Awoleski *et al* 1995, Ziegler *et al*, 1998] The regulation of MMP-2 by shear stress has been extensively studied, however there is a considerable amount of contradicting information Bassiony *et al* (1998) claim that low but not high shear stress causes significant increases in MMP-2, whilst Tronc *et al* (2000) demonstrate a significant increase in MMP-2 production in an arteriovenous fistulae model (i e high shear model) Contrary to both of these studies shear stress has also been shown to significantly decrease MMP-2 production [Palumbo *et al*, 2000] Disparities in these findings may also be attributed to differences in model (i e non-pulstaile laminar shear stress or pulsatile laminar shear stress) or differences between species Rivilis *et al* verify the results presented here, increased levels of shear were accompanied with increases in angiogenesis but with no significant changes in MMP-2 They attribute this fact to differences in the mechanism(s) by which different mechanical forces induce angiogenesis Shear stress resulting in longitudinal splitting of the capillary and not by abluminal sprouting as observed following cyclic strain [Brown *et al*, 2003]

Further examination of cyclic strain-mediated regulation of MMP-2 indicated that cyclic strain elicits its effects on MMP-2 at a number of levels Cyclic strain regulates MMP-2 expression at the level of mRNA as determined by RT-PCR, protein translation as determined by analysis of cell lysates and finally secretion as determined by zymography and western blot of conditioned media

MMP-2 is a constitutively secreted protein, and as such it is not generally regulated at the level of transcription We have found however that cyclic strain causes an up regulation in MMP-2 mRNA when compared to static controls A number of other studies have shown relatively similar increases in MMP-2 mRNA by Northern blot and cDNA microarray analysis following exposure to cyclic strain [Grote *et al*, 2003, Wang *et al*, 2003, Haseneen *et al*, 2003] Wang *et al* (2003) has also confirmed strain-induced increases in MMP-2 mRNA levels are as a result of changes in mRNA expression and not stability Pro-MMP-2 activation is believed to involve a process involving MT1-MMP and TIMP-2, where the three molecules are co-regulated However, other studies have found an up-regulation of MMP-2 mRNA without a concurrent increase in MT-1 MMP mRNA One explanation has been proposed by Grote *et al* (2003) who suggest that ROS produced from NADP(H) oxidase are responsible for the cyclic strain induced increases in MMP-2 expression

Analysis of conditioned media by western blot and zymography indicated a significant increase in both pro and active MMP-2 activity following 24h of cyclic strain It may be tempting to surmise that cyclic strain increases the activation of pro-MMP-2, however, this may not be the case as levels of active enzyme may increase proportionally with levels of pro-enzyme The fold increases as determined by both methods were different, however, levels of protein expression and protein activity may often be different The increases in pro-MMP-2 activity in conditioned media were mirrored by an increase in pro-MMP-2 protein in cell lysates This, coupled with the RT-PCR and LDH assay data suggests that cyclic strain-induced increases in pro-MMP-2 activity and protein are a result of transcriptional and translational events and is not due to accumulation of pro-MMP-2 in media due to loss of cell integrity Similarly, analysis of conditioned media following exposure to cyclic strain revealed significant increases in pro-MMP-9 activity, previous studies have also reported cyclic strain induced increases in MMP-9 [Berry *et al*, 2003, Fujisawa *et al*, 1999] This is an important observation as both MMP-2 and MMP-9 are believed to be important in angiogenesis and other vascular remodeling processes As such coincidental increases

in MMP-2, MMP-9 and EC tube formation suggest a possible link between the two phenomena, something that will be looked at in greater detail in chapter 5

Secretion of pro-MMP-2 activity was found to be time-dependent which concurs with its' constitutively secreted nature Detectable levels were observable in media as early as three hours after initiation of experiments and continued to increase over 24 hours Cyclic strain was found to amplify the time dependent release following only three hours exposure but was more defined at later time points Our data appears to concur with other studies where cyclic strain augments the temporal release of MMP-2 following 3 to 12h of strain and levels of MMP-2 remain elevated for up to 24 hours [Grote *et al*, 2003, Wang *et al*, 2003]

We have demonstrated a relationship between magnitude of cyclic strain and pro-MMP-2 secretion Our data suggests that there is a minimum threshold of cyclic strain required to promote MMP-2 production Cyclic strain of 2 5% was found to cause a slight but not significant increase in pro-MMP-2 activity However, 5% and 10% cyclic strain resulted in significant increase Wang *et al* (2003) observed that 10% cyclic strain caused no change in MMP-2 production in HUVECs and significant increases in expression and activity could only be observed following 20% cyclic strain Unlike the sinusoidal waveform employed by Wang *et al* this study used a heart pulse pressure waveform, this may result in recruitment of different mechano-transduction pathways with resultant differences in cellular response In addition to this, our study used 5% cyclic strain, which, is generally accepted as being physiological, 20% cyclic strain is likely to be more associated with pathological conditions Asanuma *et al* (2003) highlight the importance of strain regime In their study, they found that 5% uniaxial stationary strain resulted in a significant increase in MMP-2 expression and activity in SMC, however 5% uniaxial cyclic strain had the opposite effect Unlike the present study we used 5% equibiaxial strain, which means that the distribution of cyclic strain is more equal over the cell surface possibly accounting for observed differences in cyclic strain regulation of MMP-2 in BAECs

Both shear stress and cyclic strain promote EC migration to similar degrees as determined by transwell migration This response was similar to previously reported effects of cyclic strain and shear stress on EC migration [Cullen *et al*, 2002, Zheng *et al*, 2001, Shyy *et al*, 2002, Urbich *et al*, 2002, Carmeliet *et al*, 2000] Increases in EC migration are characteristic of cells with an angiogenic phenotype [Carmeliet *et al*, 2000] Thus we examined the ability of cyclic strain and shear stress to promote angiogenesis by the formation of three-dimensional structures (tubes) on collagen gels Consistent with previous reports, we found that both cyclic strain and shear stress promoted EC tube formation an established indicator of an angiogiogenic phenotype [Cullen *et al*, 2002, Rivilis *et al*, 2002, Shyy *et al*, 2002, Milkiewicz *et al*, 2001] In conjunction with this study we found that EC migration was dependent on the magnitude of cyclic strain Coincidental force dependency in MMP expression and EC migration may suggest a possible link between the two phenomena

3 4 Conclusion,

We have demonstrated that both cyclic strain and shear stress promote EC migration and tube formation In addition, we have clearly shown that BAECs constitutively produce pro-MMP-2 but can also produce pro-MMP-9 in the presence of serum Cyclic strain but not shear stress can up-regulate expression and activity of pro-MMP-2 and pro-MMP-9 We have established that 5% cyclic strain augmented constitutive secretion of MMP-2 in a time-dependent manner Finally, we have shown a positive relationship between magnitude of cyclic strain and MMP-2 secretion EC migration has also been shown to be sensitive to the magnitude of cyclic strain applied

Our data suggests that hemodynamic forces play an important role in dictating the phenotype of BAECs However, cyclic strain and not laminar shear stress appears to be the more prevalent in the regulation of MMP activity Cyclic strain-induced increases in pro-MMP-2, MMP-2 and pro-MMP-9 with coincidental increases in migration and tube formation suggest a possible link between the two phenomena This relationship is to be investigated in more detail in the following chapters

Chapter 4

4 1 Introduction

Vascular cells have the ability to respond to mechanical forces namely cyclic strain and shear stress The ability to respond to mechanical forces is facilitated by mechanically sensitive receptors or "mechanoreceptor" present in vascular cells This process by which mechanical forces are detected and converted into a cell signal to elicit a response is referred to as "*mechanotransduction*" [Lehoux *et al*, 2001] This process facilitates changes in cellular phenotype in response to changes in their hemodynamic environment Mechanotransduction requires the activation of mechano-sensitive receptors, which may be activated directly by the mechanical force, disruption of the ECM, or distortion of the cell membrane and cytoskelton G-proteins, integrins and PTKs have all demonstrated mechano-sensitivity As such they have formed the focus of this study as a means of elucidating the signaling pathway involved in cyclic strain –mediated regulation of MMP-2

Following initiation of a mechano-transduction process, downstream signaling molecules must be recruited to transduce the signal to the cell nucleus and elicit a cellular response A number of studies have demonstrated the recruitment of the Shc adapter molecule by a number of receptors in response to mechanical stimuli [Shyy, *et al*, 2002, Labrador *et al*, 2003] Similarly, the MAPK family of proteins have been shown to be recruited by both cyclic strain and shear stress [Jalil *et al*, 1998, Surapisitchat *et al*, 2001, Jo *et al*, 1997] Thus, we have examined the possible recruiutment of Shc and the MAPK pathway by G-proteins, integrins and PTKs in regulating MMP-2 production in response to cyclic strain

The specific aim of this chapter was to investigate the roles of G-proteins, integrins, PTKs, Shc and MAPK in the signaling mechanisms involved in cyclic strain-induced increases in MMP-2 expression and activity

4 2 Results

4 2 1 Cyclic Strain Increases pro-MMP-2 Activity in a Giα -protein Independent Manner

In order to determine the involvement of Gi α protein subunits in cyclic strain-induced increases in pro-MMP-2 activity, BAEC were initially pretreated for 4h with pharmacological inhibitors of Gi-proteins, pertussis toxin (PTX, 100ng/ml) or a suramin analogue, NF023 (10μM), before the cells were exposed to 5% cyclic strain for a further 24 h in the absence or presence of the inhibitors In both cases, pretreatment with either Gi-protein inhibitor did not have any significant effect on strain-induced increases in pro-MMP-2 activity or MMP-2 expression in conditioned media (Figure 4 1) Moreover, both treatments failed to significantly alter basal levels of in-gel pro-MMP-2 activity in unstrained cells

A more complete evaluation of the role of Giα-protein activation in modulating strain-induced changes in pro-MMP-2 activity, was achieved by selective inhibition of Giα (Giα 1-3) subunits with dominant negative mutants for Giα proteins (Giα1-G202T, Giα2-G203T and Giα3-G202T) BAEC were transiently transfected with the appropriate mutants before the cells were exposed to cyclic strain Although difficulties in the transient transfection of some vascular cell types exist, we have successfully transfected BAEC with high efficiency (50%) as demonstrated by co-transfection with a plasmid expressing GFP or LacZ (Figure 4 3) Transfection with the mutant constructs enhanced the expression of the specific Giα subunit in the absence of any effect on the other G proteins as compared to mock controls (Figure 4 2) [58] Over expression of mutant Giα subunits failed to alter basal levels or cyclic strain-induced increases in pro-MMP-2 activity as compared to mock transfected cells (Figure 4 2)

4 2 2 Cyclic Strain Stimulates pro-MMP-2 Activity in a Gβγ-dependent Manner

To examine the possible role of Gβγ subunits in regulating MMP-2 expression, BAEC were transfected with β-Ark-ct, a 194-amino-acid peptide which functions by binding to and sequestering the Gβγ subunit, as previously described Transfection with β-Ark-ct resulted in a minor decrease in basal levels of pro-MMP-2 activity in conditioned media Following 5% strain however, levels of pro-MMP-2 activity were reduced by 83±3% (n=3 $P<0$ 05) as compared to mock transfected cells A similar result was also observed for pro-MMP-2 in cell lysates following over-expression of β-Ark-ct (Figure 4 3)

4 2 3 Cyclic Strain Increases ERK and p38 Activity in a Gi α -independent, Gβγ-dependent Manner

Due to the involvement of Ras and the MAPK family of enzymes in strained-induced processes, the effect of cyclic strain on MAPK activity was examined in BAEC by determining phospho-ERK-1, -ERK-2 and -p38 activity by western blot in these cells Exposure to 5% cyclic strain for 24 h resulted in a significant increase in phospho-ERK-1 and -ERK-2 activity 1 7 ± 0 13 fold (n=3 $P<0$ 05) as compared to unstrained cells (Figure 4 4) However, over-expression of inhibitory Giα mutant subunit Giα2-G203T or transfection with β-Ark-ct failed to significantly alter strain-induced phospho-ERK activity in these cells as compared to mock-transfected strained cells (Figure 4 4) In parallel studies, cyclic strain significantly increased phospho-p38 activity after 24 h exposure Furthermore, transfection with β-Ark-ct significantly decreased (56±18% n=3 $P<0$ 05) the strain-induced phospho-p38 activity as compared to mock-transfected strained cells (Figure 4 5)

4 2 4 Cyclic Strain Increases pro-MMP-2 Activity and Expression in a MAPK-dependent Manner

Following the observed cyclic strain-induced increases in both phospho-ERK

and -p38 activity in these cells, the effect of pharmacological inhibition of ERK and p38 activity on strain-induced changes in MMP-2 expression was determined (Figure 4 6) BAEC were exposed to 5% cyclic strain for 24 h in the absence or presence of either PD98059 (10μM) or PD169316 (10μM), specific inhibitors of MEK and p38 kinase, respectively [322, 323] Treatment of BAEC with either PD98059 or PD169316 treatment significantly decreased cyclic strain-induced increases in MMP-2 expression by 64% ± 17% (n=3 P<0 05) and 57 ± 8% (n=3 P<0 05) respectively, as compared to unstrained cells

4 2 5 Cyclic Strain Stimulates pro-MMP-2 Expression in an RGD-independent Manner

Activation of ERK1/2 and p38 MAPK is involved in cyclic strain-induced increases in pro-MMP-2 activity We investigated the upstream signaling mechanism(s) that lead(s) to the activation of ERK1/2 and p38 MAPK in response to cyclic strain In this regard, the relevance of integrins to the transduction of mechanical stimuli into intracellular signals is particularly significant and has been highlighted in a number of studies [Frangos *et al*, 2001] The RGD sequence is the target epitope of several selective integrin receptor inhibitors including blocking antibodies and inhibitory peptides

We examined the effects of synthetic linear and cyclic RGD peptides on cyclic strain-induced changes in MMP-2 expression and activity (Figure 4 7) Pre-treatment of cells with a linear RGD peptide (H-Arg-Gly-Asp-OH) failed to significantly inhibit cyclic strain-induced increases in pro-MMP-2 activity Pre-treatment of cells with a cyclic RGD peptide (Cyclo-Arg-Gly-Asp-D-Phe-Val) also failed to inhibit cyclic strain-induced pro-MMP-2 activity, despite being capable of inhibiting BAEC wound healing

4 2 6 Cyclic Strain Stimulates pro-MMP-2 activity in a Tyrosine Kinase-dependent Manner

Protein tyrosine kinase (PTK) phosphorylation is one of the most important events that lead to the transduction of extracellular signals to the nucleus A number of studies highlight the relevance of PTKs in mechanically stimulated processes in vascular cells [Anneren *et al*, 2003] Recent evidence also suggests that PTK may mediate their cellular responses through multiple intracellular signaling pathways involving cyto-skeletal associated proteins such as FAK and including the Shc/Grb2/Sos-ERK-2 pathway [Shyy *et al*, 2002]

We investigated the role of tyrosine kinase phosphorylation and of the Shc/MAPK pathway on cyclic strain-induced changes in MMP-2 expression (Figure 4 8) To understand whether PTK plays a role in the activation of ERK and MMP-2, BAEC were pre-incubated with 50μM genistein (a PTK inhibitor) before phospho-ERK activity and pro-MMP levels were determined Gemstein caused a significant reduction in cyclic strain-induced phospho-ERK-2 activity (83±8% n=3 $P<0$ 05) (Figure 4 9) with no significant changes observed in strain-mduced increases in phospo-P38 (Figure 4 10) Moreover, gemstein significantly attenuated cyclic stram-induced pro-MMP-2 activity (73±9% n=3 $P<0$ 05) without any significant effect on baseline unstrained levels

The adaptor protein Shc is an immediate substrate of tyrosine kinase and may play an important role in linking activated tyrosine kinases to downstream signaling pathways Indeed, Shc has previously been shown to activate the ERK pathway via tyrosine kinase Grb2/SOS signaling complexes [Shyy *et al*, 2002, Sayeski *et al* 2003] Elucidation of whether Shc directly mediates cyclic strain-induced MMP-2 expression, was examined by transfection of BAEC with Shc-SH2, a dominant negative adapter protein encoding the SH2 domain of Shc (Figure 4 11) Inhibition of Shc activity resulted in a significant reduction in cyclic stram-induced increases in phospho-ERK activity (88 ± 11% n=3 $P<0$ 05) concomitant with a significant decrease in both basal and strain-induced increases in pro-MMP-2 activity and expression (Figure 4 11)

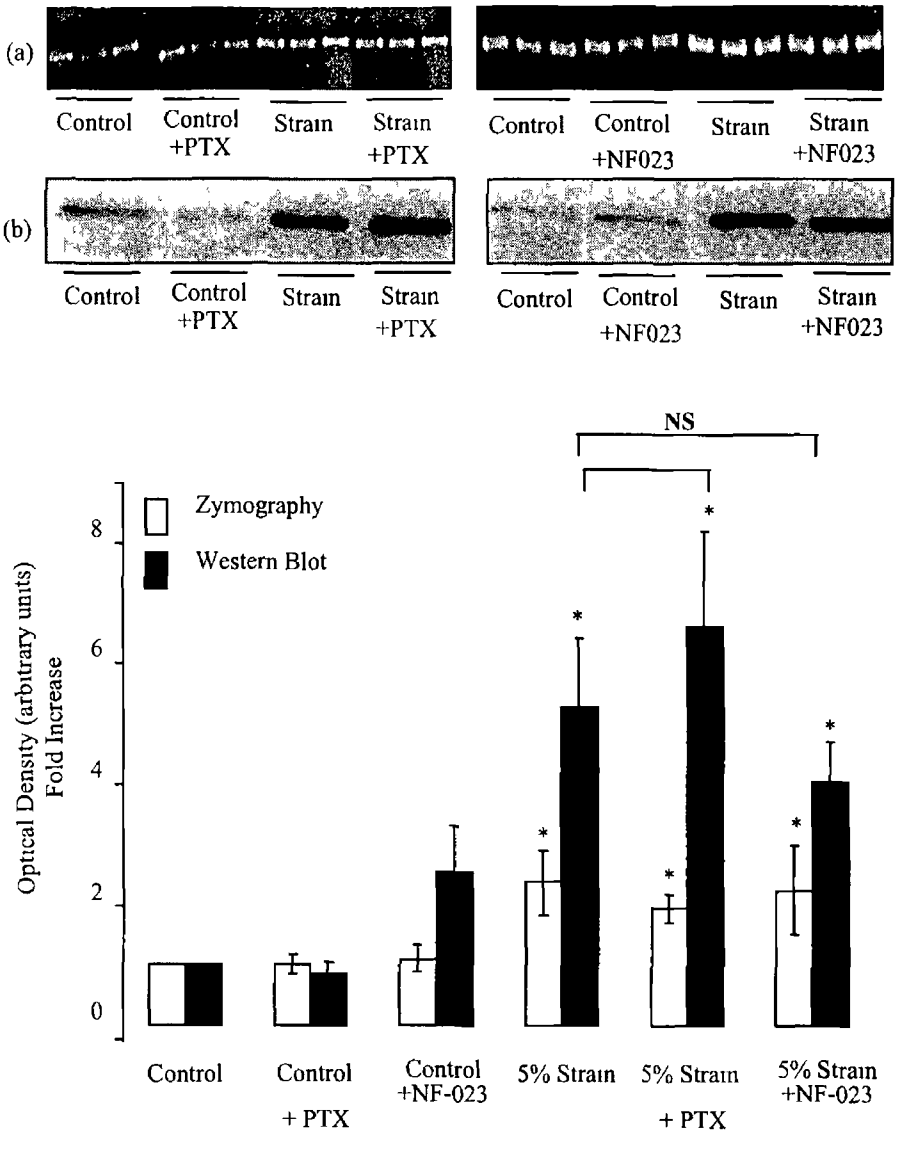


Figure 4 1 The effect of G-protein inhibitors on strain-induced increases in Pro-MMP2 activity and protein expression BAECs were pretreated with either pertussis toxin(100ng/ml) or NF023(10uM) prior to 5% cyclic strain for 24hrs (a) Zymography gels and Western blot (b) showing lack of effect of either PTX or NF023 on strain-induced increases in pro-MMP-2 activity or protein expression Histogram represents mean values from three independent experiments ± SEM, *p<0 05 compared to controls NS = not significant

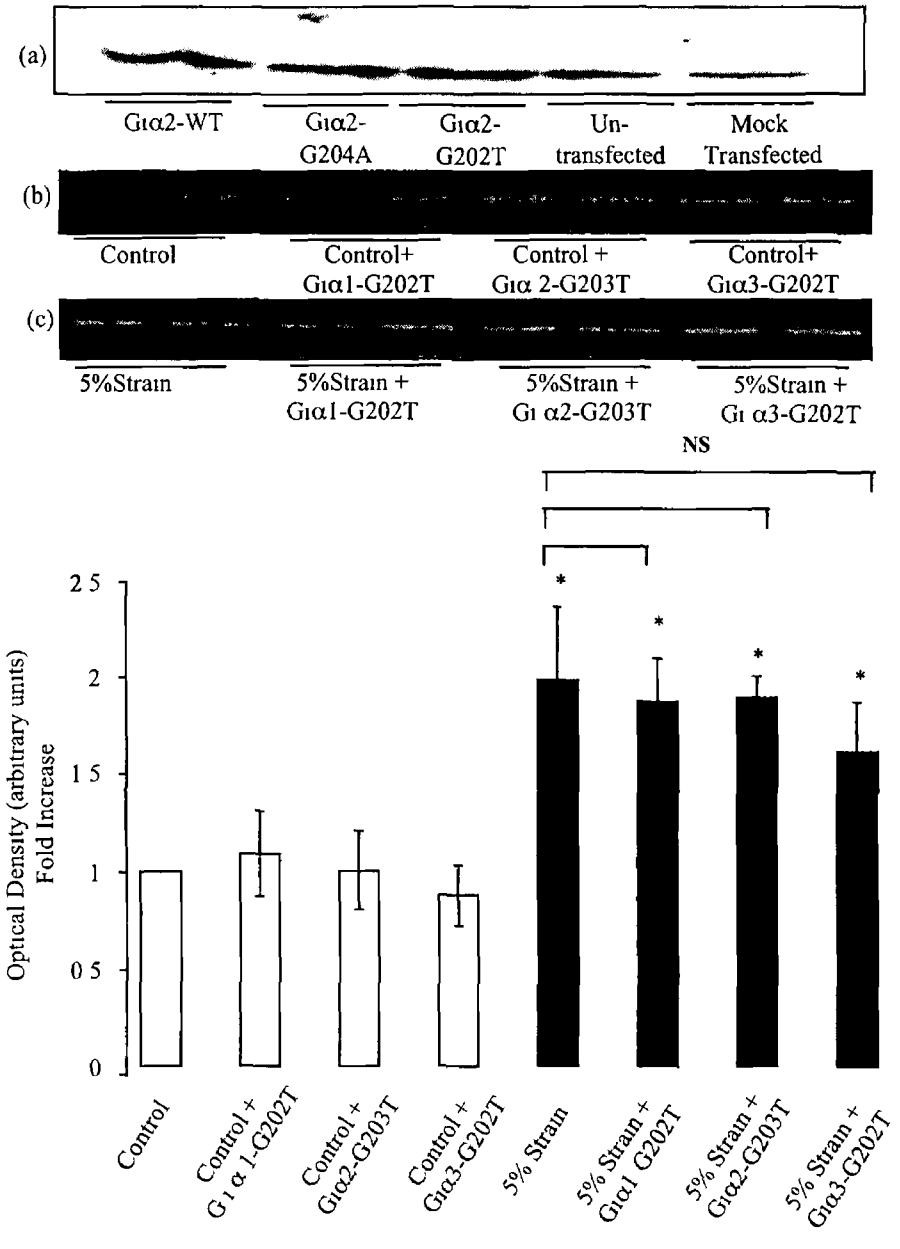


Figure 4 2 The effect of Gıα subunit inhibition on strain-induced increases in pro-MMP-2 activity BAECs were transfected as described in methods with Gı α1-G202T, Gıα2-G203T and Gıα3-G202T prior to exposure to 5% cyclic strain for 24hrs (a) Western blot showing overexpression of Gıα subunit following transfection (b-c) Zymograms showing the lack of effect of Gıα-subunit dominant negative mutants, Gıα1-G202T, Gıα2-G203T and Gıα3-G202T on strain-induced increases in pro-MMP-2 activity (conditioned media) following 5% strain for 24 h Histogram represents mean values from three independent experiments ± SEM, *p<0 05 compared to controls NS = not significant

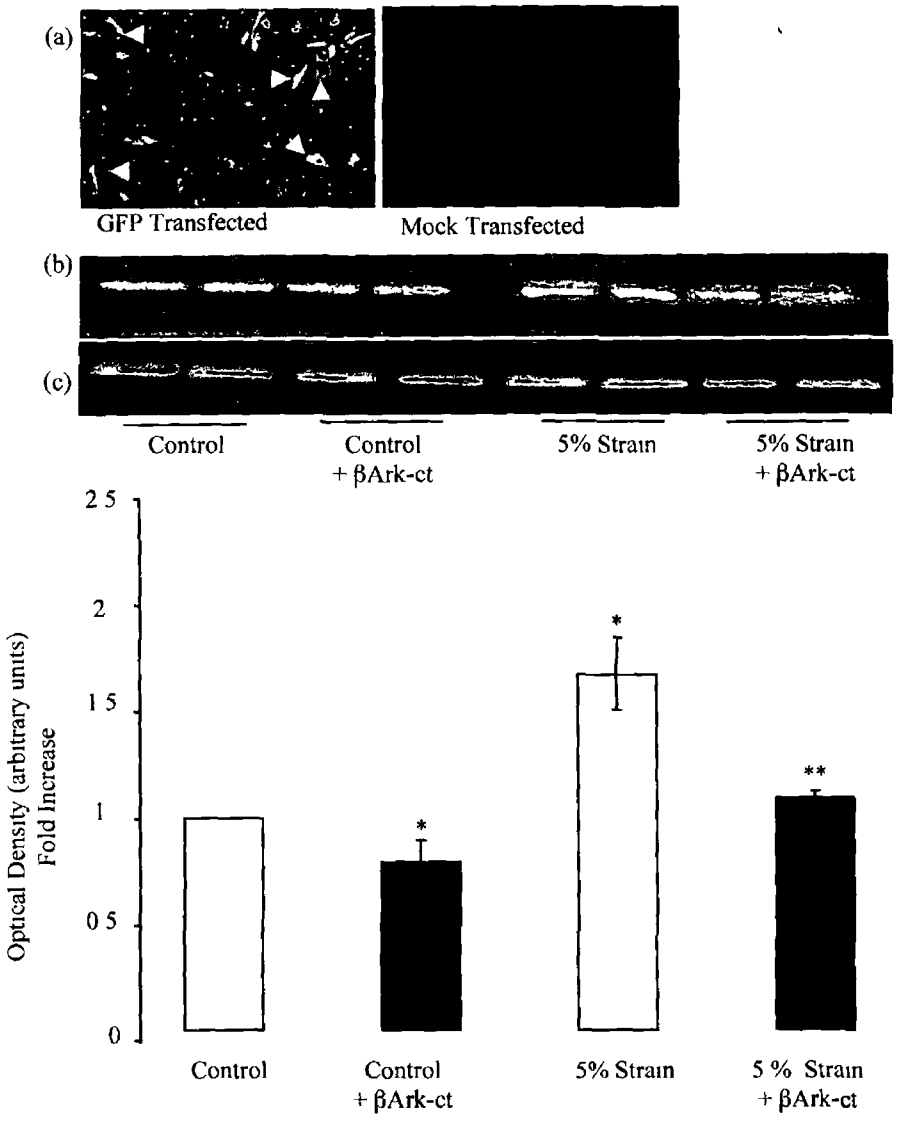


Figure 4.3 The effect of βark-ct transfection on strain-induced increases in pro-MMP2 activity BAECs were transfected as described in methods with βark-ct prior to exposure to 5% cyclic strain for 24hrs (a) Transfection effeciency was assesed by co-transfection with GFP, arrows indicate GFP transfected cells Representative zymogram of (b) cell lystae and (c) culture media Histogram represents densitometric analysis of (c) and mean values from three independent experiments ± SEM, *p<0 05 compared to controls **p<0 05 compared to 5%strain

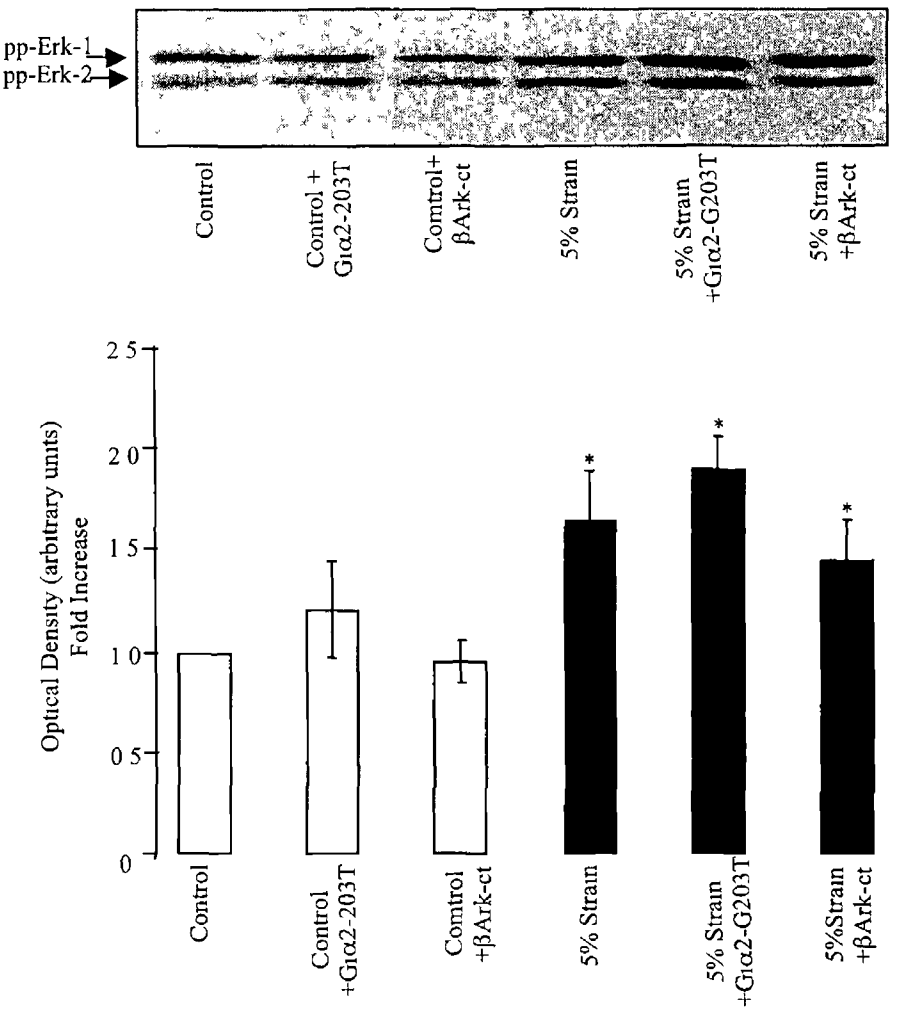


Figure 4 4 The effect of inhibitory Gια2 protein mutant and βArk-ct on cyclic strain-induced increases in ERK-1/2 activity BAEC were transfected with either Gια2-G203T, βArk-ct, or mock transfected The transfected cells were exposed to 0% (control) and 5% cyclic strain for 24 h (a) Representative Western blot shows the levels of phospho-ERK-1/2 in mock and transfected cells following exposure to 5% cyclic strain Histogram represents mean values from three independent experiments ± SEM, *p<0 05 compared to controls

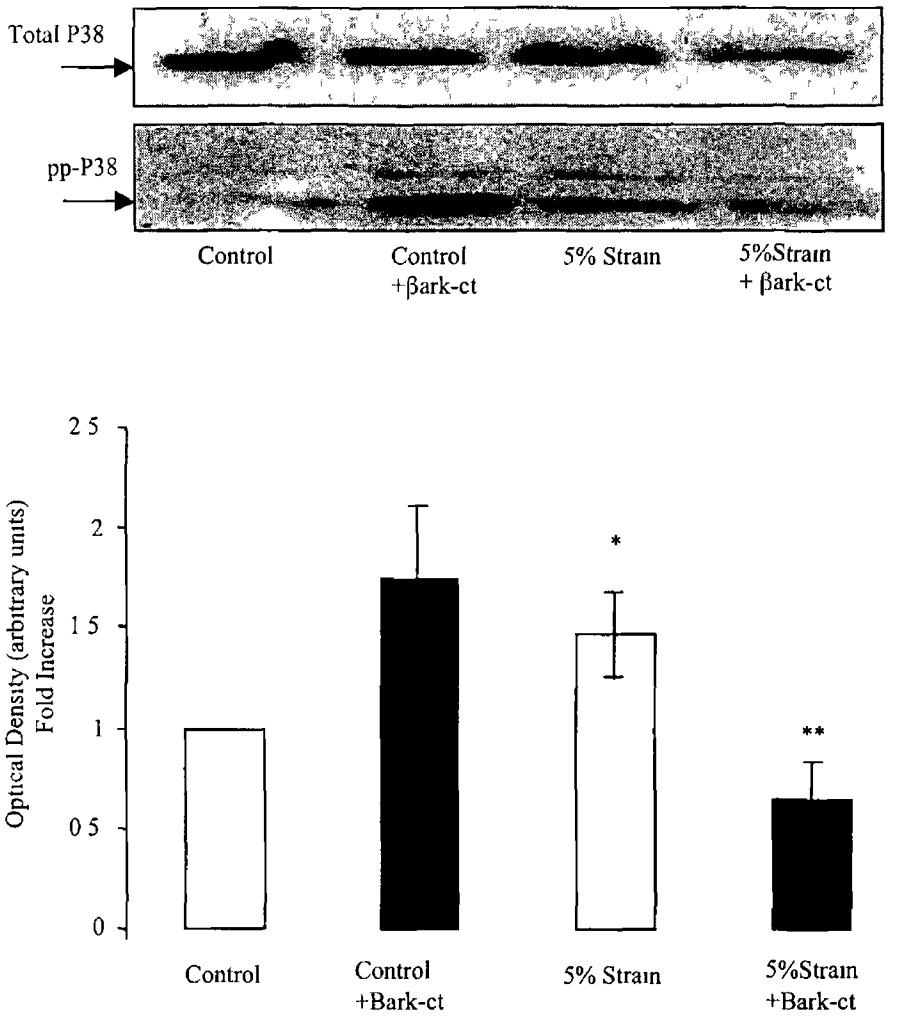


Figure 4 5 The effect of βArk-ct on cyclic strain-induced increases in p38 MAPK activity BAECs were transfected with βArk-ct or mock transfected The transfected cells were exposed to 0% (control) and 5% cyclic strain for 24 h (a)Phospho-p38 activity was determined by Western blot analysis using a phospho specific p38 antibody Histogram data represents ratio of phosphorylated to total p38 and represents mean values from three independent experiments ± SEM, *p<0 05 compared to controls, **p<0 05 compared to 5% cyclic strain

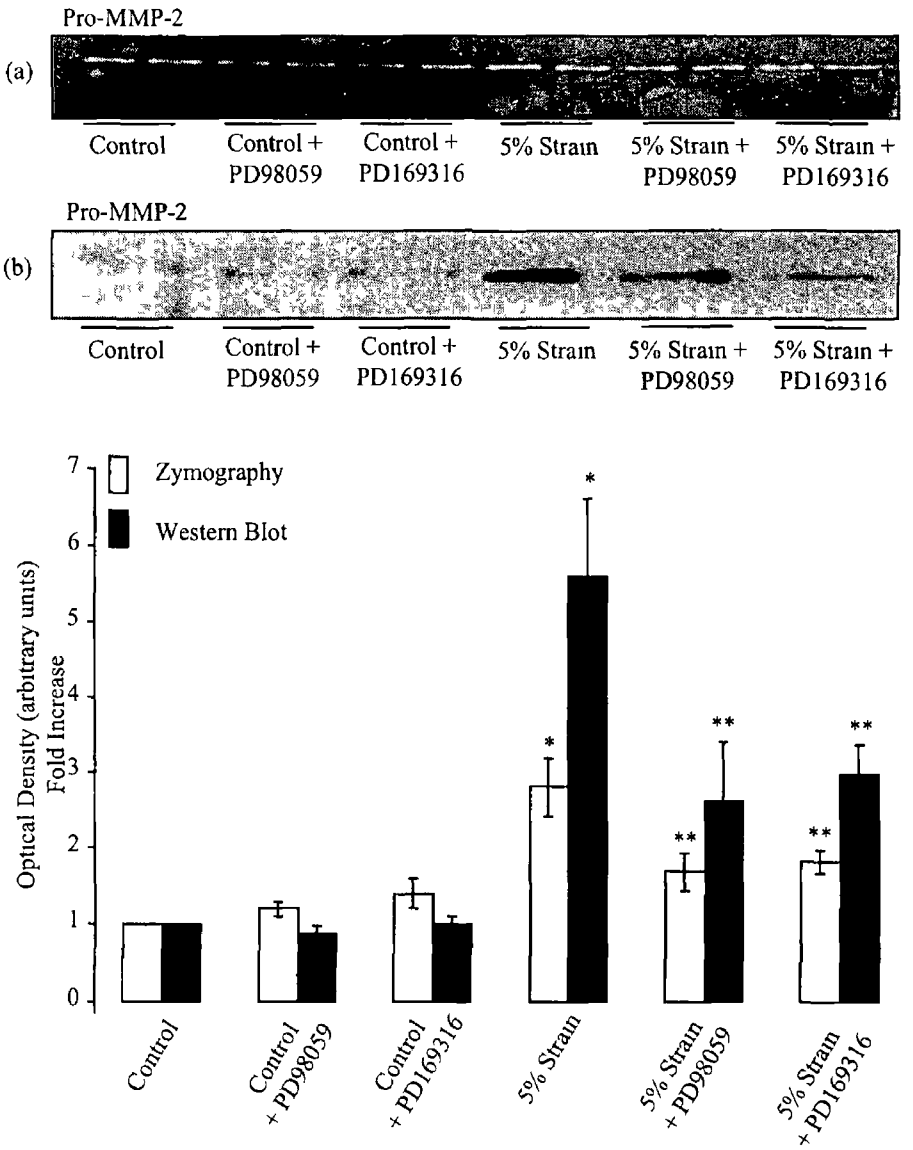


Figure 4 6 The effect of MAP kinase inhibition on strain-induced increases in Pro-MMP2 activity and protein expression BAECs were treated with either PD98059 (10μM) or PD169316 (10μM), following this, cells were exposed to either static of 5% cyclic strain conditions for 24hrs (a) Representative zymogram shows effect of inhibitors on pro-MMP-2 activity in conditioned media (b) Western blot for pro-MMP-2 protein in conditioned media following treatment with inhibitors Histogram represents mean values from three independent experiments ± SEM, *p<0 05 compared to controls, **p<0 05 compared to 5%Strain

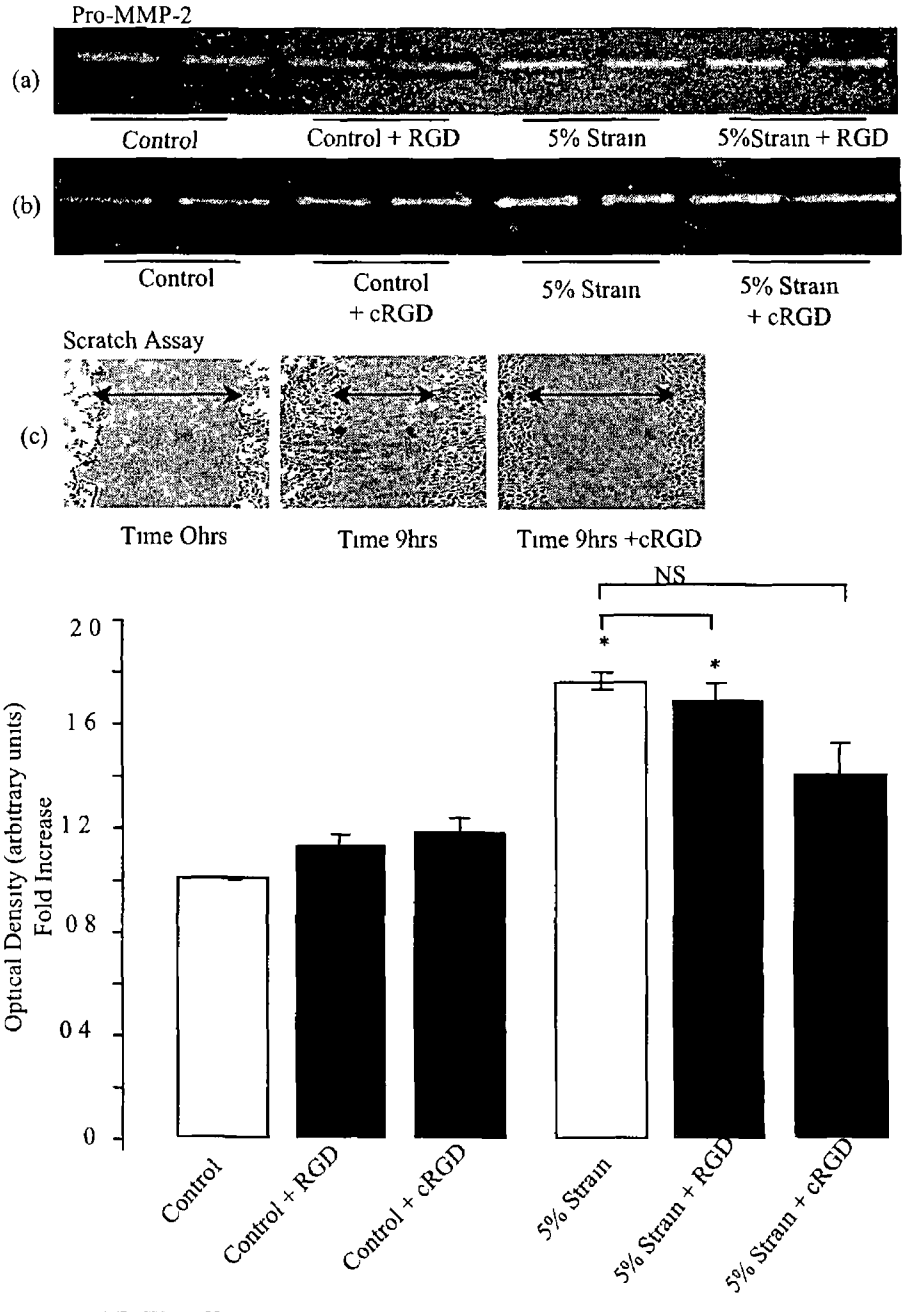


Figure 4 7 The effect of integrin blocking peptides on strain-induced increases in pro-MMP2 activity BAECs were pretreated with either (a) linear RGD peptide (0 5mM) or (b)cyclic RGD peptide (100μM) prior to 5% cyclic strain for 24hrs Representative zymograms show the detection of pro-MMP2 activity in the culture media Histogram represents densitometric analysis of zymograms and represents mean values from three independent experiments ± SEM,, *p<0 05 compared to controls cRGD peptide functionality was demonstrated in wound healing assay (c)

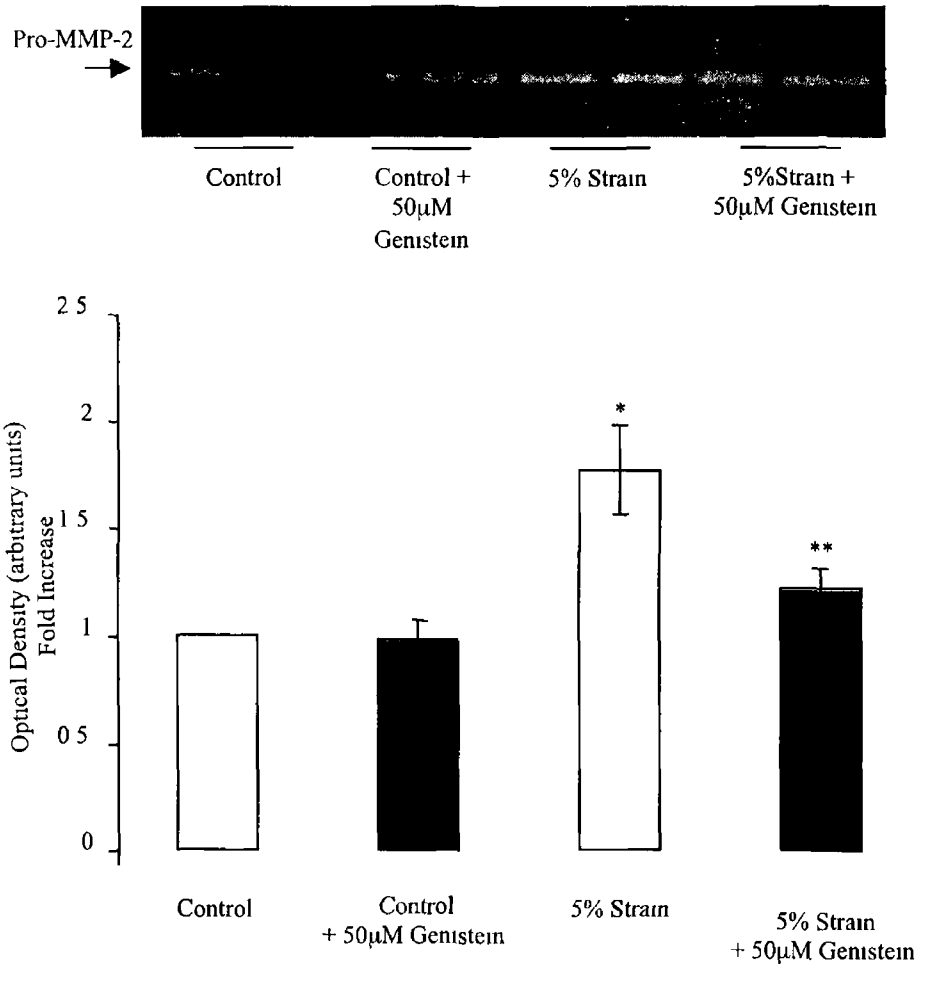


Figure 4 8 The effect of PTK inhibition on cyclic strain-induced increases in pro-MMP-2 activity BAEC were treated with 50μM gemstein and were exposed to 0% (control) and 5% cyclic strain for 24h (a) Representative zymogram show detection of pro-MMP-2 in culture media Histogram represents mean values from three independent experiments ± SEM, *p<0 05 compared to controls, **p<0 05 compared to 5% cyclic strain

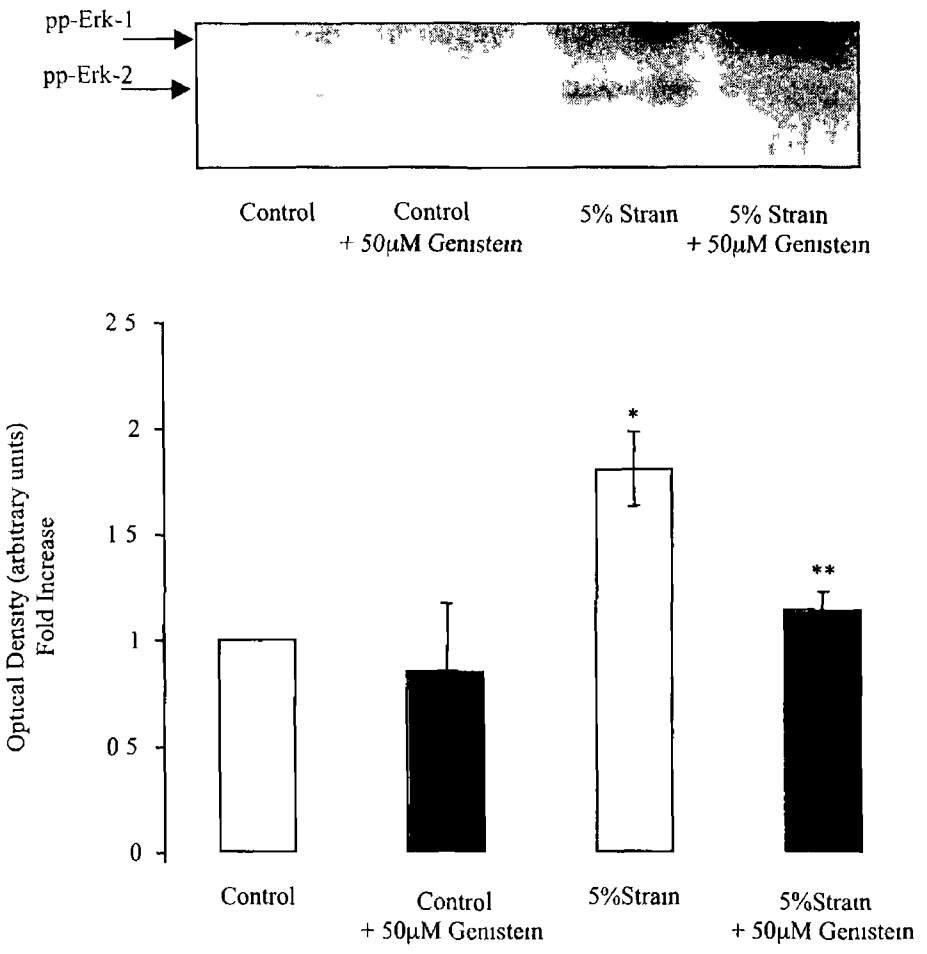


Figure 4 9 The effect of PTK inhibition on cyclic strain-induced increases in pp-ERK-1/2 BAEC were treated with 50μM genistein and were exposed to 0% (control) and 5% cyclic strain for 24 h ERK activity was then determined by Western blot using a specific phospho-ERK antibody (a) Representative Western blot of phospho-ERK-1/2 in control and genistein treated cells following exposure to cyclic strain Histogram represents mean data from three independent experiments ± SEM, *p<0 05 compared to controls, **p<0 05 compared to 5% cyclic strain

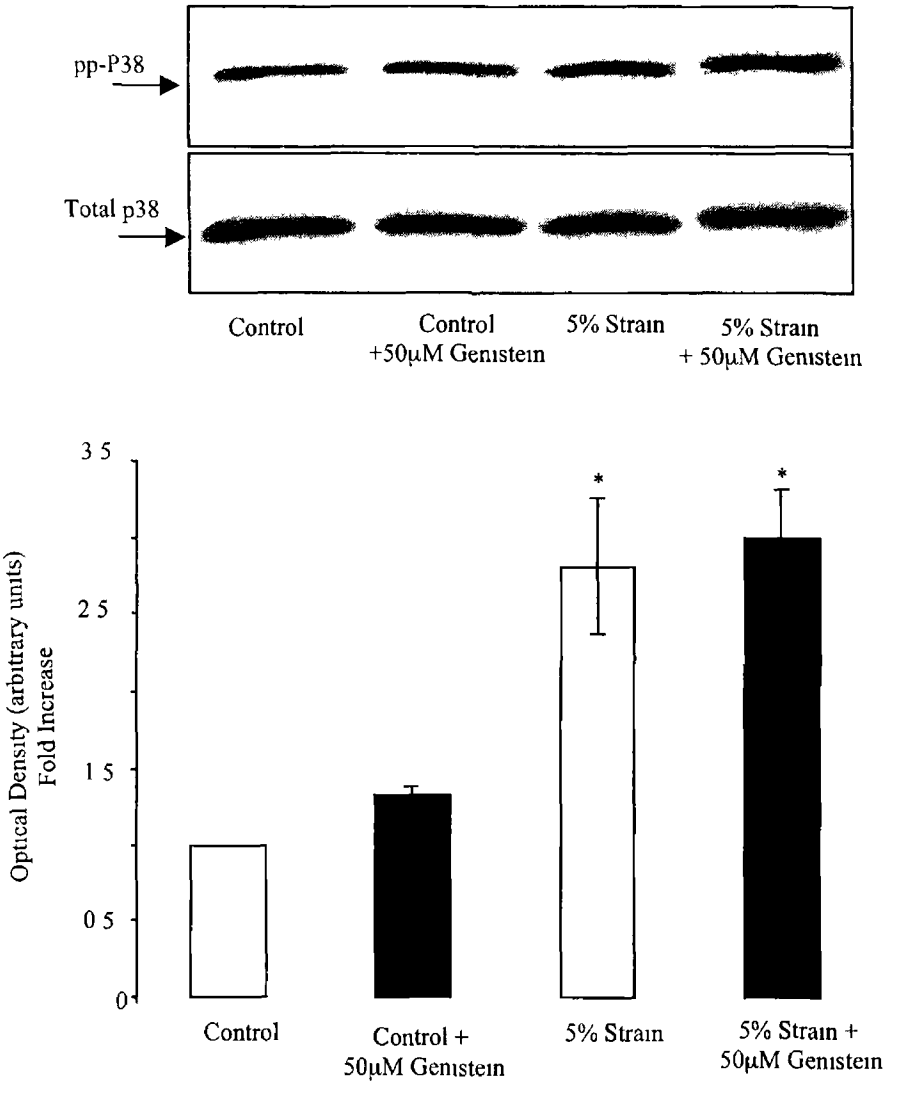


Figure 4 10 The effect of PTK inhibition on cyclic strain-induced increases in p38 MAPK. BAEC were treated with 50μM genistein and were exposed to 0% (control) and 5% cyclic strain for 24 h p38 activity was then determined by Western blot using a specific phospho-p38 antibody Representative Western blot of phospho-p38 in control and treated cells is presented Histogram represents ratio of phosphorylated to total p38 and represents mean values from three independent experiments ± SEM, *p<0 05 compared to controls, **p<0 05 compared to 5% cyclic strain

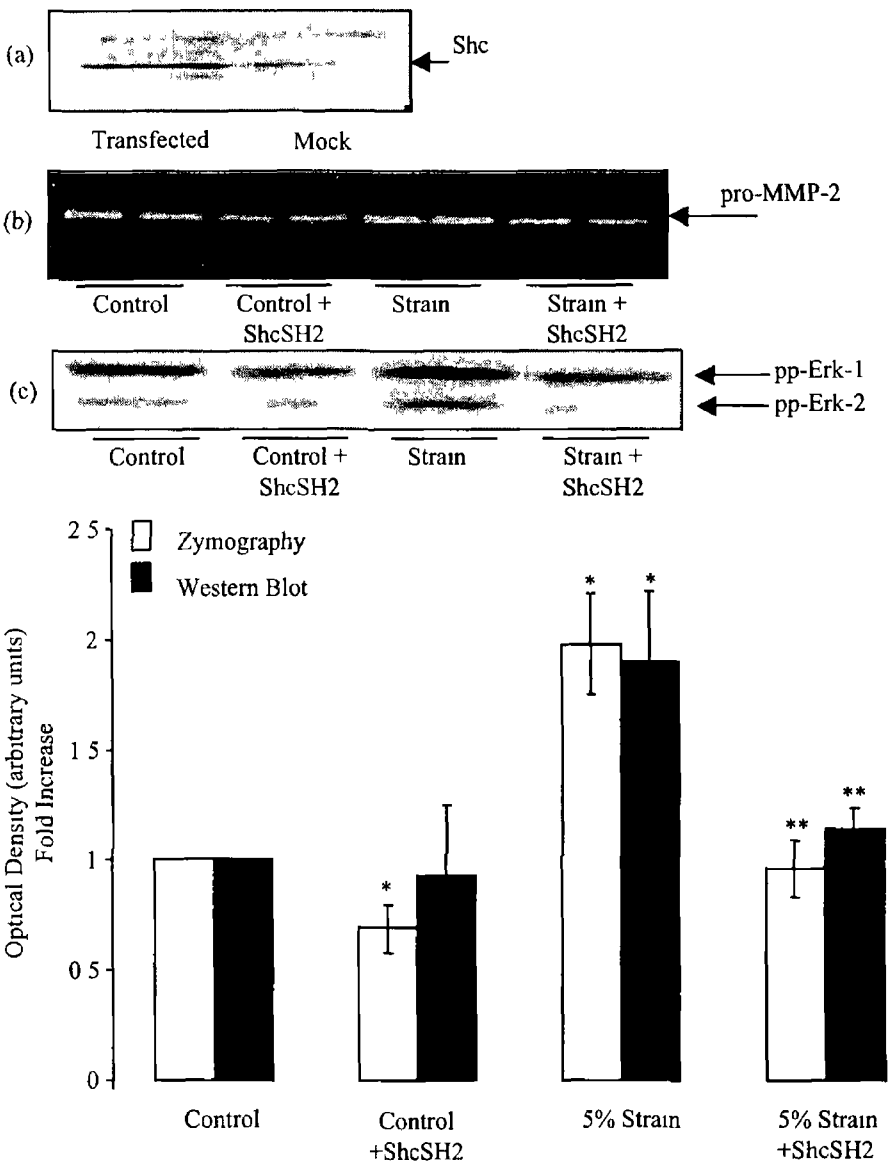


Figure 4 11 The effect of Shc inhibition on strain-induced increases in Pro-MMP2 BAECs were transfected as described in methods with Shc-SH2 (a dominant negative construct) The transfected cells were then exposed to static conditions or 5% cyclic strain for 24hrs (a) Western blot confirming overexpression of the Shc protein (b) Representative zymogram demonstrating inhibition of pro-MMP-2 activty following Shc-SH2 transfection (c) Representative Western blot revealed concomitant decreases in ppERK1/2 following Shc-SH2 transfection Histogram represents mean values from three independent experiments ± SEM, *p<0 05 compared to controls, **p<0 05 compared to 5% cyclic strain

4 3 Discussion

Structural adaptation of the vasculature occurs in response to both physiological and pathological changes in blood pressure and flow [Schwartz *et al*, 1995, Libby *et al*, 2003] We have already discussed at length the role of MMPs in vascular remodeling in section 1 5 8 2 We have clearly demonstrated that cyclic strain increases endothelial cell pro-MMP-2 activity and expression in a force- and time-dependent manner In this chapter we have investigated G-proteins, integrins, PTKs, Shc and the MAP kinases as possible components of the mechanical signalling cascade involved in mechano-regulation of MMP-2 in BAECs

With respect to Gi-proteins, previous studies have demonstrated the rapid activation of Gi-proteins in HUVEC by cyclic uniaxial strain in a time- and strain rate-dependent manner [Gudi *et al*, 2003] Moreover, pertussis toxin-sensitive G-proteins have been implicated in MMP production in several cell types [Conant *et al*, 2002, Guenzi *et al*, 2003] In the current study, selective ablation of individual Gi-protein subunits with dominant negative mutants suggests that strain-dependent upregulation of MMP-2 activity and expression was independent of Giα protein signaling These findings confirm our observations with pharmacological inhibitors of Giα proteins (PTX and NF023), and reinforce the lack of significant involvement of multiple Giα protein subunits in transduction of these events

Cyclic strain-induced pro-MMP-2 activity and expression was significantly attenuated following inhibition of Gβγ with βArk-ct implicating Gβγ signaling in transducing the strain response Recent studies suggest that the rapid, shear-induced activation of Ras is mediated through the activity of Gβγ subunits in human vascular EC [Gudi *et al*, 2003], suggesting that Gβγ activation of the Ras/ERK pathway could putatively mediate cyclic strain-induced increases in pro-MMP-2 However, this seems unlikely as the inhibitory effect of βArk-ct was independent of changes in downstream ERK activity In contrast, cyclic strain increased phospho-p38 activity in BAEC and inhibition of p38 activity with a selective inhibitor, PD169316, significantly reduced

strain-induced changes in pro-MMP-2 activity and expression Since inhibition of Gβγ functionality with βArk-ct resulted in inhibition of strain-induced phospho-p38, these data suggest that Gβγ proteins increase cyclic strain-induced MMP-2 levels, in part, via a p38-dependent pathway

Previously, two distinct and complementary signaling mechanisms mediating the induction of MMP's in fibroblasts have been reported AP-1-dependent transcriptional activation via the ERK1/2 pathway and AP-1-independent enhancement via p38 alpha MAPK by mRNA stabilization [Reunanen *et al*, 2002] As p38 inhibition attenuates strain-induced MMP-2 responses in BAECs, it is possible that activated Gβγ-subunits impact on stress activated protein kinase/*c*-jun N-terminal kinase (SAPKs/JNKs) and p38 pathways in these cells The recent findings of Wang *et al* (2003) are noteworthy in this regard as they demonstrate that selective JNK inhibition leads to ablation of strain-mediated increases in MMP-2 expression in HUVECs While SAPKs/JNKs and p38 are also activated by Gβγ-subunits in a pathway involving Rho proteins, including RhoA, Rac1 and Cdc42 [Tangkijvanich *et al*, 2003, Smith *et al*, 2003], inhibition of Rho kinase with specific inhibitors did not attenuate MMP-2 activity or expression in myofibroblasts [Tangkijvanich *et al*, 2003] In addition, since PTKs are also important regulators of MMP activity and expression in response to a variety of stimuli [Wagner *et al*, 2003, Wang *et al*, 2001] and are regulated by Gβγ subunits [Schmitt *et al*, 2002], it is possible that Gβγ signaling also affects PTK activity in these cells Moreover, because cell membrane-mediated MMP-2 activation also requires MT1-MMP and low amounts of TIMP-2 [Visse *et al*, 2003, Galis *et al*, 2002, Beaudeux *et al*, 2003] an effect of Gβγ subunits on MT1-MMP and TIMP-2 cannot be ruled out Further work will be required to delineate the precise mechanism of p38/Gβγ activation of MMP-2 in these cells following cyclic strain

Consistently, MMP activity and expression is inhibited following MAPK kinase (MEK) inhibition suggesting that ERK may also regulate strain-induced changes in MMP activity and expression [Kito *et al*, 2000, Visse *et al*, 2003, Galis *et al* 2002, Beaudeux *et al* 2003] Moreover, pharmacological inhibition with specific

ERK inhibitors significantly attenuated cyclic strain-induced changes in MMP-2 activity and expression, thereby confirming an important role for ERK in mediating these events

Integrins may serve as mechanosensors in EC leading to the activation of ERK or p38 activity in these cells Indeed, shear stress causes integrin-Shc association and assembly of the signaling complex that then leads to ERK activation [Shyy, *et al*, 2002, Labrador *et al*, 2003] Moreover, α5β1 and α2β1 integrins play an important role in transducing mechanical stimuli into intracellular signals in EC where cyclic strain led to a reorganization of α5 and α2 integrins in a linear pattern in HUVECs seeded on fibronectin or collagen, respectively [Shyy, *et al*, 2002, Labrador *et al*, 2003] In the current study, when BAEC were seeded on pronectin™ plates and exposed to cyclic strain, the strain-induced changes in pro-MMP-2 expression were unaffected following pre-treatment with linear or cyclic RGD peptides that selectively target the epitope of several integrin receptors Our data suggest that cyclic strain promotes pro-MMP-2 expression and activity in BAEC in an RGD-independent manner

Tyrosine kinases have been implicated in hemodynamic force-induced changes in EC function [Labrador *et al*, 2003] Shear stress induced a rapid and transient tyrosine phosphorylation of Flk-1 and its concomitant association with the adaptor protein Shc [Shyy, *et al*, 2002, Labrador *et al*, 2003] The adapter protein Shc is implicated in signaling via many different types of receptors, such as growth factor receptors, antigen receptors, cytokine receptors, G-protein coupled receptors, hormone receptors, integrins and tyrosine kinases [Ravichandran *et al* 2001] Based on dominant negative studies and ShcA-deficient mice, a clear role for Shc in leading to ERK activation has been established [Lopez-Ilasaca *et al*, 1998] Moreover, cyclic strain has been shown to induce PYK2 activity in EC [Cheng *et al*, 2002] Our findings are in agreement with studies demonstrating that stretch-induced increases in ERK activity were attenuated by inhibition of tyrosine kinases [Wang *et al*, 2001] Our data further demonstrated that inhibition of Shc signaling with a dominant

negative peptide significantly attenuated cyclic strain-induced changes in pro-MMP-2 expression and activity in BAEC while concomitantly inhibiting phospho-ERK activity in these cells As inhibition of ERK activity results in inhibition of strain-induced pro-MMP-2 expression, it is likely that PTK/Shc is responsible, in part, for the strain-induced pro-MMP-2 response in these cells Indeed, a role for tyrosine kinase in controlling MMP-2 expression following stimulation with magnesium has recently been reported [Yue *et al*, 2003] These data confirm the potential importance of PTK as mechanosensors for MMP-2 production in EC and suggest that protein tyrosine kinases may serve as mechanosensors to transduce mechanical stimuli into chemical signals via their association with Shc and ERK Indeed, cyclic strain has recently been shown to enhance contraction of bovine coronary arteries via through an epidermal growth factor receptor (EGFR)/srcdependent mechanism involving an NAD(P)H oxidase-mediated activation of ERK [Oeckler *et al*, 2003]

4 4 Conclusion

In conclusion, our data demonstrate that cyclic strain stimulates BAEC pro-MMP-2 activity and expression *in vitro* Moreover, the strain-induced increases in pro-MMP-2 activity were independent of Giα-protein activation but dependent on G$\beta\gamma$/p38, and PTK/ERK/Shc interactions in these cells It is tempting to speculate that strain-induced changes in endothelial MAPK signaling may functionally regulate endothelial cell phenotype *in vivo* by modulating MMP production following exposure to strain This possibility and potential targets for strain-induced endothelial MMP-2 remain to be determined

Chapter 5

5 1 Introduction

Angiogenesis occurs in an orderly sequence of events and is a tightly controlled process It requires an initiatory stimulus such as hypoxia, ischemia or changes in blood flow following which a strictly controlled set of events occurs The ability of mechanical forces to stimulate angiogenesis would strongly suggest that mechano-sensitive receptors such as G-proteins, integrins and PTKs may function in the regulation of angiogeneic processes The involvement of these mechano-receptors has been well established in a number of models [Slepian *et al*, 1998, Urbich *et al*, 2002,Gao *et al*, 2000, Benndorf *et al*, 2003, Kanda *et al*, 2000, Dallabrida *et al*, 2000, Kronenwett *et al*, 2002] We have previously demonstrated the regulatory effect of cyclic strain on BAEC migration and tube formation Here we examine the roles of G-proteins, integrins and PTKs in regulating cyclic strain-induced increases in these fate decisions

Many studies have demonstrated roles for MMPs in angiogenesis and migration of a number of cell types both *in vivo* and *in vitro* We have shown previously the mechanical regulation of MMP-2 and MMP-9 in BAEC Therefore, we have examined if MMP-2 and/or MMP-9 are involved in cyclic strain-induced angiogenesis and migration

Vascular endothelial cells due to their location in the blood vessel are subject to mechanical forces generated by blood flow These forces regulate the production of a variety of vasoactive compounds, cytokines and growth factors which may regulate cell function in a paracrine and autocrine manner [Awoleski *et al*, 1995, Wang *et al*, 2003, Wung *et al*, 2001, Sumpio *et al*, 1998, de Jonge *et al*, 2002] These molecules affect the phenotype of endothelial cells and the phenotype of the underlining SMC, this may help to explain why shear stress may cause vascular remodeling although SMC are not in direct contact with shear stress Due to their ability to interpret mechanical load and to elicit a cellular response EC may be responsible for regulating SMC function by producing vasoactive factors in response mechanical stimuli A number of studies have

demonstrated the importance of ECs in regulating SMC proliferation, migration and cell morphology [Cucina *et al*, 2003, Powell *et al*, 1998, Fillinger *et al*, 1997] We have already demonstrated cyclic strain increases levels of pro-MMP-2 and pro-MMP-9 and therefore, we have investigated the involvement of EC derived MMPs in SMC migration

The specific aims of this chapter were i) examine the roles of G-proteins, integrins and PTK in strain-induced BAEC migration and tube formation. ii) identify the involvement of MMP-2 and MMP-9 in these events iii) determine a possible paracrine role for BAEC derived MMPs on BASMC migration

5 2 Results

5 2 1 Cyclic strain increases BAEC migration in a Giα-protein-dependent, RGD/PTK-independent manner

The regulatory effects of cyclic strain on BAEC migration have been described in Chapter 3 (Figure 3 8, Figure 3 10) In this study, we have examined the roles of mechanically sensitive receptors in mediating this response To determine the role of Giα-proteins, integrins and PTKs in strain-induced BAEC migration, cells were pretreated with, PTX, cRGD peptide or genistein respectively, prior to exposure to 5% cyclic strain Pretreatment with either 100μM cRGD peptide or 50μM genistein did not result in any significant changes in BAEC migration (Figure 5 3 and Figure 5 5) However, Giα inhibition was found to significantly decrease basal (42±6% n=3 P<0 05) and strain-induced increases (117±17% n=3 P<0 05) in BAEC transwell migration (Figure 5 1)

5 2 2 Cyclic strain increases BAEC tube formation in a Giα-protein/RGD-dependent and PTK-independent manner

BAEC tube formation was monitored following the same treatments as above In this case it was found that Giα-protein inhibition also resulted in a significant (75±7% n=3 P<0 05) decrease in strain-induced tube formation (Figure 5 2) Similarly treatment with cRGD peptide caused a significant (87±8% n=3 P<0 05) decrease in strain-induced tube formation (Figure 5 4) This attenuation of tube formation may be attributed to inhibition of integrin interactions with the collagen matrix following treatment with the cRGD peptide The lack of an ECM matrix in the transwell migration assay may explain why integrin inhibition had no effect of strain-induced migration However, PTK inhibition with genistein did not have a significant effect on strain-induced tube formation (Figure 5 6)

5 2 3 Cyclic strain-induced increases in BAEC tube formation, but not migration, are MMP dependent

We have previously demonstrated that cyclic strain causes increase in pro-MMP-2 and pro-MMP-9 activity (Figures 3 1 and 3 3) with concurrent increases in migration and tube formation (Figures 3 8 – 3 10) To investigate a possible link between these two events we have used GM-6001, also known as Ilomastat, a broad-spectrum MMP inhibitor, with inhibition constants in the subnanomolar range for many MMPs We subjected cells to cyclic strain in the absence and presence of this inhibitor and assessed its effect on BAEC migration and tube formation It was found that treatment with this inhibitor significantly inhibited pro-MMP-2 activity as determined by zymography (Figure 5 7) Analysis of BAEC migration showed no significant changes in either basal or strain-induced events (Figure 5 7) However, treatment with GM-6001 caused a marked reduction (46±7% n=3 $P<0$ 05) in strain-induced tube formation (Figure 5 8) This data suggests that MMPs are involved in strain-induced BAEC tube formation but not transwell migration

5 2 4 Cyclic strain and shear stress-induced increases in BAEC migration and tube formation are independent of MMP-2

Following the observed decrease in tube formation after MMP inhibition with GM-6001, we ascertained the specific involvement of MMP-2 in cyclic strain and shear stress mediated increases in migration and tube formation siRNA targeted specifically at MMP-2 mRNA resulted in significant reduction in MMP-2 activity and expression as seen in Figure 5 9 Following transfection, cells were exposed to cyclic strain or shear stress and their migratory and angiogenic profile assessed Inhibition of MMP-2 was subsequently not found to have significantly reduced either cyclic strain (Figures 5 9-5 10) or shear stress-induced (5 11-5 12) increases in migration or tube formation

5 2 5 Cyclic strain increases BAEC migration and tube formation are MMP-9-dependent

MMP-2 knockdown was not found to be involved in cyclic strain induced migration and tube formation Previous studies have indicated the involvement of MMP-9 in angiogenesis [Pepper *et al*, 2001, Johnson *et al*, 2004], our study has shown that pro-MMP-9 activity was up-regulated in response to 5% cyclic strain, therefore, we investigated the possible role of MMP-9 in strain-induced migration and tube formation Cells were transfected with siRNA targeted to MMP-9 mRNA prior to exposure to 5% cyclic strain Following transfection, MMP-9 expression and activity was markedly reduced (Figure 5 13) Transfection with MMP-9 siRNA did not cause a significant decrease in strain-induced migration (Figure 5 14) However, a significant (40±10% n=3 $P<0$ 05) decrease in BAEC tube formation was observed (Figure 5 15) This decrease is similar to that observed following treatment with GM-6001 which may suggest that MMP-9 was the target of GM-6001 inhibition

5 2 6 Cyclically strained endothelial cell conditioned media inhibits SMC migration

Hemodynamic forces associated with blood flow play a critical role in endothelium-mediated control of vascular tone, remodelling and associated pathologies The vascular endothelial cell (EC) monolayer, by virtue of its unique location, constitutes a dynamic interface between the vessel wall and bloodstream, regulating the physiological balance between vessel wall remodelling processes and hemodynamic forces Consequently, EC-mediated mechanotransduction may impact on vascular smooth muscle cell (SMC) fate decisions such as migration and proliferation We have therefore investigated the possible role of MMP-2 released from BAEC during exposure to cyclic strain in mediating BASMC fate decisions

To investigate this hypothesis we used EC conditioned media as a chemoattractant in BASMC transwell migration assays We found that BASMC migration was markedly reduced (40 ± 5% n=3 $P<0$ 05) when conditioned media from

strained EC was used as a chemo attractant when compared to media from static controls (Figure 5 16)

5 2 7 Cyclic strain conditioned media inhibition of SMC migration is MMP-2 but not MMP-9 dependent

The data in section 5 2 6 indicates that conditioned media from cyclically strained BAEC had an inhibitory effect on BASMC migration Here we attempt to elucidate the involvement of MMP-2 in these events Our data demonstrates that inhibition of MMP-2 activty by either treatment with gensitein or targeted siRNA 'knockdown' of MMP-2 expression and activity in BAECs prior to strain was found to completely reverse the inhibitory effects of conditioned media on BASMC migration relative to controls (Figure 5 17 and Figure 5 18) Conversely, inhibition of MMP-9 with siRNA knockdown had no observed effects on BASMC migration (Figure 5 19)

5 2 8 The involvement of MMP-2 in the inhibition of SMC migration occurs during cyclic strain

To characterize the role of MMP-2 in mediating the inhibition of BASMC migration we examined baseline BASMC transwell migration in the absence or presence of recombinant MMP-2 in a concentration range of 0 – 100ng/ml (Figure 5 20) The addition of MMP-2 in this concentration range had no effect on BASMC migration Furthermore, reversal of the siRNA effect on BASMC migration was not observed when MMP-2 depleted conditioned media was supplemented with active recombinant MMP-2 (100ng/ml) prior to the BASMC transwell migration assay (Figure 5 21) These data suggest that MMP-2 is not directly involved in the BASMC migration event, rather, it is indirectly involved at the BAEC level, most likely during straining

To investigate this hypothesis BAECs which were transfected with MMP-2 siRNA were exposed to 5% cyclic strain in the absence or presence of active

recombinant MMP-2 The presence of recombinant MMP-2 during the strain regime resulted in a reversal of the siRNA effect (75 ±10% n=3 $P<0.05$) on BASMC migration (Figures 5 22a & b)

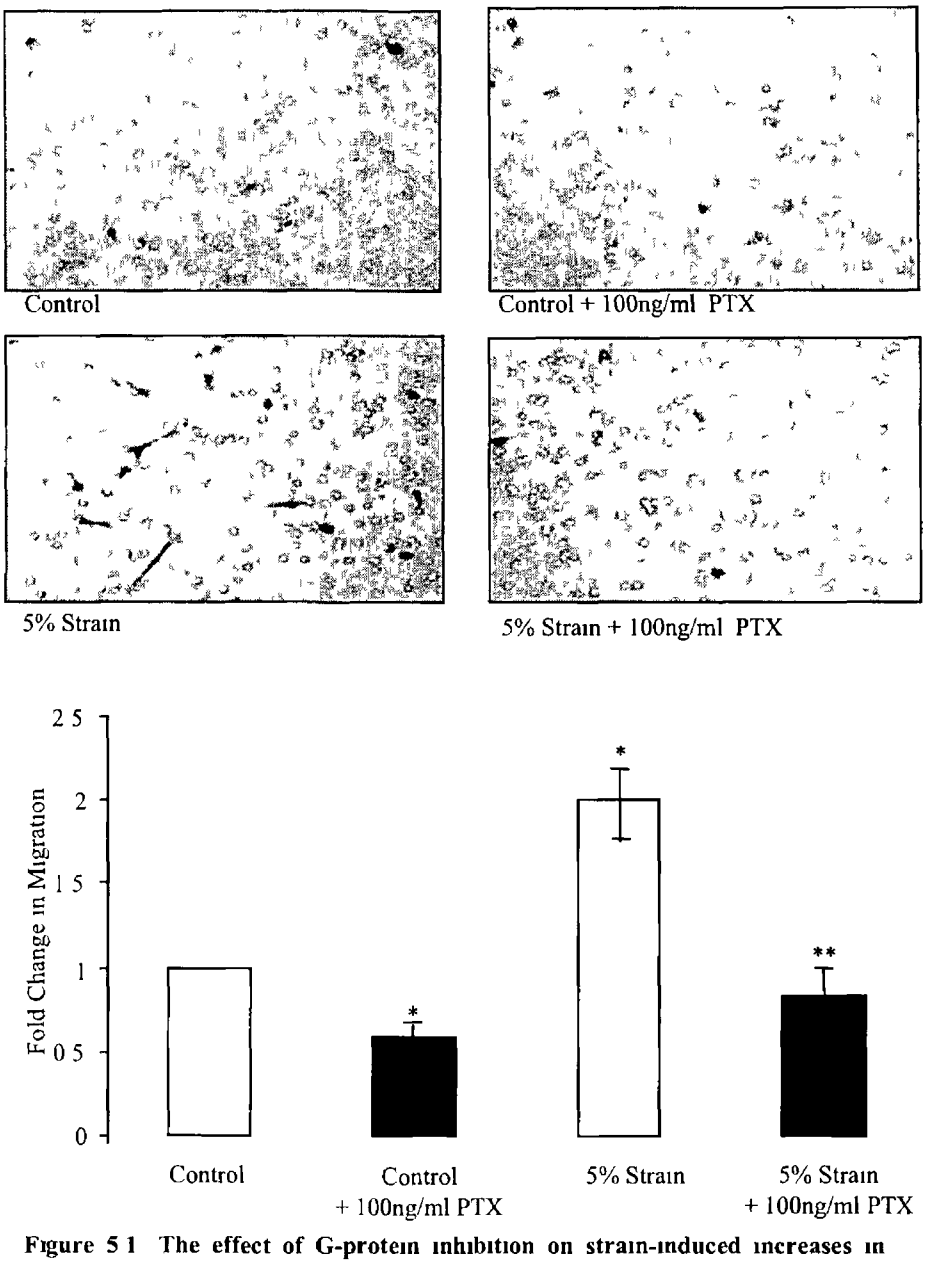


Figure 5 1 The effect of G-protein inhibition on strain-induced increases in BAEC migration Force-dependent increase in BAEC migration following exposure to 0% (control) or 5% cyclic strain in the absence or presence of pertussis toxin (100ng/ml) Cells were exposed to strain for 24 h prior to transwell migration assay BAEC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of control Representative hpf from each treatment is shown and arrows indicate the presence of migrated cells Histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls **P<0 05 compared to 5%Strain

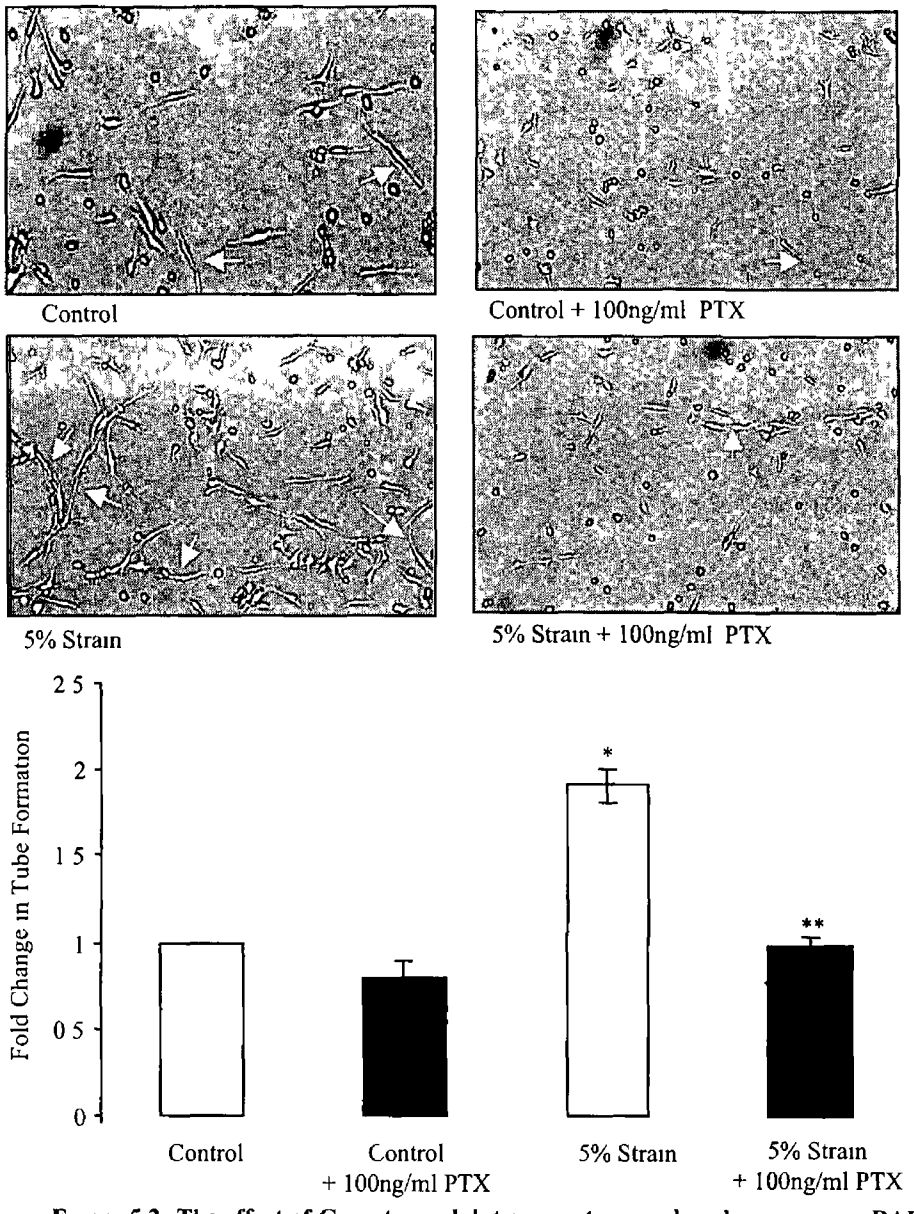


Figure 5 2 The effect of G-protein inhibition on strain-induced increases in BAEC tube formation Force-dependent increase in endothelial cell tube formation following exposure to 0% or 5% cyclic strain in the presence or absence of pertussis toxin (100ng/ml) Cells were exposed to strain for 24 h prior to tube formation assay Results are expressed as tubule formation on collagen where four random fields of vision were photographed from each gel and the mean length of tube formation was quantified by measuring the length of the network of connected cells in each well using NIH image Representative fields of vision are shown for each treatment and arrows indicate tubule formation Results are expressed as fold change relative to control unstrained cells and histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls **P<0 05 compared to 5%Strain

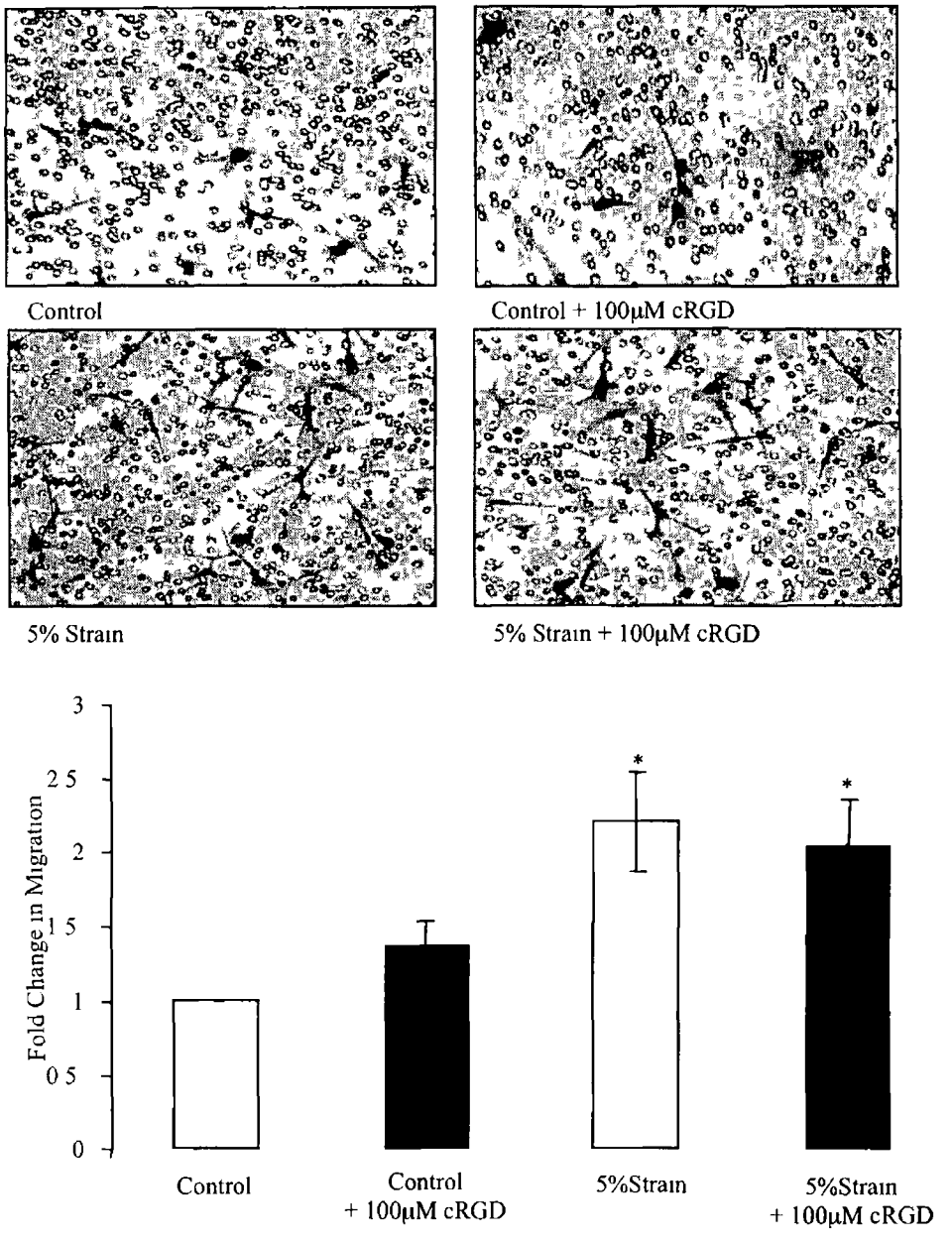


Figure 5 3 The lack of effect of integrin blocking peptide on strain-induced increases in BAEC migration Force-dependent increase in BAEC migration following exposure to 0% (control) or 5% cyclic strain in the absence or presence of cRGD peptide (100μM) Cells were exposed to strain for 24 h prior to transwell migration assay BAEC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of control Representative hpf for each treatment is shown and arrows indicate the presence of migrated cells Histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls

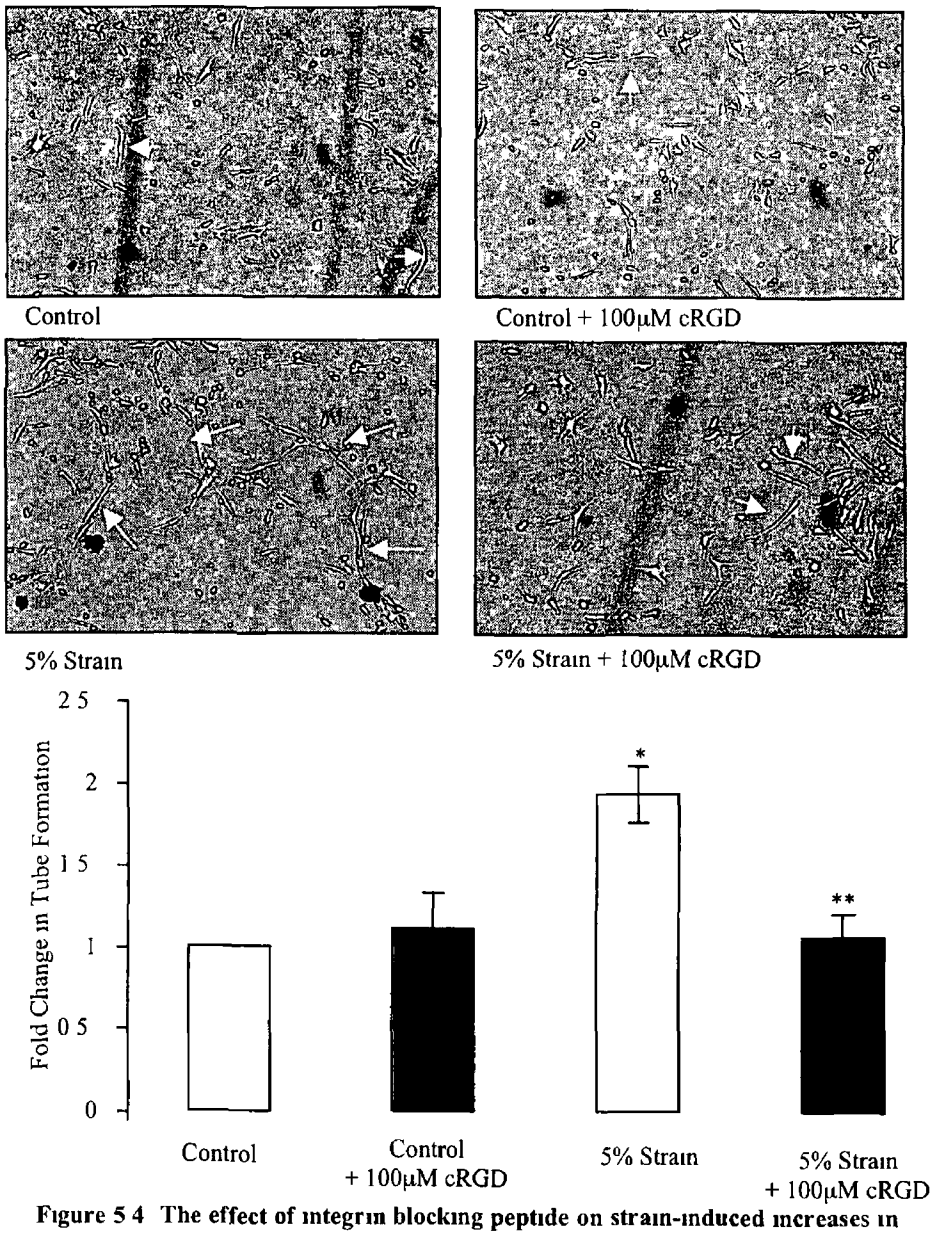


Figure 5 4 The effect of integrin blocking peptide on strain-induced increases in BAEC tube formation Force-dependent increase in endothelial cell tube formation following exposure to 0% or 5% cyclic strain in the presence or absence of cRGD peptide(100μM) Cells were exposed to strain for 24 h prior to tube formation assay Results are expressed as tubule formation on collagen where four random fields of vision were photographed from each gel and the mean length of tube formation was quantified by measuring the length of the network of connected cells in each well using NIH image Representative fields of vision are shown for each treatment and arrows indicate tubule formation Results are expressed as fold change relative to control unstrained cells and histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls **P<0 05 compared to 5%Strain

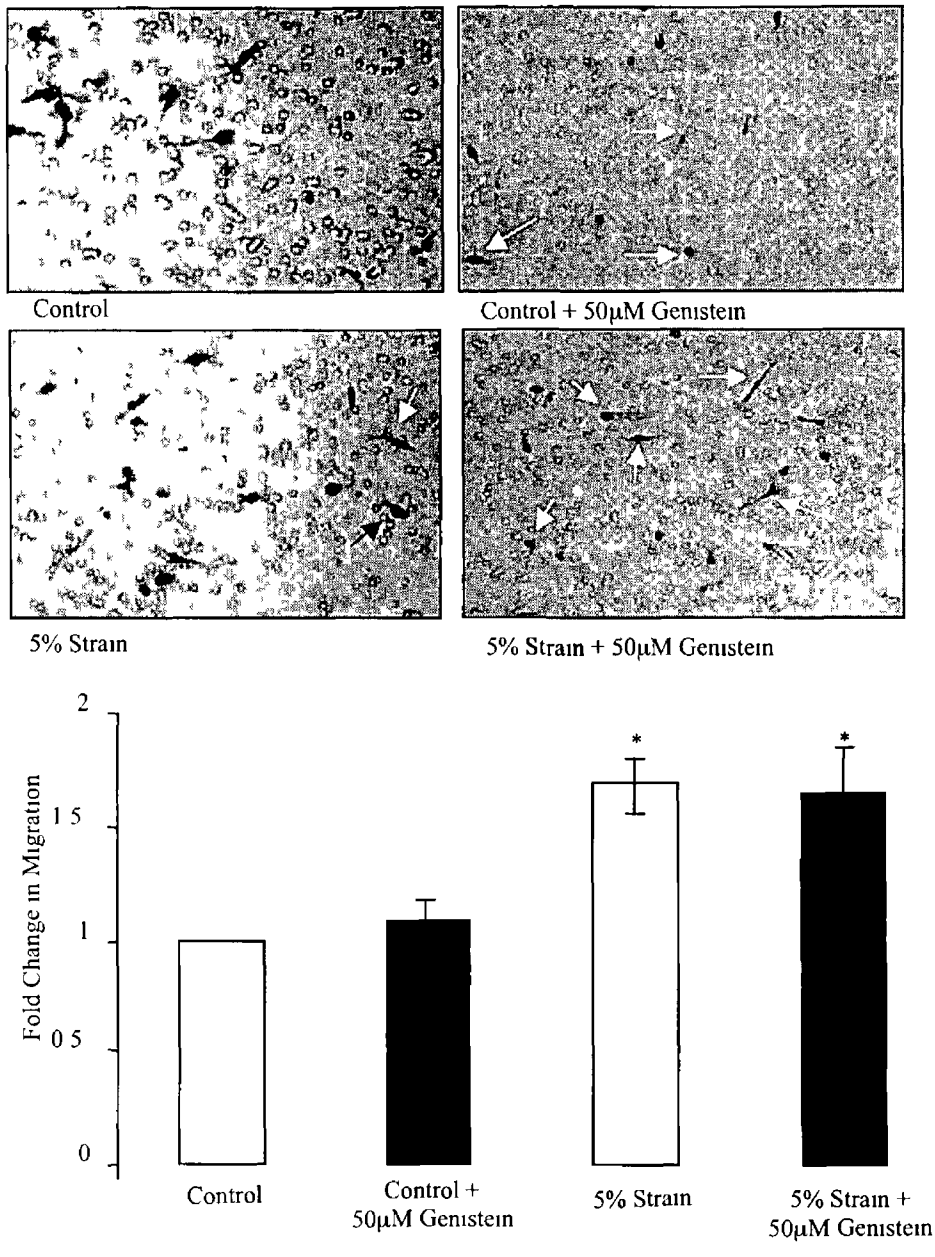


Figure 5 5 The lack of effect of genistein on strain-induced increases in BAEC migration Force dependent increase in BAEC migration following exposure to 0% (control) or 5% cyclic strain in the absence or presence of genistein (50μM) Cells were exposed to strain for 24 h prior to transwell migration assay BAEC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of control Representative hpf of each treatment are shown and arrows indicate the presence of migrated cells Histogram represents mean values from three independent experiments± SEM, *$P<0$ 05 compared to controls **$P<0$ 05 compared to 5%Strain

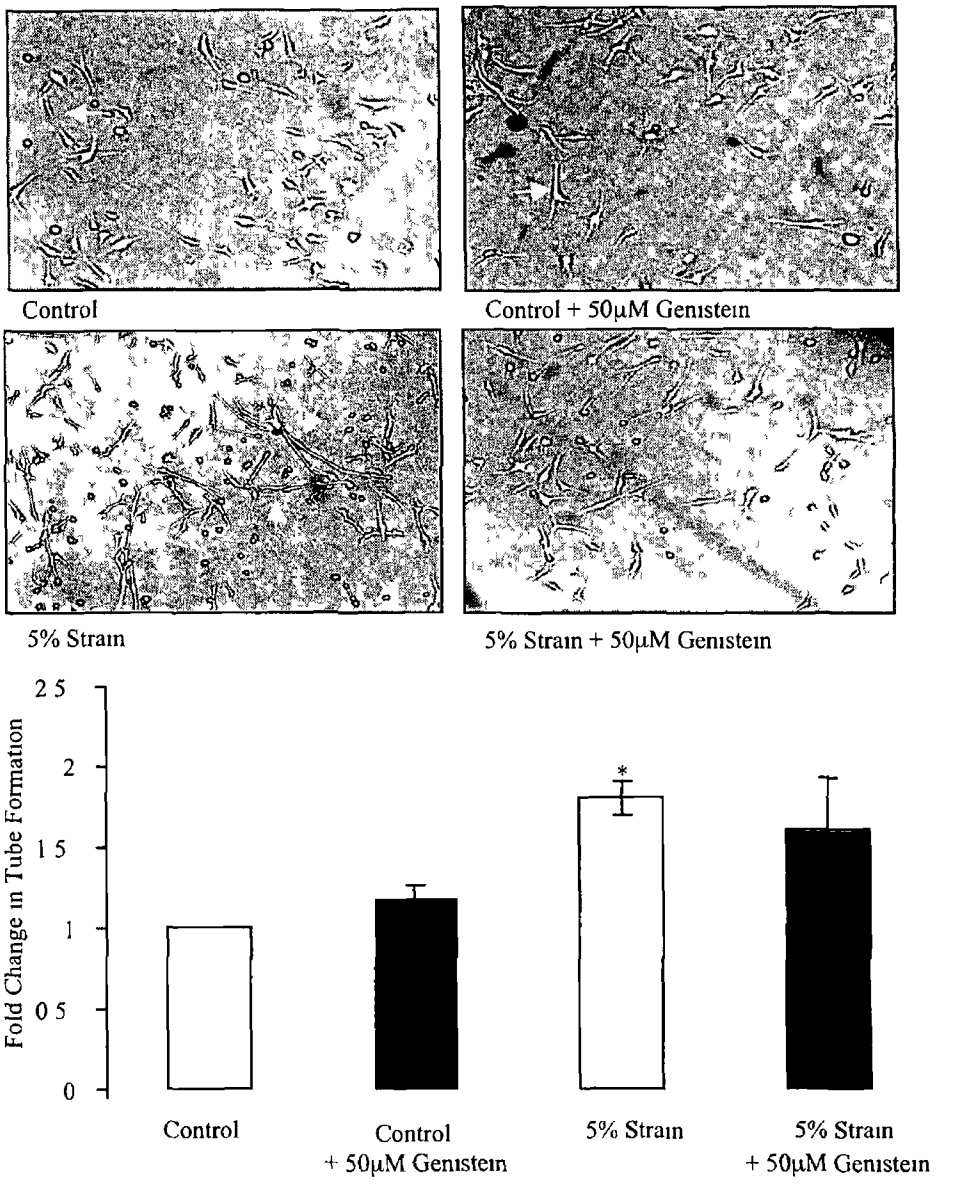


Figure 5 6 The lack of effect of genistein on strain-induced increases in BAEC tube formation Force-dependent increase in endothelial cell tube formation following exposure to 0% or 5% cyclic strain in the presence or absence of gensitein (50μM) Cells were exposed to strain for 24 h prior to tube formation assay Results are expressed as tubule formation on collagen where four random fields of vision were photographed from each gel and the mean length of tube formation was quantified by measuring the length of the network of connected cells in each well using NIH image Representative fields of vision are shown for each treatment and arrows indicate tubule formation Results are expressed as fold change relative to control unstrained cells and histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls

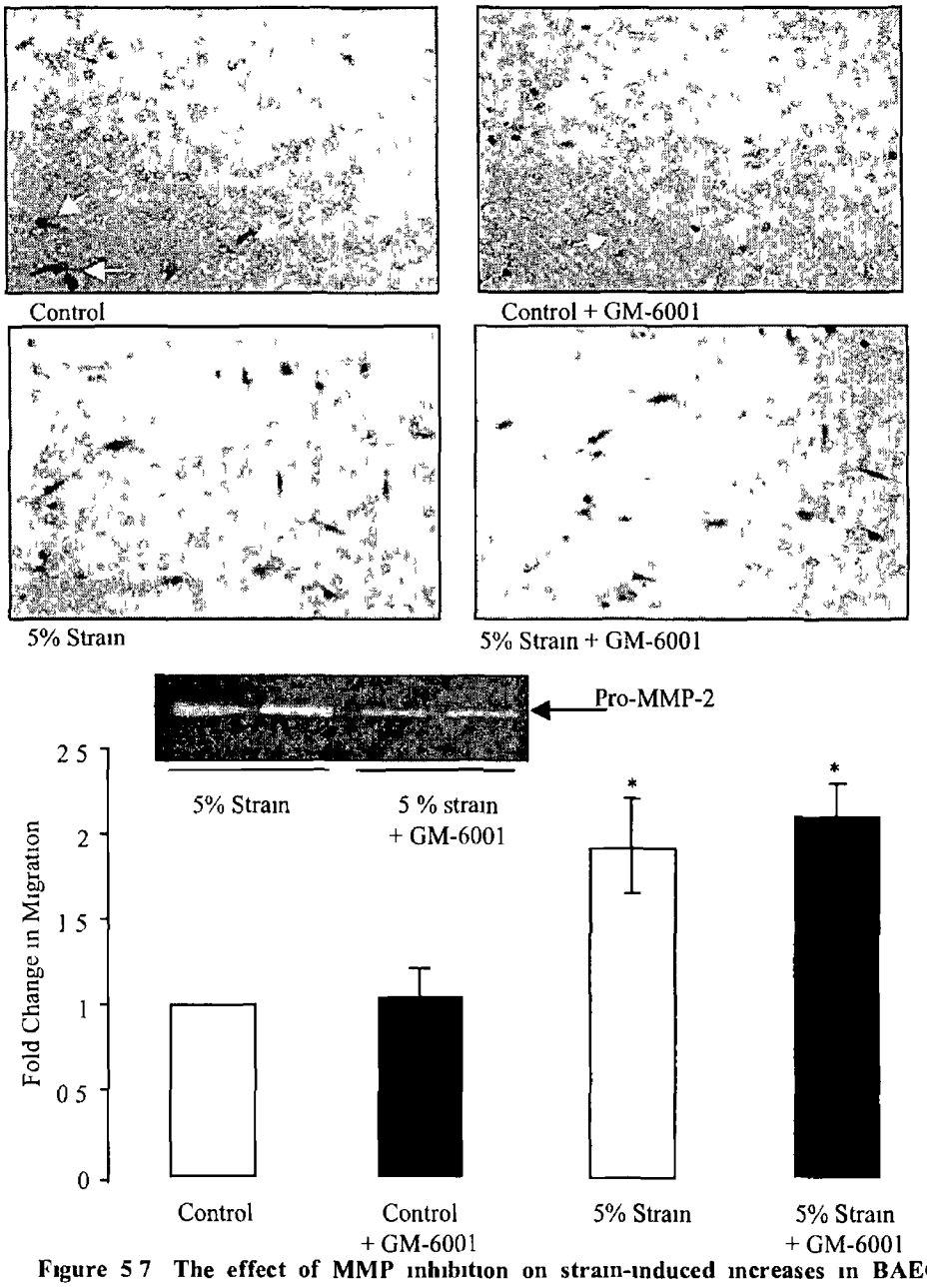
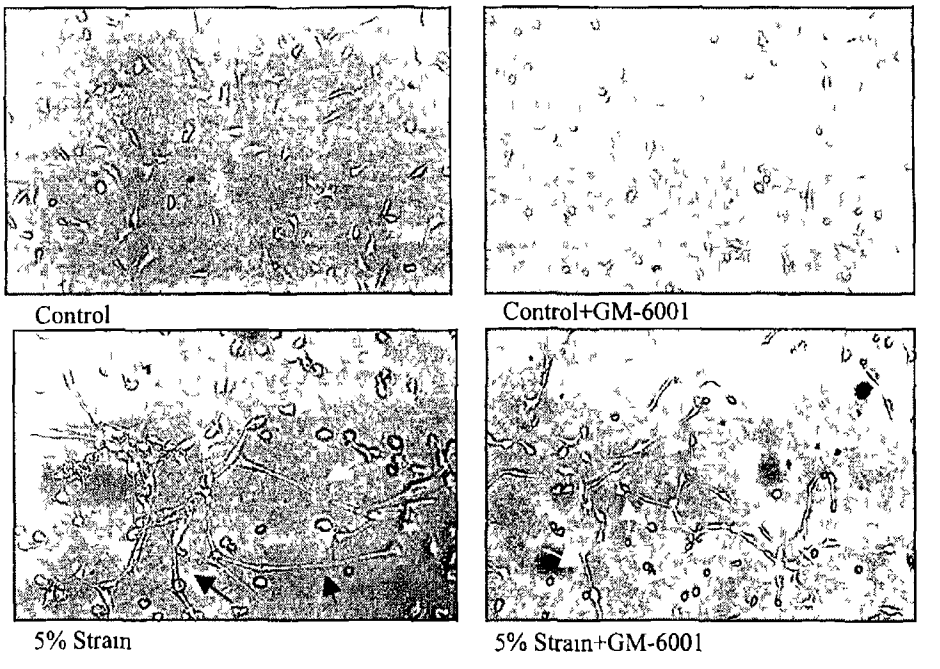


Figure 5 7 The effect of MMP inhibition on strain-induced increases in BAEC migration Force-dependent increase in BAEC migration following exposure to 0% (control) or 5% cyclic strain in the absence or presence of GM6001 Cells were exposed to strain for 24 h prior to transwell migration assay BAEC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of control Representative hpf from each treatment is shown and arrows indicate the presence of migrated cells Representaive zymogram demonstrates inhibition of pro-MMP-2 activity in conditioned media following treatment with GM-6001 Histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls



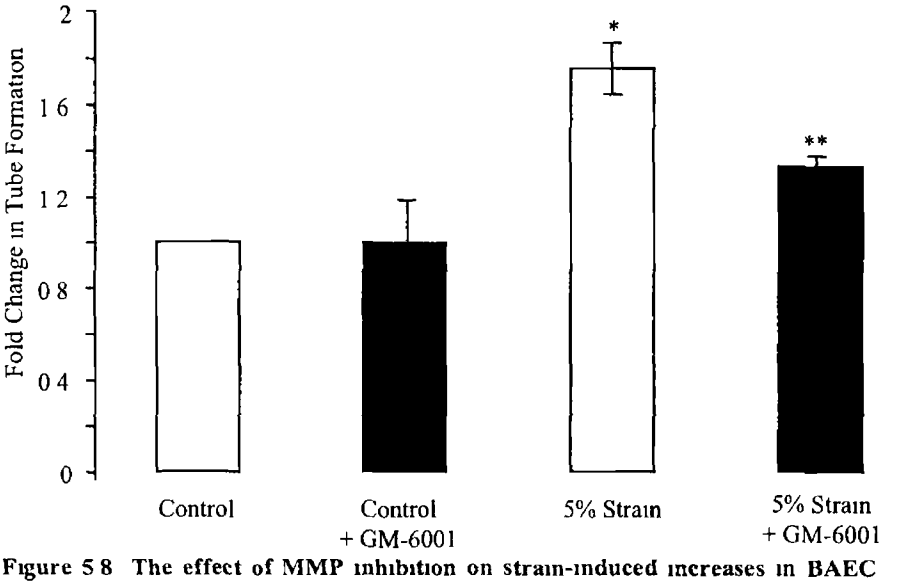


Figure 5 8 The effect of MMP inhibition on strain-induced increases in BAEC tube formation Force-dependent increase in endothelial cell tube formation following exposure to 0% or 5% cyclic strain in the presence or absence of GM-6001 Results are expressed as tubule formation on collagen where four random fields of vision were photographed from each gel and the mean length of tube formation was quantified by measuring the length of the network of connected cells in each well using NIH image Representative fields of vision are shown for each treatment and arrows indicate tubule formation Results are expressed as fold change relative to control unstrained cells and histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls **P<0 05 compared to 5%Strain

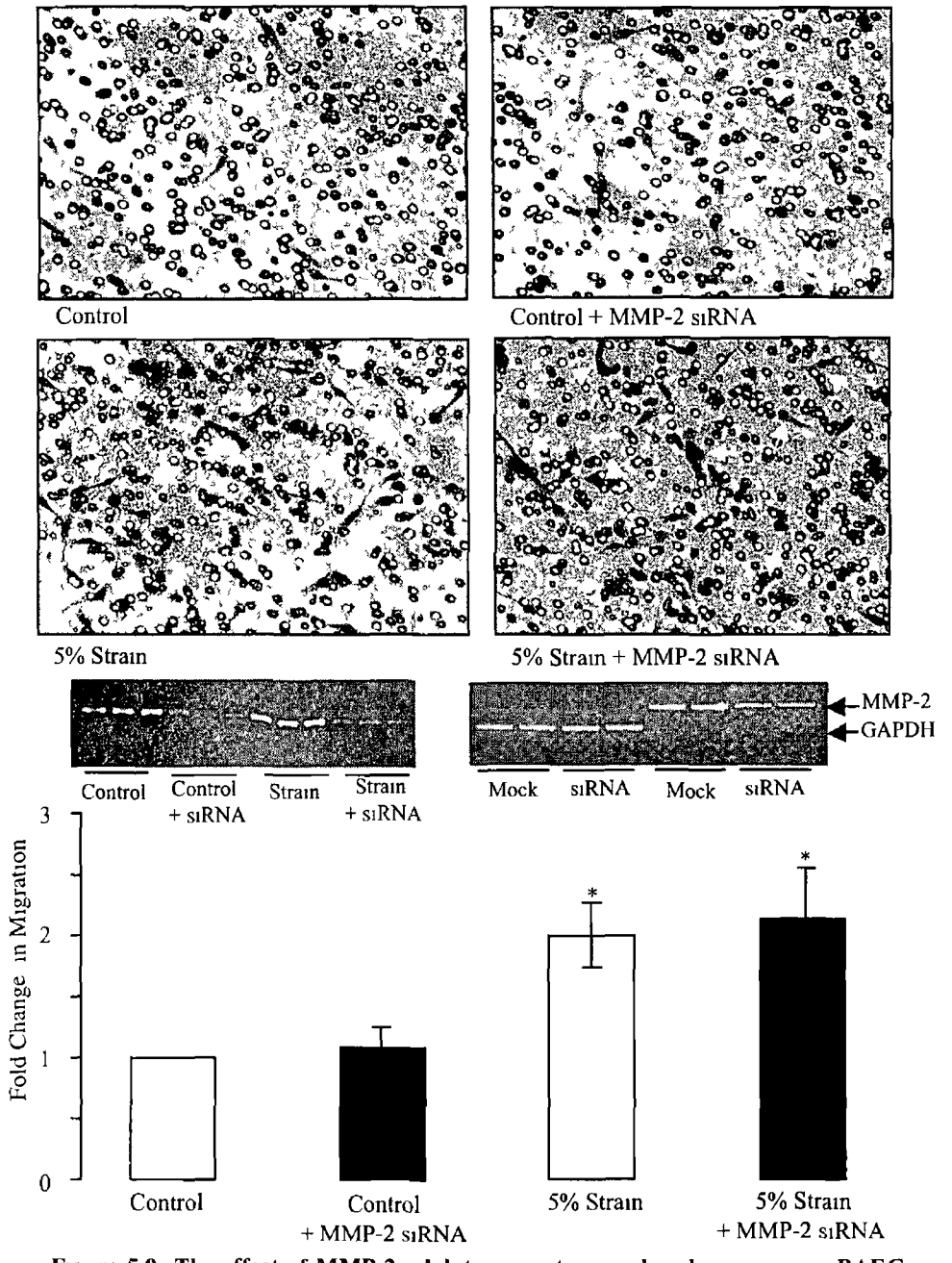


Figure 5 9 The effect of MMP-2 inhibition on strain-induced increases in BAEC migration Force-dependent increase in BAEC migration following exposure to 0% (control) or 5% cyclic strain in the absence or presence of MMP-2 siRNA Cells were exposed to strain for 24 h prior to transwell migration assay BAEC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of control Representative hpf for each treatment is shown and arrows indicate the presence of migrated cells Represntative zymograghy and RT-PCR gels demonstrate knockdown in pro-MMP 2 activity and expression Histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls

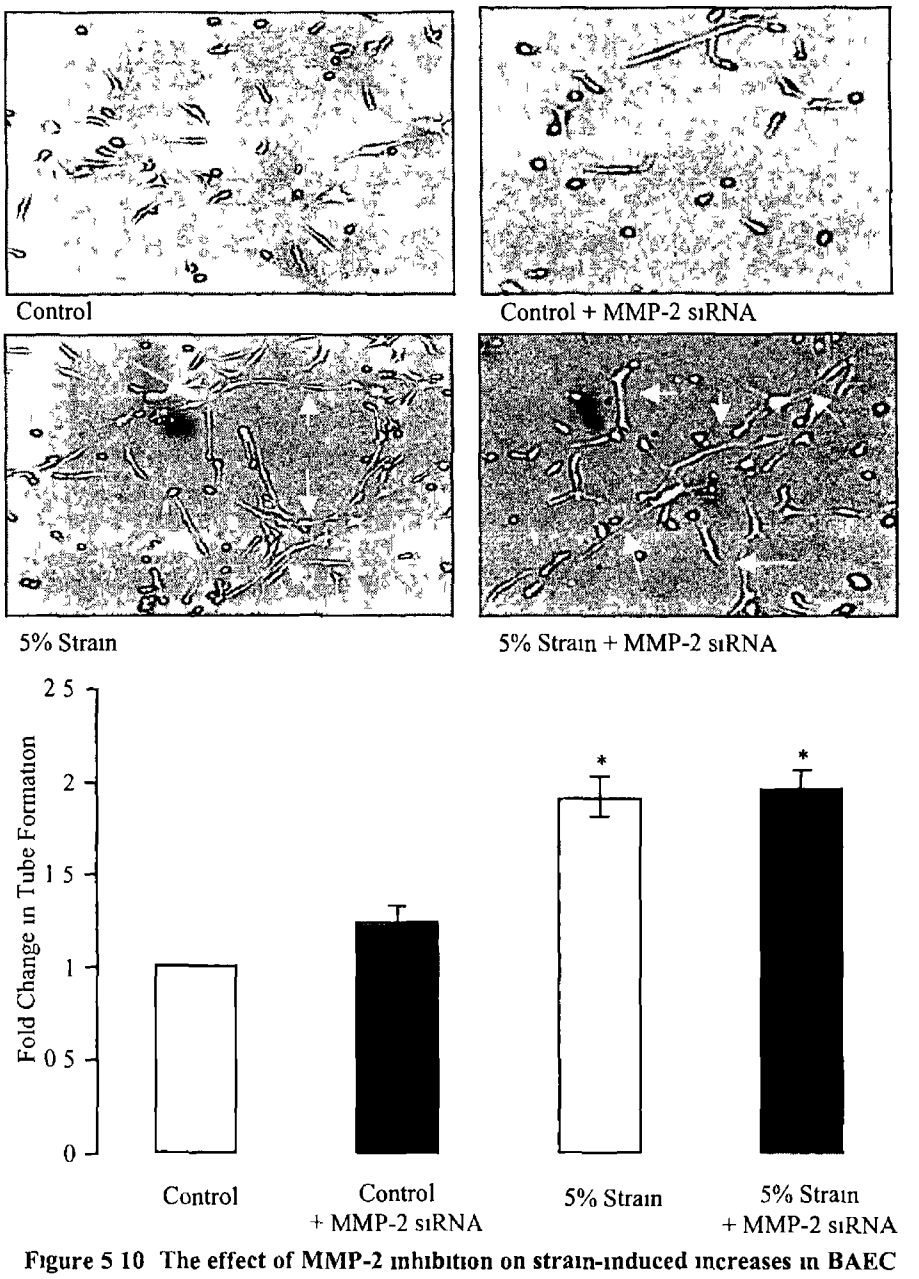


Figure 5 10 The effect of MMP-2 inhibition on strain-induced increases in BAEC tube formation Force-dependent increase in endothelial cell tube formation following exposure to 0% or 5% cyclic strain in the presence or absence of MMP-2 siRNA Results are expressed as tubule formation on collagen where four random fields of vision were photographed from each gel and the mean length of tube formation was quantified by measuring the length of the network of connected cells in each well using NIH image Representative fields of vision are shown for each treatment and arrows indicate tubule formation Results are expressed as fold change relative to control unstrained cells and histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls

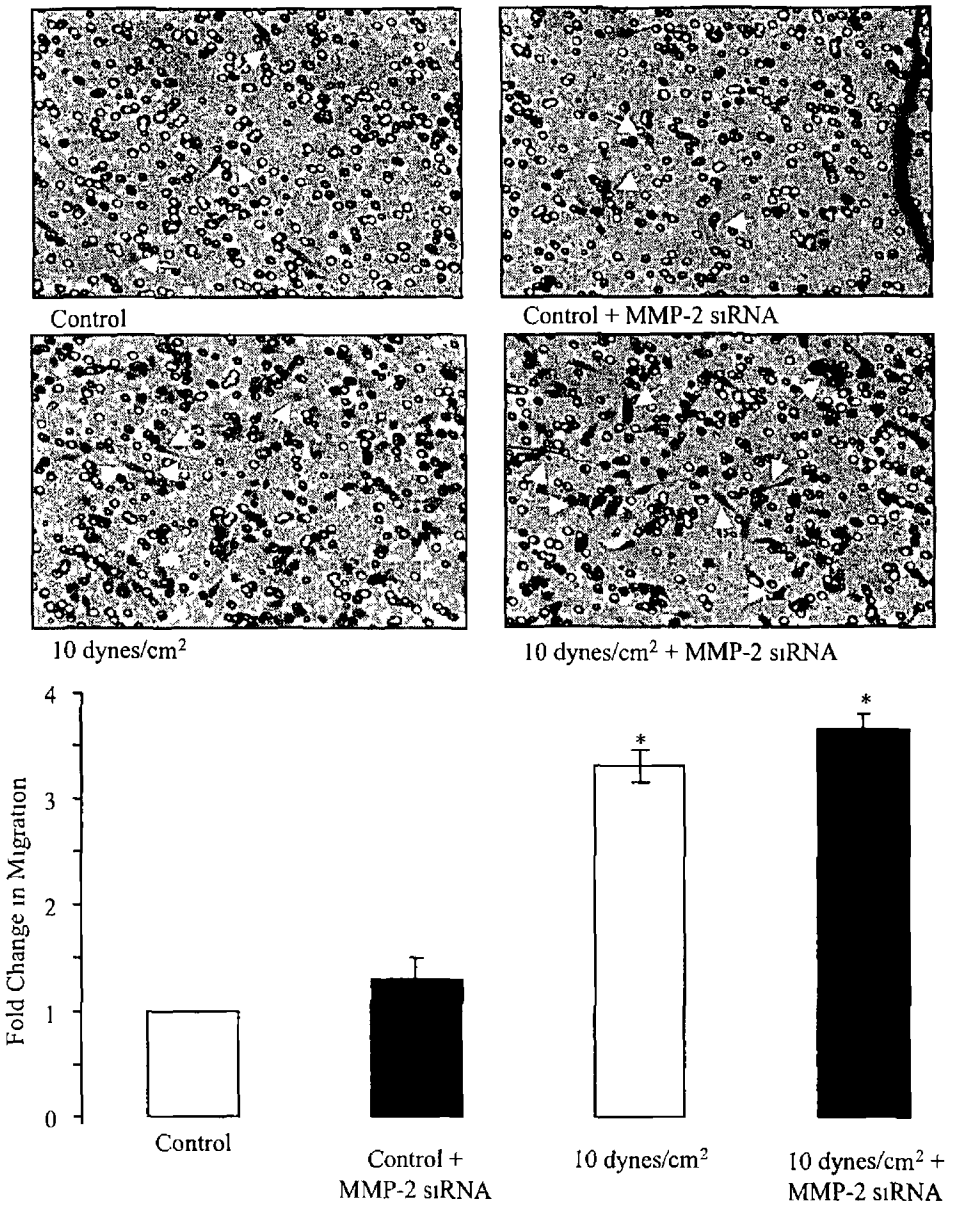


Figure 5 11 The effect MMP-2 inhibition on shear stress-induced increases in BAEC migration Force-dependent increase in BAEC migration following exposure to 0 or 10dynes/cm^2 non-pulsatile laminar shear stress in the absence or presence of MMP-2 siRNA Cells were exposed to non-pulsatile laminar shear stress for 24 h prior to transwell migration assay BAEC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of control Representative hpf for each treatment is shown and arrows indicate the presence of migrated cells Histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to control

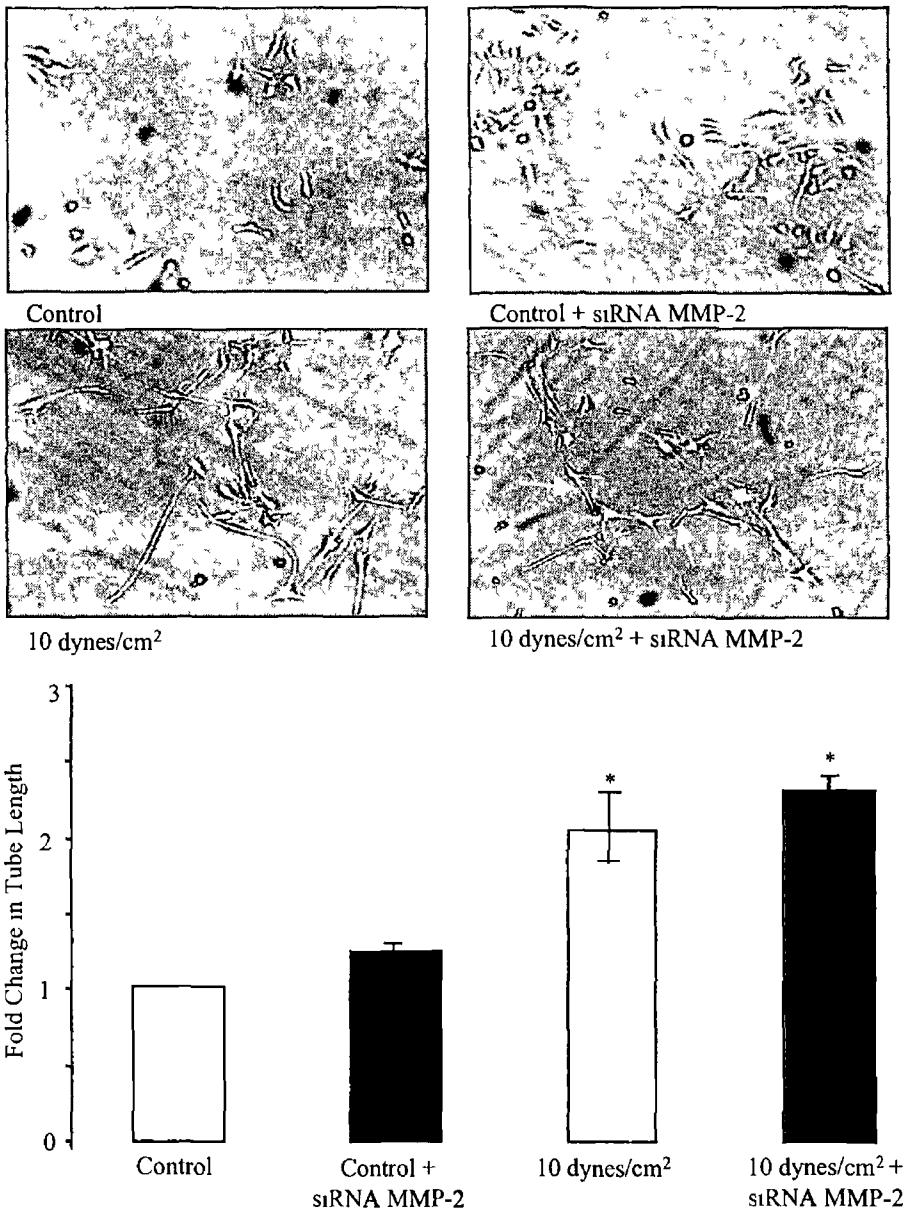


Figure 5 12 The effect of MMP-2 inhibition on shear stress-induced increases in BAEC tube formation Force-dependent increase in endothelial cell tube formation following exposure to 0 or 10 dynes/cm^2 non-pulsatile laminar shear stress in the presence or absence of MMP-2 siRNA Results are expressed as tubule formation on collagen where four random fields of vision were photographed from each gel and the mean length of tube formation was quantified by measuring the length of the network of connected cells in each well using NIH image Representative fields of vision are shown for each treatment and arrows indicate tubule formation Results are expressed as fold change relative to control unstrained cells and histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls

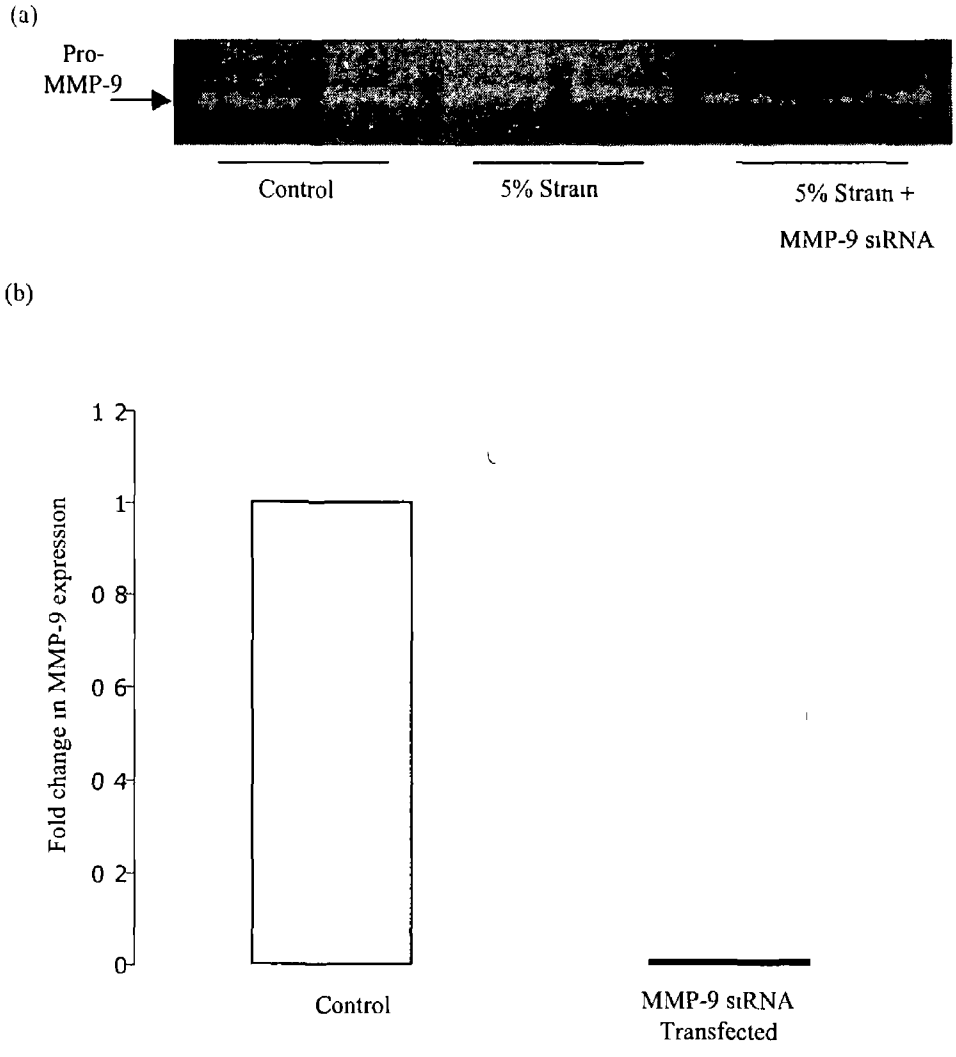


Figure 5 13 MMP-9 knockdown following transfection with MMP-9 siRNA
(a) Representative zymogram demonstrating knockdown of pro-MMP-9 activity following transfection with 100 pmoles of MMP-9 siRNA (b) Histogram representing Real-time PCR data indicating a marked reduction in MMP-9 expression post transfection

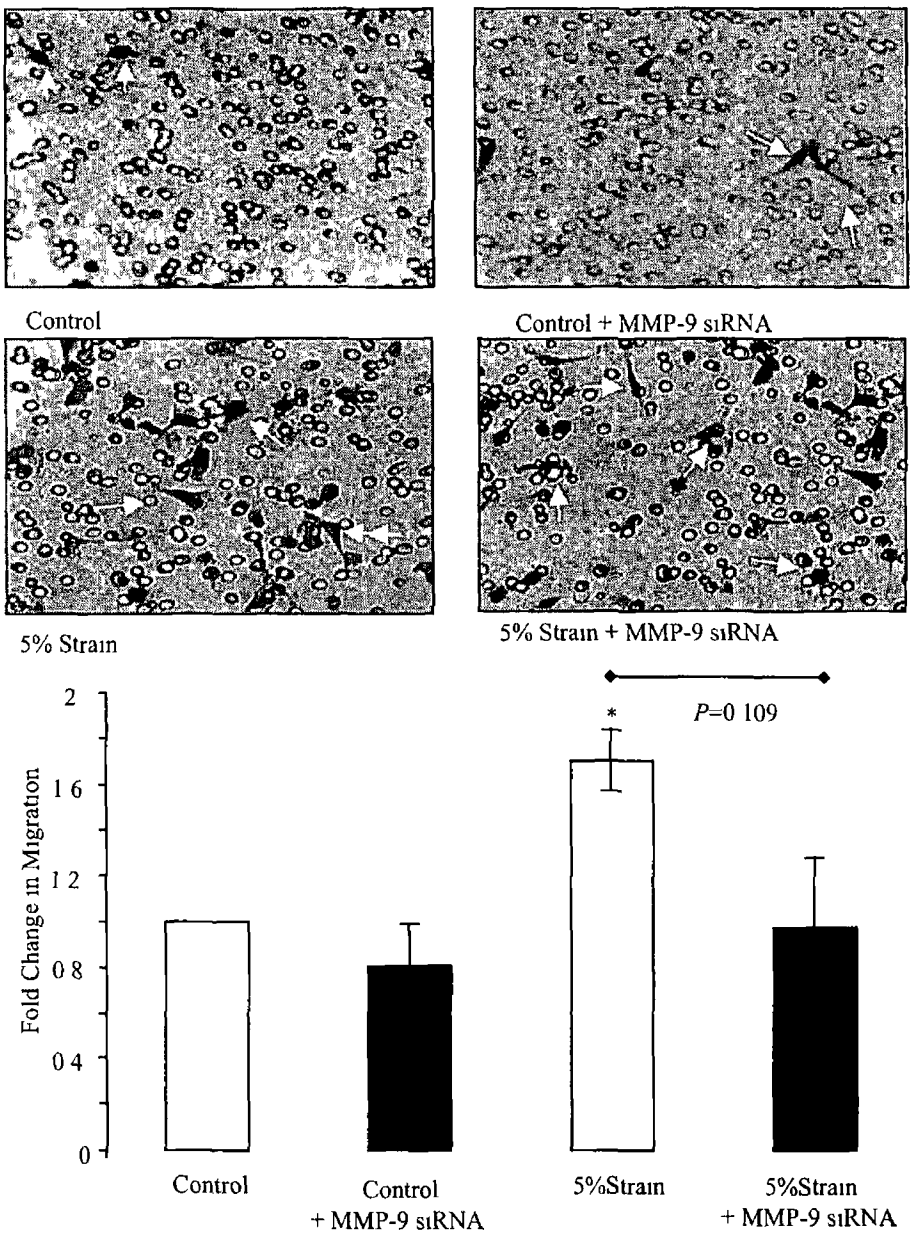


Figure 5 14 The effect of MMP-9 inhibition on strain-induced increases in BAEC migration Force-dependent increase in BAEC migration following exposure to 0% (control) or 5% cyclic strain in the absence or presence of MMP-9 siRNA Cells were exposed to strain for 24 h prior to transwell migration assay BAEC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of BAEC exposed to control Representative hpf of each treatment are shown and arrows indicate the presence of migrated cells Histogram represents mean values from three independent experiments± SEM, *p<0 05 compared to controls **p<0 05 compared to 5%Strain

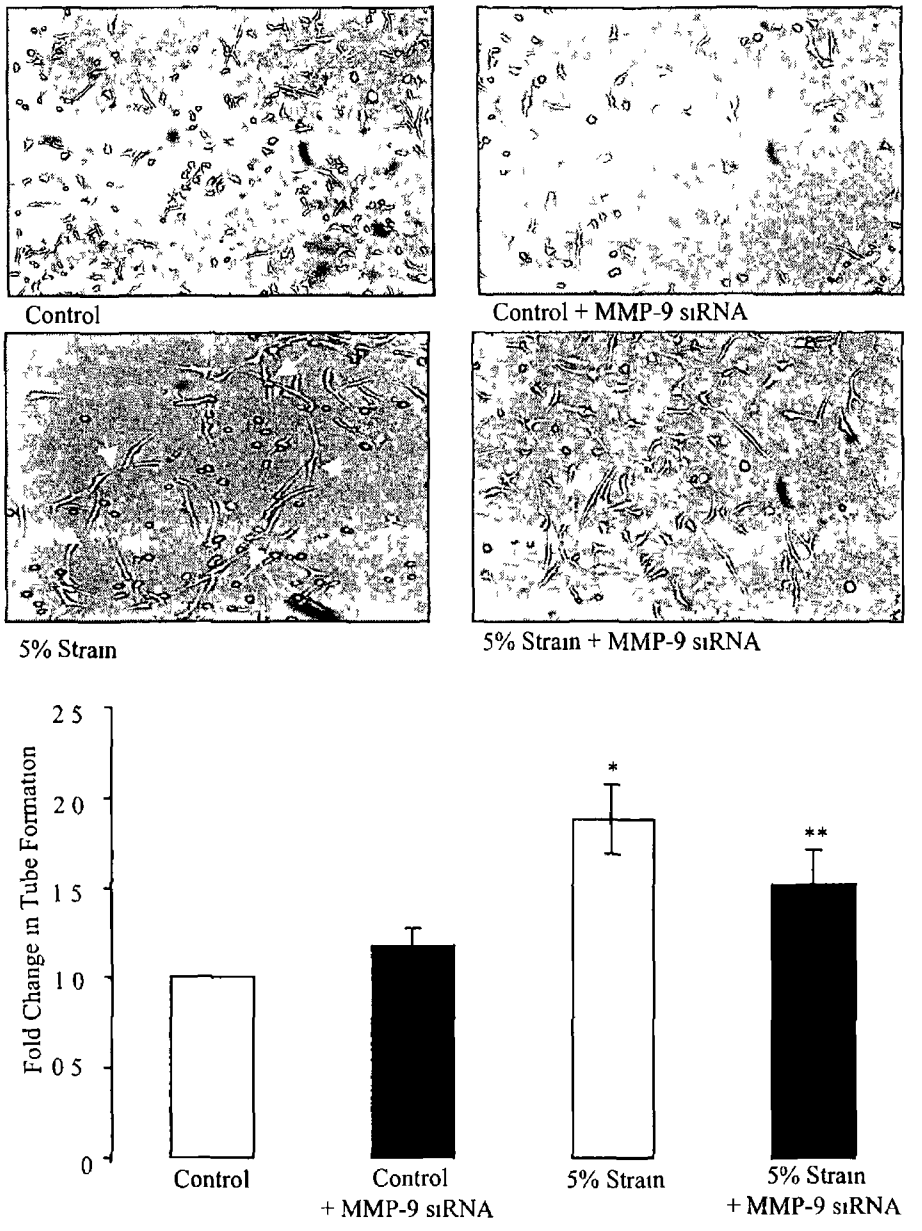


Figure 5 15 The effect of MMP-9 inhibition on strain-induced increases in BAEC tube formation Force-dependent increase in endothelial cell tube formation following exposure to 0% or 5% cyclic strain in the presence or absence of MMP-9 siRNA Results are expressed as tubule formation on collagen where four random fields of vision were photographed from each gel and the mean length of tube formation was quantified by measuring the length of the network of connected cells in each well using of NIH image Representative fields of vision for each treatment are shown and arrows indicate tubule formation Results are expressed as fold change relative to control unstrained cells and histogram represents mean values from three independent experiments± SEM, *p<0 05 compared to controls **p<0 05 compared to 5%Strain

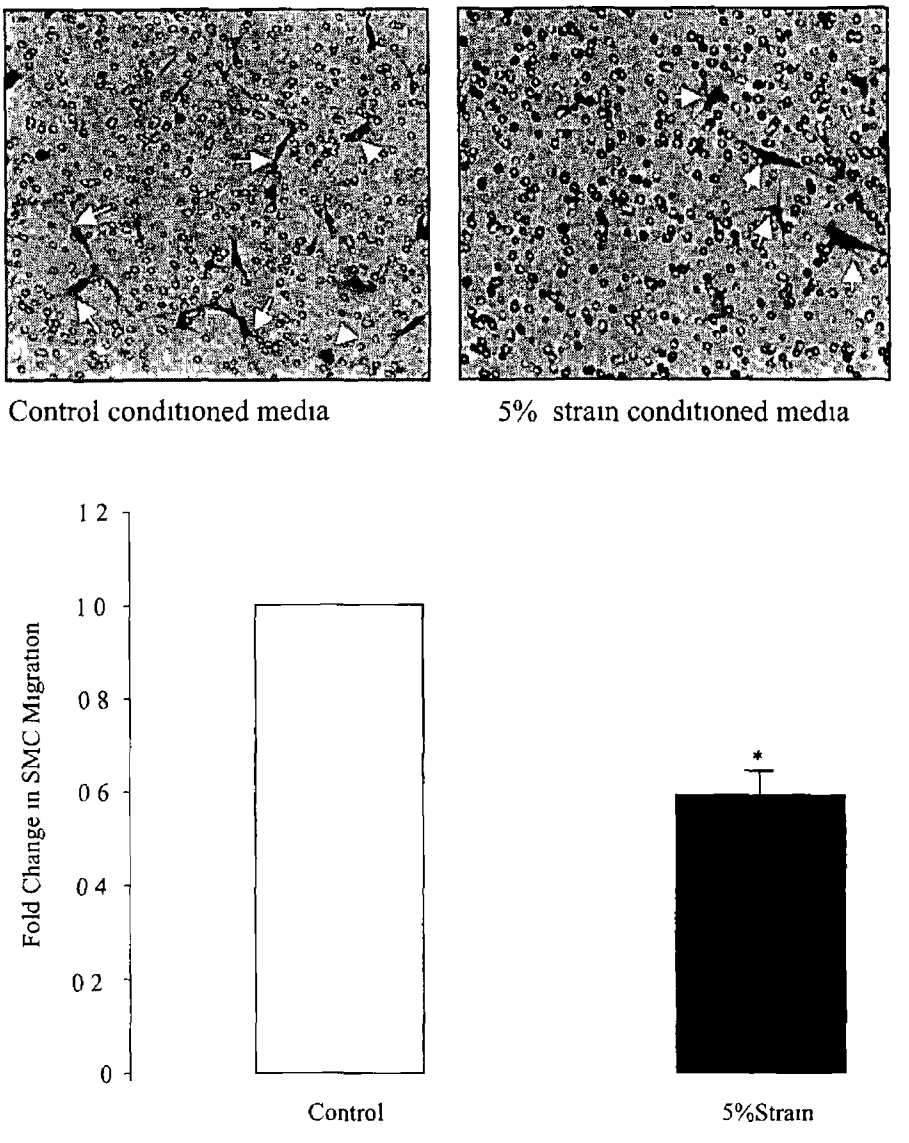


Figure 5 16 The effect of strained BAEC-conditioned media on BASMC migration Following exposure to either 0% or 5% cyclic strain for 24h the resultant BAEC *conditioned media was employed as a chemoattractant in* BASMC migration assays BASMC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of control Representative fields of vision from each treatment are shown and arrows indicate the presence of migrated cells Histogram represents mean values from three independent experiments± SEM, *$P<0.05$ compared to controls

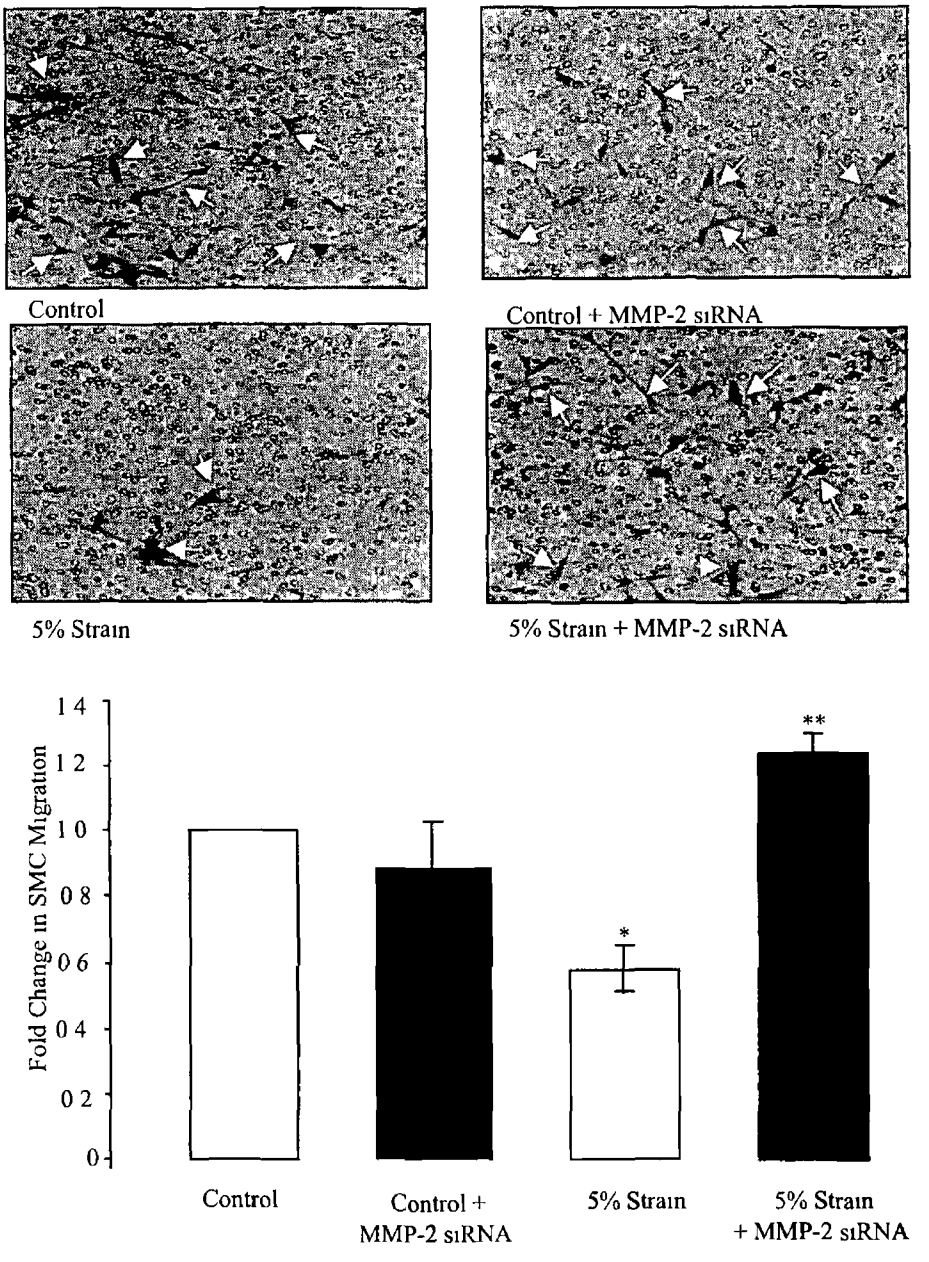


Figure 5 17 The effect MMP-2 siRNA strained BAEC conditioned media on SMC migration BAEC were transfected with MMP-2 siRNA prior to exposure to either 0% or 5% cyclic strain for 24h, the resultant BAEC conditioned media was employed as a chemoattractant in BASMC migration assays BASMC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of control Representative hpf for each treatment are shown and arrows indicate the presence of migrated cells Histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls **P<0 05 compared to 5% strain

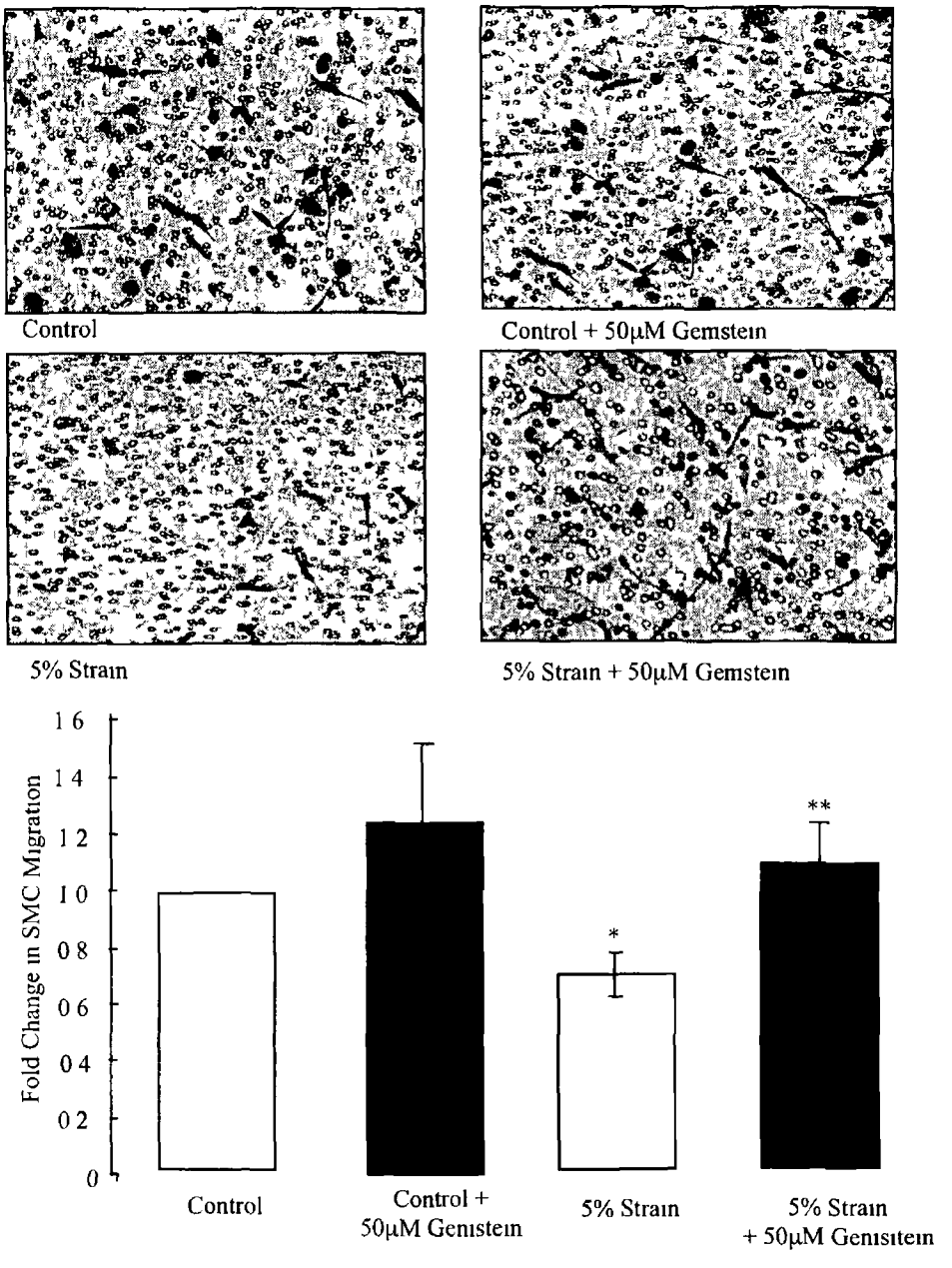


Figure 5 18 The effect of PTK inhibition on strained BAEC conditioned media on BASMC migration BAEC were exposured to either 0% or 5% cyclic strain for 24h in the absence or presence of genistein (50μM) The resultant BAEC conditioned media was employed as a chemoattractant in BASMC migration assays BASMC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of control Representative hpf of each treatment are shown and arrows indicate the presence of migrated cells Hostogram represents mean values from three independent experiments± SEM, *$P<0.05$ compared to controls **$P<0.05$ compared to 5% strain

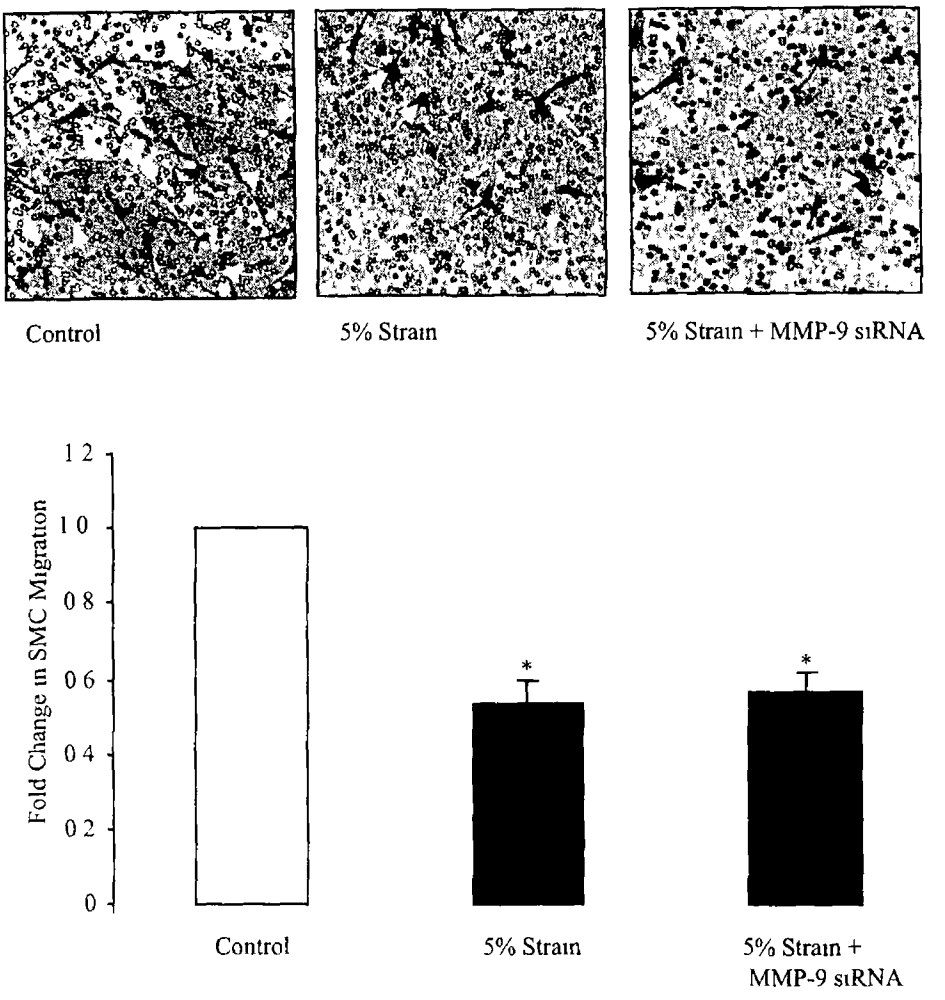


Figure 5 19 The effect of MMP-9 siRNA post strain conditioned media on SMC migration BAECs were transfected with MMP-9 siRNA prior to exposure to either 0% or 5% cyclic strain for 24h, the resultant conditioned media was employed as a chemoattractant in BASMC migration assays BASMC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage control Representative hpf of each treatment are shown and arrows indicate the presence of migrated cells Histogram represents mean values from three independent experiments± SEM *P<0 05 compared to controls

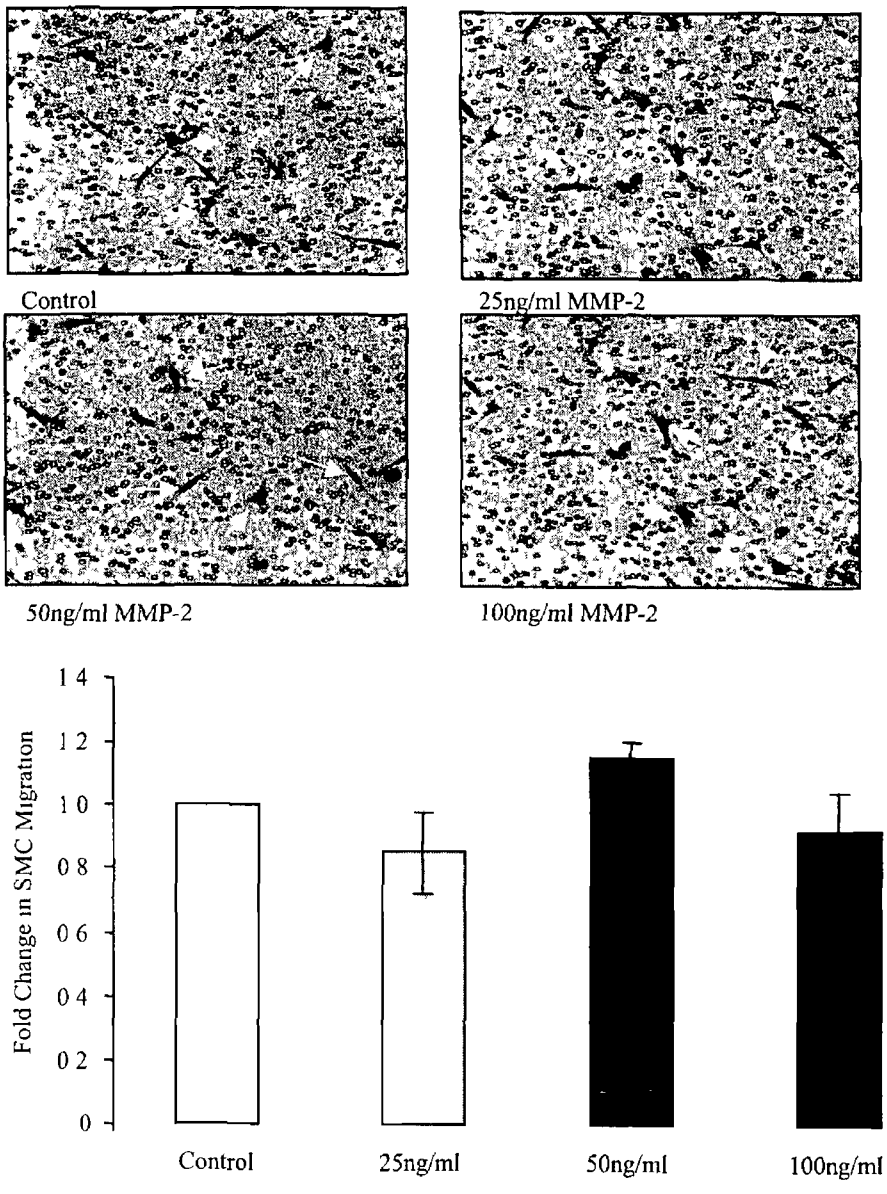


Figure 5 20 The effect of exogenous recombinant MMP-2 on basal BASMC migration BASMC migration was examined in the presence of exogenous recombinant MMP-2 (0-100ng/ml) BASMC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of control Representative hpf of each treatment are shown and arrows indicate the presence of migrated cells Histogram represents mean values from three independent experiments± SEM *$P<0$ 05 compared to controls

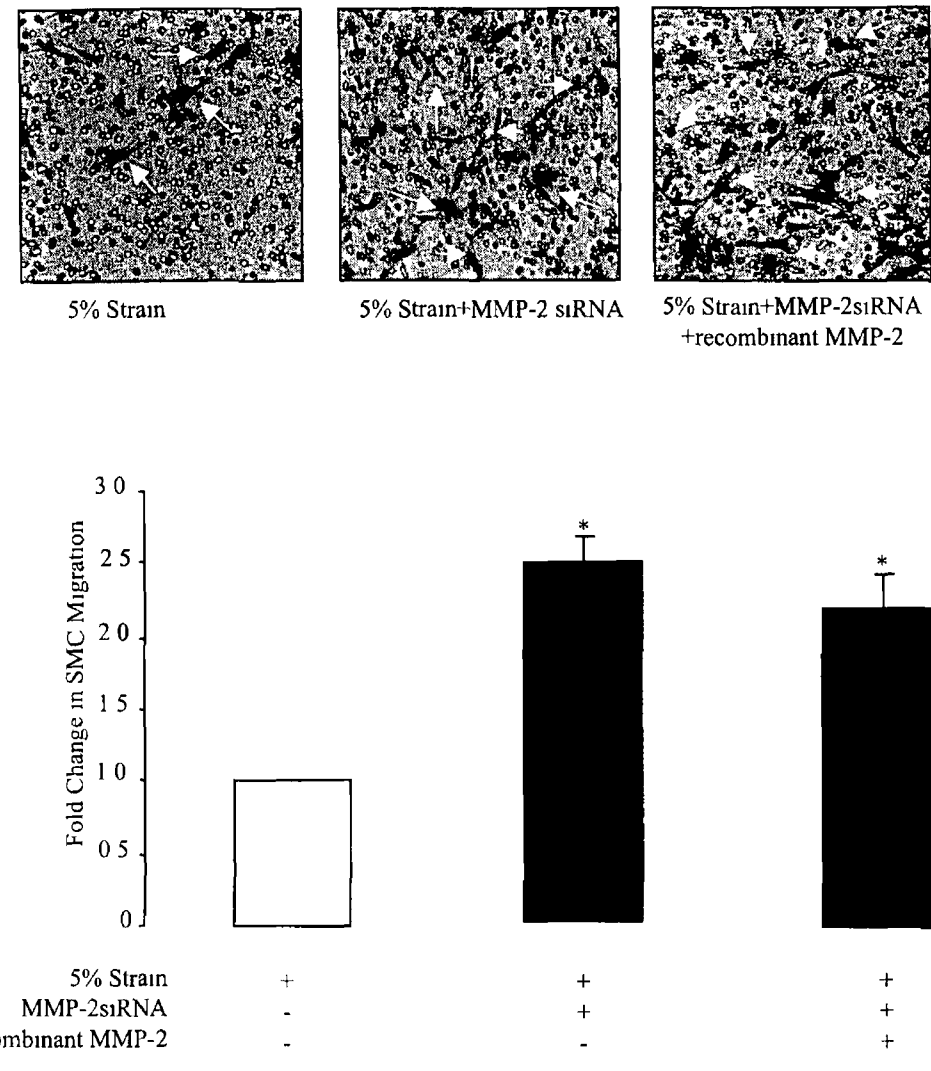
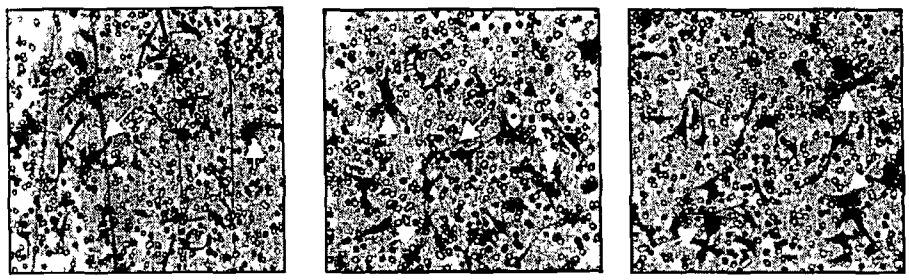


Figure 5 21 The effect of recombinant MMP-2 "add back" to post strain conditioned media on BASMC migration BAEC were transfected with MMP-2 siRNA prior to exposure to 5% cyclic strain for 24h, the resultant BAEC conditioned media was employed as a chemoattractant in BASMC migration assays 100ng/ml exogenous MMP-2 was 'added back" to MMP-2 depleted media prior to migration assay BASMC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of control Representative hpf of each treatment are shown and arrows indicate the presence of migrated cells Histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls



Control | Control + MMP-2 siRNA | Control + MMP-2 siRNA + recombinant MMP-2

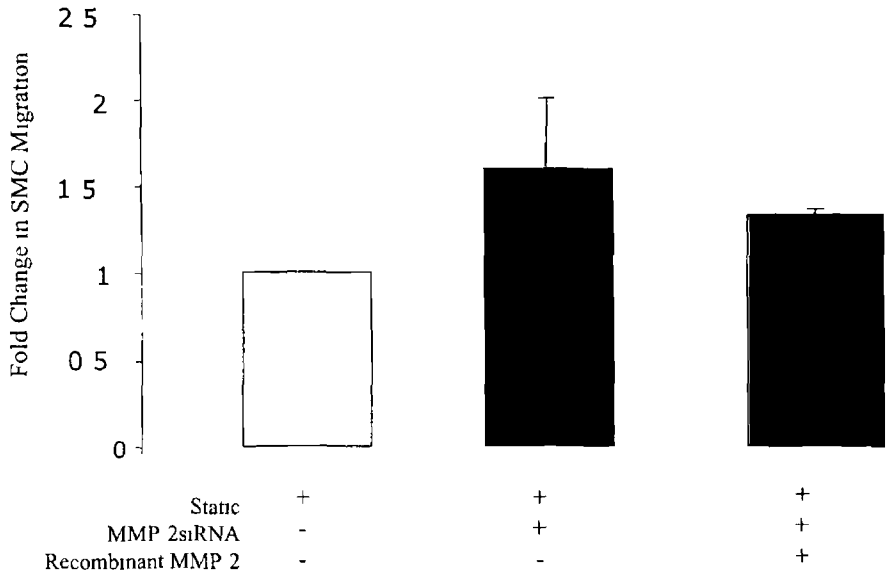


Figure 5 22 (a) To characterise the effects on BASMC migration of supplementing BAEC with recombinant MMP-2, following siRNA blockade of MMP-2 BAEC were transfected with MMP-2 siRNA prior to exposure and grown in the absence or presence of 100ng/ml recombinant MMP-2 for 24h The resultant BAEC conditioned media was employed as a chemoattractant in BASMC migration assays BASMC migration is expressed as number of cells that have migrated through the filter per 5 hpf and are expressed as a percentage of control Representative hpf of each treatment are shown and arrows indicate the presence of migrated cells Histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to controls

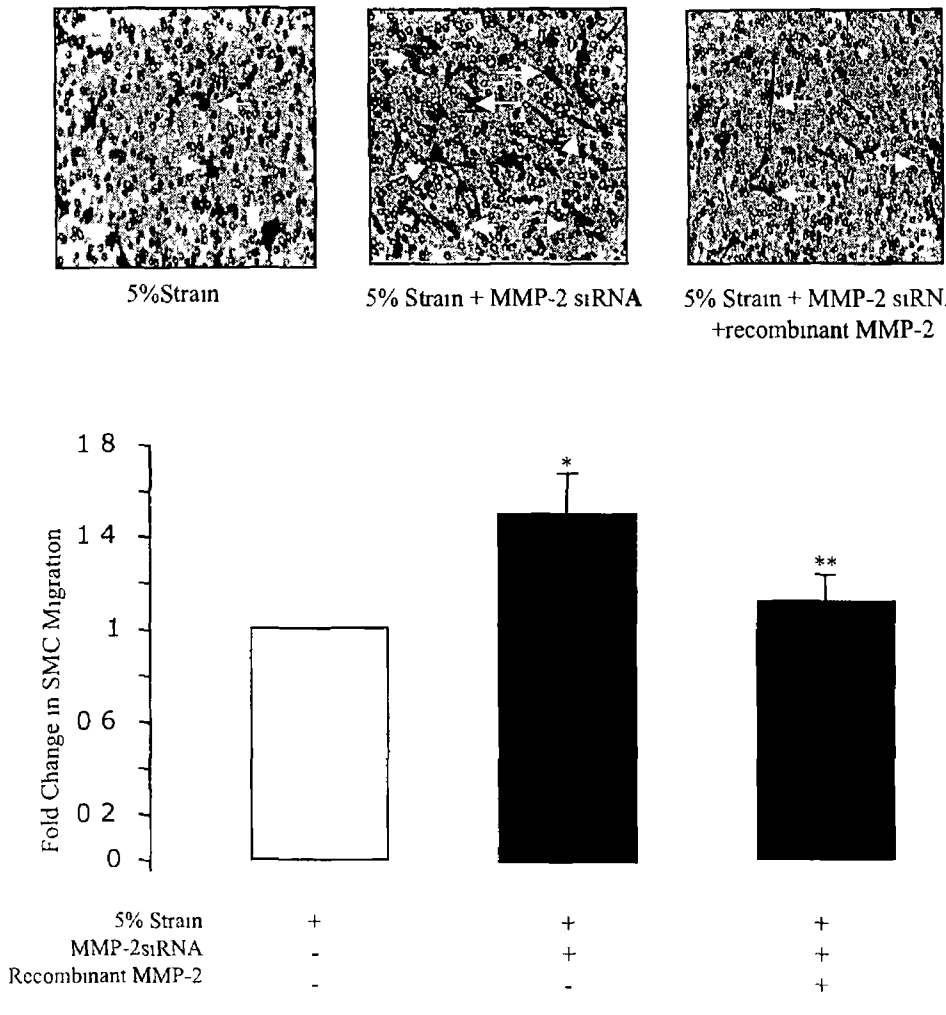


Figure 5 22 (b) To characterise the effects on BASMC migration of supplementing BAEC with recombinant MMP-2, following siRNA blockade of MMP-2 BAEC were transfected with MMP-2 siRNA prior to exposure to either 0% or 5% cyclic strain for 24h in the absence or presence of 100ng/ml recombinant MMP-2 The resultant BAEC conditioned media was employed as a chemoattractant in BASMC migration assays BASMC migration is expressed as number of cells that have migrated through the filter per, 5 hpf and are expressed as a percentage of control Representative hpf of each treatment are shown and arrows indicate the presence of migrated cells Histogram represents mean values from three independent experiments± SEM, *P<0 05 compared to 5%Strain **P<0 05 compared to 5%Strain + MMP-2 siRNA

5 3 Discussion

We have clearly demonstrated that mechanical stimuli such as cyclic strain and shear stress promote a pro-migratory and pro-angiogenic phenotype in BAECs We have also established that cyclic strain modulates MMP-2 by two different pathways involving G-protein subunits and p38 MAPK or PTK and ERK1/2 MAPK In this chapter we examine the role of G-proteins, integrins, PTKs and MMPs in modulating cyclic strain-induced increases in migration and tube formation EC can influence the phenotype and contractile features of SMCs *In vivo* EC are in direct contact with the bloodstream and release a variety of growth factors in response to shear stress and cyclic strain [Palumbo *et al*, 2002, Vouyouka *et al*, 2003] Therefore, we have also investigated the role of cyclic strain derived factors from BAEC on BASMC migration

To elucidate the signaling mechanisms involved in cyclic strain-induced increases in BAEC migration and tube formation, we examined the putative role of Gi-proteins in mediating this effect The rapid activation of Gi-proteins by cyclic strain has been demonstrated in previous studies [Gudi *et al*, 2003] Inhibition of GPCRs by PTX or Thromboxane A_2 has been found to inhibit basal EC migration and tube formation [Gao *et al*, 2000, Benndorf *et al*, 2003] Similarly small GTPases have been found to be crucial to the progression EC migration [Seasholtz *et al*, 1999] The involvement of Gi-proteins in mediating mechanically stimulated EC migration was demonstrated by Cullen *et al* This study is in agreement with these findings and as inhibition of cyclic strain-induced BAEC migration and tube formation following treatment with PTX suggests that these events are Giα-protein dependent

A vital mechanism of cell migration and tube formation is the interaction of the cell with its ECM Integrins play a pivitol role in cell/ECM interactions and so are believed to be important in these cell fate decisions Previously, it has been demonstrated that treatment with a cyclic RGD peptide will not inhibit SMC migration but will inhibit SMC invasion (where invasion requires migration through an ECM) [Slepian *et al* 1998, Kanda *et al*, 2000] Selective inhibition of the β3 subunit using

antisense oligonucleotides inhibits EC tube formation on fibrin but not on matrigel suggesting the involvement of integrins may depend on the composition of the ECM [Dallabrida *et al*, 2000, Kronenwett *et al*, 2002] Urbich *et al* demonstrated that shear stress-induced migration assessed by wound-healing assays could be attenuated by treatment with cRGD peptide In the current study it was found that cyclic strain-induced transwell migration could not be inhibited by pre-treatment with cRGD peptide, however, cyclic strain-induced tube formation was significantly attenuated In agreement with the work of Urbich *et al* we found that cRGD peptide could significantly inhibit wound-healing Taken together, these data imply that transwell migration and wound-healing migration are regulated by different mechanisms

One possible explanation for the differences between the two types of migration may relate to the fact that following injury EC phenotype may be dramatically different to that of uninjured cells The release of inflammatory cytokines such as TNF-α or interleukins in response to injury may promote migration via integrin-dependent pathways which would not occur in uninjured cells IL-1ß and IL-3 induce migration in VSMC via increases in PGE2 and VEGF respectively [Brizzi *et al*, 2001, Yamamoto *et al* 1999] A characteristic of the cytokines is the coupling of their activity to cell-cell interactions therefore blocking of these interactions with cRGD peptide may inhibit their effect In addition migration observed during wound-healing involves the migration of an EC monolayer, as such there is extensive cell-cell communication The ability of cRGD peptide to inhibit wound-healing may lie in its ability to interrupt suitable cell/cell communication Our data does not imply that integrins are not involved in the process of cell migration, however it does infer that the ability of cyclic strain to induce a migratory phenotype in BAECs is integrin independent

The observed inhibition of tube formation following pre-treatment with cRGD peptide is, in agreement with previous studies The αvβ3 integrin is a receptor for a number of proteins with an exposed Arg-Gly-Asp (RGD) tripeptide moiety, including vitronectin, fibronectin and fibrinogen *In vivo*, this receptor is not widely expressed The appearance of this tripeptide occurs predominantly on cytokine-activated

endothelial cells [Varner *et al*, 1996] The vitronectin receptors (αvβ3, αvβ5) are expressed during *in vivo* angiogenesis and markers for an angiogenic phenotype Blocking of the αvβ3 integrin leads to the inhibition of tumour- and growth factor-induced angiogenesis *in vivo* [Chavakis *et al*, 2002] There is a large amount of contradicting data concerning the involvement of integrins in angiogenesis as abalation of integrin subunits in mice failed to inhibit angiogenesis [Hynes , 2002] However other studies have demonstrated that β1, α1 and α5 integrins are involved in angiogenesis One proposed explanation is that a functional redundancy exists between integrins The ability of integrins to mediate angiogenesis may be due to their ability to effect and be affected by pro-angiogenic cytokines such as bFGF, VEGF and TGF-β1 Engagement of integrins can increase the production of pro-angiogenic cytokines such as VEGF [Chung *et al*, 2004, Murphy *et al*, 2003], moreover these cytokines can increase expression of the αv and β3 subunits in endothelial cells [Basson *et al*, 1992, Swerlick *et al*, 1992, Sepp *et al*, 1994, Senger *et al*, 1996] In addition, αvβ3 antagonists have been shown to markedly inhibit angiogenesis induced by bFGF and TNF-α in the chicken chorioallantoic membrane and rabbit corneal micropocket assays [Brooks *et al*, 1994, Friendlander *et al*, 1996] Integrin antagonists such as cRGD peptides may inhibit cyclic strain-induced tube formation by inhibiting production of pro-angiogenic factors

Angiogenesis is a carefully balanced sequence of events Vascular growth factors are capable of regulating this process in a dynamic model of blood vessel formation VEGF in concert with other angiogenic molecules including the angiopoietins effect and modulate blood vessel formation Both receptor tyrosine kinases and non-receptor tyrosine kinases have been identified as being important to the progression of angiogenesis Selective inhibition of non-receptor tyrosine kinases such as FAK or RTKs such as KDR/Flk-1, Tie-2 or the PDGF receptor inhibits angiogenesis in a number of models [Nakatsu *et al* 2003, Miura *et al*, 2004, Dudley *et al*, 2003, Banai *et al*, 1998, Kim *et al*, 2000, Bohnsack *et al* 2003] However, in this study we found that cyclic strain-induced migration and tube formation occurred independently of PTK activation, as treatment with gemstein failed to inhibit these cell fates

Overall we have demonstrated that cyclic strain-induced migration is a Gi-protein dependent process and is independent of integrins and PTK However, both Gi-protein and integrm signaling appear to be important to cyclic strain-induced tube formation Whether or not they control cyclic strain-induced angiogenesis by two different pathways has yet to be elucidated However, associations between integrins and G-proteins have been well documented [Stouffer *et al*, 2003, Fujiwara *et al*, 2004] and integrin recruitment of Gi-proteins during exposure to cyclic strain driving EC to an angiogenic phenotype is feasible Integrins may regulate cyclic strain-induced angiogenesis by concentrating Gi-proteins at focal adhesions or may orientate them so as to be in close proximity to effector molecules Alternatively GPCR which are activated by cyclic strain may subsequently activate integrin subunits

Cyclic strain leads to an increase in BAEC migration and angiogenesis with a concurrent increase in pro-MMP-2 activity However, the signaling pathways involved in mediating these responses are distinctly separate Therefore we investigated if the release of pro-MMP-2 during cyclic strain is linked to cyclic strain-induced migration and angiogenesis The involvement of MMPs in angiogenesis and specifically gelatinases has been discussed in detail in section 1 5 We have demonstrated that cyclic strain-induced tube formation but not migration is an MMP dependent event and more specifically this event is MMP-9 but not MMP-2 dependent

Cell migration is an event which requires the modification of the ECM As such it is believed that MMPs play a pivotal role in ECM modification to facilitate migration However, our data contradicts a number of reports that indicate a role for MMP-2 in migration [Gurjar *et al* 1999, Palumbo *et al* 2000, Bohnsack *et al* 2003, Johnson *et al* 2004, Kuzuya *et al*, 2003, Lee *et al* 2003] Conversely, *in vitro* studies report that inhibition of MMP-2 blocks migration across a matrix barrier, such as a collagen plug, whereas migration in the absence of a matrix barrier was unaffected by MMP inhibition [Lee *et al* 2003, Koike *et al*, 2002] These reports corroborate our observations that MMP-2 inhibition had no effect on transwell migration in the absence of a matrix barrier The involvement of MMP-2 in migration may therefore require suitable

orientation and attachment to a 3D matrix Angiogenesis requires EC to invade and migrate into the surrounding stroma, which comprises of ECM proteins such as collagen, which can bind to receptors on the endothelial cell surface Considering this model of EC migration during angiogenesis in conjunction with the data presented above, it may be inferred that interaction of EC with components of the ECM is a requirement for MMP-2 dependent migration

MMP inhibition using a broad spectrum inhibitor such as GM-6001 identified that MMP activity is an important aspect of *in vitro* tube formation Selective inhibition of MMP-2 uing siRNA duplexes indicated that MMP-2 is not a key angiogenic regulator in either cyclic strain- or shear stress–induced tube formation This observation is in agreement with the gensitein study in which MMP-2 was inhibited by PTK blockage with no effect on migration or tube formation These data contradict the work of Rivilis *et al*, (2002) who indicate that MMP-2 is required for cyclic strain but not shear stress-induced tube formetion

In addition, other studies have highlighted the importance of MMP-2 in angiogenesis In MMP-2 deficient mice, reduced retinal angiogenesis and tumour-induced angiogenesis was observed [Pepper *et al*, 2001, Matsui *et al*, 2003] Increased angiogenesis with associated increases in MMP-2 have also been observed in hypoxia-, tumour- and exercise-induced models of angiogenesis [Rivilis *et al* 2002, Brown *et al*, 2003, Ben-Yosef *et al*, 2002] However, increased MMP-2 activity may also be associated with inhibition of angiogenesis via the production of anti-angiogeneic factors such as angiostatin and endostatin in response to NO depletion or exercise [Matsunaga *et al* 2002, Gu *et al* 2004] Moreover, increases in ROS and/or NO such as those seen following cyclic strain may induce angiogenesis [Ushio-Fukai *et al*, 2002, Maulik *et al*, 2002] and may offer an alternative to MMP-2-dependent angiogenesis It can be seen that the involvement of MMP-2 in angiogenesis may depend greatly upon the initiating stimulus and model used Whereas some studies have shown cyclic strain-induced angiogenesis *in vivo* is MMP-2-dependent [Rivilis *et al*, 2002], our data demonstrates that *in vitro* cyclic strain of EC monocultures may stimulate angiogenesis

independently of MMP-2 Indeed Seo *et al* have established that TIMP-2 may inhibit angiogenesis independently of MMPs

Conversely, selective inhibition of MMP-9, using siRNA duplexes clearly identifies the involvement of MMP-9 in strain-induced tube formation Our data indicate that MMP-9 is an important factor in regulating strain-induced angiogenesis There is considerable evidence to support the opinion that MMP-9 is a key regulator of angiogenesis The inhibition of MMP-9 with cyclic peptides (His-Trp-Gly-Phe), are capable of inhibiting EC migration and invasion in a tumour model Additionally genetic studies in mice have shown that MMP-9 knockout mice had a distinct angiogenic phenotype and there was a significant reduction in bone growth plate angiogenesis [Pepper *et al*, 2001] Studies with Rip1Tag2 mice, which develop pancreatic beta-cell tumours that are neither lymphangiogenic nor metastatic, show that MMP-9 and not MMP-2 is the angiogenic switch in pancreatic beta-cell carcinogenesis [Pepper *et al*, 2001] Angiogenesis triggered by tissue ischemia requires MMP-9, which may be involved in capillary branching [Johnson *et al*, 2004] In addition, exogenous MMP-9 (but not MMP-2) has been found to cause release of VEGF from a carcinoma cell eliciting an angiogenic response with co-cultured endothelial cells [Sternlicht *et al* 2001] These studies confirm the significance of MMP-9 to angiogenesis They also substantiate our hypothesis that MMP-9 and not MMP-2 is the key angiogenic switch involved in evoking cyclic strain-induced angiogenesis

Due to their position in the vessel, ECs are exposed directly to mechanical forces As such they may act as intermediates in the mechano-regulation of SMC fate decisions by the production of growth factors, cytokines and enzymes etc which may subsequently will alter SMC phenotype Therefore, suitable communication between ECs and SMCs is essential for maintaining vessel homeostasis e g increases in endothelin-1 (a potent vasoconstrictor) expression in response to low shear stress will promote vasoconstriction to increase blood flowrate In this example SMC adopt a contractile phenotype in response to ET-1 to maintain homeostasis in the vessel We have hypothesized that conditioned media from ECs exposed to cyclic strain may

influence SMC migration A number of *in vitro* studies have demonstrated that co-culture of ECs with SMC or exposure of SMC to EC conditioned media may alter dramatically, SMC migration, proliferation, morphology, mRNA and protein synthesis [Cucina *et al*, 2003, Fillinger *et al*, 1997, Proia *et al* 2002, Vouyouka *et al*, 2003] In hemodynamic models it has been demonstrated that SMC migration is increased in the presence of conditioned media from EC exposed to non-pulsatile shear stress Conversely, SMC migration was not altered when co-cultured with EC and exposed to pulsatile flow [Palumbo *et al* 2002, Redmond *et al*, 2001]

Our data has shown that exposure of SMC to conditioned media from cyclically strained EC inhibits SMC migration compared to controls The involvement of MMP-2 in this process was demonstrated by conditioned media from MMP-2 siRNA transfected cells The absence of MMP-2 but not MMP-9 from cyclic strain conditioned media attenuated the inhibition of SMC migration However, MMP-2 does not directly influence SMC migration as addition of exogenous recombinant MMP-2 had no effect on SMC transwell migration which, is in agreement with previous studies [Lee *et al*, 2003, Koike *et al*, 2002] Thus, it appears that MMP-2 is required during the conditioning of EC media This would suggest that MMP-2 is involved in controlling the production, activation or inhibition of EC-derived migratory factors

One possible group of enzymes which may provide more insight into the involvement of MMP-2 in SMC migration is the urokinase-type plasminogen activator/inhibitor system, comprising urokinase plasminogen activator (uPA) and plasminogen activator inhibitor type-1 (PAI-1), in vascular ECs [Pepper *et al*, 2001, Proia *et al*, 2002] The extensive cross-talk between these components during hemodynamically-mediated events is highly significant Redmond *et al* (1999) have shown the combined involvement of uPA/MMP signalling in the vascular remodelling process and have clearly demonstrated a role for EC-derived PAI-1 in the reduction of shear force-induced vascular SMC migration [Redmond *et al*, 2001] Also noteworthy is the probable role of integrins in modulating interactions between MMP-2 and the uPA system Studies have consistently demonstrated transcriptional and functional inter-

dependence of integrins (e g , αVβ3) with MMP-2 [Brooks *et al* , 1998] and the uPA system [Reuning *et al* , 2003]

5 4 Conclusion

Our data demonstrates that cyclic strain stimulates endothelial cell migration and tube formation *in vitro* Cyclic strain-induced migration is dependent on Giα-protein activation but independent of integrin or PTK signaling However, both Giα-protein and integrin signaling appear to be important to cyclic strain-induced tube formation It is tempting to speculate that the strain-induced increases in BAEC migration and tube formation are associated with the strain-induced increases in MMPs described previously We have demonstrated that strained-induced increases in transwell migration occur independently of MMP activity and that strain-induced increases in angiogenesis are dependent on MMP-9 but not MMP-2 We have also established that MMP-2 derived from strained BAECs indirectly causes the inhibition of BASMC migration Overall, we have discovered that cyclic strain derived-MMPs are involved in regulating both BAEC and BASMC cell fate decisions

Chapter 6

6 1 Final Summary

Hemodynamic forces generated by the flow of blood are crucial in maintaining homeostasis within the blood vessel wall Cyclic strain and shear stress are intricately involved in vascular remodeling, a process which underlies the pathogenesis of cardiovascular diseases such as atherosclerosis and restenosis [Schwartz *et al*, 1995, Libby *et al*, 2003] Vascular remodeling requires degradation/modification of the extracellular matrix The ECM is a dynamic environment that serves as an anchoring mechanism for cells but also presents cells with an array of growth factors and other signaling molecules The ECM is important in maintaining the integrity of the blood vessel in addition, alterations in cellular interactions with the ECM brought about by changes in blood flow may contribute to vascular pathologies Normal biological processes such as growth, wound healing and angiogenesis require remodeling of the ECM to allow space for growing and advancing cells Appropriate modification of the ECM to facilitate these processes involves a number of proteinases including metalloproteinases, which collectively are capable of degrading the entire ECM Therefore, tight regulation of the MMPs by the cells from which they are secreted is necessary as deviant expression has been associated with vascular disease [Gibbons *et al* 1994, Rajavashisth *et al*, 1999, Uzui *et al*, 2002, Freestone *et al*, 1995]

Considering the importance of MMPs to ECM modification, the relevance of this to normal physiological processes such as EC migration and angiogenesis and the ability of hemodynamic forces to alter cell phenotype the initial aim of this study was to determine the mechano-sensitivity of MMP-2 to cyclic strain and shear stress Following this we determined the effect of hemodynamic forces on BAEC cell fate decisions, namely migration and tube formation Furthermore we investigated the involvement of MMP-2 and MMP-9 in these events The final component of this study was to determine a role of MMP-2 and -9 derived from strained BAEC in regulating BASMC migration

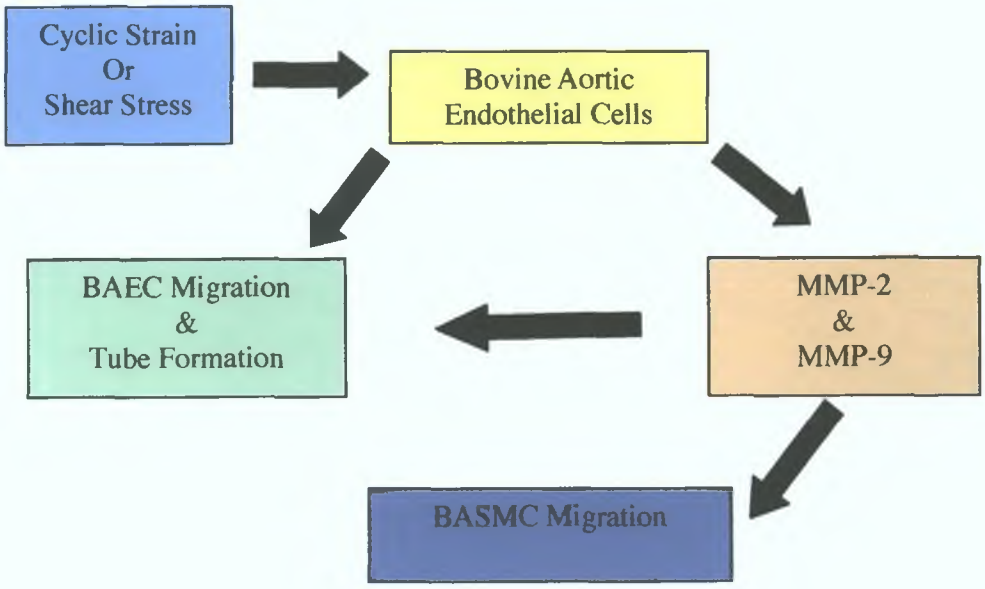


Figure 6.1: Diagramtic representation of experimental approach. The regulation of MMPs, migration and tube formation in response to cyclic strain and shear stress was investigated. Identification of signaling pathways involved in cyclic strain-induced increases in pro-MMP-2 activity, BAEC migration and tube formation The involvement of MMP-2 and MMP-9 in strain-induced migration and tube formation was determined. Finally the ability of BAEC derived MMPs in regulating BASMC migration was investigated.

This thesis describes studies on hemodynamic regulation of endothelial cell migration and angiogeneic activity and the roles of gelatinases in these processes. The initial component of this study was to investigate if the use of *in vitro* models to simulate cyclic strain and shear stress could alter BAEC derived MMPs. Analysis of conditioned media from these experiments revealed that pro-MMP-2 and MMP-2 activity were significantly up-regulated following exposure to cyclic strain but not shear stress. Moreover, the observed increase in MMP-2 activity was associated with increases in protein expression and steady state mRNA levels. We observed that both cyclic strain and shear stress resulted in significant increases in BAEC migration and tube formation compared to static controls. Cyclic strain and shear stress have

previously been reported to induce angiogenesis by differing mechanisms [Rivilis *et al*, 2002, Brown *et al*, 2003] cyclic strain-induced angiogenesis is MMP-2 dependent while shear stress-induced angiogenesis is not Enhanced pro-MMP-2 activity in conditioned media following cyclic strain was found to be a time and force-dependent process Additionally, cyclic strain was found to increase MMP-9 activity in conditioned media, this was however only observed when cells were strained in serum-containing media

Many studies have demonstrated that MMP-2 is sensitive to shear stress and indeed Bassiony *et al* (1998) believe that changes in blood flow are the most important regulators of MMP-2 expression Moreover, the sensitivity of MMP-2 to changes in shear stress has been demonstrated both *in vitro* and *in vivo* [Palumbo *et al*, 2000, Sho *et al*, 2002] Conversely, Rivilis *et al* (2002) established that changes in MMP-2 expression were associated with cyclic strain and not shear stress The differences in the regulation of MMP-2 were attributed to the differential involvement of MMP-2 in cyclic strain and shear stress-induced angiogenesis There are considerable deviations in data presented in these studies, as high blood flow appears to increase, decrease or have on effect on MMP-2 activity [Sho *et al*, 2002, Bassiony *et al*, 1998, Rivilis *et al*, 2002] The variations in MMP-2 activity in response to changes in shear stress may be linked to the method by which increased shear stress was administered Increases in shear stress brought about by AVF may have considerably different effects to changes brought about by ligation or by the administration of prazosin The magnitude of AVF-induced increases in blood flow (8 fold) [Tronc *et al*, 2000] is substantially greater than prazosin-induced increases in blood flow (2-4 fold) [Rivilis *et al* 2002] which may account for observed differences in MMP-2 activity following both treatments Exposure of EC to physiological levels of shear stress (10-70 dynes/cm^2) may not greatly alter MMP-2 activity while shear stresses outside this range may be more influential However, given the range of reported observations considerable doubt remains over the regulation of MMP-2 in response to altered shear stress Our observations lead to the conclusion that non-pulsatile laminar shear stress does not regulate MMP-2 production However, preliminary findings within our laboratory have

demonstrated that MMP-2 activity is regulated by pulsatile laminar shear stress These data in conjunction with the data presented in this thesis imply that the cyclic strain component of pulsatile blood flow is the key hemodynamic regulator of MMP-2 activity

Angiogenesis is an adaptive process that requires modification of the ECM Inhibition of MMP activities has been important in identifying their roles in angiogenesis MMP activity has been associated with both pro- and anti-angiogeneic processes [Yang *et al*, 2001, Haas *et al*, 1998] More specifically the gelatinases (MMP-2 and MMP-9) are thought to be of particular significance in angiogenesis Considerable data exists which identify MMP-2 and MMP-9 as being potent regulators of angiogenesis in a number of experimental models [Chakraborti *et al*, 2003, Fang *et al*, 2000, Pepper *et al*, 2001, Wang *et al*, 2003, Nguyen *et al*, 2001] Hemodynamic stimuli modulate both angiogenesis and MMP activity and indeed may be the most important regulator of MMP expression and activity in vascular cells Alteration in the hemodynamic environment causes upregulation of MMP-2 and MMP-9 associated with failure of saphenous vein graft failure and vascular remodelling linked to hypertension [Zucker *et al*, 1998, Godin *et al*, 2000] Since degradation of the extracellular matrix scaffold enables reshaping of tissue, the role of matrix metalloproteinases (MMPs) has become the object of intense recent interest in relation to physiological and pathological vascular remodeling A more complete understanding of the hemodynamic regulation of MMPs may advance the understanding of vascular remodeling Both shear stress and cyclic strain may stimulate angiogenic growth *in vivo* and *in vitro* Substantial evidence has identified that mechanical forces regulate EC morphology, cytoskeleton, and ECM remodeling in addition to modulation of pro-migratory and pro-angiogenic factors including VEGF, FGF, integrin subunits, TGF-β and MMPs [Matthew *et al*, 1991, Schanper *et al*, 2003, Brown *et al*, 2003, Chen *et al*, 2001, Zheng *et al*, 2001, Rivilis *et al*, 2002 Vailhe *et al*, 1996, Banai *et al*, 1994]

Bearing in mind the relevance of both cyclic strain and MMPs and specifically MMP-2 to angiogenesis, we endeavored to elucidate the signaling mechanisms involved

in cyclic strain-induced increases in pro-MMP-2 activity We examined the putative involvement of Gia-proteins in mediating this response, and determined that strain-induced increases in MMP-2 occurred independently of Gia protein subunits However, inhibition of Gβγ dimer significantly attenuated pro-MMP-2 activity in both static controls and cyclically strained cells In this study, it was observed that Gβγ inhibition did not attenuate strain-induced increases in phospho-ERK1/2 However, inhibition of Gβγ activity resulted in diminished phosho-p38 activity with concominant decreases in MMP-2 activity in response to cyclic strain Similarly, selective inhibition of p38 activity significantly reduced strain-induced increases in pro-MMP-2 activity These data suggest that strain-induced changes in MMP-2 expression and activity may in part occur via a Gβγ/p38 dependent pathway PTK inhibition significantly attenuated strain-induced increases in pro-MMP-2 activity with concurrent decreases in phospho-ERK1/2 activity but not phospho-p38 activity MMP activity has previously been linked to changes in MEK [Kito *et al*, 2000, Visse *et al*, 2003, Galis *et al*, 2002, Beaudeux *et al*, 2003], and selective inhibition of ERK significantly attenuated strain-induced changes in pro-MMP-2 activity and protein levels, together these data confirm an important role for ERK in mediating these events These data suggest that cyclic strain stimulates pro-MMP-2 expression, in part, by stimulating both p38- and ERK-dependent pathways through activation of Gβγ and tyrosine kinase in BAEC (see Figure 6 2)

Inhbition of either the Gβγ subunit or PTK both attenuate strain-induced increases in pro-MMP-2 activity by similar amounts Similarly, inhibition or either p38 or ERK1/2 attenuates pro-MMP-2 activity by comparable amounts This may suggest a possible redundancy mechanism for the regulation of MMP-2 in response to cyclic strain Alternatively and more likely the activation of both p38 and ERK1/2 is required for cyclic strain-induced increases in MMP-2 In support of this hypothesis, previous studies have identified that both p38 and Erk1/2 activation are involved in increases in MMP-2 and MMP-9 in response to a number of stimuli [Donnini *et al*, 2004, Esparza *et al*, 1999, Wang *et al*, 2002] Inhibition of PI-3 kinase, ERK1/2 or p38 all have profound effects on the MMP-2 and MMP-9 activity [Esparza *et al*, 1999] Ruhul

Amin *et al*, (2003) have demonstrated that activation of ERK1/2 and p38 is required for MMP-2 secretion in response to concanavalin-A, however they do not determine why simultaneous activation of these MAPKs is required for these events Similarly, secretion of MMP-9 following injury could only be attenuated by inhibition of both p38 and ERK1/2 [Wang *et al*, 2002] Lee *et al*, (2002) infer that p38 MAPK may have a regulatory role in ERK1/2 activation and subsequent regulation of MMP-2, MMP-9 and uPA ERK1/2 activation of Ap-1 has a stimulatory effect on MMP-2 expression [Donnini *et al*, 2004] The expression of c-fos and c-jun the two components of the AP-1 conplex are regulated by p38 [Frigo *et al*, 2004] Thus, increased expression of c-fos and c-jun via p38 signalling and subsequent activation via ERK1/2 may bring about increases in MMP-2 expression

We have demonstrated that the adapter protein Shc is important in mediating strain-induced changes in pro-MMP-2 activity and ERK-1/2 phosphorylation Previous studies have demonstrated the regulation of ERK via the recruitment of Shc by integrins ($\alpha v\beta 3$) and PTKs (Flk-1, FAK) [Giancotti *et al*, 1999, Chen *et al*, 1999] GPCRs may phosphorylate Shc leading to the activation of Ras [Gudi *et al*, 2003] which may subsequently activate p38 and/or ERK1/2 [Ruhul Amin *et al* 2003] Thus, Shc may play a pivotal role in the activation of ERK-1/2 via a PTK or the activation of p38 via $G\beta\gamma$ Previous studies have indicated the relevance of integrins in activation of the MAP kinase pathways [Shyy *et al*, 2002, Labrador *et al*, 2003] In addition integrin signaling has been associated with G-proteins and PTKs However, in this study strain-induced changes in pro-MMP-2 were found to be independent of integrin signaling

Regulation of MMP-2 secretion and activation seems to involve an intricate process We have identified two pathways of equal importance which regulate strain-induced increases in MMP-2 The reason for the existence of two pathways exists is not yet understood One explanation is that the two pathways are in fact one more complex pathway However this is speculative and will require further work to identify links between the two pathways

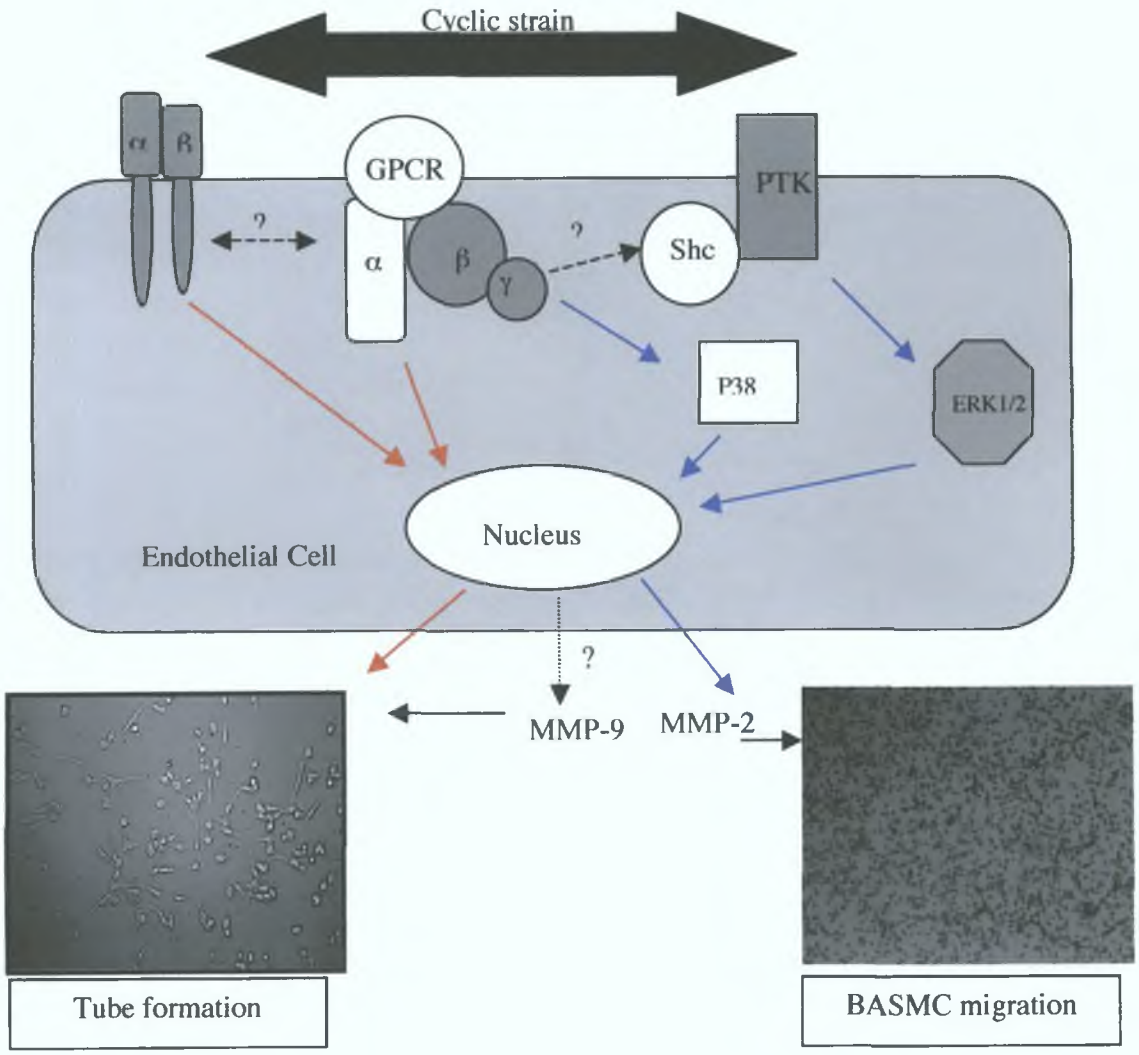


Figure 6.2: Possible EC signaling mechanisms following cyclic strain. Gβγ/p38 and PTK/ERK-1/2 regulation of MMP-2, which plays a role in SMC migration. Integrin and Giα signaling is involved in EC tube formation. Cyclic strain leads to increases in MMP-9, the signaling pathway was not elucidated, but it plays a role in strain-induced tube formation. (-------) signifies possible associations or interactions.

The mechanisms by which cyclic strain mediates changes in pro-MMP-2 expression/activity appear to somewhat different from those governing migration and angiogenic activity. Strain-induced changes in BAEC migration and tube formation

were sensitive to Giα-protein and integrin signaling Whether these receptors work in concert or independently in this context has yet to elucidated Thus, while cyclic strain-induced increases in pro-MMP-2 activity occur concurrently with strain-induced migration and tube formation they occur via independent signaling pathways To elucidate if strain-derived MMPs were involved in strain-induced migration and tube formation, we attenuated MMP by using either a broad-spectrum inhibitor (GM-6001) or selectively using siRNA targeted to either MMP-2 or MMP-9 We have established that strain-induced transwell migration was an MMP-independent process however, tube formation was MMP-dependent and more specifically MMP-9 dependent

We have determined that increased MMP-2 activity associated with cyclic strain did not contribute to strain-induced migration or tube formation However, we believe that strain-induced increases in MMP-2 activity may play a role in endothelial function Under normal physiological conditions hemodynamic forces generally offer a protective effect Exposure of EC to physiological levels of cyclic strain (5 - 10%) suppresses apoptosis via a PI-3 kinase / Akt dependent pathway Exposure to higher magnitudes of cyclic strain (20%) has been shown to induce apoptosis [Haga *et al*, 2003, Chen *et al*, 2001, Liu *et al*, 2003] The ability of physiologic cyclic stretch to inhibit EC apoptosis may provide a previously unrecognized mechanism by which hemodynamic forces exert an anti-atherogenic effect The recruitment of PI-3 kinase and Akt by integrin subunits has been well documented Suitable interaction of integrins with components of the ECM is required for cell survival and loss of ECM attachment leads to apoptosis Therefore, cyclic strain-induced increases in MMP-2 activity may play a role in suppressing apoptosis by suitably modifying the ECM to promote cell survival Cyclic strain has also been linked to increases in the proliferation of a number of cell types including endothelial cells [Vouyouka *et al*, 1998, Murata *et al*, 1996] Cyclic strain may enhance cellular proliferation by inhibiting protein phosphatase 2A as well as stimulating protein kinase C [Murata *et al*, 1996] Conversely, conditioned media from strained EC has been found to inhibit the proliferation in a TGF-β independent manner [Vouyouka *et al*, 1998] Cell growth requires modification of the ECM and increased activity of MMP-2 has been linked to increases in proliferation [Li *et al*, 2003] Thus,

strain-induced increases in MMP-2 activity may play a role in maintaining suitable levels of cell growth and cell death within the endothelial monolayer.

Vascular remodeling in response to hemodynamic forces requires SMC migration/proliferation [Galis *et al.*, 2002]. Many molecules secreted from EC in response to mechanical forces may affect SMC phenotype [Wang *et al.*, 2003; Wung *et al.*, 2001; Sumpio *et al.*, 1998; Cheng *et al.*, 1996; Cucina *et al.*, 2003; Powell *et al.*, 1998; Fillinger *et al.*, 1997]. EC-derived MMP may play a role in modifying the ECM to facilitate SMC migration. Therefore, we investigated the effect of conditioned media from cyclically strained BAEC on BASMC migration and particularly focused on the relevance of MMPs in this process. We determined that post-strain BAEC-conditioned media significantly inhibited SMC migration. Moreover, MMP-2 but not MMP-9 plays an important role in eliciting this response but does not act directly on SMCs.

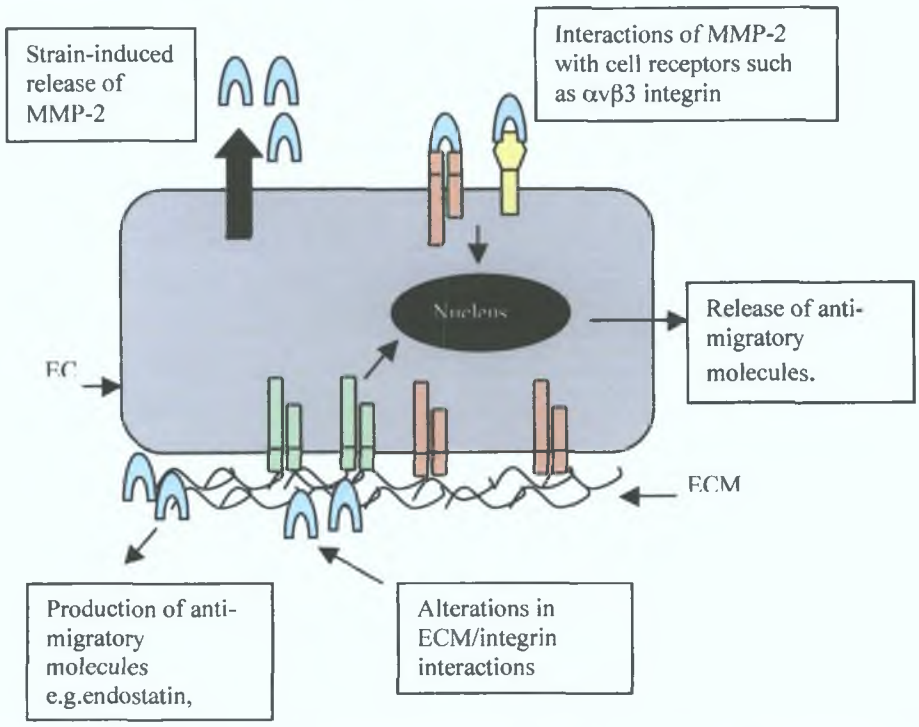


Figure 6.3: Possible mechanisms by which EC derived MMP-2 may effect SMC migration

The ability of MMP-2 to indirectly inhibit BASMC migration may come from its ability to generate anti-migratory molecules such as angiostatin or endostatin from plasminogen and collagen respectively Alternatively MMP-2 activity may effect production of anti-migratory cytokines from BAECs by altering integrin/ECM interactions MMP-2 may also bind to integrin receptors such as $\alpha v\beta 3$ on the cell surface [Sternlicht *et al*, 2001] Interactions such as this may trigger the release of migratory inhibtors

As previously discussed in Chapter 5 the uPA system may be of particular importance in the MMP-2 dependent effects on SMC migration Considerable cross talk exists between the components of the uPA system and MMPs [Siconolfi *et al*, 2003] and significant evidence exists indicating their roles in hemodynamically mediated events [Redmond *et al* 1999, Redmond *et al*, 2001] MMP-2 involvement with vasoactive molecules such as NO and ET-1, ROS and growth factors such as VEGF or bFGF may provide new insights into the ability of EC to mediate SMC fate decisions

Nitric oxide (NO) has been demonstrated to play a central role in vascular biology Endothelial NO synthases (eNOS) expression and activation is regulated in part by mechanical forces such as cyclic strain NO can modulate a number of cellular events in response to cyclic strain such as inhibition of MAPK activity or regulating MCP-1 expression [Ingram *et al*, 2000, Wung *et al*, 2001] The ability of NO to modulate SMC migration is believed to occur via regulation of MMP-2 and MMP-9 activities Inhibition of NO during AVF stimulated arterial enlargement resulting in decreased MMP activity with reduced remodeling [Tronc *et al*, 2000] Gurjar *et al*, (1999) on the other hand demonstrated that overexpression of eNOS inhibited SMC migration by inhibition of MMP-2 and MMP-9 In conjunction with our data, these findings suggest that interactions between NO and MMPs possibly in conjunction with ROS derived from cyclically strained EC may play a role in regulating SMC migration

VEGF is a potent cytokine which is sensitive to changes in mechanical forces {Brown *et al*,2003, Rivilis *et al*, 2002] This cytokine has been linked to increased angiogenesis, proliferation, and migration of both EC and SMC The expression of MMP-2 and VEGF appears to be intrinsically linked Brown *et al*, (2003) demonstrated that cyclic strain-induced angiogenesis was associated with increases in VEGF and MMP-2 The release of VEGF may also be regulated by MMP-2 [Belotti *et al*, 2003] Thus strain induced increases in MMP-2 may influence release of VEGF from EC with subsequent effects on SMC migration

This study identifies the roles of some mechanically sensitive pathways in strain-induced increases in migration, angiogenesis and MMP-2 Future work may involve a more complete analysis of the components of these pathways In addition, the mechanism by which cyclic strain-induced increases in MMP-9 occur need to be determined as it plays an important part in strain-induced angiogenesis Expansion of this study to work with human endothelial cells or *in vivo* models of enhanced cyclic strain may provide a greater insight into the relevance of these pathways in MMP regulation In addition, the use of a human cell line would permit the employment of gene chip analysis for the identification of genes regulated by cyclic strain which may be important in angiogenesis The development of a co-culture model would give a better understanding of EC/SMC interactions during exposure to cyclic strain This combined with the ability to specifically target MMPs in either of the cell types would identify the source and roles of MMPs in mediating either EC angiogenesis or SMC migration

In conclusion our data suggests that cyclic strain plays an important role in the regulation of MMPs in BAEC These MMPs are important in mediating cell functions in both BAEC and BASMC The identification of the mechanisms by which cyclic strain regulates MMPs and angiogenesis may lead to possible new drug targets that can promote/inhibit angiogenesis *in vivo* Similarly a more complete understanding of how EC and SMC interact in response to hemodynamic forces may lead to a better understanding of pathological vascular remodeling

Chapter 7

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