The role of Moesin in the vasculature; regulation and functions under mechanotransduction.

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 program of study leading to the award of Doctor of Philosophy is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, 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 work.

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Acknowledgements

This is the hardest part to write in this thesis. At the same time, here I get the poetic license to go mad with my broken english.

Heartfelt thanks to Ronan who mentored this research and made sure I was walking the line. I feel also lucky to have been exposed to diverse areas of vascular research. And for all the genuine help, support and guidance both professionally and otherwise. Thanks Phil for all the timely advice, practicality and support. Dr Bernard Degryse for his very inspirational visit. Thanks to Ger for all the help and also the banter at conferences. I would also like to say thanks to everyone including Paul Cahill, Yuvvone, Laura, Gail and others from the "old lab". Thanks to Dr Greg Foley, Dr Neal Lemon for route to bread, and Ronan again for facilitating this.

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Jeffers

Appa, Amma Y Amma

Everyone from the island Lastovo

Abstract

Cardiovascular disease is the major cause of morbidity and mortality, and is preceded by chronic irregular blood flow. The resulting pathogenesis recruit a platform of molecules, which require the reshuffle of the omnipresent cytoskeleton.

Utilizing novel techniques including microfluidics and flexible membranes we subject both endothelial and smooth muscle cells to specific shear stress and cyclic strain. We then endeavor to understand the functions, signaling mechanisms and cytoskeletal dynamics that involve moesin. We also try to expand the nascent knowledge of microRNA regulation here.

The work show global regulation of major facets of mechanotransduction (including proliferation, endothelial microparticle release, realignment), by microRNAs. As well, an array of microRNAs is found sensitive to shear stress and cyclic strain. Furthermore, expression pattern of these microRNAs are profiled.

Actin binding ERM protein moesin meanwhile, is seen as an important modulator of mechanotransduction regulated by microRNA and Rho A. In contrast to ERM protein ezrin, moesin we find is sensitive to mechanotransduction, and is atheroprotective buffering endothelial microparticle release, while subtly sensitive to thrombosis and haemostasis. The spatial and temporal dynamics of moesin, studied with mechanotransduction, demonstrates its key role, regulated by RGD-sensitive integrins.

Urokinase is known to effect the endothelial cells with cyclic strain and injury. We find novel interaction of urokinase, requiring similar biochemical change off moesin utilized during mechanotransduction. We delineate the different transducers including FPRL1, αVβ3 / α5β. Moesin under these transducers is critically involved in chemotaxis, angiogenesis and endothelial barrier integrity. Moesin thus we discover is indispensable towards the normal functioning of the vasculature.

Peer reviewed poster presentations

2009 International Symposium of the Blood Brain Barrier, London, UK
The Role of MicroRNA regulation of cortical actin binding dynamics at the endotheial membrane; Moesin's perspective. Britto, M., Fitzpatrick, P., Murphy, A., Cummins, P., Meade, G., Murphy, R.P.

2008 North American Vascular Biology Organization (NAVBO)- Boston, US
The Role of Hemodynamic Stimuli on Endothelial Cell microRNA Expression Signature: Potential New Regulators of Actin Binding Proteins. Britto, M∗., Fitzpatrick, P∗., Meade, G., Barron, N., Cummins, P.M., Murphy, R.P. ∗equal contribution

2008 Irish Society of Gene and Cell Therapy (ISGCT) - Cork, Ireland
The Role of Haemodynamic stimuli on Endothelial cell microRNA expression signature: Potential new targets for Cardiovascular gene therapy. Britto, M∗., Fitzpatrick, P∗., Meade, G., Barron, N., Cummins, P.M., Murphy, R.P. ∗equal contribution

2007 International Society of Thromobosis and Haemostasis (ISTH) - Geneva, Switzerland
Molecular and Cellular dynamics of Moesin in vascular cells via mechanotransduction. Britto, M., Fitzpatrick, P., Meade, G., Cahill, P.A., Cummins, P.M., Murphy, R.P.

2007 International Society of Thromobosis and Haemostasis (ISTH) - Geneva, Switzerland
Mechano-Regulation of LASP-1 in the pathogenesis of Cardiovascular disease Fitzpatrick, P., Britto, M., Cahill, P.A., Cummins, P.M., Meade, G., Murphy, R.P.

Peer reviewed oral presentations

2009 International Society of Thromobosis and Haemostasis (ISTH)- Boston, US

Bio-mechanical regulation of microrna expression and endothelial cell phenotype in vitro Murphy, A∗., Britto, M∗., Fitzpatrick, P., Barron, N., O'Gorman, D., Moyna, N., Cummins, P., Murphy, R.P. ∗equal contribution

2007 Irish Society of Gene and Cell Therapy (ISGCT) - Galway, Ireland

The Role of the Actin binding proteins LASP-1 and Moesin, in the pathogenesis of Cardiovascular disease Fitzpatrick, P∗., Britto, M∗., Ferguson, G., Cummins, P.M., Cahill, P.A., Meade, G., Murphy, R.P. ∗equal contribution

Other Conferences and Oral presentations showcasing this research

Keynote Lecture at the opening of the new Waterford Institute of Technology Biomedical Research Centre, 27th February 2009. The Cell and Molecular Physiology of Vascular Health Research

IFOM-IEO CAMPUS, European Institute of Oncology, Milan, Italy, May 2006. Integrin Modulation- From Hematopoietic to Vascular Cells.

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Health Research Board Project Grant 2009-2012 (HRA/2009/122). "The Role of microRNA in Modulating Vascular Smooth Muscle Cell Phenotype: Novel Regulators of Vascular Physiology and Disease".

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First name papers in preparation

Temporal and Spatial dynamics of the ERM Protein, Moesin, in the Vasculature and its Role in the Biogenesis of Endothelial Microparticles.

Moesin involves integrins and FPRL1 and is critically required to mediate Urokinase mediated migration, angiogenesis and barrier integrity of endothelial cells.

Second name papers in preparation

The Role of Lasp-1 in Cell-Cell, Cell-Matrix Adhesion Dynamics in Endothelial Cell.

Regulation of Lasp-1 by Hemodynamic Forces with in Endothelial Cells and its Role in Actin dynamics.

The role of microRNA in Integrin Signalling: Novel players in vascular inflammation.

Abbreviations

2-D-DIGE	2-D Fluorescence Difference Gel Electrophoresis
Ago	Argoanute
AREs	AU-rich elements
ATF	Amino Terminal Fragment
ATP	Adenosine triphosphate
BAEC	Bovine Aortic Endothelial Cells
bFGF	Basic Fibroblast Growth Factor
CaM	Calmoudulin
CAM	Chick Chorioallantoic Membrane
CAP23	Cytoskeleton-associated protein23
CAPS	Ca^2+ dependent activator protein
CDC42	Cell division control protein 42 homolog
CHO	Chinese Hamster Ovary Cell
CK2	Casein kinase-2
COPI	Coat protein complex I
COPII	Coat protein complex II
CVD	Cardiovascular Diseases
DAG	Diacylglycerol
DGCR8	DiGeorge syndrome critical region 8 protein
EC	Endothelial Cells
ECM	Extracellular Matrix
EGF	Endothelial Growth Factor
EGFR	Epidermal Growth Factor
Egr-1	Early Growth Response Factor 1
EMP	Endothelial Microparticles
ERK	Extracellular Signal-regulated Kinase
F-actin	Filamentous Actin
FAK	Focal Adhesion Kinase
FERM	(F for 4.1 protein, E for ezrin, R for radixin and M for moesin)

fMLP	Formyl-Methionly-Leucyl-Pheynylalanine
FPRL1	Formyl Peptide Receptor 1
FRET	Fluorescence Resonance Energy Transfer Analysis
Fyn	Proto-oncogene tyrosine-protein kinase Fyn
G-actin	Globular Actin
G-protein	Guanine nucleotide binding protein
GAP43	Growth-associated protein 43
GAPs	GTPase activating proteins
GDIs	Guanine Nucleotide Dissociation inhibitors
GDP	Guanine di Phosphate
GEFs	Guanine Nucleotide Exchange factors
GFP	Green Fluorescent Protein
GMC proteins	(GAP43, MARCKS and CAP23 proteins)
GPCRs	G-protein-coupled receptors
GPI	Glycosyl-phosphatydil-insositol
GTP	Guanine Triphosphate
HAECs	Human Aortic Endothelial Cells
HCC	Hepatocellular Carcinoma
HDL	High Density Lipoproteins
HHV8	Human herpesvirus 8
HIV	Human Immunodeficiency Virus
HKa	High molecular-weight kininogen
hnRNPC	Heterogeneous Nuclear Ribonucleoprotein C
Hp	*Helicobacter pylori*
HSPG	Heparin Sulphate Proteoglycans
ICAM	Inter-Cellular Adhesion Molecule
ILK	Integrin Linked Kinase
IP3	IInositol1,4,5-trisphosphate
ITAM	Immunoreceptor Tyrosine based Activation Motif
JAK	Janus Kinases

JNK	c-JUN N-terminal Kinase
LASP-1	Lim and SH3 protein 1
LDL	Low density lipoproteins
LDLR	Low Density Lipo Protein Receptor
LPP	Lipoma preferred partner
LRP-1	Lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase
MARCKS	Myristoylated alanine-rich C kinase substrate
MBS	Myosin Binding Subunit
Mef2	Myocyte-enhancing Factor 2
MMP-2	metallaproteinase2
MP	Microparticles
miRISC	miRNA-induced silencing complex
miRNA	microRNA
MLCK	Myosin light-chain kinase
MMPs	Matrix metalloproteases
Moesin	Membrane-organizing Extension Spike Protein
MRTF	Myocardin-related Transcription Factor family
Myo X/ Myo 10	Myosin 10
MyoD	Myogenic differentiation factor D
NFkB	Nuclear factor kappa-B
NO	Nitric Oxide
PAF	Platelet Activating Factor
PAI-1	Plasminogen Activator Inhibitor
Pak	p21-activated kinase
PARs	Protease Activated Receptors
PC	PhosphatidylCholine
PDGF	Platelet Derived Growth Factor
PE	Phosphatidylethanolamine
PECAM-1	Platelet/endothelial cell adhesion molecule-1

PH	Pleckstrin Homology
PGK	phosphoglycerate kinase
PLC	Phospholipase
PI3-K	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIPKIg661	Phosphatidylinositol Phosphate Kinase Isoform g 661
PKC	Protein Kinase C
PKC	Protein kinase C
PKN	Protein kinase N
PLC	Phospholipase C
PLSCR1	Phospholipid Scramblase
PMA	Phorbol 12-myristate 13-acetate
PP2C	Type 2 protein phosphatase
PS	Phosphatidylserine
PTB	Phosphotyrosine binding
PTPL1	Protein Tyrosine Phosphatase
RBC	Red Blood Cells
RGD	Argenine-Glycine-Aspartic acid
SAA	Serum Amyloid A
SDF-1α	Chemokine Stromal Cell-Derived Factor 1α
siRNA	Silencer RNA
SMB	Somatomedin B domain
SRFs	Serum-response factors
SRSRY	(S for serine, R for arginine and Y for tyrosine) peptide
ssRNA	single-stranded RNA
TFPI	Tissue Factor Pathway Inhibitor
TGFβ	Transforming Growth Factor
TIMP-1	Tissue Inhibitor of MMPs
TNFα	Tumour Necrosis Factor α
tPA	Tissue-type plasminogen activator

uPA	Urokinase
uPAR	Urinokinase plaminogen activator receptor
UTR	Untraslated region
VCA	verprolin-cofilin-acidic
VCAM1	Vascular cell-adhesion molecule 1
VEGF	Vascular endothelial growth factor
Vn	Vitronectin
VSMCs	Vascular Smooth Muscle Cells
vWF	von Willebrand factor
WASP	Wiskott−Aldrich syndrome protein
WAVE	Wiskott−Aldrich syndrome family Verprolin-homologous protein
WHO	World Health Organization
ZAP-70	Zeta-chain-associated protein kinase 70

Units

Å	Angstroms
bp	Base Pairs
cm	Centimeters
cm^2	Centimeter Squared
c^o	Degree Celsius
g	Grams
h	Hours
kDa	Kilodaltons
L	Litre
M	Molar
mg	Milligrams
min	Minute
ml	Mililitre
mm	Milimetre
mM	Milimolar
ng	Nanogram
OD	Optical Density
pM	Picomolar
rpm	Revolutions Per Minute
sec	Seconds
U	Enzyme Units
μg	Microgram
μl	Microlitre
μm	Micrometre
μM	Micromolar
VN	Vitronectin
V/v	Volume per volume

W/v	Weight per volume
V	Volts
W	Watts
x g	G force

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Chapter 1

Introduction

1.1 Pathogenesis of the Vascular system

Introduction to a thesis in vascular biology, usually starts by appreciating, how deadly pathogenesis of the cardiovascular system can be. These stem from certain other facts, which needs to be appreciated more.

1. Unhealthy diet
2. Physical inactivity
3. Tobacco use
4. Weight

Commonly referred to as modifiable facors, these contribute to 80% of deaths due to cardiovacular diseases (CVD). This is no small factor, when according to the World Health Organisation (WHO), CVD is the number one cause of global deaths $http://www.who.int/topics/cardiovascular_diseases/en/$.

The above list chronically leads to intermediate risk factors which are raised blood pressure, raised blood glucose, raised blood lipids and obesity. Research into the pathogenesis of the cardiovascular system is the only check point that could challenge the projected 20 million deaths by 2015 due to CVD (Van Gaal

et al., 2006; Coca *et al.*, 2008; Forman and Bulwer, 2006; Sniderman and Furberg, 2008)(Van Gaal *et al.*, 2006)

The human circulatory system sustained by current medical advances, is a system run beyond its evolved capacity. The blood vessels and heart are constantly challenged by obesity, diabetes and chronic smoking. Meanwhile we are living ever longer. Age alone outweighs all the other modifiable factors in its ability to increase risk (Sniderman and Furberg, 2008). Classing it as a modifiable factor is currently beyond technological capability, as we can't actually modify it. Hundred years ago human beings didn't have the lifespan we enjoy. Rapid advance in research, has led to our ability to counter considerably at a medical level. Age related concerns though, need a different perspective of technology. Stem cell derived artificial vessels, might be in the right direction. Stem cells, have been found to utilize mechanotransduction towards differentiating as vascular cells. Studies into stem cell derived grafts, also find flow to be an indispensable requirement for graft retention (McIlhenny *et al.*, 2009; North *et al.*, 2009; Stolberg and McCloskey, 2009).

Body was not evolved to meet age related challenges it is presented with. Considering these factors, it has to be appreciated this feat of engineering, that is able to self-maintain, grow and adapt to its environment is truly remarkable. Vascular vessels are not simply inert plumbing that delivers blood, but rather is a dynamic organ. It has the ability to remodel and transform itself to a changing environment. Over long periods of time, conduit vessels will remodel considerably, according to the needs of a particular agonist. This is the means by which pathogenesis takes hold, after exposure to modifiable factors over a lifetime (Martinez-Lemus *et al.*, 2009).

1.1.1 Vasculature

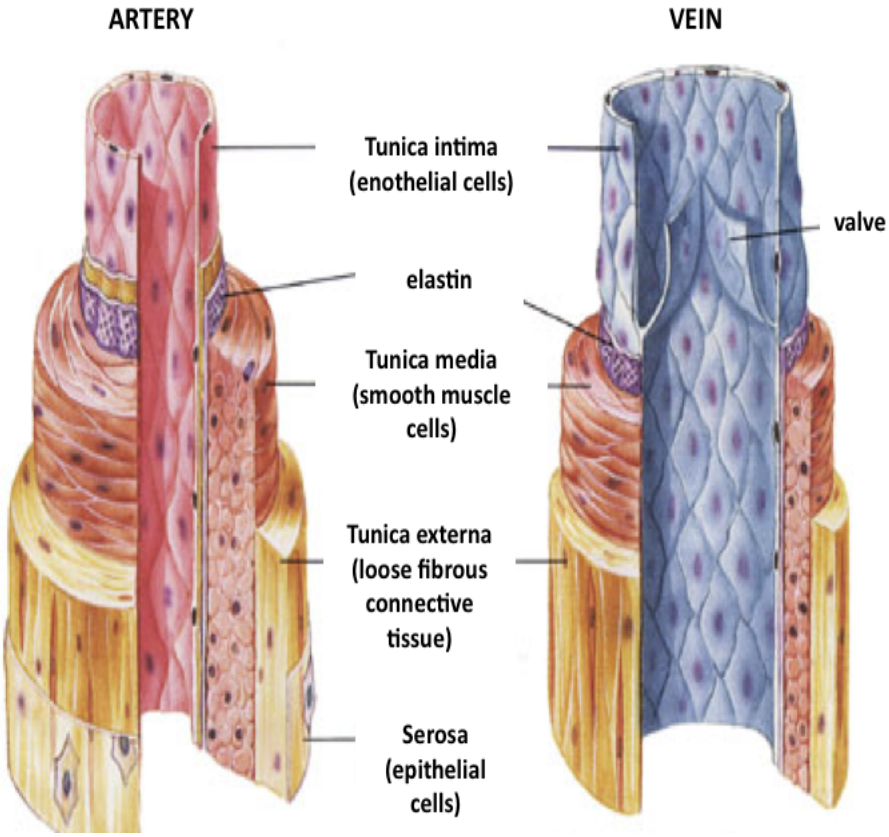

Figure 1.1: Cross Section of the Vasculature
The figure shows diagrammatic representation of an artery and vein, showing different layers of the vessel. Note that the innermost layer is the endothelial cell, which is a contact inhibited confluent monolayer of cells (Fox, 2002).

1.1.2 Endothelium

The first line of cells (Figure 1.1), the endothelium, is unique in its ability to sense blood flow, which results in many different stimuli being constantly relayed to it (Rizzo, 2009). Derived from hemangioblasts, they maintain "regional specialisation" dependent on the local needs. During pathogenesis,

there occurs activation of endothelium by tumour necrosis factor-α (TNF-α) and other molecules, resulting in inflammation that counter pathogenesis (Rask-Madsen and King, 2007; Koh, 2008; Kubo *et al.*, 2005).

As the role of the endothelium goes, it is primarily a component of the vasculature, in direct contact with blood while surrounded by smooth muscle cells, connective tissue (elastic and collagen fibers) and extracellular matrix (ECM). It is non-thrombogenic in nature and with the help of thrombomudulin, tissue factor pathway inhibitor (TFPI), protein C, heparin sulphate proteoglycans (HSPG) it promotes fibrinolysis. It is also involved in the production of prostacyclin, nitric oxide (NO), tissue-type plasminogen activator (tPA), urokinase (uPA) and CD39 and inhibits platelet aggregation and clotting cascade activation. It exists as a quiescent monolayer (Rask-Madsen and King, 2007; Koh, 2008).

Endothelial cells signaling mechanisms constantly strive to achieve homeostasis rapidly in case of injury or trauma. With respect to flow, it clearly aligns in the direction of blood flow (Goldfinger *et al.*, 2008). Any disturbances in flow, clearly disrupts the brushed alignment in the direction of flow. This is also true with respect to studies that interfere with signaling pathways, that mould the cells to align in this way. Actin and associated cytoskeletal proteins play pivotal role towards this (Chien, 2008; Vartanian *et al.*, 2008). Release of prothrombotic factors including Von Willebrand Factor (vWF) with trauma is a prelude to achieving homeostasis (Jacquemin, 2009; Oliveira *et al.*, 2008). There are other evidences in the literature that show vWF release with mechanotransduction (Kearney *et al.*, 2008). Weibel-palade bodies store and secrete vWF (von Willebrand factor) in endothelial cells (Rondaij *et al.*, 2008; Huang *et al.*, 2008).

Endothelial cells also maintain a selective barrier to the onslaught of various factors from blood especially, when lining organs. Endothelium controls the selective permeability of water and solutes. The barrier/permeability specializes according to the needs of the cell, and thus is strictly dependent on where the endothelial cells line. The endothelium is found to maintain a strict barrier between the blood and brain (Weksler *et al.*, 2005; Janzer and Raff, 1987).

Glomerulus in the kidney is another area where endothelium maintains a selective barrier (Satchell and Braet, 2009). Integrin αvβ3 is found to be a modulator of endothelial barrier properties. Which of course recruit cytoskeletal adapter molecules, that mould the cells for this specialisation. These are examples of "regional specialisation" that endothelium undergoe dependent on the organ's need (Li *et al.*, 2008).

Endothelium dependent vasodilation is an atheroprotective mechanism that induce synthesis of nitric oxide (NO) and prostacyclin (Davignon and Ganz, 2004; Mitchell *et al.*, 2008). Endothelial cells also derive endothelin-1, angiotensin II, platelet derived growth factor (PDGF). Uniform linear laminar flow of blood and physiological cyclic strain is another atheroprotective measure. Pathogenic hemodynamic flow conditions, will alert by downstream signaling the cells to adapt and alleviate the circumstances. Normal physiological haemodynamic flow results in NO and prostacyclin production. Transforming growth factor (TGF)-β (transforming growth factor), is also induced by laminar shear stress which counter inflammation (Libby *et al.*, 2002; Koh, 2008).

Endothelial cells, due their contact with blood is in direct contact with leukocytes. During inflammation and attack by pathogens, leukocytes are attracted by endothelial cells by presentation of its inflammatory status. These chemotactic and adhesive molecules, help leukocytes maintain a selective binding capability on endothelial cells (Vestweber, 2007; Baluk *et al.*, 2007). A key player in this are the polysaccharide glycosaminoglycans, in the form of proteoglycans. Cell adhesion molecules such as selectins (P-selectins, E-selectin) and immunoglobulin superfamilies (ICAM-1, PECAM-1) have roles in the leukocyte interaction. Its important to be selective in endothelial cell's stickiness to leukocytes, while still maintaining blood flow (Ley *et al.*, 2007; Woodfin *et al.*, 2009). This is to maintain them in circulation, which is controlled by endothelium. This is significant, as chronic activation of endothelium at certain sites lead to future plaques focussed to these areas. Without finite regulatory mechanisms in place, the flow of blood would be impeded constantly ending in premature death (Koh, 2008; Vestweber, 2007; Baluk *et al.*, 2007).

Remodeling

Chronic inflammatory stimuli, will lead to the remodeling of the vasculature. Dependent on the stimuli, endothelial cells mediate the adaptive response. Non-uniform blood flow is consistently found to be the major factor. Chronic hypoxia is also an usual suspect that induce remodeling (Chiang *et al.*, 2009b; Laughlin and Roseguini, 2008; Hahn and Schwartz, 2009). This remodeling obviously starts a genetic program ending in expression of pro-angiogenic factors. Recently deceased distinguished scientist, Judah Folkman first to describe angiogenesis and its uncontrollable continuous growth in tumours. He suggested inhibition of angiogeneis to contain tumour growth. This has since been used starve tumour and is a promising method to combat cancer (Koh, 2008; Folkman, 2006, 2007). Clinical trials of various drugs are underway that target angiogenesis in this manner (Crawford and Ferrara, 2009; Giaccone and van Cruijsen, 2009; Giraudo and Hanahan, 2009).

Angiogenesis

During angiogenesis, vascular endothelial growth factor (VEGF), PDGF, basic fribroblast growth factor (bFGF) are produced. This is regulated by utilizing negative factors such as angiostatin and TGF-β. Also involved is uPA, matrix metalloproteases (MMPs), plasminogen activator-inhibitor (PAI-1), tissue inhibitors of MMPs which digests the basement membrane and extracellular matrix (Lac, 2007; Neal *et al.*, 2006; Goh *et al.*, 2007; Shetty and Idell, 2007; Wong *et al.*, 2009). This paves the road forward by digestion, allowing new vessels to sprout. Endothelial cells also regulate vascular tone and compliance, primarily in response to blood flow (Reneman *et al.*, 2006; Birukov, 2009b). Cyclic strain and shear stress signaling mechanisms also thus feed into angiogenesis (Von Offenberg Sweeney *et al.*, 2005; Gee *et al.*, 2009).

1.1.3 Vascular Smooth Muscle cells

Vascular smooth muscle cells (VSMCs) (Figure 1.1), are elongated and bipolar. They are pluripotent and retain their plasticity, which is dependent on environmental cues. They play roles in contraction, owing to actin-myosin fibers anchored to dense bodies. They also have roles in immune regulation, laying the matrix and in cytokine production (Ruan *et al.*, 2006; Newby, 2006; Clempus and Griendling, 2006). They are normally arranged in palisading rows, that are part of tunica media (Figure 1.1). In pathogenic conditions with vascular injury, they can be exposed to the neo-intima. They exist in higly differentiated state, while retaining the ability to migrate. Contrasting studies exist regarding their proliferation, though cyclic strain has been shown to regulate VSCM's proliferation (Rudijanto, 2007; Birukov, 2009b)

Normally, VSMCs sense haemodynamic force of shear stress by means of paracrine signaling. This is mediated by endothelial cells, which in turn directly sense shear stress. Vascular smooth muscle cells, also generate increased microparticles in response to diseased conditions (Ess, 2005). These signals, also translate in endothelial cells due to the close proximity to VSMCs. VSMCs meanwhile, are able to directly sense circumferential strain due to blood flow. In an injured artery, such as after balloon angioplasty when endothelial cells are scraped exposing smooth muscle cells, VSMCs are able to directly sense shear stress (Fitzgerald *et al.*, 2008; Haga *et al.*, 2007). Their characteristic response to stretch is to produce vasoconstrictors and growth factors. With stretch they also regulate ion-channels and signaling enzymes. It regulates the membrane potential (E_m) to four ions K^+,Cl^-, Na^+ and Ca^{2+} (Haga *et al.*, 2007). Cell-cell and ECM-cell, paracrine interactions all feed into VSMC behaviour. In conclusion VSMCs maintain vascular tone and drives vascular remodeling (O'Rourke *et al.*, 2006; Haga *et al.*, 2007)

1.1.4 Flow of blood

Haemodynamic forces, result from the flow of blood, a primary reason, for which the circulatory system evolved. There is the circumferential wall tension, originating from the blood pressure. There is also the force of shear stress, resulting from friction due to the blood dragging on the endothelium.

At the macro level, flow plays an essential role and is not a waste of energy due to friction. The circulatory system utilises friction and flow. Flow mediated friction is used to sense underlying problems, by means of various receptors on the endothelial cells. At the micro level, this information is processed into well established signaling pathways. When there are disturbances in flow of blood, these signaling mechanisms adapt to alleviate flow abnormalities (Hahn and Schwartz, 2009). With chronic pathogenesis, these mechanisms even lead to remodeling of the system. The end goal of which, is to maintain continuous supply of oxygen and nutrients throughout the body (O'Rourke *et al.*, 2006; Haga *et al.*, 2007; Hahn and Schwartz, 2009).

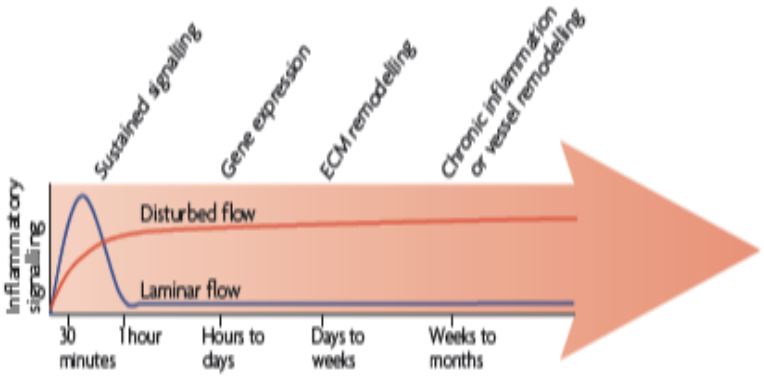

Figure 1.2: Chronic non-uniform flow and its effects
The effects of chronic dysfunctional flow, over time in contrast to normal laminar flow.
(Hahn and Schwartz, 2009)

Current knowledge about flow of blood suggests intervention in term of good diet and excersice at an early age. Even a moderate level of excersice has shown to increase the NO and prostacyclin at areas prone to fatty streak de-

position (thus prone to plaque formation) (Belardinelli *et al.*, 2006). These are areas where non-laminar flow, low flow and sometimes oscillatory flow exists. These turbulent flow patterns are usually realized in points of branching (convex areas of branching) (Hahn and Schwartz, 2009). Over time dysfunctional hemodynamic forces, cause vasculature to remodel and cause it to become diseased (Figure 1.2). These fatty streaks are usually the map of areas prone to future artherosclerotic beginnings (Figure 1.3) (Koh, 2008).

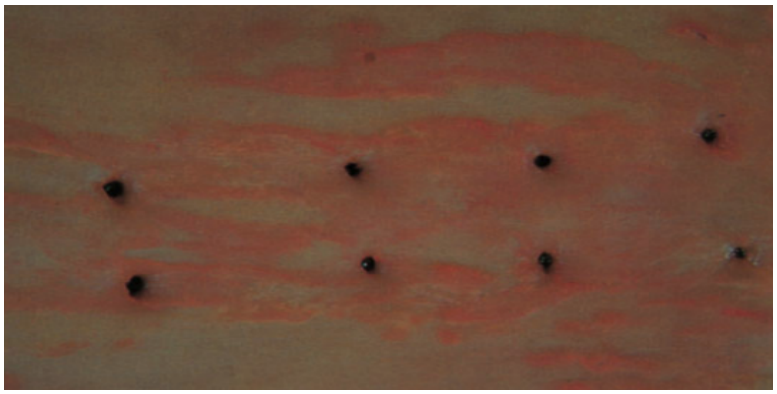

Figure 1.3: Fatty streak prone to future atherosclerosis
The map of fatty streak in endothelial cells, which is where, future atherosclerotic beginnings occur with dysfucntional flow. Raised lesions later develop, in same areas catalysed by modifiable factors and non-uniform flow. Fatty streaks are stained in red with Oil Red-O for lipids, of a young male who died of accidental cause.
(Koh, 2008)

It is hopeful, to note that atherosclerosis is reversible, if the modifiable factors are regulated early. Scientists are increasingly of the consensus, that modifiable factors need to addressed at an early stage of life, such as childhood. Data gathered over decades, has led to this insight. (Helen H. Hobbs plenary lecture oral communication, ISTH 2009).

1.2 Molecular Players

The different signals percieved by the endothelium, with regard to flow and other biochemical factors, are in turn controlled by intricate molecular mechanisms. These classically start from the genes to the removal of the protein. Increasingly research has found diversions in any of these steps. Thus understanding in detail the molecular players, help to understand the complexities of pathogenesis.

1.2.1 Rho GTPases

GTPases switch back and forth between two forms. The active form is guanine tri-phosphate (GTP) bound, while the inactive form is guanine di-phophate (GDP) bound. The shuttle between these forms are controlled by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). This is a set of three proteins whose brief function is listed below (Figure 1.4) (Etienne-Manneville and Hall, 2002).

1. GEFs: Encourage the binding of GTP and dissociating the GDP from Rho and thus activating in its role. (Rossman *et al.*, 2005)

2. GAPs: Increases the intrinsic GTPase activity of Rho protein, which in turn inactivates the Rho GTPase (Etienne-Manneville and Hall, 2002).

3. GDI: As the name suggests it inhibits the dissociation, whereby the GDP form of Rho is kept locked from dissociating. They, as well are able to bind to C-terminal prenyl groups on some Rho proteins. This sequesters them in the cytoplasm away from their regulators holding them in an inactive form (DerMardirossian and Bokoch, 2005).

Rho GTPases number around 20, and are members of the Ras superfamily of small G proteins. Their name Rho, is an abbreviation of Ras homologs. The regulators of the Rho GTPases number in hundreds, thus enabling one to

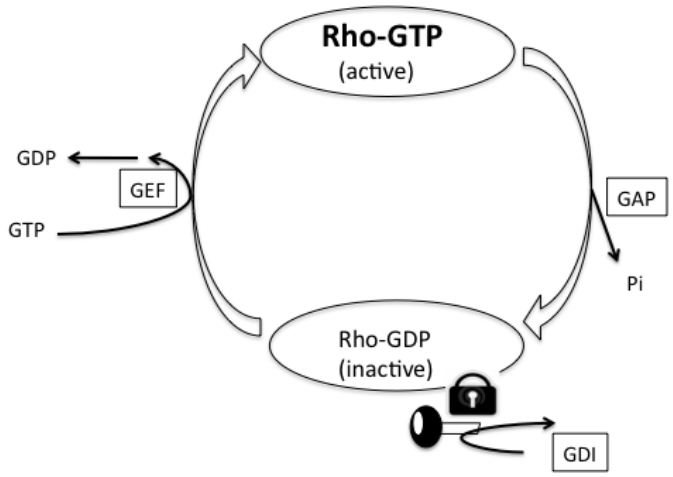

Figure 1.4: Rho cycling between GTP and GDP stages
The roles of GEFs, GAPS and GDI are shown in context of Rho cycling between GTP and GDP stages.

appreciate the permutation and combination in which these interactions exists and is controlled. Rho GTPases were discoverd in early 1990s, as important regulators of the cytoskeleton. Since then, its role in many other areas are increasingly appreciated. It has such diverse roles, some of which are in gene regulation, cell cycle progression, blood pressure, platelet activation, wound healing, cell adhesion, cell migration, barrier regulation and leukocyte extravasation (Etienne-Manneville and Hall, 2002; Hall, 2005).

In simplified terms, there exists three classes, which is also highly conserved between different organisms. These are Rho, Rac and CDC42 with differential role on actin control. The explosion in discovery, has also led to some new members, which include the atypical Rho. They are not regulated by GEFs or GAPs, instead regulated by phosphorylation states, genetic expression, protein stability such as Rnd proteins, RhoH and CDC42 homologue1 (WRCH1), also known as RhoU. Due to their atypical regulatory system, they came to be called the atypical Rho GTPases. Including these, there exists a total of six groups. These are Rho, Rac, Cdc42, Rnd, RhoD and TTF encompassing their own members (Jaffe and Hall, 2005; Chardin, 2006).

The well characterized three classes include Rho (RhoA, RhoB, and RhoC), Rac (Rac1, Rac2, and Rac3, which is also known as Rac1B [RhoG]), Cdc42 (Cdc42Hs, Chp, G25K, and TC10). Rho is involved in the formation of stress fibers, Rac regulates lamellipodia formation and CDC42 caters to filopodia moulding. Specific inhibitor of all three isoforms of Rho, clostridial enzyme C3 transferase has been an invaluable tool in our understanding of Rho signaling. The inhibitor is substantially more potent towards Rho (Novakofski *et al.*, 2009; Kumar and Lassar, 2009). All three are implicated with integrin signaling, and thus in focal adhesion formation and matrix adhesion. Rho, infact induces the formation of focal adhesion (Wennerberg and Der, 2004; Etienne-Manneville and Hall, 2002; Hall, 2005).

Rnd proteins, the member of the atypical Rho GTPases are very similar to RhoA. Due to their lack of GTPase activity, they are constitutively in the active state. The name Rnd stands for round, and thats what it does when Rnd is overexpressed (Chardin, 2006). It rounds cells, acting antagonistically to Rho and Rac.

This is very interesting, as their expression state determines their activity, with possible routes for microRNA based control as well. Generally, activity of Rho proteins is regulated by cytokine activity and hence its receptors, G-coupled receptors, tyrosine kinases or via adhesion and integrin clustering (Etienne-Manneville and Hall, 2002; Hall, 2005). Rho GTPases are also known to be post translationally modified. This results in specific sub-cellular localization (Rho *et al.*, 2009). There are also, well known mechanisms of microRNA regulation such as p250GAP which is a direct target of miR132 (Wayman *et al.*, 2008).

1.2.2 The control of actin

The cytoskeleton's main component is actin. It is an omnipresent protein, that senses all mechanical load and capable to convey mechanical energy. Actin is indeed, very important structural component of the cytoskeleton. Actin, also

has many binding partners. It is such a molecule, that it requires an ability to be regulated with plasticity and reversibility, which in fact is specific to its role. Functionally, it is involved in almost everything, in line with its omnipresence. Some examples include endocytosis, pathogenic entry, any sort of movement, cyclic strain, shear stress in endothelial cells, woundhealing, entry or exit of cargo from cells (Kaksonen *et al.*, 2006; Burkhardt *et al.*, 2008; Wang and Thampatty, 2006).

Actin changes from the globular (G) actin to filamentous (F) actin; G-actin to F-actin. In the filamentous form it is of a flat conformation, which encourages the subnunits to adhere, forming a helix (Heinrichs, 2009). Actin meshwork, is anchored to the extracellular matrix by means of different transmebrane proteins, such as integrins. Integrin adhere to extracellular matrix, which itself is a dynamic process, which will be discussed later in the section covering with integrins. Integrin clustering, leads to focal complexes. This in turn recruits a functional platform of kinases and phosphatases. These include integrin binding proteins and other scaffolding proteins. Kinases such as focal adhesion kinase (FAK) are involved in this process, further leading to focal adhesions (Le Clainche and Carlier, 2008).

Actin polymerisation actually generates mechanical energy, as Brownian-ratchet models prove (Kaksonen *et al.*, 2006). Polymerisation also leads to activation of the ATPase function of actin. The change from G-actin to F-actin, brings closer the crucial Gln137 residue in the ATPase region and the γ-phosphate together. This might allow the bound ATP to be hydrolysed (Heinrichs, 2009).

The scaffolding proteins involved in the cytoskeleton, can harness this mechanical force, generated by the Arp 2/3 activation/inhibition as the polymerisation /de-polymerisation occurs. A defining functional example would be during migration. The nucleation by Arp 2/3 complex is rate limiting and N-WASP and cortactin activate Arp 2/3 (Kaksonen *et al.*, 2006). Meanwhile, integrins are controlled from the inside, commonly referred to as "inside out signaling". There also exist outside in signaling, whereby cues from the out-

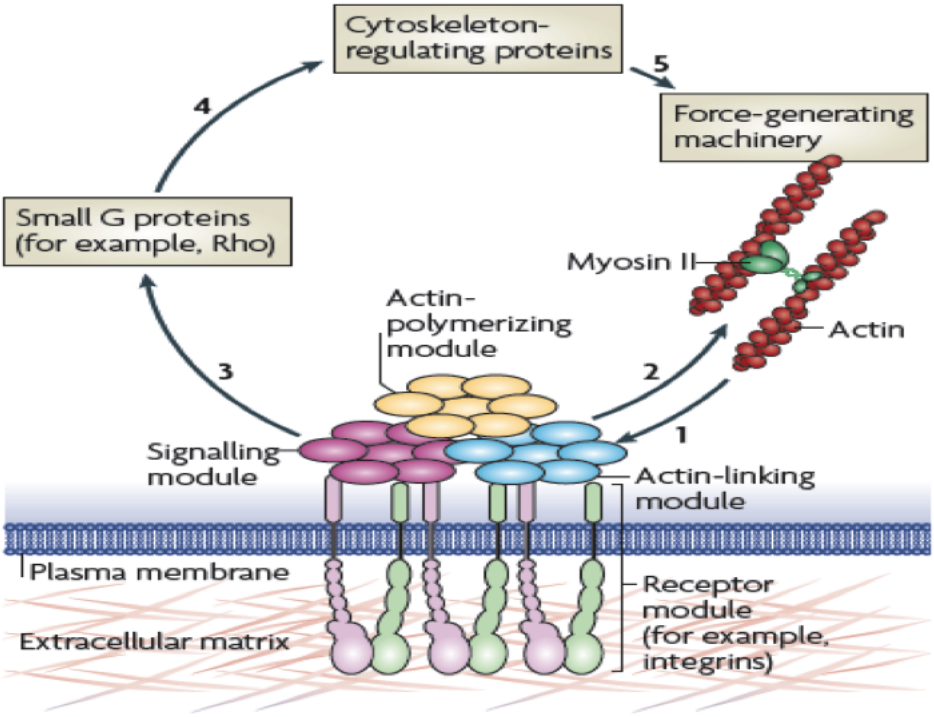

Figure 1.5: Actin and Force Generation
The various steps resulting in the generation of force via actin (Geiger *et al.*, 2009)

side is transduced to the inside. Actin remains sensitive to these signals, and moulds the cell according to the force requirement. The inside out signaling is catalysed by disocciation of complexes between GTP-bound Rho-GTPases and Rho- GDI. This extends the membrane, by the release of active Cdc42 and Rac1 (Del Pozo *et al.*, 2002).

This actin machinery is conserved across eukaryotes. So is most of the machinery between functions, such as endocytosis, lamellapodia formation, motility of cells (Kaksonen *et al.*, 2006). The lipids such as phosphatidylinositol 4,5-bisphosphate (PIP_2) are also important.

Pipmodulins are another class of molecules that are a group of proteins called "GMC" proteins, that include GAP43, MARCKS and CAP23. They regulate actin by controlling the local availablity of PIP_2. This is because many actin

binding proteins bind to PIP_2. PIP_2, by binding to GMC proteins, is sequestered inhibiting its function. When PKC activate GMC proteins by phosphorylation, it releases these groups of PIP_2 (Figure 1.6). The binding to calmoudin (CaM), also release PIP_2. There are other feedback loops that can stimulate production of PIP_2. This free PIP_2, by binding to actin binding proteins of which moesin is a member, stabilises them. The binding domain is the pleckstrin homology (PH) domain, of which another example is talin, moesin.

This spur in PIP_2 production is another controller of actin dynamics (Figure 1.6) (Laux *et al.*, 2000; Lanier and Gertler, 2000).

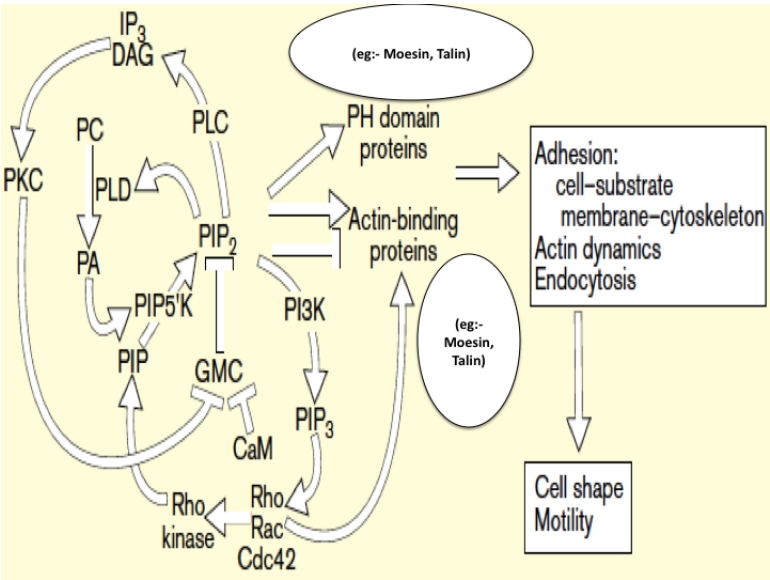

Figure 1.6: PIP_2 signaling and actin
PIP_2 dynamics feed into actin machinery, with implications in various functions such as cell motility, endocytosis, possibly in microparticle formation. The figure was taken from (Lanier and Gertler, 2000), but contextually changed.

The integrins meanwhile are able to control a multitude of proteins, that link to the actin and regulate it. This includes FERM domain containing protein talin and moesin, integrin linked kinase (ILK) via α-parvin and β-parvin. α-actinin with and without vinculin also link integrins to actin. Vinculin can also

associate with talin. Talin is controlled by PIP_2 like moesin. By binding to PIP_2, talin increases association with integrins. Like moesin, specific localization of talin near membrane depends on PIP_2 binding (Martel *et al.*, 2001).

There are many other molecules involved, such as formin-1 that nucleate actin linearly, while arp 2/3 is acutally for branched actin filaments (Figure 1.7). Ena/Vasp Proteins encourage continued elongation of existing filaments.

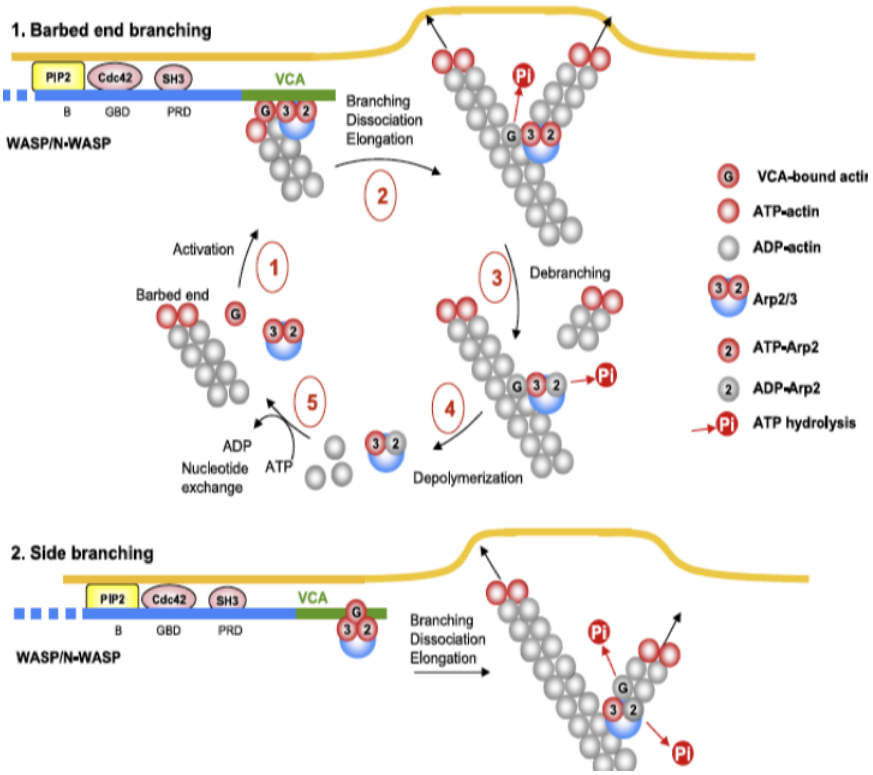

Figure 1.7: Regulation of actin Top(1):- The steps involved in cyclic barbed end branching. This involves binding to the verprolin-cofilin-acidic (VCA) domain of WASP/N-WASP (or WAVE isoforms) by the barbed end of the mother actin filament. Bottom(2):- Side branching model. Arp 2/3 is activated by the COOH-terminal domain of WASP/N-WASP (or WAVE isoforms), which binds to the side of an actin filament. Figure was taken from (Le Clainche and Carlier, 2008)

This is due to its prevention of binding of capping proteins, by binding to the quickly growing barbed ends (Barzik *et al.*, 2005). There are other transmembrane receptors, such as cadherins, ICAMs which connect to actin, via molecules such as α-actinin.

It is not only vinculin or α-actinin, but many other adapters swarm here, such as zyxin, extracellular signal-regulated kinase 1/2 (Erk1/2), mitogen-activated protein/extracellular signal-regulated kinase kinase 1 (MEKK1), protein kinase N (PKN) and the p85 subunit of phosphatidylinositol- 3 kinase (PI3-K). FAK actually inhibits α-actinin binding to actin by phosphorylation. Integrins also bind to filamin which enable orthogonal linkage to actin networks. This also works in parallel. Integrins stimulate signaling to actin eg: via FAK and Src-like kinases. This complex choreography of molecules thus regulate and is controlled by actin (Brakebusch and FaÈssler, 2003).

1.2.3 Thrombin

Thrombin is a key enzyme of the blood coagulation system. This serine protease of the trypsin family, is also a key player in inflammation. Inflammation is a neccessary response of the body to regain homeostasis (Medzhitov, 2008). Underlying hyperlipidemia is cited to cause atherogenesis. Research has meanwhile shown skewed mechanical stimuli and inflammation causing hyperlipidemia. This chronically in concert with other factors lead upto atherogenesis (Libby *et al.*, 2002).

Thrombin is a 36 kDa molecule formed when the zymogen prothrombin is cleaved, by a prothrombinase complex. It is well characterized for its roles in fibrinolysis, tissue repair, blood coagulation and wound healing. As well, it is involved in platelet and endothelial cell activation and the progression of neoplasia. Protease activated receptors (PARs) are thrombin receptors and PAR1, PAR3, PAR4 are the receptors for thrombin. PAR receptor, is a G-protein and is expressed in endothelium as well as in other cells (Mann, 2003). PAR1 is the major mediator of thrombin, in inflammatory responses. This receptor, is acti-

vated with cleavage by thrombin. This is unlike receptors of factor Xa, protein C, and urokinase, that need not be cleaved for their downstream processing (Strukova, 2001; Mann, 2003). As well, it dilates the vasculature, by releasing NO which is mediated by PAR1 (Motley *et al.*, 2007). This results in increased vascular permeability which leads to edema (Laposata *et al.*, 1983). Vascular injury furthers thrombi formation. This is enhanced in instances of oscillatory and low flow. Arthereosclerotic plaque formed by chronic hyperlipidemia, deposits lipids and lipid-laden macrophages (foam cells) which can then rupture (Figure 1.8). The shedding of granules by platelets further to their accumulation, ultimately leads to thrombus incorporating fibrin.

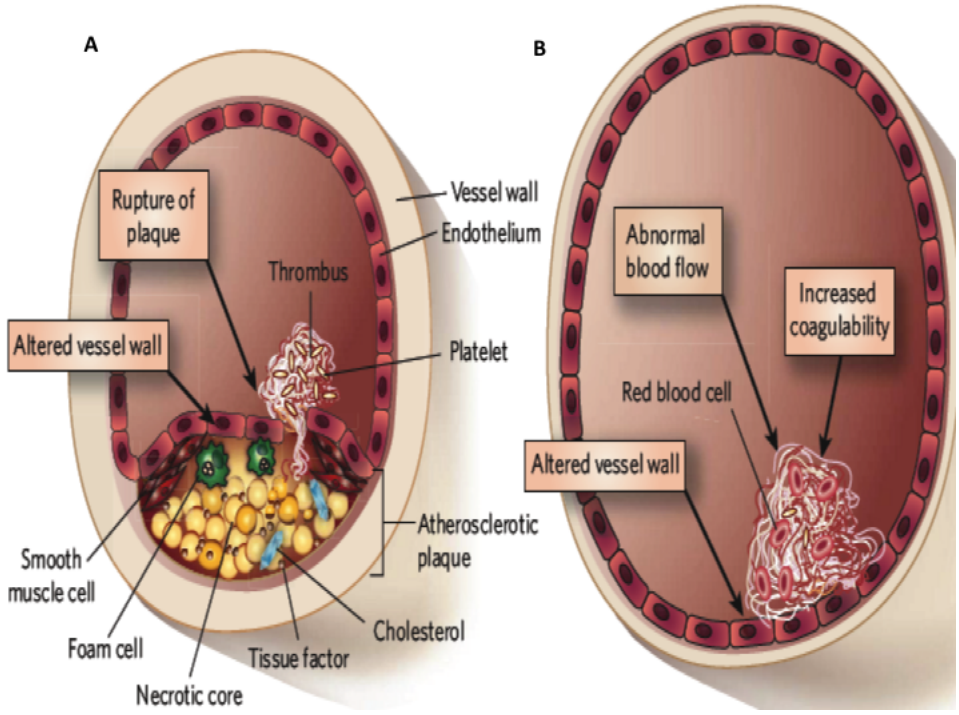

Figure 1.8: Thrombosis of the Artery and the Vein
A) Representation of various factors leading upto arterial thrombosis. B) Representation of various factors leading upto venous thrombosis.
(Mackman, 2008).

Chemotaxis, is induced on monocytes and neutrophils by thrombin, from

the endothelium (Zimmerman *et al.*, 1985). This also increases the inflmmatory response, inducing intercellular gap formation (Mann, 2003). Thrombin is also expressed highly in platelets, and is a major activator of the same (Leger *et al.*, 2006; Crawley *et al.*, 2007). Chemokines, such as platelet activating factor (PAF) via PAR1 are generated by thrombin. This encourages further inflammatroy response, inducing previously stated chemotaxis (Mann, 2003). Thrombin induces angiogenesis by activating VEGF. This is achieved by increasing the receptors for VEGF (Tsopanoglou and Maragoudakis, 1999).

Cytoskeletal proteins are rapidly recruited downstream of thrombin. The proteins, thus associated with thrombin, needs the required sensitivity. Moesin has been shown to associate dynamically with platelet cytoskeleton, within, a minute of thrombin addition. This response is due to moesin phosphorylating transiently, reaching basal levels soon after (Shcherbina *et al.*, 1999b). Though often associated as a pro-inflammatory molecule, physiologically thrombin acts to restore homeostasis (Strukova, 2001). Recruitment of other cells to the area of inflammation, is a step towards this repair process. Integrins such as $\alpha M\beta 2$ (Mac1 or CD 11b/CD 18), are expressed downstream of thrombin exposure, on activated monocytes (Flick *et al.*, 2004). Thrombin, thus enables exposure of various adhesion molecules in various cells, including endothelium.

Thrombin is a mutifaceted molecule with sometimes contrasting roles, inducing vasoconstriction by release of endothelin-1 (Marasciulo *et al.*, 2006). Thrombin also induce microparticle release (Simoncini *et al.*, 2009). The group, had previously shown thrombin to generate microparticle by ROCKII, activated in turn by caspase-2 (Sapet *et al.*, 2006). These molecules also regulate various cytoskeletal molecules, unravelling of which would be of interest. For instance, there still doesn't exist any drug that can prevent coagulation without promoting bleeding.

1.3 Integrins and the vascular cells

The cell needs molecules that communicate and interact at various levels, thus sensing their outside and inside environment at the same time. This helps the cell to regulate its growth efficiently and safely. Integrins (Figure 1.9) are proteins, that provide such a requirement for the cells.

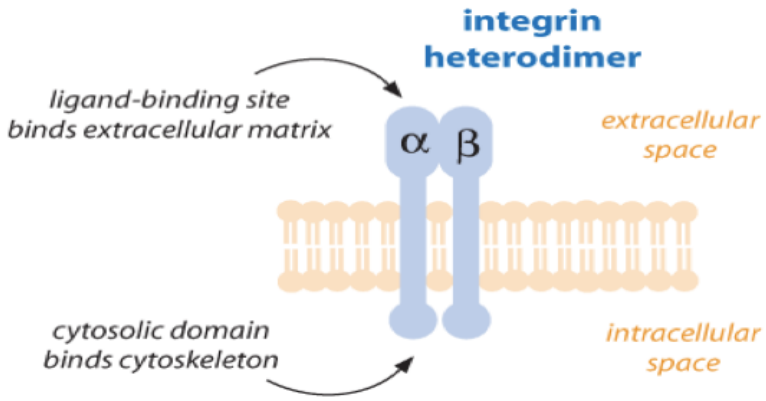

Figure 1.9: Basic Structure of Integrin Heterodimer
Over view of the basic structure of integrin heterodimer. A cell surface receptor. $http://www.chemie.tu-darmstadt.de/bt/Endothel.html$

1.3.1 Integrins

Integrins are mainly involved in forming specialised complex structures, termed focal contacts or focal adhesions. These are formed between the cell and extracellular complexes (ECM) (Jockusch *et al.*, 1995). Studies in this field, has shown very clearly that cell adhesion complexes are not simply "static architectural entities", but offers a more dynamic integrating system capable of controlling and regulating a range of cellular functions (Inoue *et al.*, 1977). In the process it interacts with various signaling molecules, the understanding of which is important.

Integrins exist as heterodimers of an α and a β subunit. More than 24 dif-

ferent types of integrins are formed by the association of 18α subunits and 8β subunits (Hynes *et al.*, 1999; Brakebusch and FaÈssler, 2003; Brakebusch *et al.*, 2002). The β integrins form heterodimers to form αIIβ3 and αvβ3 integrins. The former is specific for the megakaryocytic lineage and latter is expressed on endothelial cells, vascular and uterine smooth muscle cells, monocytes, macrophages, osteoclasts (where it contributes to bone resorption), certain subpopulation of lymphocytes, Islets of Langerhans (during morphogenetic movements), neural crest cells (Baker and Zaman, 2009; Ballestrem *et al.*, 2001; Sadeghi and Bender, 2007; Cirulli *et al.*, 2000; Eliceiri and Cheresh, 1999; Shattil *et al.*, 1995; Avraamides *et al.*, 2008).

The ligand binding domain of the integrins are globular regions near the N-termini of the α and β subunits and are connected to the transmembrane domains by long stalk like structure. Integrins are specific in binding to extracellular matrices (ECM). α5β1 binds to fibronectin and αvβ3 to fibronectin but also to vitronectin, fibrinogen and osteopontin (Luo *et al.*, 2007; Hynes *et al.*, 1999)

1.3.2 'Outside-in' and 'Inside-out' signaling by integrins

Integrin signaling is a bi-directional process (Luo *et al.*, 2007; Shattil *et al.*, 1995). Outside-in signaling is when integrins are clustered following engagement to the ECM. This is in turn acts to recruit various cytoskeletal proteins and signaling molecules. Molecules recruited thus affect cell growth, differentiation, gene expression and programmed cell death. Inside-out signaling occurs vice-versa, whereby the intracellular interactions have a profound effect on adhesion, progression and motility of the cell (Shattil *et al.*, 1995). Thus the understanding of components which immediately and directly binds to integrins, is important to our understanding of the downstream events that regulate all major cell modulating functions. The direct interactions, might be also important as a therapeutic target against cancer metastasis (Ricono *et al.*, 2009).

1.3.3 Binding partners of integrin receptors

Integrin cytoplasmic domain bind directly to many different cytoskeletal proteins, such as talin, which in turn act as adaptors to recruit signaling molecules (Anthis *et al.*, 2009). It has been reported that the β1A, β3 and β1D cytoplasmic domains bind to talin, β1A tail bind to α-actinin, β1A,β2 and β7 tails bind to filamin and the α4 tail binds to paxillin. Of these, filamin, paxillin associate with other adaptors, signaling molecules and is important in effecting the signaling (Stossel *et al.*, 2001; Turner, 2000). The extracellular domain, cytoplasmic domain are both involved in bidirectional signaling. Transmembrane domains also involve in integrin signaling. They have been shown to be significant to the heterodimer formation (Anthis *et al.*, 2009). Integrins, thus provide a regulated link for cytoskeleton to the outside of a cell. Integrins are also a communicative tool, enabling cytoskeletal molecules to interact with the extracellular matrix and to the outside of the cell.

1.3.4 Significance of integrin receptors

Integrins thus recruit and localize various proteins. They help connect cytoskeleton to the various organelles with their enormous surface area. This makes integrins an ideal binding partner to regulate the cellular machinery of integrin adhesome (Janmey, 1998). This encompasses multitude of molecules and is is complex. This is represented in (Figure 1.10).

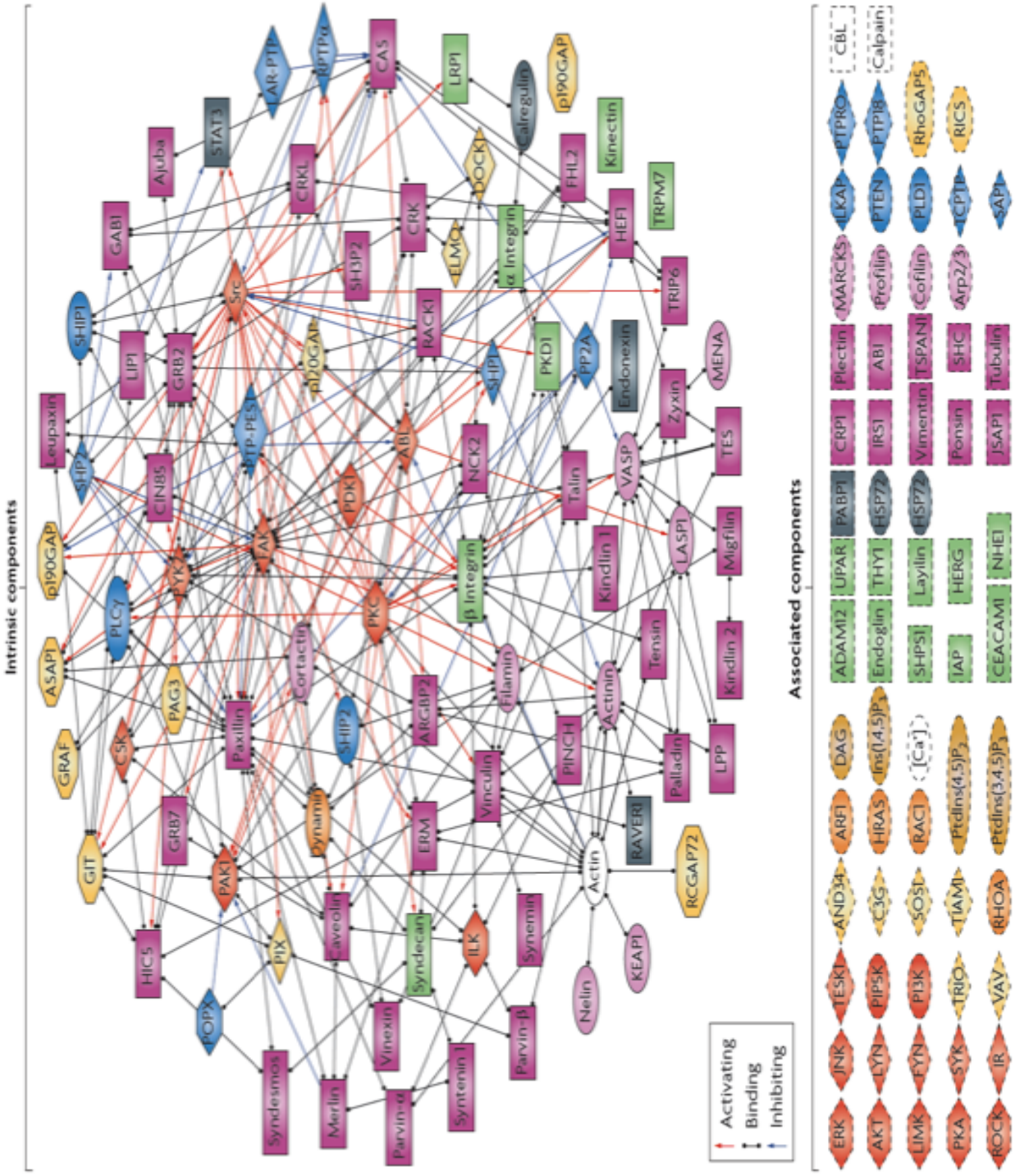

Figure 1.10: The integrin adhesome
Here the various integrin-mediated adhesions, linking extracellular matrix to the actin cytoskeleton is shown, with 160 distinct components. The entire adhesome consists of 700 links from which 55% are binding interactions, the rest being modifying interactions. This shows the significance of the integrins in the biological context and its hyper environmental sensitivity. Many different kinases, phosphatase, G-proteins, regulators all mix, regulate each other pulling and tugging at the actin mediated by this integrin-mediated adhesome.
(Geiger *et al.*, 2009)

As integrins are a binding partner to the cytoskeleton, and because it is linked to ECM, integrins, take cues from ECM and inside of the cell, enabling them to be regulator of the cellular activities. Thus integrins are seen to be essential in various processes, like cell migration and motility, angiogenesis and apoptosis (Degryse *et al.*, 2001). The ability of integrins to help a cell migrate during normal cell development is hijacked for metastasis by tumour cells (Ricono *et al.*, 2009).

1.3.5 Role of kinases in Integrin function

Kinases are important regulatory proteins with respect to integrins. They respond to mitogenic signals from growth factors, which are activation signals, which trigger specialised cellular responses (such as secretory response in neuroendocrine cells and platelet aggregation), adhesion signals promoted by the extracellular matrix, and signals which stimulate cell motility and progression (Slack-Davis *et al.*, 2009; Zou *et al.*, 2007)

Studies have demonstrated integrin cytoplasmic domains to bind with protein tyrosine kinases. Focal adhesion kinase (FAK) was reported to bind to peptides from β1 subunit (Romer *et al.*, 2006). The sequence utilized for FAK binding, is the same sequence in β1 that Talin exploits (Lewis and Schwartz, 1995). Phosphorylated FAK has been reported to directly associate with β5 cytoplasmic domain, which is critical for β5-mediated cell migration (Romer *et al.*, 2006).

The tyrosine kinase Syk meanwhile, bind directly to the β3 cytoplasmic domain. The activation of the Syk is seen on the αIIβ3 integrin's clustering. This is a key upstream event in the activity of the platelets and locomotion of heamopoiteic cells (Woodside *et al.*, 2001). This interaction is mediated by moesin and ezrin, which directly interact with Syk in an ITAM-dependent manner (Urzainqui *et al.*, 2002). A related kinase, ZAP-70, also has been implicated to bind with β3 cytoplasmic tail via their tandem SH2 domain (Woodside *et al.*, 2001). Integrin linked kinase (ILK) also associate with the cytoplasmic do-

main of β1 integrin (Zervas *et al.*, 2001; Hannigan *et al.*, 2005).

β3-endonexin is a protein, which has been shown to be very specific in binding to β3 integrin. It was discovered by a yeast two hybrid system, where the β3 tail was used as a bait. Point mutation studies of the β3-cytoplasmic tail where a serine to proline mutation at position 752 (associated with a bleeding disorder) exhibited markedly reduced spreading and focal adhesion formation, following adhesion to fibrinogen (Shattil *et al.*, 1995). Meanwhile αvβ3 is able to provide a sustained ERK response (Kim *et al.*, 2003). This has consequence in cell proliferation possibly involving Cyclin D1 (Walker and Assoian, 2005).

Src-family kinases such as p120c$^{\text{c-cbl}}$(Cbl) and PI-3 meanwhile have been implicated with β1 signaling (Meng and Lowell, 1998). SH3 domain of Src is the region of interaction with the β subunit at its cytoplasmic domain (Arias-Salgado *et al.*, 2003). Src family of kinases meanwhile phosphorylate ezrin (member of FERM domain containing proteins), with implication in adhesion and migration (Srivastava *et al.*, 2005). This can lead to interference in heterodimer formation between other ERM proteins (such as moesin).

1.3.6 Integrins and Angiogenesis

Angiogenesis is the process by which new blood vessels are made. This process is very active in the normal foetal development, also while healing wounds, for restoring blood flow to tissues after injury/insult (Folkman, 2007)

1.3.6.1 Molecules that drive angiogenesis

Angiogenesis depends on specific molecular interactions between the components of ECM and the vascular cells. It is a complex process of interaction between growth factors, their receptors and as well, ECM and their receptors the integrins (Tucker, 2006). Integrin αvβ3 is able to recognize a number of ECM molecules in context of RGD adhesive sequence (Arginine-Glycine-Aspartic acid) (Serini *et al.*, 2006). αvβ3 integrin binds fibronectin, vitronectin, fibrinogen, thromabospondin, proteolysed collagen, vonWillebrand factor and osteo-

pontin which have exposed RGD sequence. It is also binds to metallaproteinase-2 (MMP-2) in a non-RGD dependent manner (Brooks *et al.*, 1994, 1996).

1.3.6.2 Different Integrins, Different signaling

The involvement of the integrin and its cell-matrix interactions, is significantly felt in vascular remodeling. This is because angiogenesis involves the invasion of the ECM and migration of endothelial cells through the ECM. Integrin mediated angiogenesis, largely depends on the matrix the extracellular domains occupy. Thus, with fibronectin its major receptor $\alpha 5\beta 1$ integrin comes to action. While $\alpha 6\beta 4$ integrin is utilized with increasing laminin-5 in the extracellular matrix. The involvement of different integrins with different matrix is central to controlled angiogenesis. Similarly, different agonists involve different integrins for the same matrix (Figure 1.11) (Serini *et al.*, 2006; Tucker, 2006).

In endothelial cells exposed to growth factors such as bFGF (Basic fibroblast growth factor) and VEGF (Vascular endothelial growth factor) it is noticed that Von Willebrand factor, vitronectin, fibronectin, and fibrin that binds to $\alpha \mathrm{v}\beta 3$, is expressed in large quantities (Yan *et al.*, 2008; Presta *et al.*, 2005; Shizukuda *et al.*, 1999; Tsou and Isik, 2001).

Integrins such as $\alpha \mathrm{v}\beta 3$, $\alpha \mathrm{v}\beta 5$, and $\alpha 6\beta 4$, are found to be activated driving angiogenesis mainly with tumour progression. $\alpha \mathrm{v}\beta 3$ integrin drives angiogenesis in such scenarios, both positively and negatively depending upon insoluble or soluble vitronectin respectively (Serini *et al.*, 2006; Tucker, 2006; Folkman, 2007). $\alpha \mathrm{v}\beta 3$ mediates endothelial cell attachment, migration and spreading and thus gets transiently localized to endothelial cells at the tips of the capillary sprouts during wound repair (Beauvais *et al.*, 2009; Degryse *et al.*, 2001; Soldi *et al.*, 1999).

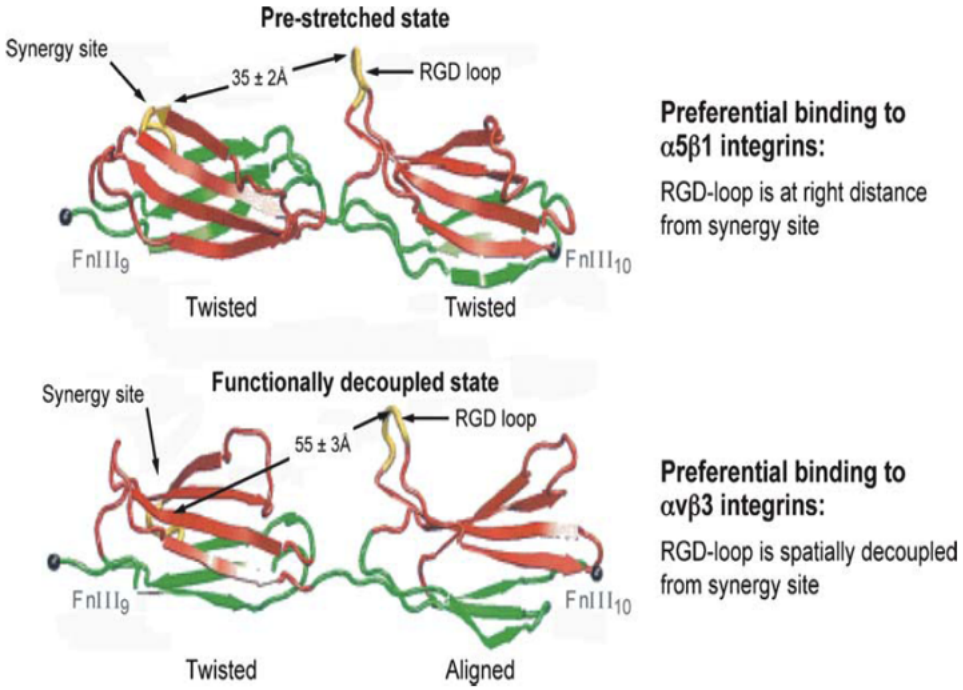

Figure 1.11: Different integrins involved with force transduction in fibronectin
Force, as an example of different integrins involving at different stages of a cell. Integrins $\alpha 5\beta 1$, αIIb$\beta 3$ recognize the synergy site. This results in enhanced binding to fibronectin, when the two sides are presented at the right distance. This is changed with stretch, where, synergy-RGD distance is increased from 35 Å to 55 Å. Here, αv$\beta 3$ is found to be more specific.
(Vogel, 2006)

$\alpha v\beta 3$ is also heavily concentrated in granulation tissue than in normal skin tissue. Newly formed blood vessels induced by bFGF in the CAM (chick chorioallantoic membrane) assay show robust $\alpha v\beta 3$ expression, this is not seen in untreated CAMs (Chandra, 2003; Eliceiri *et al.*, 2002). Antibodies against $\alpha v\beta 3$ meanwhile affect the bFGF induced vessel sprouting in the CAM, whereas there is no effect seen on the preexisting vessels. The role of $\alpha v\beta 3$ in blood vessel growth is also felt in embryonic vascular development (Eliceiri and Cheresh, 1999).

Integrins such as the collagen receptor integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are induced by VEGF. Antibodies against them drastically reduce the angiogenic processes driven by VEGF. Studies using antibodies against $\alpha 5\beta 1$ restricts the angiogenic process specific to certain other growth factors but not that of VEGF (Kim *et al.*, 2000). Antibodies against $\alpha v\beta 5$ has been shown to suppress VEGF stimulated angiogenesis (Hynes, 2002b). In addition to different matrices regulating different integrins, different growth factors also seem to recruit distinct integrins to grow new blood vessels.

These different integrins, initially by its differential adhesion to different ECMs, drive angiogenesis. There are other molecules it interacts with both extracellularly and intracellularly. The molecules both in the inside and the outside of a cell, can't communicate to each other without integrins. uPAR is one such extracellular molecule that lacks a transmembrane and cytoplasmic domain (Degryse, 2008). Meanwhile innards of the cell is spanned by the cytoskeleton. This is moulded by recruiting cytoskeletal adapter proteins such as vinculin, $\alpha-$actinin, profilin, cofilin and FERM domain containg protein talin. *In-vivo*, blockade of ezrin (a FERM domain containing protein) has been shown to increase transplanted endothelial cell proliferation and angiogenesis in a mouse hind limb ischemia model (Hood and Cheresh, 2002; Kishore *et al.*, 2005)

There are as well, different regulatory mechanism employed. There are GTPases such as Rap1 that regulate integrin activation with respect to angiogenesis (Carmona *et al.*, 2009). With uniform laminar flow, increased NO down-

stream regulates αvβ3 integrin mediated angiogenesis (Lee *et al.*, 2000). Meanwhile NO has been shown to be detrimental to Rap1. While Rap1 is also known to regulate αIIβ3 activity. Integrins are thus able to be modulated according to the angiogenic cues both pro and otherwise, from inside and outside of a cell.

αvβ3 integrin knockout mice exhibit vessel abnormalities and hemorrhaging in brain and intestinal vasculature. The role of αvβ3 integrin is not just in binding to the ECM, but is seen in binding to matrix metalloproteinase-2 and localizing the active form of the enzyme to the tips of the angiogenic blood vessels (Brooks *et al.*, 1996).

αvβ3 is postulated to locally degrade ECM, then adhering to the modulated matrix mediates endothelial cell migration (Hynes *et al.*, 2004). αvβ3 binding also induce mitogen activated protein kinase activation and suppresses apoptosis in endothelial cells (Eliceiri and Cheresh, 1999). αvβ3 also, helps endothelial cell survival by mediating intracellular pathways that promote proliferation and activation.

1.3.6.3 Angiogenesis and tumour growth

As the development of new blood vessels for any growing cell is important, so it is for tumour cells. Angiogenesis in non tumorous cells is important. It stabilises in normal circumstances and changes to accommodate remodeling of tissues. In tumor cells angiogenesis are critically important as this is a high metabolic area. In normal tissue blood vessels supply nutrients and remove waste. Angiogenesis meanwhile is very essential in high density fast growing cancerous tissue mass. Unlike normal cells, here angiogenesis is more active and proliferative (Papetti and Herman, 2002). There are stimulants and inhibitors of angiogenesis in normal cells. These molecules tightly regulate the process of angiogenesis via complex interactions. But, in tumour cells the stimulants are overexpressed, which surges the blood supply helping to proliferate cells. Therapeutic approach to use inhibitors or other mechanisms to regulate the stimulants, will be a good approach for curtailing the growth of tumour

(Folkman, 2007)

1.4 Ezrin, Moesin, Radixin (ERM) proteins and Actin

Cell shape is a dynamic process, with plasticity essential in attaining its diverse functions. Cytoskeleton by its very nature provides this plasticity to the cell. Cell adhesion, migration, cell division, docking of cells and immunological synapse are some of the functions influenced by cytoskeleton. This is enabled by fluidity and reversibility of cytoskeleton, provided by actin and actin associated proteins (Chhabra and Higgs, 2007; Niggli and Rossy, 2008; Dustin, 2005).

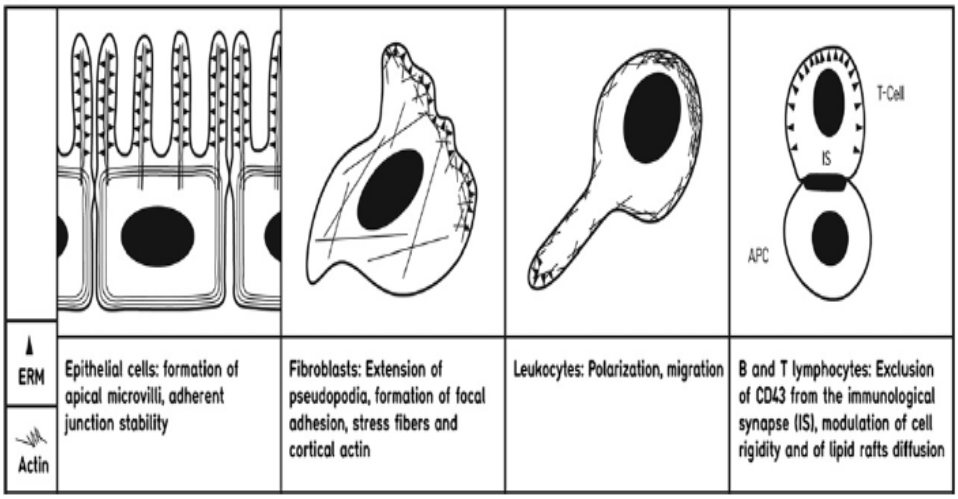

Figure 1.12: Roles of ERM proteins
Roles of ERM proteins with respect to its actin binding capability. (Niggli and Rossy, 2008)

1.4.1 ERM proteins

Ezrin, Moesin, Radixin (ERM) proteins are a family of proteins that are membrane associated. They are members of the erythrocyte protein 4.1 superfamily, providing linkage between the plasma membrane and the actin based cytoskeleton (Sato, 1992). These function to regulate specific domain in the cell cortex by conformational regulation (Pearson *et al.*, 2000a). Originally characterized 20 years ago, ERM proteins links plasma membrane and the actin-based cytoskeleton. They are found in actin-rich cell surface structures, such as microvilli, membrane ruffles, filopodia and cleavage furrows of various cell types (Figure 1.12) (Sato, 1992).

These proteins are characterized by a shared FERM (Four-point one, Ezrin, Radixin, Moesin) domain (300-residue globular N-terminal) (Bretscher *et al.*, 2002). Ezrin was discovered at the cell surface such as microvilli and membrane ruffles (Pakkanen *et al.*, 1987). While radixin was discovered at adherens junctions, and moesin as a protein which binds to heparin (Tsukita *et al.*, 1989; Lankes *et al.*, 1988). Merlin is another protein that shares the FERM domain, while its carboxy terminal is more distantly related (James *et al.*, 2001). Other proteins of the 4.1 superfamily are talin, several tyrosine kinases and phosphatases.

1.4.2 Structure of ERM proteins

ERM proteins binds to membrane proteins and F-actin via its N-terminal and C-terminal (ERMAD) regions respectively. The N-ERMAD being the conserved domain spanning the ERM proteins referred to as the FERM domain (Gary and Bretscher, 1995; Reczek and Bretscher, 1998; Turunen *et al.*, 1994).

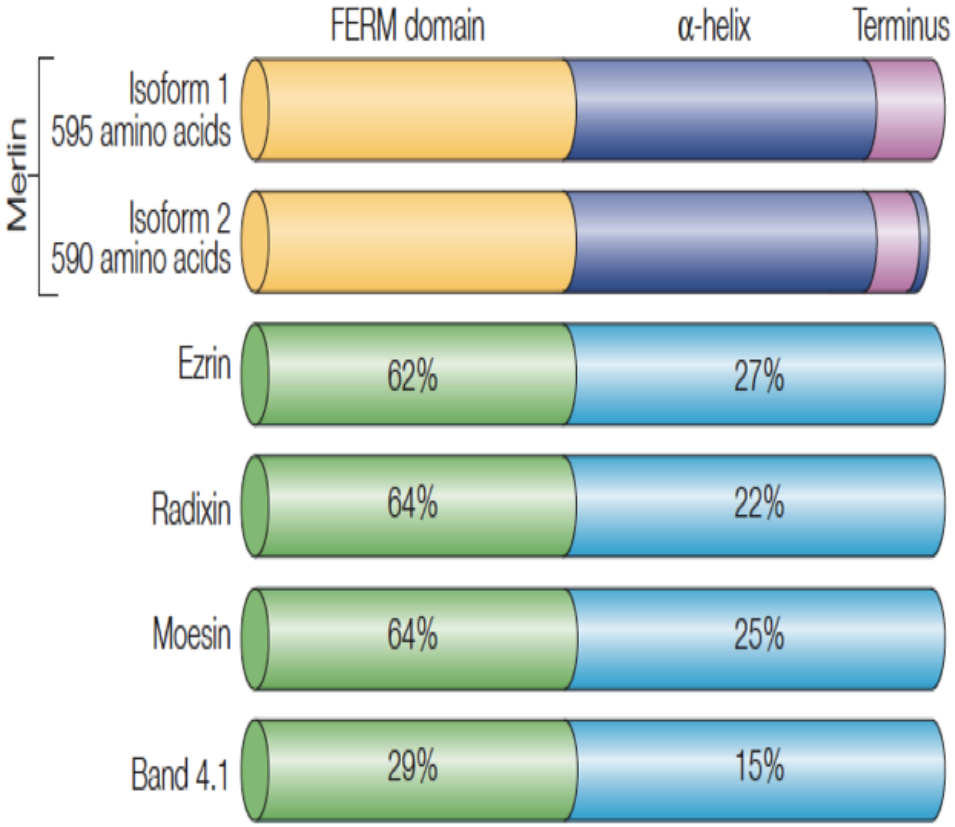

Figure 1.13: ERM proteins
ERM proteins show very high conservation of sequence across each other. Merlin has two isoforms, and are also more divergent from other ERM proteins. Isoform 1 of Merlin ends in 16 residues, while isoform 2 ends in 11 residues. The The ERM proteins are able to form intramolecular association via the N-ERMAD contained in the FERM domain with C-ERMAD. Merlin cannot bind to actin as it lacks the F-actin binding site, while moesin is able to bind to F-actin. Moesin meanwhile, lacks the proline rich area found within radixin and ezrin (Ramesh, 2004).

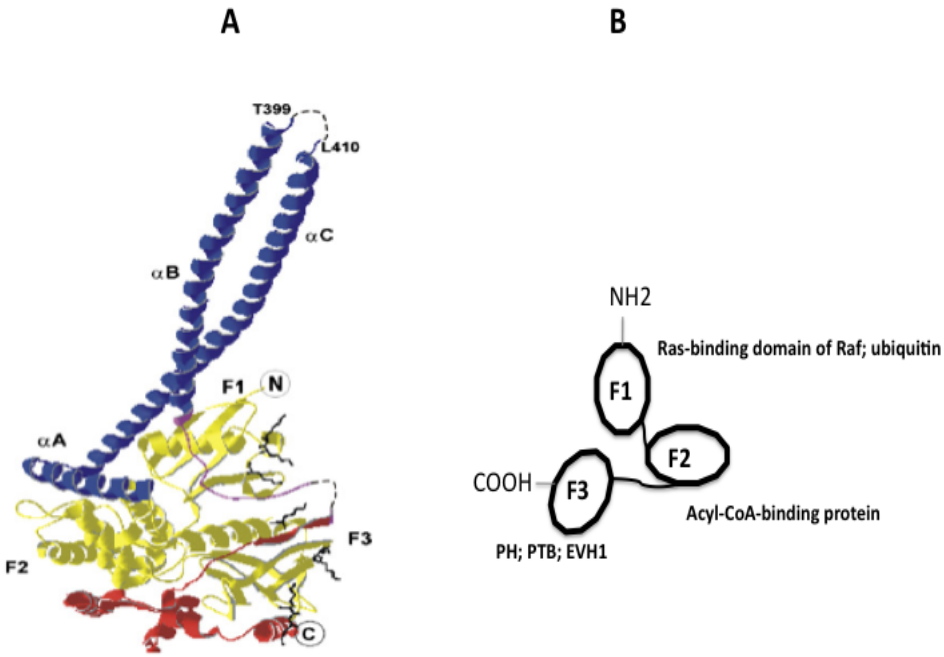

Figure 1.14: Structure of Moesin
A) Ribbon structure of Moesin (from (Niggli and Rossy, 2008)). There are three lobes in the FERM domain referred to as F1, F2 and F3 (coloured yellow). The α-helical domain is represented in blue colour. This extends and folds into three helices, αA, αB and αC. These have residues that have contact with FERM domain. There is a linker region (violet colour, residues are 461-472), which basically links the α-helical domain and the C-terminal domain (in red, and associated with lobes F2, F3 of the FERM domain). The linker region meanwhile, interacts with the F1 lobe of the FERM domain. Moesin binds to the membrane by adhering to PIP_2, the region utilized for this is indicated in black (top to bottom: lysines 64, 63, 60, 278, arginine 253, lysines 254, 262, 263). B) Cartoon representation of moesin crystal structure modified from (Bretscher *et al.*, 2002). FERM domain is a composite domain with F1 domain containing ubiquitin, Ras-binding domain of Raf. Meanwhile, F2 domain has Acyl-CoA-binding protein and F3 domain has phosphotyrosine binding(PTB), a pleckstrin homology (PH) and an enabled/VASP-1 (EVH1) domain respectively. The structure of ERM proteins are heavily conserved across most of them. Note the compositeness of all domains, which makes ERM proteins an ideal "super" adapter protein.

The FERM domain is a globular structure, composed of three subdomains (F1, F2 and F3) and arranged like a clover leaf (Figure 1.14 (Edwards and Keep, 2001). The carboxy terminal on the other hand, has a linear structure composed of a α-strand and six helical regions, extending and covering the FERM domain (Pearson *et al.*, 2000b; Edwards and Keep, 2001). The structure of the ERM Protein is conformationally dependent. The N-terminal, C-terminal regions is divided by an extended coiled –coil region (Bretscher *et al.*, 2002).

The sizes of these different regions are 300 residues, 200 residues and 100 residues respectively for N-terminal, central coiled and C-terminal respectively (Pearson *et al.*, 2000b). Also, the 3 lobes F1(4-82 residues), F2(96-195 residues) and F3 (204-297 residues) of moesin show homology to the proteins ubiquitin, acyl CoA binding protein and phosphotyrosine binding(PTB), a pleckstrin homology (PH) or an enabled/VASP-1 (EVH1) domain respectively. The F3 lobe homologue protein, enabled/VASP-1 (EVH1) domain has been found to bind peptide and lipid ligands in both cytoskeletal proteins and signaling (Pearson *et al.*, 2000b; Hamada *et al.*, 2000; Niggli and Rossy, 2008)

1.4.3 Self Association and Regulation

The N-terminal (N-ERMAD) region of an ERM protein, binds with high affinity to the C-terminal (C-ERMAD) region. This state of the protein is that of a non-active state, not enabling the C-terminal region to bind to the actin. Activation of the protein is usually enabled by the phosphorylation of its threonine residue 558, located on helix C of the tail (in moesin). The interaction between the C-ERMAD and FERM is weakened at this stage due to both electrostatic and steric effects (Gary and Bretscher, 1993; Pearson *et al.*, 2000b).

Hence, phosphorylated ERM proteins are found in cell-surface structures. Thus phosphorylation at this specific threonine residue, dissociates the binding between C-ERMAD and N-ERMAD, activates ERM protein (Figure 1.15) (Niggli and Rossy, 2008; Gautreau *et al.*, 2000).

In the inactivated state where it doesn't bind to other proteins or actin, ERM

proteins are mostly found as monomer in the cytoplasm, whereby it is able to bind its C-ERMAD to its own N-ERMAD. In homodimer formation, the ERM protein form dimer with the same protein. Meanwhile interestingly, ERM proteins are also found as heterodimer whereby it binds to another ERM protein (eg:- ezrin with moesin). There are also oligomers known between different proteins sharing the FERM domain (Gary and Bretscher, 1993; Berryman *et al.*, 1995; Bretscher *et al.*, 1995). Merlin is one such oligomer forming molecule, that actually has more affinity towards ERM proteins (McClatchey and Fehon, 2009)

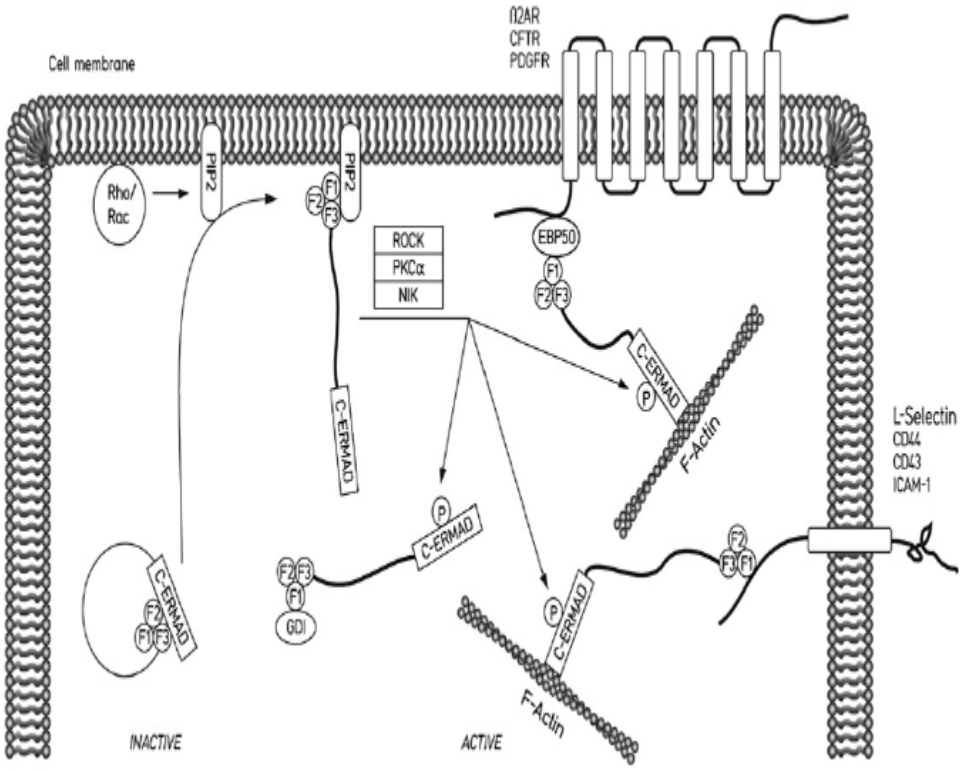

Figure 1.15: Regulation of ERM proteins
Regulation of ERM proteins. The phosphorylation of ERM proteins (active state) is regulated by various kinases. PIP_2 the major binding partner at the FERM domain stabilise ERM protein for this phosphorylation. (Niggli and Rossy, 2008)

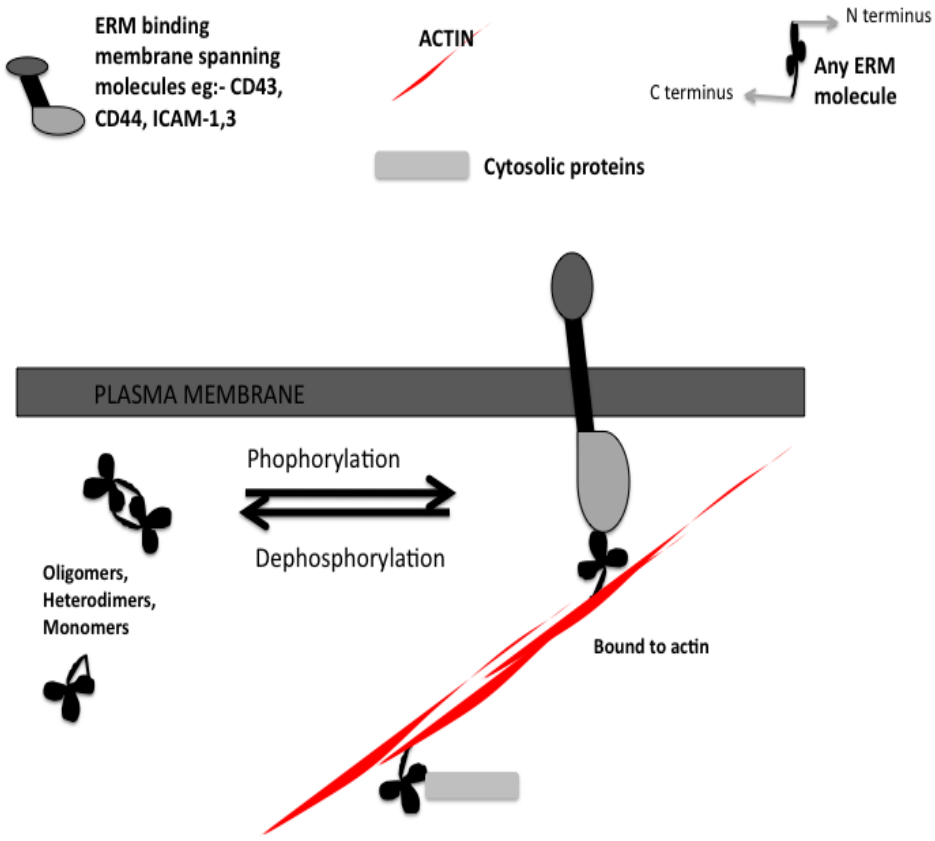

Figure 1.16: Phosphorylation-Dephosphorylation of ERM proteins
ERM proteins are activated by phosphorylation, prior to this it exists monomers (associated to itself), homodimers (eg:- moesin to moesin), Heterodimers (such merlin to moesin); note that merlin actually has more affinity to other ERM proteins than to itself. There are some evidences towards even oligomer formations. The association in all cases is via the C-ERMAD to N-ERMAD. The oligomers can also be either homo-oligomer, or hetero-oligomer (only monomers, heterodimer or homodimers are shown in the figure).

Crystal structures of moeisn N-ERMAD—C-ERMAD complex, has revealed C-ERMAD to be an elongated structure masking a large surface of the globular N-ERMAD domain(Pearson *et al.*, 2000b). Threonine residues that need to be phosphorylated to activate ERM protein varies, i.e, Thr567 in ezrin, Thr564 in radixin, Thr 558 in moesin. And, conversely dephosphorylation achieves deactivation of ERM proteins (McClatchey and Fehon, 2009; Ivetic and Ridley, 2004).

1.4.4 Phosphorylation of Moesin

Introduction of negatively charged phophoryl group at threonine residue, positioned opposite to negatively charged FERM domain, will destabilise it (Nakamura *et al.*, 1995). Mutating the threonine residue to Aspartic acid does not produce the same effect . This is due to the difference in steric effect, as there is not sufficient room to accommodate phosphoryl group among the tight side chains surrounding the threonine residue. Phosphorylation in moesin was initially noted in platelets at threonine 558 (T558) residue. This was found to reduce the affinity of C-ERMAD for the FERM domain. Phosphorylation of other residues, in every ERM proteins, elicited a similar response. The significance of this lies in the fact, that phosphorylated ERM proteins are found in cell-surface structures . As a corollary, dephosphorylation has been found to breakdown the microvilli due to anoxia or apoptosis, and the binding of phophorylated moesin to actin is inactivated by purified protein phophatase 2C (Hishiya *et al.*, 1999; Niggli and Rossy, 2008; Ivetic and Ridley, 2004; McClatchey and Fehon, 2009).

There are other complexities involved in the regulation of ERM proteins. FERM domain binds to ATP, phosphatidylinositol 4,5-bisphosphate, phosphatidylserine, calmodulin and P55 (Diakowski *et al.*, 2006) . It is also found to mediate attachment of protein 4.1 to the plasma membrane, by binding to the cytoplasmic domain of the transmembrane protein's glycophorin A, glycophorin C, band3 and CD44 (Marfatia *et al.*, 1995; Nunomura *et al.*, 2000), and also con-

nect cell-surface transmembrane proteins such as CD44, CD43, ICAM-2 and ICAM-3 to the actin cytoskeleton (Louvet-Vallée, 2000; OLSSON, 1999). Three clusters of lysines, part of FERM domain are a requirement for ERM activation via phophatidyl inositol-4-5-bisphosphate (PIP_2) (Hamada *et al.*, 2000). The lysine residues of the FERM domain important for PIP_2 binding has been indicated to be K63,K64,K253,K254,K262 and K263. PIP_2 is a requirement for conformational change of ERM proteins, in synchronisation with phosphorylation (Barret *et al.*, 2000; Blin *et al.*, 2008). Cell survival in epithelial cells is achieved by phosphorylation of the tyrosine residue (Tyr 353 in Ezrin), which in turn activates the phophatidylinositol 3-kinase (PI3-K)/Akt pathway (Yeh *et al.*, 2009; Youn *et al.*, 2009).

ERM proteins also seem to regulate each other. An evidence in this regard is the C-ERMAD of merlin having a stronger affinity for the N-ERMAD of ezrin: merlin-ezrin heterodimers thus have been detected (McClatchey and Fehon, 2009; Bretscher *et al.*, 2002). Active Rho elicits a response from moesin, to form focal adhesions. Rho kinase ROCK is seen to phophorylate ezrin. Also, for Rho exchange factors Net and Dbl to be activated there is a requirement for the activation of Ezrin (Kim *et al.*, 2009; Tomar and Schlaepfer, 2009; Geiger *et al.*, 2009)

Merlin acts quite differently to the other ERM proteins, namely ezrin, radixin and moesin. Hypo-phosphorylated merlin is found to be active as a growth suppressing molecule, while phosphorylation of a C-terminal serine residue (S518), weakens self-assocaition and inactivates the growth suppressing activity of merlin. Predominantly merlin is found in monomers. The role of other ERM proteins in this regard, is to form stabler heterodimers/oligomer with merlin (as mentioned earlier). (Hennigan *et al.*, 2009; McClatchey and Fehon, 2009)

Rac1 is another activator of merlin, found upstream and downstream to merlin activation. Rac1 is an important regulator of merlin, with active Rac1 found to activate merlin's C-domain. As well, Rac1 and Rho GTPases interact and RhoA GTPases phophorylates other ERM proteins (32). Thus there is a

great amount of cross talk between ERM protein (including merlin) with various lipids, kinases and proteins and each other. Eliciting the interactions at a molecular level will bring out the complexities at play. Functionally, this will lead to better understanding in areas where cytoskeletal exerts influence (Shaw *et al.*, 2001; Hennigan *et al.*, 2009).

1.4.5 Need for promiscuity; other partners & possible partners

Myo-x

It is the nature of things whereby evolution, universally has conferred specific roles to proteins. Some of which provide molecular niche in regulating and driving biological processes. Over the course of evolution redundancy is often common in proteins, especially in complex eukaryotic organisms, they still confer uniqe roles in specialized cells and in specialized functions. When it comes to the nature of things, rapid deployment, adaptablity is the nature of ERM proteins, like most other adapter proteins. Any molecule that has a FERM domain, is potentially sticky to any molecule that has a C-ERMAD.

Thus an interesting molecule that has not been charactherised much with respect to moesin, is Myosin-X or commonly referred to as Myo-10. An intra filopodial molecule that is able to bind to both micotubules with its MyTH4 domain, and to actin. It is also found to bind to PIP_2 by means of its FERM domain (Wang *et al.*, 2009a; Bennett *et al.*, 2007). This is potentially a binding partner for moesin due to the route it takes, making them plausible traveling partners. It will be interesting to understand what roles they play due to their interactions. (Berg *et al.*, 2000; Berg and Cheney, 2002)

1.4.6 Dephosphorylation

Due to its regulation by phosphorylation, studies still need to be undertaken, to understand more about the kinases that phosphorylate moesin and other ERM proteins. There is also a need to understand the role played by phos-

phatases that dephosphorylate moesin. The second event of dephosphorylation is often unappreciated but is important, as would be evident from some of our results. Dephosphorylation, basically, leads to moesin lose its binding to actin (Figure 1.16)(Niggli and Rossy, 2008). The results obtained from our experiments find the dephosphorylation mechanism an indispensable immediate step, with both stretch and uPA induction. We discuss contextually about plausible dephosphorylation in the discussion section. Furthermore we demonstrate events that elicit dephosphorylation off moesin.

1.4.7 L-selectin

Expressed on leuckocytes where moesin is also heavily present, the finding that this is a binding partner for moesin is important. This molecule tethers and allows association between endothelial cells and leukocytes (Killock *et al.*, 2009). With its roles in cell adhesion, and the differential way in which it binds with ezrin and moesin, L-selectin takes up a unique niche during pathways that require their seperate role. The interaction with moesin meanwhile occurs at the N-terminal domain. PMA stimulation allows only moesin and not ezrin to bind to L-selectin. CD95/Fas also binds moesin and ezrin differentially (Ivetic and Ridley, 2004).

1.4.8 Actin

The attention on moesin is due to its most important binding partner actin. Ability to bind to actin, combined with moesin's ability to transiently phosphorylate/dephosphorylate, adapt and recruit other molecules provide plasticity to the cell. This enable the cells to do various functions that depend on the contractibility of the actin cytoskeleton.

The binding proteins of actin are classed into nucleation-promoting factors, the severing proteins, motor proteins and the linker proteins. The nomenclature is derived from the primary function that these proteins are involved in. Experiments with antisense oligonucleotides in mouse thymoma cells (L5178Y)

against the ERM proteins, has shown a dramatic disruption of its microvillar structure. Thus moesin is considered as a linker protein. Far from being an inert linker, moesin and other ERM proteins due to lipid interaction, also play a role in receptor presentation or lack of it (McClatchey and Fehon, 2009).

Moesin with its ability to bind to PIP_2 by means of its FERM domain at the N terminus, is able to hold the membrane and the cytoskeleton. The motor central to this, is its ability to form heterodimer/oligomer with all ERM proteins. This is controlled by its phosphorylation/dephosphorylation. Most of its binding partners also associate with actin, and thus is choreographed an elegant movement of cytoskeleton. Meanwhile, lack of merlin in mouse keratinocytes lead to loss of junction-cortical actin interface between the cell (Lallemand *et al.*, 2003). Merlin as discussed earlier, has more affinity towards other ERM molecules than to its own monomer. This is a powerful interference, taking into respect merlin's inability to bind to actin! Moreover merlin is activated by Rac in contrast to other ERM proteins (Pearson *et al.*, 2000a; McClatchey and Fehon, 2009).

In areas of fine actin filaments devoid of surface structures, it has been reported not to have not a heavy expression (Franck *et al.*, 1993). This is not fully appreciated in papers recently published. Studies inspired from these earlier observations might pave way for myo-10, which is still not fully appreciated in its versatility.

FERM domain containing protiens such as Talin, provide malleability to the cell. Spectrin-actin complexes are tightly adhered to the erthrocyte membrane via Protein 4.1, a member of the FERM-domain protein (this provides it with its elliptical shape) (Sheetz *et al.*, 2006). Though fully developed erythrocyte lacks moesin, megakaryocyte has increasing moesin with the number of nucleus it possess. ERM proteins provide an important linkage between PIP_2 and actin. Moesin also co-localise with the mitotic spindle, provides structural integrity via actin binding in a dividing cell. Phosphorylated moesin is thought to provide the continuous cortical flow, which help the astral microtubules to seperate the two asters of the spindle (Vilmos *et al.*, 2009; Neisch and Fehon,

2008). Moesin via phosphorylation by the Ste20 protein kinase S, lends its carboxy terminal for actin binding (Hipfner *et al.*, 2004). This route is similar to the classic Rho kinase effect on moesin, while Slik is thought to be the other moesin mediator required for cell rounding prior to cell division (Rosenblatt, 2008).

Focal adhesion Kinase

With its FERM domain, focal adhesion kinase like other FERM domain containing molecules is a potential partner to moesin. This kinase which is involved in focal adhesion formation, might latch onto the C-ERMAD of moesin. Irrespective of the kinase activity, this in itself might bring moesin to the loci of focal adhesions. The FERM domain of FAK scores a score of 153 out of a maximum expected score of 158 when compared to moesin's FERM domain. Thus the FERM domain has an exceptionally high homology to moesin's own FERM domain (Dunty *et al.*, 2004).

1.5 Molecular biology of the cell biomechanics & intracellular force transduction

1.5.1 Shear stress

The dragging of blood on the endothelium is derived as shear stress (Figure 1.17). This is represented as frictional force per area. Unless injured, this force is not experienced by vascular smooth muscle cells (VSMCs) (Haga *et al.*, 2007; Orr *et al.*, 2006). Though due to the thin layer of endothelium, some amount of this force could be transferrd to VSMCs.

There is still a co-operative paracrine exhange of information between endothelium and VSMCs (Malek *et al.*, 1999). The vascular vessels are elastic, bigger the vessels, greater being the elasticity. This elasticity maintains a uniform flow of blood between the peaked pressure of (sytole) & deflation of pressure

(dystole) in smaller arteries. The elasticity of large vessels, thus compensates for lack of elasticity of smaller vessels (Hahn and Schwartz, 2009).

Endothelium in culture show some remarkable phenotype under flow and without flow. It is an often noticed feature of endothelium, whereby cells of endothelium aligns in the direction of shear stress with flow. Meanwhile primary cilia, a constituent of kidney cells which senses low shear levels, is seen in endothelium under static conditions (Iomini *et al.*, 2004). Blood flow once established as parabolic, exerts laminar flow. This though requires, a straight vessel and can then be expressed as

$\tau=4\mu Q/\pi r^3$

(μ is viscosity, Q is the flow rate, r the vessel radius and τ being shear stress) (Lehoux and Tedgui, 2003).

This always is not the case, due to the branched architecture of the vasculature. These abnormal flow patterns give rise to non-laminar, low shear stress, non-uniform flow pattern giving rise to chronic inflammation. This chronic inflammation due to these factors are even seen in newborns and is thus a basic characteristic (Hahn and Schwartz, 2009). Meanwhile, physiological values of shear stress is around 10-15 dynes/cm^2 in most animals except mice (Lehoux and Tedgui, 2003). Though there is variation depending on the arteries (higherst in small arteries (60-80 dynes/cm^2) and veins, with large veins often at being less than 1 dyne/cm^2, while small venules at 20-40 dynes/cm^2 (Ballermann *et al.*, 1998).

1.5.2 Cyclic strain

Cyclic strain is the result of our pressurised blood pumping system, whereby circumfrential cyclic strain is derived from the blood pressure (Figure 1.17). Unlike hydrostatic pressure mentioned in the next section, blood pressure is the highest in the aorta and decreases as it travels further. Blood pressure being hydraulic pressure, it is defined as force per area. The cyclic stretch is a normal physiological aspect of both endothelium and vascular smooth muscle

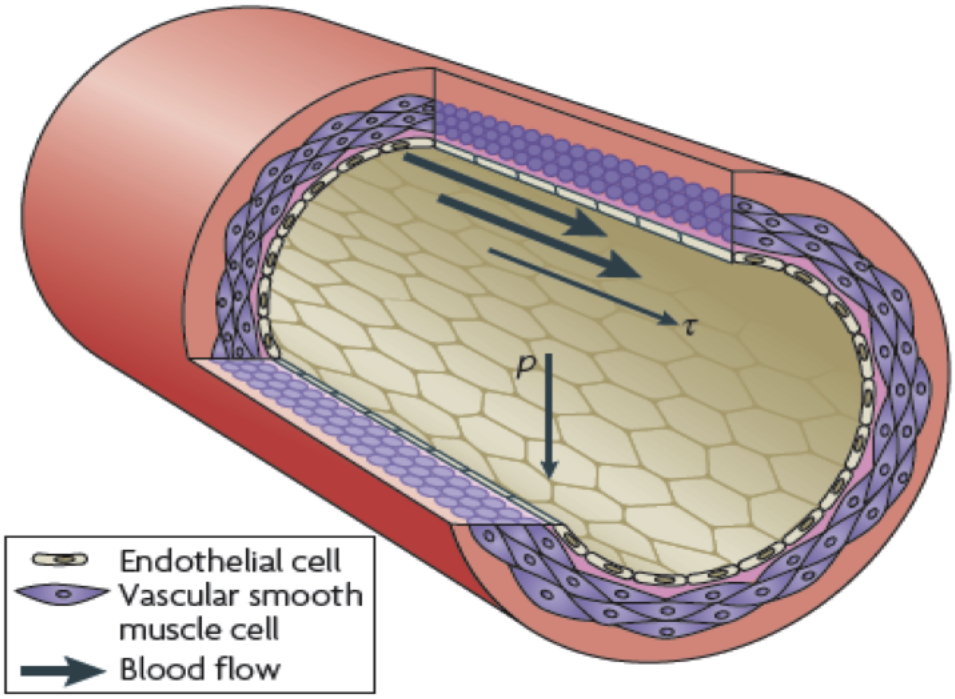

Figure 1.17: Hemodynamic Forces
Where τ is shear stress and P is pressure that results in circumfrential stretching, in all directions of the vascular circumference.
(Hahn and Schwartz, 2009)

cells (Haga *et al.*, 2007). Wall tension is the force per unit length of the vessel.

Mathematically represented as

T=Pr

(where P is the blood pressure, r the radius of the vessel and T being wall tension).

Meanwhile circumfrential strain can be expressed as

T=Pr/h

(where 'h' represents the thickness of the vessel bearing the the circumfrential strain).

Thus, strain is felt only partly by the various constituents of the vessel wall

such as VSMCs and ECs (Lehoux and Tedgui, 2003).

1.5.3 Hydrostatic pressure

This is also a force sensed by endothelium, basically, the volume of blood on top of the endothelium. In humans, lower limbs thus are exposed to higher hydrostatic pressure than upper limbs. This is an area under investigation. This force is also felt by cartilage, knee meniscus, temporomandibular joint disc, intervertebral disc, bone (Elder and Athanasiou, 2008; Padilla *et al.*, 2009).

At a cellular level fungi and plants utilise hydorstatic pressure based mechanism for protrusion (Harold, 2002; Messerli and Robinson, 2003; Money and Harold, 1992). This is one of the major force intracellularly, whereby the cytoplasm exerts a certain pressure on the plama membrane. Here cytosol acts as the conductor for hydrostatic pressure. There are locally acting pressure differences on the membrane. This results in the contractile, elastic network of membrane being infiltrated by cytosol (Charras *et al.*, 2005). These differences provide niche compartments for molecules, that then thrive in such circumstances. Hydrostatic pressure has to said, has been found to have much little effect compared to shear stress or cyclic strain in terms of blood flow (Hahn and Schwartz, 2009). Nevertheless, only further investigation into the molecular events will lead to hydrostatic pressure's real significance.

1.5.4 Molecular determinants & regulators of flow

1.5.4.1 Remodeling with flow

With flow, higher or lower blood pressure over long periods of time lead to remodeling of our vasculature. The vasculature accomodates the change by structural changes to itself. Vascular smooth muscle cells, play a major role in directing this remodeling due to high flow with increased blood pressure. This leads to the thickening of vasculature due to constriction by VSMCs, eventually leading to loss of elasticity. "Laplace's law: This law states that tension in the vessel wall equals the difference in pressure across the vessel times the radius of the vessel, divided by the thickness of the wall. Thus, higher blood pressure or vessels of larger radius require thicker walls to be mechanically stable" (Hahn and Schwartz, 2009).

At the same instance, higher shear stress felt by endothelium induces NO release, which then relaxes the VSMCs. VSMCs meanwhile, protect the endothelium from NO-induced apoptosis by reducing bcl-2 activity in endothelium (Haga *et al.*, 2007).

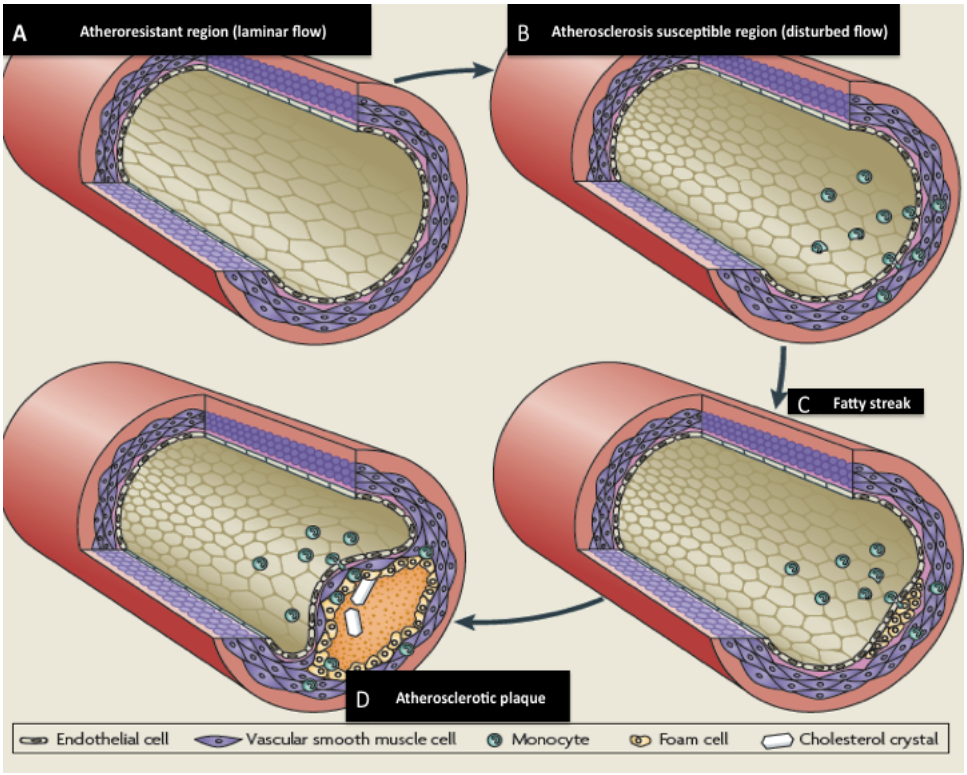

Figure 1.18: Progression to atheroscerosis
A)Endothelial cells normally align in direction of flow. The expression of adhesion receptors, cytokines is kept to a minimum. The proliferation rate is quite low, owing to quiescent nature of endothelium. Apoptosis is also at a minimum at this stage. B) With susceptibility to atherosclerosis, owing to disturbed flow, ECs get misaligned. Note that this misalignment is a prelude to further disease. This results in inflammatory response. As the cause is disturbed flow, the inflammation does not subside. Which would be the case with pathogenic entry. Higher cell turnover, expression of leukocyte adhesion receptors (E-selectin), vascular cell-adhesion molecule 1(VCAM1), intracellular adhesion molecule 1 (ICAM1) and cytokines occur. The leukocytes, meanwhile migrate into the vessel wall. C) Fatty streaks are formed in these regions with deposition of lipoproteins (low density lipoproteins (LDL) and very low density lipoproteins). Further to this, cholestrol, triglycerides are taken up. The reason high density lipoproteins (HDL) is seen as the "good molecule", is becuase it essentially, carry lipids away and oppose deposition (HDL in this circumstance is considerably reduced, due to the earlier uptake of LDL). Macrophages meanwhile is laden with considerable amount of lipid (referred to as foam cells). The activation of cells, meanwhile continues to increase resulting in secretion of cytokines, enzymes. These rearrange the ECM, resulting in increased migration, thickening of the VSMCs. D) Finally, is formed atherosclerotic plaque. Previously appreciated, modifiable risk factors determine, how fast this stage is reached. (Hahn and Schwartz, 2009)

Increase in stress meanwhile, induces smooth muscle cell hypertrophy. Also, the wall undergoes atrophy when circumfrential stress is substantially reduced (Bomberger *et al.*, 1980).

Vascular cells shed extracellular matrix complimentary to the type of flow that they experience. Collagen IV, laminin, nidogen and proteoglycans are the normal constituents of ECM for endothelium. With injury, fibronectin, fibrinogen and thrombospondin are also incorporated. ECM also allow the cells to sense stiffness. A decrease in substrate stiffness thus reduce formation of focal adhesions and stress fibers. As well, they play a vital signaling role, allowing the cells to sense, adapt and remodel (Chiang *et al.*, 2009a; Wang and Thampatty, 2006; Hahn and Schwartz, 2009).

In situations with chronic hyperlipidaemia, an abuse of the modifiable factors over chronic period leads to atherosclerosis (Figure 1.18). Further long term implications are calcification of the vasculature. Meanwhile, thrombus formation is activated when these atherosclerotic plaques rupture, ending in vessel occlusion (Hahn and Schwartz, 2009).

1.5.4.2 The transducers

Endothelial cells effect a range of signaling pathways with flow. Including nitric oxide production, phosphoinositide 3-kinase (PI3K), extracellular signal-regulated kinase 5 (ErK5/ MAPK7)) pathways. To sense the shear stress and cyclic strain, endothelial cells are equipped with various receptors which include integrins, heterotrimeric G proteins, receptor tyrosine kinases, ion channels (Figure 1.19) (Malek *et al.*, 1999; Hahn and Schwartz, 2009; Lehoux and Tedgui, 2003).

Meanwhile the underlying cytoskeleton, relays the stimuli throughout the cell. Mechanical stimuli is faster than chemically induced stimuli, reaching the nucleus in less than 5μs, while the latter takes ~5s (Wang *et al.*, 2009b). This is a huge difference, which enables faster gene expression. This allows the cells to assert a finer control, with varying blood flow.

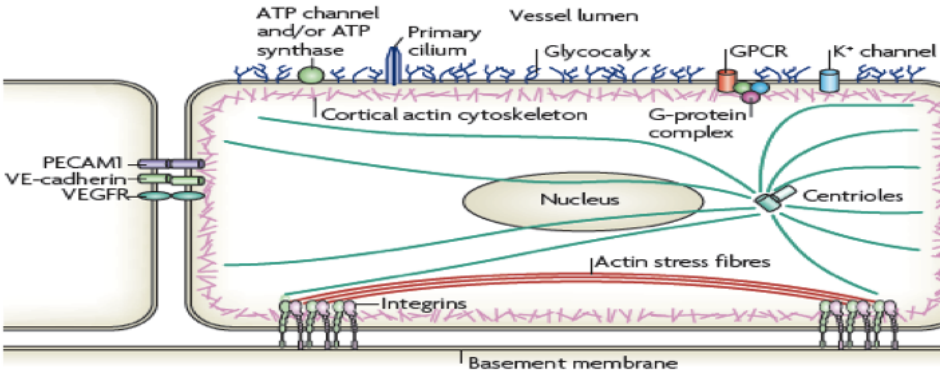

Figure 1.19: Mechanotransducers
G-protein-coupled receptors (GPCRs), heterotrimeric G proteins, ion channels, glyocalyx, ATP channels transduce various mechanical stimuli. Laterally cell membrane utilize PECAM-1, ECAM-1, VE-Cadherin to communicate to nearby cells. Cytoskeleton spans inside transmitting force by means of cortical actin cytoskeleton, actin stress fibers, microtubules. Integrins, meanwhile connect the cell to ECM (Hahn and Schwartz, 2009).

As well, the cells lying adjacent to each other are connected by means of PECAM-1, VE-cadherin and vascular endothelial growth factor receptor 2 (VEGF2). These cell-cell junctional proteins are also recruited for transmission of forces. Glycocalix is a layer of carbohydrate-rich proteins generating a lot interest recently, that can mediate shear stress (Jaalouk and Lammerding, 2009).

1.5.4.3 Flow dependent signaling

Cytoskeleton touches all areas of a cell, thus with actin and microtubules, it enables the transmission of mechnanical forces. Actin-binding proteins play a huge role bringing together different molecules. Actin stress fibers in BAECs have shown to increase with stretch, aligning perpendicular to stretch (Kaunas *et al.*, 2006; Wang *et al.*, 2002). Jnk is re-activated with realigning stress fibers, due to stretch (Chien, 2007). Focal adhesion sites then recruit proteins, which results in activation of MAPK. This downstream regulates transcription factors AP-1, NF-B, Sp-1 and Egr-1. MicroRNA regulation has not been shown with mechanotransduction. Changes in gene expression though have been well portrayed (Chien *et al.*, 1998; Chien, 2007).

Rho GTPases regulate proteins that are critical to signaling via mechanotrasduction. They have been shown to cause cell contraction. This is achieved by regulating myosin light chain and mDia incorporating Rho Kinase. Profilin and Src-tyrosine kinase takes over, regulating focal adhesion turnover and actin polymerisation. Stretch meanwhile is garnered by PECAM-1, relaying information to adjacent cells by activating respective Src-tyrosine kinase (Chrzanowska-Wodnicka and Burridge, 1996; Satoh *et al.*, 2000; Watanabe *et al.*, 1999; Newman, 1997). PECAM is also phosphorylated by Fyn, a tyrosine kinase. This enables maintainence of a quiscent monolayer of endothelium (Chiu *et al.*, 2008). Hydrostatic pressure can also activate the Src family. FAK is also activated downstream of hydrostatic pressure. This in turn phosphorylates α-actinin, this increases adhesion of the cells. Though the studies were carried out in colon cells, the critical need of $\alpha 5\beta 1$ integrin proved interesting

for vasculature (Craig *et al.*, 2007). Cyclic strain has been shown previously to reorganise $\alpha 5\beta 1$ in endothelium, parallel to FAK being phophorylated. This tyrosine phosphorylation of FAK is similar to paxillin's phosphorylation of the tyrosine residues. The same group also noted $\alpha 2\beta 1$ reorganising with cyclic strain (Yano *et al.*, 1997a).

The activation of FAK and Src occurs within ten minutes of stretch, which leaves room for more rapid adapter proteins to bring together the big party needed for focal adhesion. Vinculin, zyxin, talins (FERM domawolfn), Kindlins and as such yet to be identified components, are brought together in seconds. The primary transducer for these are the integrins, which sense inside of the cell, adjusting the adhesion to the ECM (Katsumi *et al.*, 2005). In parallel, stretch also opens up the cells to a calcium influx, via stretch activated cation channels. As well, nuclear factor kappa-B (NFkB), Ras, Rac-1 and the previously mentioned RhoA are activated (Lehoux *et al.*, 2006; Asparuhova *et al.*, 2009). Shear is found to activate the potassium channels (Lehoux and Tedgui, 2003). There is increasing evidence for direction effecting change in gene expression. The recently discovered sustained gene expression control by MAL (member of myocardin-related transcription factor family (MRTF)), via serum response factor with stretch is just one example. What about microRNA control? Interestingly Rho A is the mediator of the MAL based pathway (Asparuhova *et al.*, 2009).

How do these molecules get transmitted to the nucleus with mechanotransduction? The transmission lines are actin based cytoskeleton (Asparuhova *et al.*, 2009). Does that mean, the adapter proteins get translocated to the nucleus with stretch? Zyxin, is an example of stretch, inducing a cytoskeletal protein to translocate to the nucleus. This elegant study showed most of the zyxin in the cell, in nucleus within 30 minutes of stretch induction (Cattaruzza *et al.*, 2004). This is a protein that also is an usual suspect in stretch induced focal adhesions.

Another factor to note is that multiple pathways open up with mechanotransduction. There might be otherwise very useful mechano-transmission

proteins, which, might find itself redundant in this context. There are niche mechano sensitive proteins such as in ear, that differ from endothelium (Orr *et al.*, 2006). The opening up of previously mentioned cation gates and the influx of calcium activates calpain, which cleaves multiple proteins (Glading *et al.*, 2002). What about microparticle formation in this scenario?

Integrin linked kinase (ILK) can bind to Kindlins. This is antother stretch induced transducer, activating Rho A within 5minutes (Schwartz and DeSimone, 2008). Kindlin binds to NPXY motif of the β integrin cytoplasmic tail. Though, it has be noted that talin does not bind to the same NPXY motif, rather, utilizes its FERM domain to bind with another NPXY motif. The binding of talin is critical in inducing a conformational change to the integrin. This converts the $\alpha\beta$ heterodimer to a high affinity state from an inactive conformation. Kindlin needs the co-operation of talin to activate integrins (Geiger *et al.*, 2009). There are other areas of functionality of a cell that provide insight into this. Osmotic stress is shown to activate FAK and Src, where incidentally moesin is shown to be activated (Di Ciano-Oliveira *et al.*, 2006; Schwartz and DeSimone, 2008). It has been demonstrated that chloride channels are opened when $\beta 1$ integrins are engaged mechanically. It is interesting to note that for calcium influx, under similar conditions, a dependence on the type of ECM is seen. It has to be noted that shear stress also shows similar activation of FAK and Src (Schwartz and DeSimone, 2008).

This is but a slice of various signaling induced by shear, cyclic strain and hydrostatic pressure. The shear volume though huge, is a fine interplay, with every single molecule sensing each other regulating and adapting to the need of the flow.

1.5.5 Migration of Cells

1.5.5.1 Cell migration

The cell is spanned by an extensive network of cytoskeleton with a large surface area, connected to all the major organelles on which a large number of

proteins can dock and regulate one another. Integrins give external cues to inside, by outside-in signaling and from the inside to the outside, by inside-out signaling (Janmey, 1998).

Integrin cytoplasmic domain bind directly to many different cytoskeletal proteins, which in turn act as adaptors to recruit signaling molecules (Liu *et al.*, 2000). It has been reported that the$\beta 1A$, $\beta 3$ and $\beta 1D$ cytoplasmic domains bind to talin, $\beta 1A$ tail bind to α-actinin, $\beta 1A$,$\beta 2$ and $\beta 7$tails bind to filamin and the $\alpha 4$ tail binds to paxillin. Of these filamin, paxillin associate with other adaptors, signaling molecules and effect migratory signaling (Stossel *et al.*, 2001; Turner, 2000).

1.5.5.2 Importance of cell migration

Under normal physiological conditions, directed movement of a cell is necessary for processes like angiogenesis and leukocyte extravasation (Brakebusch *et al.*, 2002). Leukocytes are able to infiltrate into the tissues from a blood vessel in case of infection. The infection promotes inflammatory signals, which then attract the free moving leukocytes. These move to the endothelial cells, toward the infected area. Then via integrin signaling, they are able to infiltrate into the surrounding tissue and attack the infectious substance (Fujimi *et al.*, 2002).

1.5.5.3 Understanding the movement mechanism

In studies conducted using green fluorescence protein tagged to$\beta 3$ subunit of integrins, migration of cells is seen to be initiated by cell polarizations and the formation of membrane protrusions to the ECM (Laukaitis *et al.*, 2001; Ballestrem *et al.*, 2001)

At the cell front, adhesion sites containing $\alpha \mathrm{v} \beta 3$ integrin remain stationary, whereas at the rear of the cell they slide inward (Ballestrem *et al.*, 2001). At the cell front, actin cytoskeleton is recruited which triggers the association of many different signaling molecules at the focal contacts, and then, integrin signals stimulate cell contraction. While at the rear of the cell, it detaches from

the substratum by integrin inactivation and the disassembly of the adhesion complexes (Brakebusch *et al.*, 2002; Brakebusch and FaÈssler, 2003).

Adhesion via integrins is dependent on its ligand binding property. Integrins exist in high affinity and low affinity conformations. The high affinity forms are necessary at the leading end of the cell, where they attach firmly to the ECM. The high affinity binding, is regulated by the binding of regulatory intracellular molecules, to the cytoplasmic domains of the integrins. The study to understand the binding partners and the regulatory mechanisms of the cytoplasmic domains, thus has great significance. Huge array of different proteins, are currently known to bind the intracellular domains of the integrin molecules. And a lot more indirectly in the regulatory cascade (Brakebusch *et al.*, 2002; Brakebusch and FaÈssler, 2003; Rose *et al.*, 2007)

Cell attachments not just regulate by "affinity regulation", but also by avidity regulation, whereby the integrins in low affinity increase adhesion because of clustering. These clusters are seen in focal adhesions and readily detectable cell-matrix contacts, studied extensively in fibroblast cells. Lateral mobility of the integrins, also thus must have an important role in cell migration and adhesion (Brakebusch and FaÈssler, 2003; Luster *et al.*, 2005)

Experiments have shown that in leukocytes, where the integrin molecules have been attached to actin cytoskeleton when released by proteolytic digestion, results in increment of integrins resulting in lateral mobility. This also increases the chances for integrins to meet specific ligands, which facilitates integrin aggregation and high avidity clusters (Constanin, 2000).

1.5.5.4 Molecular basis of cell migration

Locking integrins into a high affinity state by mutations or activating antibodies inhibit cell migration. Different studies have shown us, that detachment of integrins at the cell's rear is an important step in the process of cell migration. Detachment processes appear to be sensitive to the Ca^{+} levels. Migrating fibroblasts have increased Ca^{+} levels in the rear. $\alpha v\beta 3$ integrin is seen to de-

tach by a Ca^{+} induced mechanism, whereby calcineurin is activated disrupting the interactions between $\alpha v\beta 3$ and cytoskeleton (Pierini, 2000). The unbound integrins from the rear end or the trailing edge is transported to the leading edge by endocytosis, which is probably needed to replace the fast depleting amounts of integrins (Brakebusch and FaÈssler, 2003).

Matrix metalloproteinases (MMP) are important enzymes in the process of metastasis, tumour invasion and angiogenesis. These zinc dependent endoproteases are important in partially destroying the surrounding ECM. Various integrin dependent pathways, regulate these enzymes. One mechanism is whereby, integrins bind to the ECM molecules and to MMPs, thus bringing these molecules closer. This enzyme is also important in wound healing (Hangai *et al.*, 2002; Marshall *et al.*, 2003; Xu *et al.*, 2001; Brooks *et al.*, 1998) Regulating proliferation of cells by integrins is also an important role that MMP undertake. $\alpha v\beta 3$ integrin and bFGF signaling is an important requirement of the cell cycle progression (Roovers and Assoian, 2003; Welsh *et al.*, 2001).

1.5.5.5 Regulatory mechanisms

Affinity and avidity of the integrins are regulated by growth factors and chemokine signaling (Brakebusch *et al.*, 2002). Phosphatidylinositol 3-kinase (PI3-K) is an important regulator of the integrin signaling mechanism, and prior to migration increase integrin avidity (Trusolino *et al.*, 2000).

In mast cells, PI3-K is activated by PDGF-R (Platelet derived growth factor-R), which then increase the affinity of the $\alpha 5\beta 1$ integrin. In breast cancer cells also, PI3-K is seen to upregulate the increased metastatic properties and adhesion (Salh *et al.*, 2002; Brakebusch *et al.*, 2002).

Extravasation process of leuokocytes require tight adhesion of itself to the endothelium, and into inflamed tissues for which chemokines are an important regulatory mechanism. Viruses affect the chemokine signaling and enter the cells by evading the immune system (Brakebusch *et al.*, 2002; Brakebusch and FaÈssler, 2003; Poli, 1999).

The Ras (small GTP binding proteins) and Rho (small GTP binding proteins), GTPases though very specific to cell type and integrin, also regulate the affinity of the integrins. In Chinese hamster ovary (CHO) cells, H-Ras (a subset of Ras protein family) inhibits activation of β1 and β3 integrins. R-Ras promotes integrin activation in the myeloid cells but has no effect in a lymphoid cell line (Kinashi *et al.*, 2000). PI3-K is required for integrin activation by both H-Ras and R-Ras (Mora *et al.*, 2007; Yoshimura *et al.*, 2006).

Rap1 meanwhile, regulate the integrin high affinity state in lymphoid and endothelial cells (Nolz *et al.*, 2008). Integrins also regulate each other, such as when αIIβ3 downregulates α2β1, α5β1 mediated adhesion (Rose *et al.*, 2007; Brakebusch *et al.*, 2002). Secreted molecules have been shown to reduce the affinity of integrin β1 to other ligands, or by directly interacting with it, thus producing a conformational change. An example of this is the Galectin-8, a galactoside binding protein. Meanwhile Galectin-3 has been implicated with β3 integrin (Diskin *et al.*, 2009; Lei *et al.*, 2009). Urokinase is another secreted molecule, that laterally interact with integrins via its receptor uPAR (D'Alessio and Blasi, 2009). This is a highly chemotactic mechanism, leading to cell migration (Degryse *et al.*, 2001; Degryse, 2008).

1.6 MicroRNA regulation

There is the constant wrestle between control and chaos, whose ratio in an organism defines life or death. The classic studies by Rosalind Franklin, which James Watson, Francis Crick and Maurice Wilkins confirmed, revealed DNA as the information blue print of an organism (Maddox, 2003)

From initial postulation of the three letter codon by George Gamow, ending in the pioneering work by Har Gobind Khorana revealed all the codons (Nanjundiah, 2004). Since the advent of the genetic code, we have come to understand other levels of control to the synthesis of the final building block of an organism, the protein (Khorana *et al.*, 1966). Post translational modification, protein stability, transcription factors are all mutiple layer of control an organism exerts.

MicroRNA is one such arsenal of control, that acts upstream at the level of post transcription, on the mRNAs repressing translation of proteins. Though called microRNA, the impact of microRNAs are quite global and macro in nature (Filipowicz *et al.*, 2008). This is because in animals each microRNA can have multiple protein targets, as long as they have 2-7 basepair seed region that are complimentary. With one third of genes under regulation of microRNA, we have considerable amount of work in front of us (Selbach *et al.*, 2008; Lewis *et al.*, 2005). They also provide fodder to the saturated research of drug discovery, bringing out new routes of drug targeting.

1.6.1 Biogenesis of microRNA

Fire and Mello were the pioneers in the field, with their studies in C. elegans (Ambros, 2008).“According to the current convention, a miRNA is defined as a singlestrandedRNA of ~22 nucleotides in length, which is generated by the RNase-III-type enzyme Dicer from an endogenous transcript that contains a local hairpin structure” (Kim, 2005). This being a rapidly advancing field, more knowledge is bound to add to our understanding.

Small RNA molecules such as tRNA are transcribed by RNA polymerase

III, while microRNAs are transcribed by RNA polymerase II (Lee *et al.*, 2004). The very long primary transcripts, pri-miRNAs that are kilobases long cannot be transcribed by RNA polymerase III. The pri-miRNAs are located in intergenic locations, introns as well as in exons. The pri-miRNAs can be generated using its own promoter region. They are polyadenylated and capped (MGpppG) (Zhang, 2008; Kim, 2005). The resulting pri-miRNA contains a hair loop structure and is cleaved by the nuclear RNase III Drosha. This sheds fragments that are thought to be degraded in the nucleus. Drosha a large protein ~160 kDa, forms a large protein complex ~650 kDa for this process (Lee *et al.*, 2003). This complex referred to as the microprocessor complex, also contain the DiGeorge syndrome critical region 8 (DGCR8) protein. This interaction is postulated to be important in substrate recognition. DGCR8 ~120 kDa is referred to as Pasha in *Drosophila melanogaster* and *Caenorhabditis elegans* (Han *et al.*, 2004).

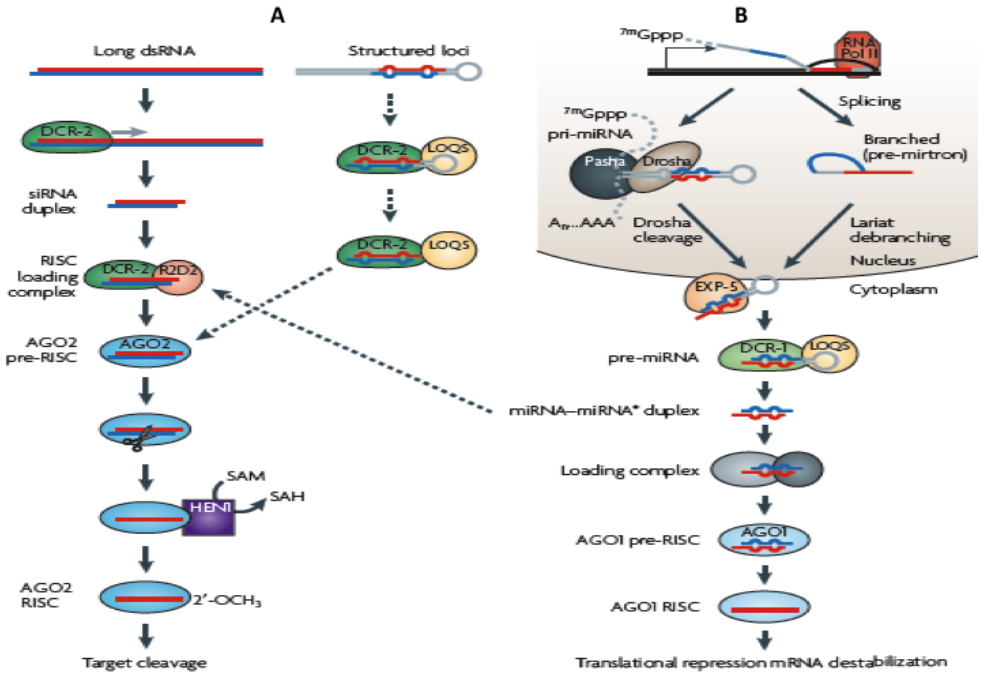

Figure 1.20: Biogenesis of microRNA
The figure shows the various steps involved in microRNA biogenesis.
A) siRNA processing; siRNA do not exist in animal cells. Its processing uses the common machinery as microRNA processing. B) microRNA processing.
(Ghildiyal and Zamore, 2009)

From the microprocessor complex emerges the precursor of miRNA (pre-miRNA). This pre-miRNA located in the nucleus, has to be critically transported to the cytoplasm. It is here that the final miRNA is generated. The export of pre-miRNA is carried out by Exportin-5 (Kim, 2004). This molecule, has been previously implicated in tRNA transport when its primary export factor exportin-t is overloaded (Bohnsack *et al.*, 2002). Pre-miRNAs are transported across nuclear pore complexes, in conjunction with Ran-GTP. Exportin 5 is also found to increase the stability of pre-miRNA, without which it is highly unstable. The whole complex is referred to as the exportin complex (Murchison and Hannon, 2004; Weis, 1998).

In the cytoplasm the Ran-GTP when hydrolyses to Ran-GDP, results in the expulsion of the pre-miRNA (Murchison and Hannon, 2004). The final processing is carried out by Dicer. Dicer is highly conserved in eukaryotes and has been experimentally proven to dice the ~70-nucleotide pre-miRNAs to the mature ~22-nucleotide miRNA (Bernstein *et al.*, 2001). This ~200 kDa protein(Mallory and Vaucheret, 2009) shares the PAZ domain with argonaute proteins. This domain binds to the 3' end of small RNAs (Yan *et al.*, 2003). Even though dicer is known to interact with various proteins, including argonaute, it is independent in its cleavage function. This was studied with purified Dicer, which was analyzed for its function (Zhang *et al.*, 2004; Kim, 2005).

The complex with the mature miRNAs are referred to as miRISC (miRNA-containing RNA-induced silencing comples). This contains both the guide strand and the passenger strand, where, the latter strand is degraded. In the complex there is also the argonaute proteins and might have other unknown proteins. Thermodynamic stability difference between the strands decides which is the passenger strand (Ghildiyal and Zamore, 2009). Argonaute (Ago2) cleaves the passenger strand wherein exists only the guide strand. This strand goes through methylation in plants and flies which increase their stability. As well, the guide strand is loaded on to the Ago complex. The RISC is referred to as mature-RISC, while the former state is referred to as pre-RISC (Ghildiyal and Zamore, 2009; Kim, 2005).

Further to this occurs target selection, where only very few mammalian miRNAs are fully complementary to the mRNAs, which is contrary to plants. As mentioned previously, the target is selected based on the complimentarity of the 'seed region' which is the 5´ end of the miRNA to the mRNA (Grimson *et al.*, 2007). There is nothing micro about the global effect a single miRNA can have. The ability to control many different genes are unmatched by any other post transcription effector. As well, the actual repression can start at the initiation step or the elongation step of the mRNA translation (Ghildiyal and Zamore, 2009).

Argoanute (Ago) proteins number 1 in S.pombe to 27 in C.elegans and 8 in humans (Sasaki *et al.*, 2003). The mature miRNA is loaded onto the Ago by unknown helicase, and there is much speculation about the actual loading (Peters and Meister, 2007). Nevertheless we know, PAZ domain in Ago proteins bind specifically to the 2 nucleotide 3' overhangs of miRNAs. This domain, as previously mentioned, is available in Dicer where it utilises the same mechanism for specific binding (Kim, 2005). The thermodynamic instability of the 5', whereby the less stably paired strand (guide strand) is incorporated into Ago (Ago1) complex while the other passenger strand is degraded. Ago proteins are the final effectors in the miRNA mediated translational repression. Thus there are variations in the actual effect it induces depending on a multitude of factors, including which particular Ago protein was used. This Ago complex, thus is referred to as the effector complex (Mallory and Vaucheret, 2009).

1.6.2 MicroRNA in cardiovascular system

The ~22 nucleotide tiny microRNAs might be dimunitive in stature, but controls diverse areas such as developmental timing, haematopoiesis, organogenesis, apoptosis, cell proliferation,and metastasis. Vascular cells are controlled at cell differentiation, growth, proliferation and apoptosis by microRNAs. Angiogenesis is under microRNA guidance as well. All this advance took place only in the last while, as people started to look into microRNA in cardiovascu-

lar cells only from 2005 (Zhao *et al.*, 2005).

MicroRNAs are highly expressed in vascular cells, and are tissue specific in their expression (Lagos-Quintana *et al.*, 2002). MicroRNA expression have been found to differ between arteries and heart. mir-1 expressed in the heart while not as much in the artery, plays a role in cardiac myocyte differentiation. This microRNA is also a target of serum-response factors (SRFs), MyoD (myogenic differentiation factor D) and Mef2 (myocyte-enhancing factor2) (Zhao *et al.*, 2005). It has been shown experimentally that miR-23a, miR-23b, miR-24, miR-195 or miR-214 as well miR-1 to have roles in hypertrophy of the heart (van Rooij *et al.*, 2006). miR-21 has been shown to increase proliferation in vascular smooth muscle cells (VSMCs) (Zhang, 2008). Different miRNAs have been shown to be also involved in endothelial migration eg:- miR-21, miR-221, let-7 and mIR-222 (Suarez *et al.*, 2007; Poliseno *et al.*, 2006; Kuehbacher *et al.*, 2007).

In the case of angiogenesis, Dicer knock down mice proved miRNA to be involved (Zhao *et al.*, 2007). miR-17-92 is an miRNA that has has been shown to increase tumour angiogeneis (Dews *et al.*, 2006). miR-221, miR-222, miR-27b, let-7f are other miRNAs implicated in tube formation (Poliseno *et al.*, 2006; Kuehbacher *et al.*, 2007). Dicer knock out endothelial cells have been very useful in understanding miRNAs roles in angiogenesis (Yang *et al.*, 2005; Suarez *et al.*, 2007). It has been shown in endothelial cells that siRNA mediated down regulation of drosha doesn't affect angiogeneis. Meanwhile, Dicer downregulation seems to prove that microRNAs mediate angiogenesis (Kuehbacher *et al.*, 2007). It has been shown that there are drosha independent pre-miRNAs, and hence Dicer is processing point that all miRNAs equally pass through (Ghildiyal and Zamore, 2009). There are also endothelial specific miRNAs such as the miR-126 that mediate developmental angiogeneis *in-vivo*. With this miRNA lacking, there occurs leaky vessels, hemorrhaging and partial embryonic lethality (Wang *et al.*, 2008).

There are gaping holes in areas such as mechanotrasduction, with respect to cyclyc strain, shear stress in microRNA research. We were the first to show that an array of microRNA, was regulated by cyclic strain and shear stress.

1.7 Endothelial derived Microparticles

Artheroprotective effects are enforced under physiological forces of blood flow, under maintenance of an intact monolayer endothelial cell barrier. Release of substances *in-vivo* that promote anticoagulation, that inhibit inflammation, cause vasodilation are encouraged under such conditions. Activation or injury of the endothelium leads to a variety of inflammatory disorders including the release of microparticles (MP) (Chironi *et al.*, 2008).

Cellular microparticles (MP) are sub-micron membrane vesicles that are released from cells upon activation, apoptosis, injury (Figure 1.21). They constitute a heterogeneous population of submicron elements, differing in cellular origin (such as platelets, leukocytes, endothelial cells), number, size, antigenic composition and functional properties (Hugel *et al.*, 2005; George, 2008).

Initially the MPs were considerd inert 'cell dust', while recent evidences suggest otherwise. They have been found to interact remotely, acquiring a pathophysiological potential (Freyssinet, 2003). Peter Wolf *et al*, in 1967 using ultracentrifugation and electron microscopy identified microparticles, while referring to as "platelet dust". He found these capable of facilitating thrombin generation, in the same manner as that of intact platelets (Wolf, 1967).

Found in the plasma of healthy subjects, they increase under pathogenic conditions. Endothelial microparticles (EMP) not only constitute an emerging marker of endothelial dysfunction, but are also considered to play a major biological role in inflammation, vascular injury, angiogenesis, and thrombosis (Chironi *et al.*, 2008).

As the name implies, endothelial microparticles are derived from endothelial cells. MPs are widely considered as bi-layered membranes, with antigenic composition very similar to the cell membrane of origin. There is this loss of ability to maintain normal phospholipid architecture, which normally is assymetric (Dignat-George *et al.*, 2004). The production of microparticles is very stringent. There are MPs in circulation from necrotic cells with loss of membrane integrity, originating from mechanical destruction of cells following in-

jury (Hugel *et al.*, 2005).

1.7.1 Structure and generation of microparticles

1.7.1.1 The assymetric plasma membrane

Generation of microparticles are effectively caused by the remodeling of the plama membrane under pro-coagulant, pro-inflammatory or apoptogenic stimulation. Resting cell membrane exists in an assymetrical design with various phospholipids. In this arrangement, phosphatidylserine (PS) and phosphatidylethanolamine (PE) are kept inside with phosphatidylcholine (PC) and sphingomyelin kept externally. The enzymatic equilibrium between flippase, floppase and scramblase maintain this assymetry. Sudden increase in cytosolic calcium disturbs the transmembrane steady state. Basic mechanism of microparticle generation was studied *in-vitro* on platelets. This is similar in all cells except for the differring antigenic composition (VanWijk *et al.*, 2003; Hugel *et al.*, 2005; Boulanger *et al.*, 2006; Piccin *et al.*, 2007). Different antigenic turnover and phospholipid content is clearly seen. This depends on the microparticle lineage (Geo, 2008; Heijnen *et al.*, 1999). The differing antigenic composition also depends on the mode of initial activation. Such as whether its due to calcium ionophore, ATP depletion, lipopolysaccharides, thrombin, complement, cytokines or autoantibodies or similar mechanisms. Thus microparticles play an important role in carrying slice of information that are locally important for the attention of the cell remotely located (Piccin *et al.*, 2007; Horstman *et al.*, 2009; Bertolini *et al.*, 2006; Sabatier *et al.*, 2009).

Activation of the cells results in release of calcium by the endoplasmic reticulum, resulting in downstream calpain activation. The precise players in the mechanism of microparticle release has not been fully elucidiated. The calpain activation ensures cytoskeletal cleavage and further blebbing, shedding of the MP. Loss of assymetry leading to the PS being externalized is a prelude to the activation of the cytosolic enzymes including calpain (Piccin *et al.*, 2007).

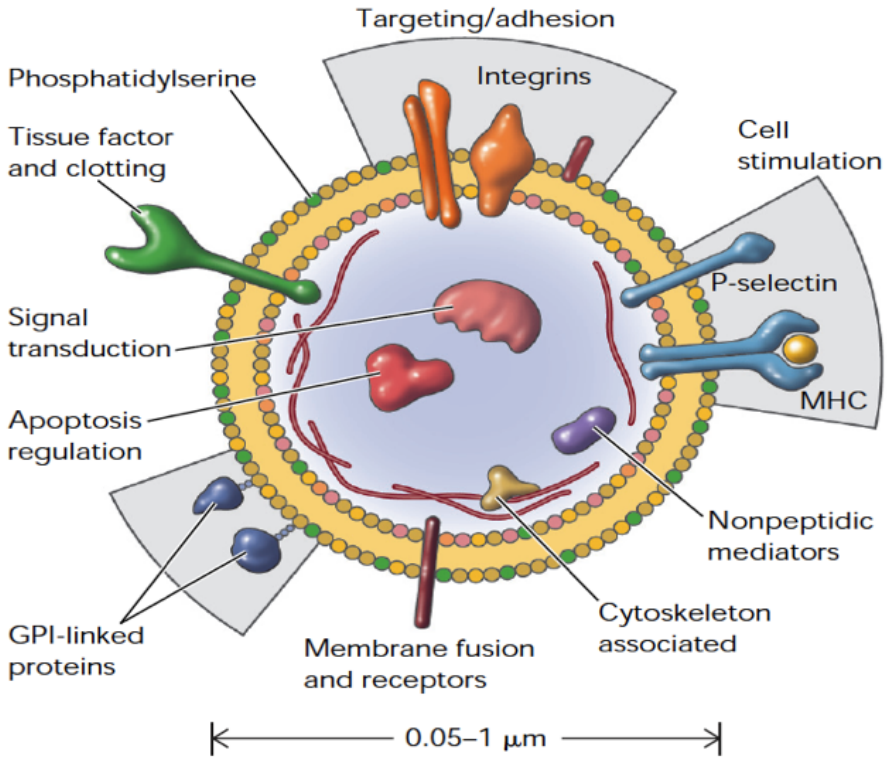

Figure 1.21: A Microparticle
Sub-micron particles that are microparticles, are shed constantly. Under pathogenesis, they increase. Different agonists induce microparticles with different antigenic components. They play vital role in priming the an organism to the state of affairs with every hear beat. The figure shows the arrangement of various proteins, associated molecules in a microparticle. Note that p-selectin is a marker for microparticles, once isolated from plasma. Understanding of the composition of MPs have profound diagnostic value. Though, molecules are associated with different agonists, there are common structural components. These could include cytoskeletal molecules, actin. Our knowledge of microparticles, its composition, activation pathways, enumeration with different agonists are all limited. The image was taken from (Hugel *et al.*, 2005).

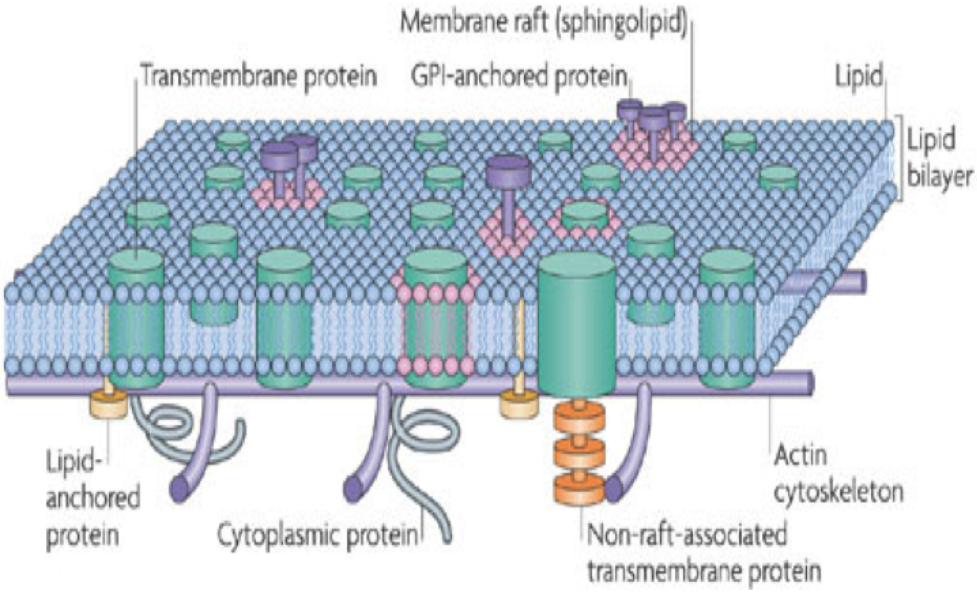

Figure 1.22: Plasma Membrane plasma membrane normally exists as an asymmetric lipid bilayer. Proteins and lipids help architecturally with non-covalent interactions. Sphingolipids, glycosphingolipids and phosphatidyl-choline are decorated at the outer leaflet while glycerophospholipids exist in the inner leaflet. Receptors such as the glycosylphosphatidylinositol (GPI)-anchored proteins (shown in purple, such as uPAR) exist extracellularly doesn't have a transmembrane domain or a cytoplasmic domain. There are other receptors such as integrins that span across, touching intracellularly and extracellularly. There are nanodomains (less than 5nm) that organize the lipid rafts (they are highly heterogeneous, highly dynamic, sterol− sphingolipid enriched domains). Inside the plasma membrane, cytoskeletal proteins provide the necessary adhesiveness by binding to proteins and lipids directly associated with the plasma membrane. Loss of this asymmetry is leads to microparticle formation. (Viola and Gupta, 2007)

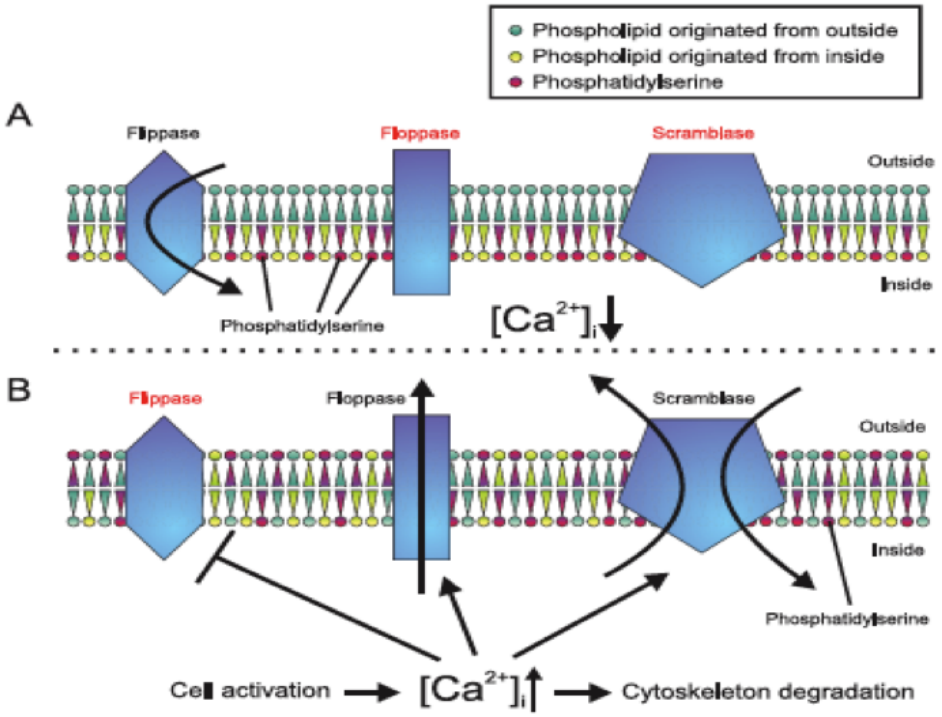

Figure 1.23: Flip Flop Scramble
The loss of assymetric composition of the plasma membrane leading to microparticle formation.
(Burnier *et al.*, 2009)

1.7.1.2 Flip, flop, scramble

Assymetry of the eukaryotic plasma membrane is ubiquitous. There is an external and internal leaflet maintained at the expense of energy. Adenosine triphosphate (ATP) dependent amino phospholipid translocase transports PS and PE from the external leaflet to the internal leaflet.This transport is much faster for PS than for PE, as this is against the concentration gradient actively spending energy. The transportation time is between 5 minutes to 10 minutes half-time for PS (Zwaal *et al.*, 1989). Mainainence of assymetry is achieved with PS and PE in the inner leaflet with sphingomyelin and PC kept externally, and is under the control of a couple of factors. ATP dependent amino phospholipid-specific translocase also referred to as flippase is rapid and specific. There is also the slower ATP-dependent nonspecific lipid floppase, responsible for transportation from cell's inner to external leaflet. There is also the scramblase, Ca^{2+} dependent nonspecific lipid transporter randomly moving lipids across both leaflets (Figure 1.23).

1.7.1.3 Loss of Assymetry and Microparticle Generation

Under physiological condition, the Flippase is the predominant player among the three, enabling PS and PE to be continuously moved internally. While floppase without specificity, and at a slower rate moves the phospholipid externally. Ca^{2+} concentration under normal physiological concentration, doesn't stimulate scramblase. The assymetry is mainly lost under stimulation of scramblase, under a sustained calcium increase externally with release from inracellular stores. This also affect negatively the translocase or otherwise referred to as the flippase. With the loss of the assymetry there occurs PS randomisation across the plasma membrane, which is then decorated externally.

The assymetry loss can be experienced within one minute of "lipid scrambling". Sustained calcium release is essential for functional scrambling. Calpain activation is one of the essential preludes to the microparticle release, occurring via blebbing by cytoskeletal breakdown (Figure 1.24). Calpain also ir-

reversably degrade translocase, which otherwise can be restored, by removing intracellular calcium that stop scrambling.

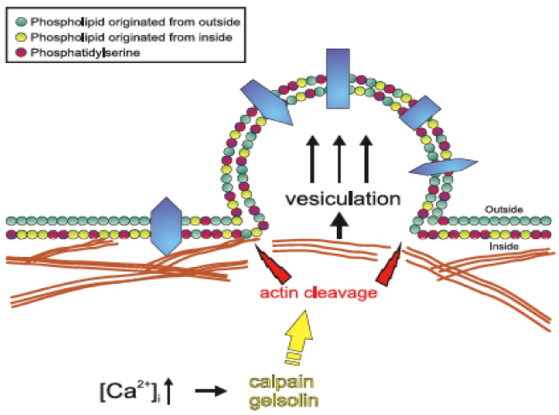

Figure 1.24: Distal elements in Microparticle formation
The cleavage of actin and its associated proteins, leading to microparticle formation. (Burnier *et al.*, 2009)

Such a controlled methodology for microparticle generation is not always the case eg: microparticle release from necrotic cells. More importantly the lack of assymetry, PS externalisation, encapsulation defines microparticles. Exosomes are a subset of microparticles that are generated, not from the plasma membrane, but rather from other internal membranes. Exosomes have highly enriched tetraspaning molecules and also play significant role in antigen presentation (Thery *et al.*, 2002). Each microparticle from even the same same cell type, have differing protein composition. This is essentially due to the fact that microparticles are a slice of information for the interest of remote cells, continuously relaying information. "There is no news like bad news" and pathogenic conditions increase the microparticle content.

1.7.2 Role of Proteins

Earlier experiments show the thermodynamic incompatibility of cytoskelal proteins in maintaining the assymetric plama membrane (Frickenhaus *et al.*, 1998). Labs have been able to maintain the lipid distribution while lacking any cy-

toskeletal proteins. This was maintained with no plasticity and as an inert distribution (Bevers *et al.*, 1999). In a dynamic cell, undergoing differerent pressures plasticity is essential while at the same time maintaining the assymetricity. To maintain the plasticity of the membrane help of cytoskeletal proteins cannot be excluded (Sheetz, 2001).

Interplay between lipids and proteins is essential for membrane remodeling. Like a dynamic fitting glove, these mechanisms are used by the cell to generate, sense and stabilise local regions of membrane curvature. Areas of high membrane curvature frequently exist, but for only limited periods of time. Surrounding proteins are utilized to change the morphology. Thus in the formation of highly curved vesicles, the curvature is induced by the effects of membrane-associated proteins, the 'coat proteins'. The curvature is readily reversible when the coats dissociate, leaving the vesicle more fusogenic (as their curvature is not stabilized) and the coat proteins can now be reused in a further round of vesicle formation (giving an efficiency to protein usage) (Sheetz, 2001; McMahon and Gallop, 2005).

Membrane curvature is no longer seen as a passive consequence of cellular activity, but an active means to create membrane domains and to organize centres for membrane trafficking. Curvature can be dynamically modulated by changes in lipid composition, the oligomerization of curvature scaffolding proteins and the reversible insertion of protein regions that act like wedges in membranes. On a larger scale, membrane curvature is a prime player in growth, division and movement (McMahon and Gallop, 2005).

As discussed earlier the cell membrane bilayer leaflet interaction is more than a brownian movement (Kusumi *et al.*, 2005). The lipid properties exert a permissive state for membrane curvature. The properties of the lipids, their headgroup binding to the membrane proteins aid in the recruitment of proteins necessary to generate the curvature (Bi *et al.*, 2002). Head group of phophoinositides are easily modified and thus are key players in the lipid-protein interaction in the membrane, leading to its curvature (Litman, 1973). PtdIns(4,5)P2 (PIP_2), the moesin phosphorylator is essential for budding, and

so is PtdIns-3-OH kinase with which Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and other FYVE domain proteins interact (Fernandez-Borja *et al.*, 1999; Gruenberg and Stenmark, 2004). Clathrin, COPI and COPII coat proteins, that interact with these lipids work in collaboration with other proteins, as they do not have direct membrane associations (Ford *et al.*, 2001; Bi *et al.*, 2002). Caveolin is another protein, but membrane associated, which oligomerises to form the coat (Razani and Lisanti, 2001).

1.7.3 Cytoskeletal Influences

Reversible membrane curvature is either categorised as positive or negative. Membrane that curve inwards towards the cytoplasm as positve (shallow pits, ealy stages of vesicle budding) and budding out of the cell as negative curvature (McMahon and Gallop, 2005). Of the many mechanisms involved in the deformation of the membrane, actin and cytoskeletal protein's also has a role. Actin polymerisation and the pulling of the tubules by motor proteins curve the membrane. Branching, bundling and treadmilling of actin filaments are involved in the generation and remodeling of many areas of high membrane curvature, including filopodia, pseudopodia, phagocytic cups and axonal growth cones (McMahon and Gallop, 2005). There is a propulsive power involved with the actin cytoskeleton mechanics due to the conductance of the membrane tension, both contributing to it and affected by it. *In-vivo* and *in-vitro* work have also implicated the microtubules, as well as implicating actin mediated transport of vesicles via myosin (Buss *et al.*, 2002). Curvature dependent banana-shaped lipid-binding BAR domain proteins also have links to actin and microtubule polymerization machinery (Peter *et al.*, 2004). Curvature induced localization of GAP or GEF activities leads to tight regulation of small G-protein GTP/GDP status. This has been proposed to result in selective actin polymerization or signaling pathway activation, as an molecular ecological niche (McMahon and Gallop, 2005).

In terms of microparticle release, the loss of adhesion between the lipid bi-

layer and the cytoskeleton, including the breakdown in the cytoskeleton is a culminating event in the blebbing process (Sheetz, 2001). As mentioned earlier the target of the calpain and other cytosolic enzymes is primarily the cytoskeleton (Chironi *et al.*, 2008).

1.7.4 Exoskeletal Influence

On the exoskeleton, dynamin family sculpt the membrane topology into tubular shape from the outside (Hinshaw and Schmid, 1995). They also via GTP hydrolysis, induce membrane fission (Marks *et al.*, 2001). These are part of the scaffolding proteins that form helical oligomers. Previously mentioned coat proteins Clathrin COPI, COPII and Claveoli are also classed as exoskeletal proteins. On binding to phospho inositols they oligomerise scaffolding the membrane into tubular shape. BAR domain proteins have preferential binding to these curved membrane structures. This is due to their nature of binding, which is electrostatic, leading to more avidity (multiple low strength bonds). Dynamin (which is a dimer) binds to amphiphysin (a BAR domain protein) more strongly when amphiphysin is a dimer (McMahon and Gallop, 2005; Peter *et al.*, 2004).

Thus the shape of the membrane also drives downstream binding or not, of other proteins. Other examples of these very important BAR domain containing proteins are endophilins, BRAPs and nadrins (Peter *et al.*, 2004).

Other domains such as the PH or the PX act as important targeted domains, that take them to specific compartments. BAR domain containing proteins are a sensor for positive curvature, with BAR domain lipid-binding mutant even with the PH domains, unable to bind to the membrane. Studies on the exosekeletal proteins are more elucidiated due to the obviousness of these proteins, but the cytoskeletal proteins though unappreciated, plays a significant role (Peter *et al.*, 2004).

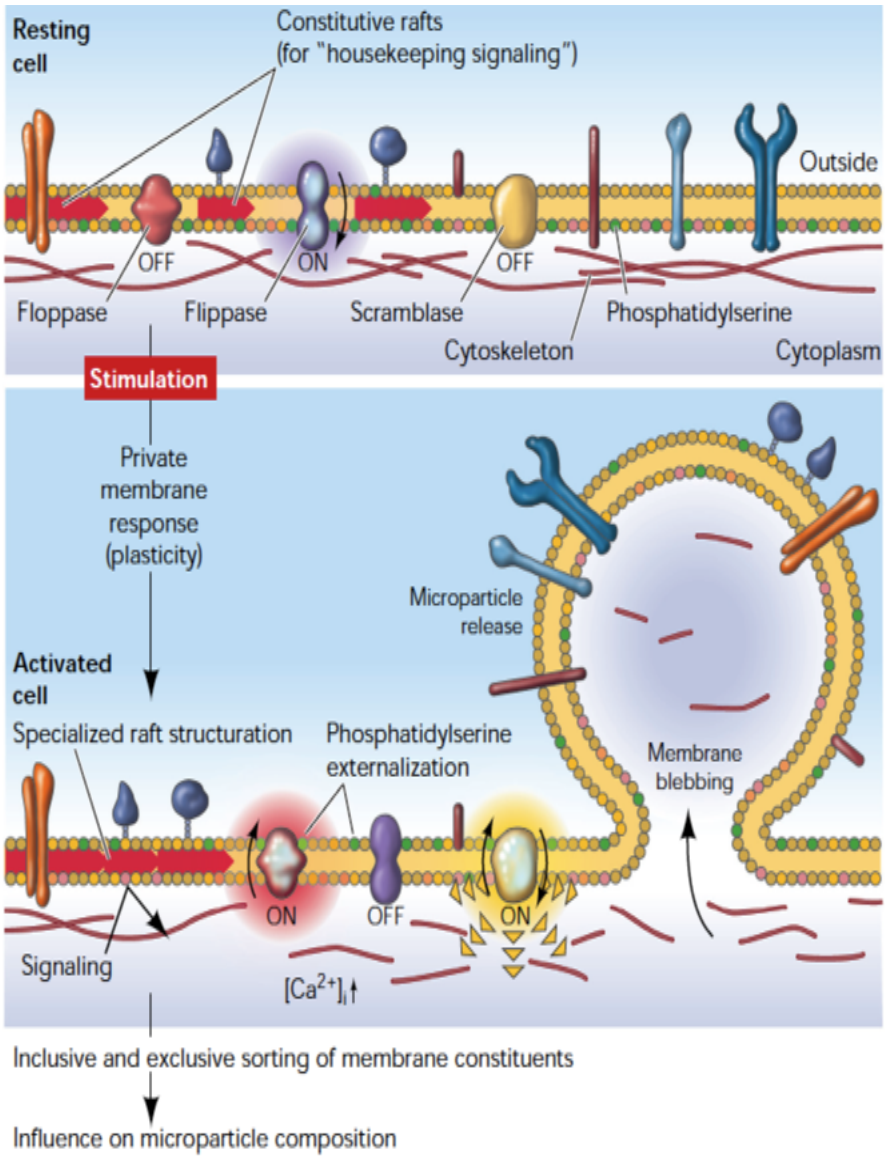

Figure 1.25: Microparticle formation
The plasma membrane is arranged in its normal asymmetric form by means of "lipid rafts". Whereby, there is movement of lipids actively. There are proteins both cytoskeletally and exoskeletally involved in maintaining this status quo. Transient change in any of these proteins, would thus automatically lead to formation of blebs, finally forming MPs. The image was taken from (Hugel *et al.*, 2005).

1.7.5 PIP_2 in the Membrane

1.7.5.1 Structure and Production

With respect to membranes, phosphoinositides are acidic phospholipids found at the inner leaflet of cell membranes, primarily in the cytoplasmic leaflet. They border the specific subcellular membrane compartments. Phosphoinositides act as membrane recognition sites for specific cytoplasmic proteins, and act from the membrane to modulate the activities of some membrane proteins. They have myo-inositol in the head group and their parent compound is phosphatidylinositol (PI). PI can become phosphorylated on the 3, 4, and 5 positions of the inositol ring in every combination. Seven low-abundance polyphosphoinositides are possible in this manner, with very specific subcellular localizations such as plasma membrane, golgi complex, endoplasmic reticulum, late and early endosomes (Suh and Hille, 2008; Di Paolo and De Camilli, 2006).

PIP_2 is the most abundant poly-phosphoinositide in the plasma membrane. Though constituting only 1% of the total acidic lipid in a cell, it is all primarily found in the membrane. PIP_2 with one phosphodiester linkage has a phosphate group and additionally two more phosphate groups (McLaughlin *et al.*, 2002). Classically PIP_2 is cleaved by the enzyme phospholipase C (PLC), a reaction that produces the two classical second messengers, soluble inositol 1,4,5- trisphosphate (IP3) and membrane-delimited diacylglycerol (DAG). IP3 and DAG go on to release Ca^{2+} from intracellular stores and recruit and activate protein kinase C (PKC), respectively. PIP_2 is a primary product, that when cleaved leads to two major signaling pathways (Hilgemann, 2007; McLaughlin *et al.*, 2002; Xu *et al.*, 2003).

1.7.5.2 Implicating PIP_2

The signaling role for PIP_2 was not appreciated till the 1990s. PIP_2's ability to serve as targeting anchor for proteins, that catalyze endocytosis and exocytosis is now well known. So is its ability to anchor small molecular weight GTPases and actin (Hilgemann, 2007; Suh and Hille, 2008). The significance of PIP_2 in

phosphorylating moesin has been discussed in depth earlier. When it comes to microparticles, losing the membrane assymetry is a crucial factor, and PIP_2 is significantly present in the membranes. It is an obvious candidate in these circumstances, for example, red blood cells (RBC) exists as a biconcave discoid body with the crenation of these cells holding unique functionality. PIP_2 converting to phophatidylinositol (this molecule has a smaller polar head group) occurs at the same instance in accordance with the classic bilayer model proposed in 1974 (Ferrell Jr, 1984; Sheetz and Singer, 1974).

Also, PIP_2 specifically and not other phosphoinositides is essential in the redistribution of the membrane lipids on addition of Ca^{2+} (or at ten times more concentration Mg^{2+}). The specificity of PIP_2 when compared to other phophoinositides is interesting, but they inferred that no protein to be involved in the scrambling process. This was because both sides of the erythrocyte membrane could respond to Ca^{2+} provided they contained enough PIP_2, (since then, phospholipid scramblase (PLSCR1) has been cloned that exhibits Ca^{2+}activated PL scrambling activity *in-vitro*). There was also no increase in cytosolic Ca^{2+} on addition of calcium externally (Sulpice *et al.*, 1994). This is important as increase in the cytoplasmic Ca^{2+} more than 1μM inhibits translocase (Bitbol *et al.*, 1987; Zachowski *et al.*, 1986).

Indirect implication of PIP_2 in microparticle release in unnecessary, with both exocytosis and endocytosis directly influenced by PIP_2. Membrane blebbable particles are docked to the membrane, when levels of PIP_2 is increased. It acts in priming the vesicles and the plasma membrane by acting in cis to the plasma membrane proteins, such as Ca^{2+} dependent activator protein (CAPS), a priming factor in neuroendocrine cells. While it acts in trans on the vesicle proteins such as synaptotagmin, a Ca^{2+} sensor for exocytosis (Martin, 1998; Bai *et al.*, 2003). The secondary products of PIP_2 cleavage DAG and IP3 are important in priming, regulating calcium response respectively. Endocytic proteins are selective and PIP_2 is an important co-receptor and regulator in their recruitment. They bind to dynamin and to all known endocytic clathrin adaptors such as epsin,AP-2, AP180/CALM (Wenk and Camilli, 2004; Owen *et al.*, 2004).

1.7.5.3 The Cytosolic Interaction

Regulation of integral membrane proteins or recruitment of membrane cytoskeletal and signaling components, is achieved through the binding of their head groups to cytosolic proteins or cytosolic domains of membrane proteins. The phosphate(s) on the inositol ring of these phosphoinositides have negative charge, thus electrostatic interactions modulate the interaction with cytosolic proteins. In some cases, adjacent hydrophobic amino acids strengthen the interaction through a partial penetration into the bilayer (Di Paolo and De Camilli, 2006) .

Actin regulatory proteins like profilin, with clusters of basic residue and folded modules such as the pleckstrin homology domain are ideal candidates. FERM domain proteins, like moesin have these PH domain in their F3 lobe. Myosin X (Myo X) is another protein with the F3 domain containing PH domain, in addition to the two more it has (Berg, 2000). The repertoire of phosphoinositide binding modules is rapidly expanding, thus increasing the understanding of the scale of signaling potential via phophoinositides (Lemmon, 2003; Balla, 2005; Hurley and Meyer, 2001; Di Paolo and De Camilli, 2006).

A wide range of actin binding proteins are recruited by the phosphoinsositides, they utilise small GTPases for these purposes (Hilgemann, 2007; Suh and Hille, 2008). Actin polymerisation via ARP2/3-mediated nucleation of actin networks is well studied, wherein PIP_2, in cooperation with the small GTPase Cdc42, binds N-WASP and triggers a conformational change that allows its binding to and activation of the ARP2/3 complex (Rohatgi *et al.*, 2000; Pollard and Borisy, 2003). The PIP_2 effects are further amplified by the recruitment of N-WASP activators such as Toca in concert with CDC42 and other phosphinositides (Ho *et al.*, 2004). Even in the CDC42 independent mechanism of actin nucleation via Nck-stimulation, PIP_2 is synergestically required (Rohatgi *et al.*, 2001).

PIP_2 also mediates the dissociation of capping proteins, such as gelsolin and CapZ. This results in actin filament elongation via the addition of G-actin

monomers at the plus end. For this pupose G-actin monomers are pumped into the reaction pool by PIP_2 dissociating the actin-monomer–profilin complexes via binding to profilin (Yin and Janmey, 2003), (Pollard and Borisy, 2003). Also, adaptor proteins such as the FERM domain proteins, such as moesin, are released from their homodimer state into phosporylated active monomers by PIP_2. This also results in the important adaptor protein talin to strengthen its binding to the integrins. Type I phosphatidylinositol phosphate kinase isoform g661 (PIPKIg661), an enzyme that makes PIP_2, is targeted to focal adhesion by binding to Talin at its FERM domain. This is an instance of ensuring spatial specificity in phosphoinositide signaling (Ling *et al.*, 2002; Di Paolo *et al.*, 2002). Pathogens sometimes inject a protein similar to mammalian inositol phosphatase (eg: Shigella and Salmonella), which eases its entry, due to the loss of adhesion between the membrane and the actin cytoskeleton. This results from the phosphoinositide imbalance as noted from the depletion of PIP_2, from the base of membrane ruffles in the proximity of the bacterial invasion (LeBrasseur, 2002).

PIP_2 and the Microparticle

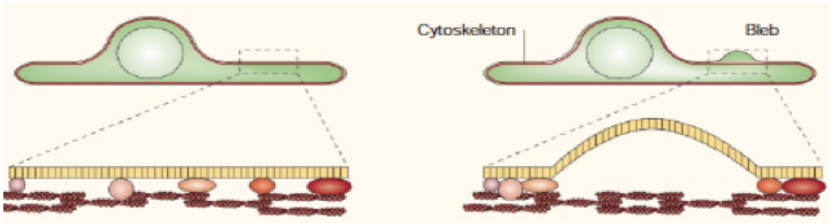

Figure 1.26: PIP_2 and intracellular adhesion of actin
PIP_2 by means of its actin binding proteins, maintain cell membrane adhesion to actin. Loss of this, leads to bleb formation. This is utilized in endocytosis, exocytosis and microparticle formation.
(Sheetz, 2001)

PIP_2 contributes to the strengthening of adhesion between the membrane and the cytoskeleton, as noticed by the directly proportional increase in membrane-cytoskeleton adhesion energy. While, Cytochalasin a potent inhibitor of actin polymerization, as well as overexpression studies using PH domain (binds specifically to PIP_2) results in reduction of the membrane-cytoskeleton adhesion energy. Expression studies using PH domains result in PIP_2 being sequestered (Raucher *et al.*, 2000). Transfection studies using IpgD, a potent PIP_2-phosphatase IpgD (virulence factor from *Shigella flexneri*), also results in similar reduction in adhesion via dephosphorylation of PIP_2 (Niebuhr *et al.*, 2002). Numerous studies have looked into membrane-cytoskeletal loss of adhesion (Sheetz, 2001). As loss of cortical actin is associated with protrusions of the plasma membrane. This is then followed by vesicle formation which is preceded by reduction of membrane-cytoskeleton adhesion (Flaumenhaft, 2006).

Actin binding proteins such asα-actinin, ezrin, radixin, and moesin bind with high affinity to PIP_2 (Flaumenhaft, 2006). Membrane and cytoskeleton adheres mostly via avidity (more numbers than the strength of the binding) than affinity, especially with PIP_2 present at a concentration of 1,000–10,000 molecules per μm^2 of membrane (Sheetz, 2001; Flaumenhaft, 2006). Spectrin and talin also contribute to the adhesion process this way, with studies showing difficulty in extracting them from membranes enriched in phosphatidylserine (Manno *et al.*, 2002; Shiffer and Goodman, 1984).

The loss of adhesion betweeen cytoskeleton and membrane results in bleb formation. Studies with PIP_2 depletion, PIP_2 sequestration, ATP depletion, all results in loss of membrane-cytoskeletal adhesion resulting in blebs (Sheetz, 2001). PIP_2 on addition to RBC promotes Ca^{2+} dependent transbilayer movement of phosphatidyl serine with subsequent redistribution in the phospholipids (Sulpice *et al.*, 1994).

Clear evidence so far is the reduction of microparticle formation in platelets pre-incubated with PIP_2 via the thromin-collagen or Ca^{2+} ionophore pathway with upto 90% reduction, with other phospholipids lacking a similar effect.

1.8 The Urokinase System

Other receptors, unlike integrins, that lack a transmembrane domain and cytoplasmic domain also play important roles in adhesivness. These molecules though less well characterized, are also integrators of focal adhesion dynamics. Molecules such as syndecan-4, uPAR (urokinase plaminogen activator receptor), layilin, transmit their niche signals by utilizing integrins among various other molecules, as well adapters which act to stablise this information passage between extracellular matrix and the membrane and inward into the cell (Geiger *et al.*, 2001).

Similarly specific internal cues are transmitted outward utilizing the inside out signaling of integrins. This is effected using adapters that might have unique roles in effecting a change in these receptors. These rapid exchange of information is regulated by cleavages of molecules, phosphorylation / dephosphorylation and recruitment of scaffolds (Ragno, 2006). Existence of these molecules in evolutionary terms have roles concurrently to their uniqueness.

One of the best characterized receptor of this kind is the uPAR. Binding integrins, EGF receptor, high molecular weight kininogen, caveolin and the G-protein-coupled receptor FPRL1, uPAR orchestrates various functions of a cell (D'Alessio and Blasi, 2009). uPAR is a critical component of the plasminogen activation system (Blasi and Carmeliet, 2002). This receptor was identified 24 years ago in monocyte like cells, being able to sense and accumulate its ligand urokinase-type plasminogen activator (uPA) on the membrane. Though famous for its enzymatic and thus proteolytic properties, last decade has shed new light on this versatile molecule, found overexpressed during inflammation and in most metastatic cells. Its importance in angiogenesis comes down to ECM remodeling utilizing downstream matrix-metalloproteases (MMPs) to remodel tissues. Its ability to exert control over integrin binding to fibronectin, collagen as well the innate ability to bind to vitronectin, lends regulatory function of adhesivness of cells (Madsen and Sidenius, 2008).

Urokinase (uPA) is the most well characterized ligand of uPAR, together

with tPA (Tissue plasminogen activator) they activate plasminogen. Combined uPA / tPA-deficient mice have more profound effect resulting in severe spontaneous thrombosis, the effect similar to plasmin deficient mice. Plasminogen is present in plasma and extracellular fluids and the plasminogen cascade remodels the ECM extensively. Cleavage of plasminogen at the Arg561-Val562 bond, generates the serine-protease plasmin, able to degrade various ECM such as fibrinogen, fibronectin and vitronectin. uPA and tPA, the physiological activators of the plasminogen, both yield two chains held together by a disulphide chain. They are both released from the endothelium while plasminogen is produced in the liver (O'Rourke *et al.*, 2006). Initially released as a single chain, uPA in this form is a zymogen. In this form uPA is inactive and differs from tPA . tPA activity is potentiated on binding to fibrin resulting in fibrinolysis, while uPA's differing roles are cell migration, tissue invasion, such as inflammation angiogenesis and tumor invasion.

1.8.1 Structure of uPAR

uPAR is attached to the cell surface sensing the extracellular space via a peripheral glycosyl-phosphatydil-insositol (GPI) tail at the Gly 283 residue of the uPAR, at the C terminus. This tail doesn't span cell membrane and only peripherally anchors the uPAR.

This necessitates collaboration with receptors that bore deep into the cytoplamic regions of a cell (Ploug *et al.*, 1991). uPAR is a single polypeptide chain of 313 amino acid residues decorated with a 21-residue signal peptide. Gly 283, the site of GPI attachment is exposed on removal of 30 C-terminal residues. This removal is by a postranslational mechanism. GPI-anchor consists of phophatidylinositol attached to a carbohydrate chain via the C-6 hydroxyl of the inositol, connected to the protein through and ethanolamine phosphate moiety (Blasi and Carmeliet, 2002). uPAR is arranged into folded homologous with almost globular structures referred to as D1,D2 and D3 from the beginning of the N-terminus respectively. Each of these folded regions have im-

portance when binding to various ligands and in peripheral association with other receptors. These structures are members of Ly-6/uPAR/α-neurotoxin protein domain family consisting of 90 amino acids with 4 to 5 disulphide bonds (Ploug *et al.*, 1993; Behrendt *et al.*, 1991a).

These three domains individually fold as three finger structures, similar to other proteins of the same family (Llinas *et al.*, 2005). Previous evidences have shown it to contain as well multiple structures, similar to metastasis-associated C4.4A (Ploug *et al.*, 1993; Behrendt *et al.*, 1991a). D1,D2 and D3 domains exist as β-pleated sheets arranged into the three finger structures, ending with a small C-terminal loop (Llinas *et al.*, 2005).

There is a 19 Å, central ligand docking cavity tapering towards the D3 region. Topology/structural preference due to the cavity, as well the existence of the ligand peptide here, draws in the ligand to this void of attraction with half of it binding to the D1. The ligand peptide provides multiple anchorage to the ligand, while the linker regions between D1, D2 is especially proteolytically sensitive, thus is exposed to the large outer surface. Additionally uPAR incorporates five N-linked glycosyation sites (N52,N162,N172,N200 and N233), also in the large outer surface. This enables it to bind multiple ligands while holding uPA inside the cavity (Blasi and Carmeliet, 2002; Llinas *et al.*, 2005; Illemann *et al.*, 2009)

Observations *in-vitro* has shown the sensitivity of uPAR to enzymes such as trypsin, chymotrypsin, elastase, cathepsin G, metalloproteases as well by plasmin and uPA. These enzymes generate varying products. Truncated forms lacking D1 is referred to as c-uPAR, with the varying differences at the N-terminus amino acid region 88-92. c-uPAR has also been found *in-vivo*, in both normal and cancerous cell types, with evidence to show uPA can be similarly proteolytic *in-vivo*. The 88-92 amino acid region that is affected is especially pertinent to cell migration (Illemann *et al.*, 2009; Mondino and Blasi, 2004; Ragno, 2006)

The full length as well the cleaved form also exists as soluble uPAR and is often shed from a cell. In this form its referred to as suPAR or c-suPAR cor-

responding to the existence of D1 or not. The full length uPAR form is generated by the juxtamembrane cleavage *in-vivo* by glycosylphosphatidyl-inositol-specific phospholipase C or D (Ploug *et al.*, 1991; Wilhelm *et al.*, 1999; Beaufort *et al.*, 2004b,a). There is evidence to this soluble full length suPAR's ability to be cleaved further, to produce c-suPAR. The enzymes implicated in this process are metalloproteases, cathepsin G and elastase (Montuori *et al.*, 2005b).

1.8.2 Genetic regulation of uPAR

The human uPAR gene is at the locus 19q13 (long arm of chromosome 19) with seven exons spanned by six introns, all together 23 kb in size (Casey *et al.*, 1994; Suh *et al.*, 1994). A GC rich region lacking TATA and CAAT boxes with consensus elements for SP, AP1, AP2, PEA3 and NFkB has been identified as the major transcription start site (Soravia *et al.*, 1995; Wang *et al.*, 2000). This region within 188bp from the major transcription start site of the uPAR gene between -141 and +47 bp relative to the transcription initiation site is implicated as a silencing agent. Experimental evidence has shown the deletion of PEA3/Ets motif, to impair the β3-integrin mediated downregulation of uPAR expression (Hapke *et al.*, 2001). In colon cancer cells uPAR receptor is overexpressed by transcrptional regulation via Kruppel-like transcription factor, utilizing multiple sites in the uPAR promoter (Wang *et al.*, 2004). Post transcriptional regulation, is another level of control over uPAR expression with several sequence elements identified. AU-rich elements (AREs) in the 3' untranslated region (UTR) of the uPAR transcript, functioning to make it significantly unstable (Ragno, 2006). Meanwhile, ELAV protein HuR and heterogeneous nuclear ribonucleoprotein C (hnRNPC) bind uPAR ARE, stabilising uPAR mRNA (Tran *et al.*, 2003; Shetty, 2005). In small lung carcinoma a stabilising factor binds, upregulating the uPAR, while this factor is not hugely present in normal cells (Montuori *et al.*, 2001, 2003). Meanwhile, phosphoglycerate kinase (PGK) in bronchial epithelial cells drive uPAR mRNA degradation by binding specifically to a regulatory element in the coding region (Shetty *et al.*, 2004; Shetty

and Idell, 2000). Urokinase (uPA) upregulates uPAR expression in MDA-MB-231 breast carcinoma cells by increasing SP1 binding activity, while in lung carcinomas cells by increasing uPAR mRNA stability (Montuori *et al.*, 2003; Zannetti *et al.*, 2000). Thus the major ligand of uPAR, uPA regulates the uPAR's expression both transcriptionally and post-transcriptionally. Unidentified rapid cytoskeletal elements, recruited by uPA, obviously has implications.

1.8.3 Structure of Urokinase (uPA) and its association to uPAR

Urokinase, the pro-enzyme is a 54 kDa single chain, and is inactive referred to as pro-uPA or sc-uPA. This is converted into the active form by a single proteolysis at Lys158-Ile159. This active form is commonly referred to as uPA, now consisting of two chains.

Urokinase both in the form of pro-uPA and uPA folds into three parts. There is an amino terminal fragment (referred to as ATF, residues 1-135) in which exists the first two domains (Behrendt *et al.*, 1991b; Llinas *et al.*, 2005). Epidermal growth factor (EGF)-like domain (residues 4-43,GFD) exists in the noncatalytic amino-terminal A chain, after which lies the tripledisulphide containing structure called kringle domain (residues 47-135) (Vassalli *et al.*, 1985; Stoppelli *et al.*, 1985). The ATF has strong specificity to the uPAR . The last part of the folding topology is a βchain with serine-protease domain, which doesn't bind to the uPAR. This is the C-terminal domain, referred to as the low molecular weight uPA (Behrendt *et al.*, 1991b; Llinas *et al.*, 2005).

As disucssed earlier uPA binds to D1 and with decreasing affinity to D2 and D3. Thus a full length uPA (active) has been found to be significantly more specific (1500 times) than to just D1 alone (Behrendt *et al.*, 1996). Further to these factors there seems to be a need for D1 and D3 to be proximal for strong binding to uPA, which enhances affinity of the multiple binding site (Ploug, 1998). In the uPA EGF domain is the receptor binding part of uPA, thus ATF, as well pro-uPA and uPA, all have high affinity to uPAR (Ploug and Ellis, 1994). The association of uPA to uPAR monomers also results in uPAR dimers which

then preferentially moves into the lipid rafts (Ragno, 2006).

1.8.4 The other ligands of uPAR

Vitronectin (VN) was identified as another binding partner to uPAR in 1994 (Wei *et al.*, 1996). VN concentrates and stabilises active PAI-1 which inhibits the plasminogen activation system. VN is well known as being able to bind and interact with the integrin family (αvβ1, αvβ3,αvβ5,αIIβ3) (Hynes, 1992; Preissner, 1991) via the RGD motif (Pytela *et al.*, 1985). As the plasminogen system took importance with respect to uPAR, uPA was increasingly considered incorrectly to be the only ligand. Including vitronectin, it also binds to high molecular mass kininogen (HKa). HKa as well as VN both require a full length uPAR for efficienct binding, differing with uPA over binding sites being in D2 and D3 (Ragno, 2006). Another aspect in which uPA binding differs to vitronectin binding is in its more efficient binding to uPAR dimers, as well association with lipid raft (Cunningham *et al.*, 2003). uPAR binds VN at the somatomedin B domain (SMB) of vitronectin (Binder *et al.*, 2007). uPA induced binding to VN requires the extracellular protein kinase casein kinase-2 (CK2), to phosphorylate vitronectin.

uPAR is abe to adhere to vitronectin possibly as part of the initial adhesive mechanism, but cannot spread or migrate without the integrins. Specific abolishment of CK2 was found to totally abolish uPA induced vitrnoectin binding to uPAR (Stepanova *et al.*, 2002; Madsen and Sidenius, 2008)

Hka and VN due to theire similar binding regions in uPAR is competitive, with Hka binding inhibiting VN binding (Chavakis *et al.*, 2000). PAI 1 (Plasminogen acitvator-inhibitor 1), an important regulator of uPA-uPAR association also inhibits VN binding to uPAR. This is due to similar association areas of PAI1 on VN as uPAR, also proximal to the integrin binding site (Dellas and Loskutoff, 2005). Vitronectin circulates the blood as a monomer, but on stabilizing as ECM turns multimeric. Similar to uPAR, VN plays functional roles in regulating cell adhesion, migration and proliferation (Hayman *et al.*, 1983;

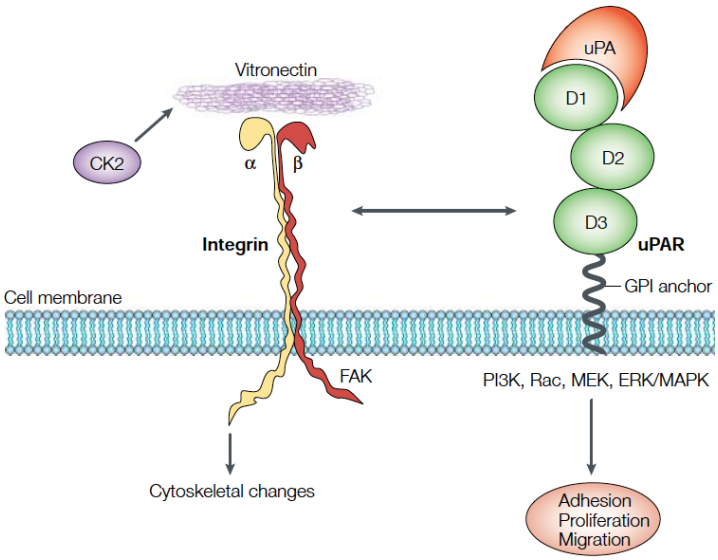

Figure 1.27: Vitronectin binds to uPAR
Casein kinase-2 (CK2) by phosphorylating vitronectin (Vn) regulates uPA-uPAR association with vitronectin. Integrins are recruited to effect cytoskeletal changes as uPAR lacks cytosolic domains.
(Blasi and Carmeliet, 2002)

Reilly and Nash, 1988; Blasi and Carmeliet, 2002; Ossowski and Aguirre-Ghiso, 2000).

1.9 Transducers of uPAR signals to the cytoplasm

The extracellular receptors that has the cytoplasmic domain, such as integrins, caveolin and G-coupled receptors can interact with uPAR (Blasi and Carmeliet, 2002). These interactions utilize the need for information transmission inwards, into the cytoplasm of the cells. These outside-in signaling mechanisms internally recruit adapters specific to the cues transferred, and transmembrane receptors used (Figure 1.28).

1.9.1 Integrins and uPAR interactions

Integrins have been discussed previously in great detail and are cell surface receptors that bind to ECM and pass information bidirectionally. The association of integrins and uPAR, has led to considerable excitement in the field (Figure 1.28). This has brought to focus other signaling molecules associated with integrins and plausible effect on uPAR of these molecules. Different techniques have proven the the interaction of uPAR with the β1, β2, β3 and β5 integrins. Experiments such as FRET (Fluorescence resonance energy transfer analyzis), co-immunoprecipitation, immunolocalization, uPAR binding peptides from phage libraries, have all gathered evidence in this regard (Ragno, 2006; Blasi and Carmeliet, 2002; D'Alessio and Blasi, 2009). The ability to transduce signals to the internal compartment of the cell from outside the cell membrane, resulted in the nomenclature "integrin" (Giancotti and Ruoslahti, 1999). Integrin signaling is now well established regarding its bidirectionallity i.e, the cytoplasmic domain interactions of the integrins being able influence interaction of its extracellular domains as well as vice versa.

Migration due to uPA-uPAR interactions and other ligand-uPAR interactions have been found to recruit integrins. Antibodies against uPAR has been found to block cell adhesion, migration as well as proliferation, whilst interacting with various ligands. This effect is similar to when antibodies targeting integrins are used (Wei *et al.*, 1996; Yebra *et al.*, 1996; Degryse *et al.*, 2001). Beyond proving uPAR-integrin signaling leading to migration, the complexities are still left to be eliucidiated. These include understanding the induction of all cytoplasmic and transmembrane adapters. The need to stabilize them transiently and reversibly bring back the machinery that continue migration (May *et al.*, 1998; Waltz *et al.*, 2000).

Leukocytes in uPAR -/- mice were extensively limited in their migration capacity via a β2 integrin pathway to inflamed peritoneum (May *et al.*, 1998). Similarly, other integrins have been implicated in uPA-uPAR mediated migration, such as α5β1 in Chinese hamser ovary cells (CHO). Interestingly they showed

temperature sensitivity, as well a requirement for activation of mitogen-activated protein kinase (MAPK) activation. Epithelial cells, endothelial cells, myeloid cells have all been shown to be similarly responsive to uPA induced cell migration (Busso *et al.*, 1994; Odekon *et al.*, 1992; Waltz *et al.*, 1993; Gyetko *et al.*, 1994). With respect to uPA, the non-proteolytic domain and the Kringle domain have been found to not affect migration.

The integrins that interact with uPAR include integrin β2 members, uPAR in fact, was first identified bound to these integrins in resting neutrophils (Xue *et al.*, 1994). Spontaneous migration led uPAR to dissociate from Mac-1(αMβ2) with uPAR staying in lamellipodia while Mac-1 polarised into uropods (Kindzelskii *et al.*, 1996). Mac-1 also known as CR3, CD11b/CD18 is an integrin heterodimer, which is also a receptor for fibrinogen (Kindzelskii *et al.*, 1996). Integrins have also been shown to be associated with signaling complexes with uPAR (Bohuslav *et al.*, 1995). αvβ5 and αvβ3 are other heterodimer integrins implicated to associate with uPAR, which are also receptors for vitronectin (Montuori *et al.*, 1999). α4β1, α6β1, α9β1, αvβ3 are integrin heterodimers shown to associate with suPAR (Binder *et al.*, 2007). In their work, interestingly they have proven the specific binding between glycosyl phosphatidylinositol-anchored uPAR on the cell surface. This binding was to integrins on the apposing cells.

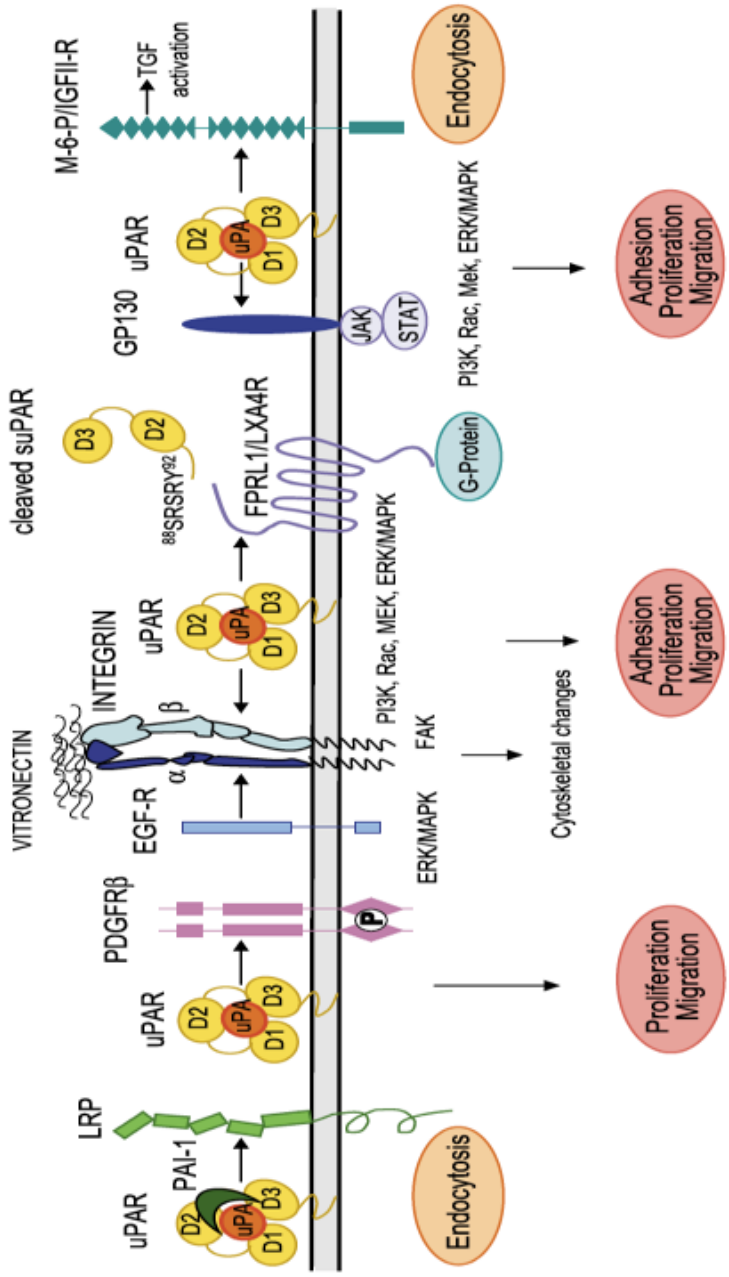

Figure 1.28: uPA-uPAR transducers
The various transducers of uPA-uPAR interactions and their functional significance. Note the interaction with integrins and G-coupled proteins.
(Binder *et al.*, 2007)

This adds new evidence suggesting that uPAR-integrin interaction may mediate cell-cell interaction (trans-interaction) (Binder *et al.*, 2007; D'Alessio and Blasi, 2009).

These interactions that control migration or proliferation have roles in angiogenesis in which the associated integrin is αvβ3 (Brooks *et al.*, 1998; Nykjaer *et al.*, 1997; Prager *et al.*, 2004). Details of this process have been discussed previously. Though able to bind many different integrins, "uPAR has the highest affinity for the bondafide fibronectin receptors α3β1 and α5β1" (Blasi and Carmeliet, 2002).

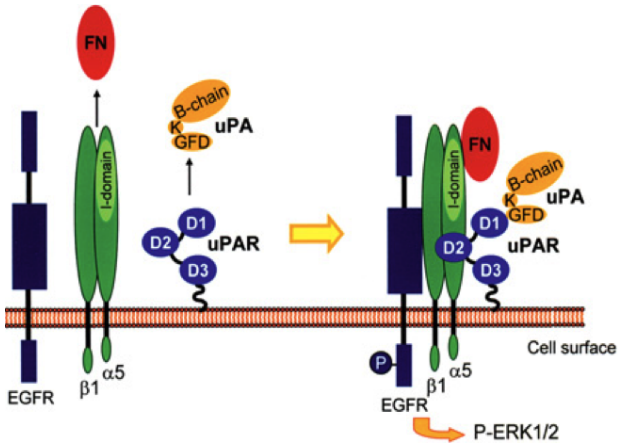

Figure 1.29: α5β1 integrin interacts with urokinase increasing ERK activation uPA-uPAR as well as promoting α5β1 integrin activity, it also leads to phosphorylation of EGF receptor (EGFR). The EGFR association with α5β1 integrin is also tightened. The EGFR phosphorylation in this circumstance is independent of other EGFR ligands.
(Ragno, 2006)

1.9.2 G-coupled receptors

FPR (N-formyl-peptide receptor) and its homolgues FPR-like 1 (FPRL1) and FPR-like2 (FPRL2) are seven transmembrane domain G-coupled receptors. N-formyl- methionyl-leucyl-phenylalanine (fMLP), is a peptide which is formylated and of bacterial origin. This peptide stimulate chemotaxis, activating the

FPR and its homogues. The fMLP receptors initially identified in leukocytes has a broad expression pattern, including in endothelial cells (Carriero *et al.*, 2009; Degryse, 2008)

FPR has the highest affinity for fMLP among its homologues, while FPRL2 doesn't bind and FPRL1 has very low affinity. FPRL1 can be activated by lipoxin A4, serum amyloid A, the prion peptide PrP106–126, human immunodeficiency virus-type 1 (HIV-1) envelope peptides. *Helicobacter pylori* Hp(2–20) peptide and certain synthetic peptides can bind both FPRL1 and FPRL2 (Le *et al.*, 2002; Nedellec *et al.*, 2009)

In monocytes it was discovered that FPRL1 is activated by c-suPAR (Figure 1.28) (explained under structure of uPAR, is the cleaved soluble form of uPAR with a role in migration *in-vivo* and shed in fluids from diseased subjects) (Resnati *et al.*, 2002b; Montuori *et al.*, 2005a). There is infact a specific peptide that activates FPRL1 referred to as SRSRY representing S for serine, R for arginine and Y for tyrosine. This peptide is exposed when uPAR is cleaved and is part of the linker region connecting D1 domain of uPAR to the D2 (Blasi and Carmeliet, 2002).

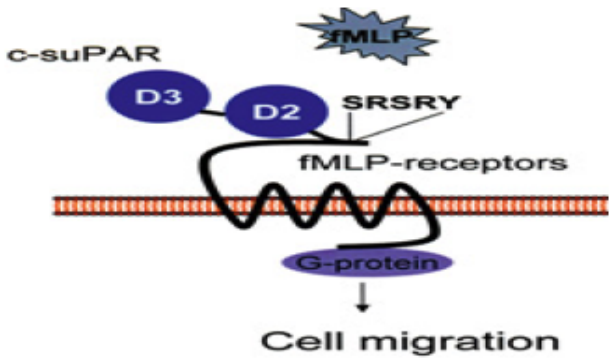

Figure 1.30: Urokinase induce cell migration
The cleaved uPAR exposes the SRSRY sequence of amino acids from the D1-D2 linker region. This region interacts with fMLP receptors inducing cell migration.
(Ragno, 2006)

This peptide sequence provides directionality to cell. With cells moving towards uPA, pro-uPA and ATF gradients (Resnati *et al.*, 1996). This active D2D3 fragment with the exposed SRSRY goes on to bind FPRL1 (Resnati *et al.*, 2002b). Downstream to binding to FPRL1, different signaling molecules are recruited. This plausibly involves some adaptor proteins utlilising the inside out signaling route to elicit chemotaxis via integrins. Full length uPAR has been demonstrated not to bind to FPRL1, even though it can bind to integrins and vitronectin (Resnati *et al.*, 1996; Fazioli *et al.*, 1997; Resnati *et al.*, 2002b). The SRSRY linker region between D1 and D2 though commonly referred to as "exposed eliciting a chemotactic resonse", there is another reason as well. This region differs in conformation between full length uPAR and c-suPAR. This was understood from differential antibody binding to this region (Høyer-Hansen *et al.*, 2001)

1.10 The effect on cyoskeleton

1.10.1 uPAR, integrins and the cytoskelton

Internally, in the cell different adaptors and signaling kinases, phosphatases and molecules are recruited to effect specific directed change. Integrin as described in detail earlier recruits a whole slew of adaptors, the list of which is still growing. uPAR lacks a transmembrane domain and a cytoplasmic domain requiring its lateral association with various receptors including integrin.

Corneal fibroblast were used to study wound healing, where the authors have found evidence of $\alpha v\beta 3$ associating with uPAR. Here they discovered that uPA-uPAR ligation leading to its association with F-actin, along the length of the actin filament. They also found focal adhesions localised with uPA-uPAR and $\alpha v\beta 3$. This association, still requires $\alpha v\beta 3$ to recruit its own set of adapters to link to F-actin (Bernstein *et al.*, 2004). Experimental evidence has proven integrin binding proteins such as vinculin and FAK to be present in focal adhesions and to localise with uPA-uPAR complex (Myöhänen *et al.*, 1993)

A characteristic of uPAR, which it shares with tPA, uPA and PAI-1 is the lack of mRNA production in quiescent endothelial cells (Reidy *et al.*, 1996). uPAR, uPA is expressed heavily in migrating cells though. This feature is shared by FERM-domain containing F-actin binding protein moesin, though moesin is still expressed at basal levels (Batchelor *et al.*, 2004). This protein is a main focus of our study. Moesin has been found to induce stress fiber assembly, cortical actin polymerization and focal complex formation in response to activation of Rho and Rac (Mackay *et al.*, 1997). In the results section we show how moesin is a downstream target of uPA-uPAR interaction and a functional trasducer. Furthermore, mechanotransduction has been shown to increase uPA in otherwise quiescent endothelial cells (Sweeney *et al.*, 2004; Von Offenberg Sweeney *et al.*, 2005). It will be interesting if moesin is similarly increased with mechanotransduction.

Lim and SH3 protein 1 (LASP-1) is another protein studied extensively in our lab. This protein is associated with actin cytoskeleton. This protein has been shown to be mechanically sensitive (personal communication Paul Fitzpatrick). Meanwhile, Salvi *et al* in Italy have shown recently LASP-1 and uPA to have a coordinated expression pattern in human hepatocellular carcinoma (HCC) derived cells (Salvi *et al.*, 2009). Lasp-1 is also implicated by Han Zhang et al to be involved in cell migration and tumour formation. They have proposed focal adhesion protein lipoma preferred partner (LPP) to be involved in this process (Zhang *et al.*, 2009).

uPA activates a number of tyrosine kinases involved in cytoskeletal regulation. Src, Hck, FAK, Gardner Rasheed feline sarcoma viral oncogene homologue (Fgr) as well serine/threonine kinases (ERK/MAPK) are all shown to be activated downstream of uPA (Resnati *et al.*, 1996; Degryse *et al.*, 2001, 1999; Nguyen *et al.*, 2000). uPA activates Rac (member of Rho family GTPases) a major regulator of cytoskeletal changes and an effector of moesin and LASP-1. uPA is also able to effect membrane translocation of Rac as well Raf, Src and FAK. This happens while localizing ERK/MAPK1/2 to the nucleus (Degryse *et al.*, 2001, 1999; Nguyen *et al.*, 2000; Webb *et al.*, 2000; Kjøller and Hall, 2001).

The functional importance of this has to be appreciated as there seems to be an array of kinases dowstream of uPA. Activation of Ras, Myosin light-chain kinase (MLCK) , Protein Kinase C (PKC) and PI3k are some other kinases involved (Nguyen *et al.*, 2000, 1999; Busso *et al.*, 1994; Degryse *et al.*, 2001). There is also evidence towards molecules, such as α-actinin and JAK-STAT (pathway pertinent to haematopoesis), to co-immunoprecipitate with uPAR (Koshelnick *et al.*, 1997; Dumler *et al.*, 1998). uPA induces cell shape changes, reorganises the cytoskeleton, ruffles the membrane, forms actin rings, induces laemellipodia and extends uropodia (Degryse *et al.*, 1999). Functionally, cells deficient in the kinases listed above, will prove difficult to effect their function dowstream of uPA. As, experimental evidence has proven Src, MAPK and ERK kinase in these (Resnati *et al.*, 1996; Degryse *et al.*, 2001, 1999; Nguyen *et al.*, 2000).

With respect to ERK, increased firbronectin adhesion correlated with increased uPAR expression due to α5β1 integrin.

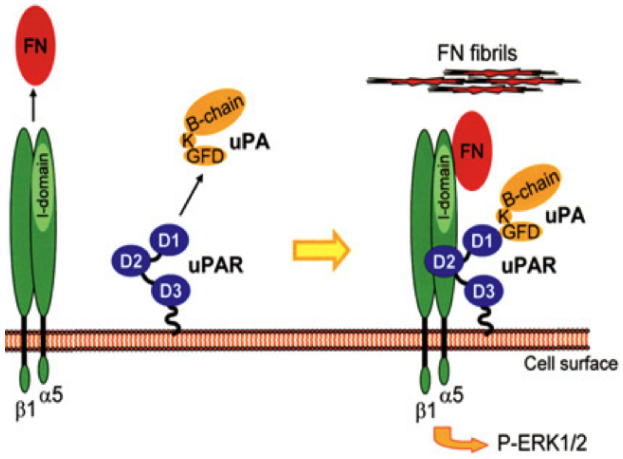

Figure 1.31: α5β1 integrin interacts with urokinase increasing ERK activation
uPA-uPAR has been shown to promote α5β1 integrin activity, wherein uPAR is able to bind both α5 and β1 subunit via its D2 subunit. This also leads to formation of insoluble fibronectin fibrils and ERK 1/2 activation.
(Ragno, 2006)

This was shown to be controlled by ERK phosphorylation levels. It was able to block this increased adhesion by the uPAR peptide P25, which inhibits

uPAR – integrin interactions (Aguirre-Ghiso *et al.*, 2003; Aguirre Ghiso *et al.*, 1999; Aguirre-Ghiso *et al.*, 2001).

1.10.2 uPAR, G-coupled receptors and the cytoskeleton

FPRL1 was found to act upstream in similar fashion to integrin in signaling. FPRL1 induces signaling pathways involving cytoskeletal molecules. This regulaton is necessarry as FPRL1 effects a chemotactic response on binding to c-suPAR. This requires regulation, contraction of the actin cytoskeleton. FPRL1 acts upstream to integrin as shown with experimental evidence with regard to Hck and ERK/MAPK 1/2 (Resnati *et al.*, 1996; Degryse *et al.*, 2001; Fazioli *et al.*, 1997).

FPRL-1 receptor couples to Gαi/Gα0 proteins and generally found to mobilise calcium and generate reactive oxygen species with induction of chemotaxis upon activation and calcium mobilization (Elagoz *et al.*, 2004; Bae *et al.*, 2003). Calcium mobilisation is an aspect where it differs, with respect to FPRL1. Normally the ligands for FPRL1 mobilise calcium including the first such chemotactic ligand identified for FPRL1 serum amyloid A (SAA) as well as fMLP among other ligands (Su *et al.*, 1999). While this is not the case with the SRSRY exposed D2D3 upar (c-suPAR) also a chemotactic ligand when it binds to FPRL1 (Resnati *et al.*, 2002a; Furlan *et al.*, 2004). uPA can induce calcium moblisation but at higher concentration requiring initially for uPAR to cluster together (Resnati *et al.*, 2002a; de Paulis *et al.*, 2004; Blasi and Carmeliet, 2002). uPA and the ATF of uPA can also induce cell migration mediated by FPRL1 (Resnati *et al.*, 2002a; de Paulis *et al.*, 2004). This still requires uPAR to be present, as shown by experiments with uPA in cells lacking uPAR (Resnati *et al.*, 2002a). FPRL1 based signaling with respect to uPAR is still not fully elucidiated.

A study utilizing proteomics, Karsten Boldt *et al* identified in polymorphonuclear neutrophils cytoskeletal changes downstream of FPRL1 (Boldt *et al.*, 2006b). They did not study the effect of uPAR based effect on FPRL1, rather used the ligands such as the synthetic ligand W-peptide and sCK8-1. Groups

have shown extracellular-regulated kinase 1/2 (Erk1/2), phospholipase D (PLD), c-JUN N-terminal kinase (JNK), and mitogen-activated kinase p38 (p38) to be activated in neutrophils mediated by FPRL-1

Boldt went on to show L-plastin was phosphorylated while moesin, cofilin, and stathmin was dephosphorylated in neutrophils utilizing proteomics based 2-D Fluorescence Difference Gel Electrophoresis (2-D-DIGE) method. This work was pioneering with its usage of 2-D-DIGE. There are limitations to this method, as listed by Alberto Paradela *et al*. Testing by western blotting would be an ideal confirmation (Paradela and Albar, 2008).

These studies were important in our understading with regard to FPRL1. There needs to be further studies that put this in perspective, with c-suPAR based association with FPRL1. Further understanding in this matter pertains to integrin signaling and whether these pathways are linked. There is also a lack of understanding in other cell types, such as in the vascular system in endothelial cells. It will be interesting to know if these changes produce a migratory response, instigating the inside out signaling with respect to integrins.

1.10.3 Other receptors for uPAR

LDLR family

uPAR is a ligand, receptor, member of supramolecular complexes and an important player at the extracellular domain with ability to effect multiple targets (D'Alessio and Blasi, 2009).

There are other molecules to which it is associated, such as the low-density lipoprotein receptor-related protein (LRP-1). This protein is able to bind and mediate endocytosis and can activate Rac1. This Rac1 activation is also shown to be uPAR dependent. Endocytosis involves dynamic actin movement with the involvement of clathrin (Binder *et al.*, 2007). Here clathrin coated pits are changed into clathrin coated vesicles and is involved in the internalisation of receptors including G-coupled receptors (Slepnev and De Camilli, 2000; Merrifield *et al.*, 2005). Moesin, one of the cytoskeletal proteins discussed earlier, is

involved in this process. With experimental evidences, it has been proposed that moesin is a clathrin-coated vesiclelinker acting on rab5-clathrin-coated vesicle pulling at the actin, while bound to phosphatidylinositol (4,5)- bisphosphate and actin cytoskeleton (Barroso-Gonzalez *et al.*, 2009). Previous to this paper ezrin was also implicated in clathrin mediated endocytosis of $\alpha 1\beta$-adrenergic receptor (Stanasila *et al.*, 2006). There is a reciprocal modulation of uPAR dependent biological responses, which can simultaneously involve EGFR and integrins, GPCR, members of the LDLR-family and integrins or matrix metalloproteases (Ragno, 2006).

1.10.4 The uPA-uPAR system in angiogenesis

Kaposis's sarcoma is is a well known pathological condition in which uPAR plays a role in the angiogenesis (Morris *et al.*, 2008). This disease is usually prevalent concurrently to AIDS and is caused by Human herpesvirus 8 (HHV8) (Casanova *et al.*, 2009). Experimental evidences has also characterized the role of uPA-uPAR interaction in angiogenesis. Antibodies against uPAR clearly blocks angiogenesis (Gondi *et al.*, 2007). In the section covering endothelial cells are listed pro-angiogenic factors and anti-angiogenic factors. The balance between these molecules are tightly controlled. Increase in angiogenesis leads to a dramatic increase in urokinase, thus the mechanisms, interactions required to recruit cytoskeletal molecules, is important to our understanding of angiogenesis itself (Rao *et al.*, 2005; Basire *et al.*, 2006).

VEGF is clearly one of the more potent pro-angiogenic factors and on association with its receptor VEGF-2, induces pro-uPA on endothelial surface. It acts initially to allow the crossing of plasma proteins (Linderholm *et al.*, 2008). This lays scaffold for endothelial cells to adhere to. Meanwhile release of VEGF results in transcriptional activation of also many other proteolytic enzymes (Prager *et al.*, 2006; Conn *et al.*, 2009; D'Alessio and Blasi, 2009). Then proteolysis ocurrs whereby MMPs, chymase and heparanse families play a role in degradation. uPAR thus plays an initial role in this. There is a seemingly para-

dox role played by PAI-1 in these steps. The logical conclusion should be, with more PAI-1 less angiogensis occurs, due to its inhibition of uPA-uPAR association. Clinically a poor prognosis is conferred to patients that have cancer with increasing PAI-1 and tissue inhibitor of MMPs 1 (TIMP-1). The paradox is explained by increased expression of uPA and uPAR at the leading migratory front (Labied *et al.*, 2008; Blasi and Carmeliet, 2002; Basu *et al.*, 2009). This is supposed to overcome the inhibition by PAI-1 of uPA-uPAR mediated proteolytic breakdown of the vessel wall. There are as such unknown mechanisms which might also explain this paradox. This degradation results in PAI-1 being removed from the vitronectin rich matrix. Initially LDL receptor family is involved in internalising uPA-PAI-1-uPAR complex, which is then degraded. Another interesting aspect is the requirement of PAI-1 for this internalisation process (Dass *et al.*, 2008; Ha *et al.*, 2009; Binder *et al.*, 2007; Diebold *et al.*, 2008)

Integrins, as explained earlier in the section covering angiogenesis is a major player in angiogenesis. Whereby, uPA-uPAR complex correctly positions the integrins to high affinity binding site in the matrix (Kreidberg, 2008). This is because the degradation exposes integrin binding regions, to which it has high affinity. Thus, it actually encourages integrin-dependent endothelial adhesion and migration. Focal contacts that are established by this integrin mediated event is continuously re-established. At the trailing edge, integrins allow the cells to continue moving by being internalised due to endocytosis. Integrins are then re-distributed thus continuing the process. uPAR is recycled back to these focal contacts. There are still answers needed to understand the full mechanism with respect to integrins. NPXY motif found in β subunits via clathrin coated endocytotic machinery is understood to be the major mechanism in place (Binder *et al.*, 2007; van Hinsbergh and Koolwijk, 2008; Blasi and Carmeliet, 2002).

The underlying mechanism involves cytoskeletal proteins. There is a need to better understand these proteins, that can then be regulated by integrins, uPAR and in processes such as endocytosis.

1.11 Rationale

Moesin with ezrin and radixin is an highly redundant ERM protein. It is highly expressed in the endothelial cells compared to its cousins ezrin and radixin. We want to investigate how this protein is affected by the hemodynamic force, generated by the blood conducting through the arteries. We elucidate if moesin is translocated to nucleus with cyclic strain and if this protein is phosphorylated. Our investigations here lead us to suspect moesin regulation by microRNA. We see if this is the case, meanwhile elucidating an array of microRNAs regulated by hemodynamic forces. Moesin's role in endothelial microparticle release is also investigated. We investigate if this is in conjunction with microRNA regulation. Studies previously carried out in the lab has shown uPA increase with cyclic strain and its effects on both migration and angiogenesis. From our knowledge of moesin expression with cyclic strain, we investigate if moesin and uPA interact. Furthermore we elucidate their synergy in migration, wound healing, angiogenesis and also its possible implications in endothelial barrier upkeep.

Chapter 3

Aim of this chapter is to elucidate if and how moesin is affected by hemodynamic forces.

Chapter 4

Aim of this chapter is see if moesin is regulated by microRNA with cyclic strain, as well to elucidate microRNAs regulated by shear stress and cyclic strain.

Chapter 5

Aim of this chapter is to investigate moesin's role in pathogenesis of endothelial cells, with regard to endothelial microparticle release. We also investigate microRNA regulation's need for endothelial microparticle release.

Chapter 6

Aim of this chapter is to investigate moesin and uPA interaction in a slew of arterial functions, with primary focus on migration and angiogenesis.

Chapter 2

Materials & Methods

2.1 Materials

All reagents used in this study were of the highest purity commercially available and were of cell culture standard when applicable. Specific materials, not shown below is listed with corresponding methods.

Materials

AGB Scientific (Dublin, Ireland)
Whatmann Chromatography paper

Abcam (Cambridge, UK) ab3196 mouse anti-Moesin antibody [38/87]
ab6728 secondary antibody, anti-mouse IgG-HRP

Amersham Pharmacia Biotech (Buckinghamshire, UK)
ECL Hybond nitrocellulose membrane
ECL Hyperfilm

BD Biosciences (Oxford, UK)
BD FACSflow™
FACS Caliber Flowcytometer

BioRAD (Alpha Technologies, Dublin)

iScript™cDNA Synthesis Kit

Bio-Sciences Ltd (Dun Laoghaire, Ireland)

DEPC-treated water

Trizol® Reagent

Cell signaling (Boston, US)

3363S mouse anti-Dicer antibody

Dako Cytomation (UK)

Dako mounting media

Dunn Labortechnik GmBH (Asbach, Germany)

6-well Bioflex® plates

Flexcell International Corp. (Hillsborough, NC)

Flexercell® Tension Plus™- FX-4000T™- system

Invitrogen (Groningen, Netherlands)

Vybrant™CFDA SE Cell Tracer Kit

Lonza, Cologne, Germany

Basic Nucleofector® Kit for Primary Smooth Muscle Cells (SMC)

MWG Biotech (Milton Keynes, UK)

Moesin forward primer	5′-ATC ACT CAG CGC CTG ATC TT-3′
Moesin reverse primer	5′-GAT ATT CCA GGA CAG CAT CC-3′
Ezrin forward primer	5′-GTT TTC CCC AGT TGT AAT AGT GCC-3′
Ezrin reverse primer	5′-TCC GTA ATT CAA TCA GTC CTG C-3′
Radixin forward primer	5′-CGA GGA AGA ACG TGT AAC CGA A-3′
Radixin reverse primer	5′-TCT TGG TTT CAT CTC TGG CTT G-3′
GAPDH forward primer	5′-TGC TGA CTA TGT CGT GGA GT-3′
GAPDH reverse primer	5′-GCA TTG CTG ACA ATC TTG AC-3′

Pierce Chemicals (Cheshire, UK)

BCA protein assay kit

Supersignal West Pico chemilumescent substrate

Roche (West Sussex, UK)

Complete Protease Inhibitor Cocktail Tablets

Santa Cruz Biotechnology, Inc (Santa Cruz, US)

sc-12895 goat anti-p-Moesin antibody (Thr 558)

sc-2304 secondary antibody, anti-goat IgG-HRP

Sarstedt (Drinagh, Wexford, Ireland)

1.5 ml eppendorf tubes

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
cryovials

Imaging software, (Image J)

Image J (Abramoff *et al.*, 2004)

Sigma Chemical Company (Poole, Dorset, England)

β-glycerophosphate
β-mercaptoethanol
Acetic Acid
Acetone
Agarose
Ammonium Persulphate
Acrylamide/Bis-acrylamide
Bovine Serum Albumin
Brightline Haemocytometer
Chloroform
DMEM
DMSO
DPX Mounting Media
EDTA
EGTA
Ethidium Bromide
Foetal Calf Serum
Glycerol
Glycine
Hematoxylin
Hydrochloric acid
Isopropanol
Lauryl Sulphate
Lithium Chloride
Methanol
PBS tablets
Penicillin-Streptomycin (100x)
Phosphatase Inhibitor Cocktail
Ponceau S
Potassium Chloride
Potassium Iodide
Protease Inhibitor Cocktail
RPMI-164040
SB216763
Sodium Acetate
Sodium Chloride
Sodium Doecly Sulphate
Sodium Hydroxide
Sodium Orthovanadate
Sodium Phosphate
SYBR Green Taq Ready Mix ™
TEMED
Tris Base
Tris Chloride
Tween 20
Triton X-100
Trypsin-EDTA solution (10x)

2.2 Methods

2.3 Cell culture methods

2.3.1 Cell culture

Cell culture was conducted in highly sterile environment using a Bio air 2000 MAC laminar flow cabinet. Cells were visualized using an Olympus CK30 phase contrast microscope unless otherwise stated. RVSMCs was purchased from Cell Applications Inc.(CA, USA). BVSMCs was purchased from Coriell Cell Repository, New Jersey, USA. BAECs were obtained from Coriell Cell Repository, New Jersey, USA. These cells were maintained in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. HAECs was purchased from Promocell, Germany. HAECs were grown in proprietary low serum media (C-22020 Endothelial Cell Growth Medium MV), obtained from Promocell, Germany.

All cells were adherent cells and required trypsinisation for subculturing and harvesting. For trypsinisation, growth media was removed from the flask and the cells were gently washed two times in Hanks buffered saline solution (HBSS)/Phosphate buffered saline solution (PBS) to remove macroglobulin, a trypsin inhibitor present in FBS.A suitable volume of trypsin / ethylenediamine tetracetic acid (EDTA) (1% v/v trypsin EDTA in HBSS/PBS) was added to the flask and incubated for 5 min 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 700g for 5 min (eppendorf centrifuge). Cells were then resuspended in culture medium and typically diluted 1:4 for HAECs/VSMCs and 1:5 for BAECs into culture flasks, or cryogenically preserved. Cells were incubated in a humidified atmosphere 5% v/v 37°C humidified atmosphere of 5%CO_2/95% air in a Hera water jacketed cell culture incubator.

Cells were cultured in T175 cm^2, T75 cm^2, T25 cm^2 flasks 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. Bioflex® plates were coated with fibrnonectin at 50 μg/ml for all cyclic strain experiments unless otherwise stated (Liu *et al.*, 2007). In the case of dextran permeability studies, cells were grown on 24 mm round polyester filters, (0.4 μm pore size, 6 well format). Cells between passages 5–10 were used in experiments.

2.3.2 Cryogenic Cell Storage and Recovery of Cells.

The cells were maintained long-term in liquid nitrogen in a cryofreezer unit. Cells to be frozen were trypsinized from the flask, centrifuged down in serum-containing media for 5 min at 700g, and the pellet was resuspended in 20% (v/v) FBS containing dimetylsulphoxide (DMSO) at a final concentration of 10% (v/v). HAECs were resuspended in serum free proprietary solution from Promocell (Germany). This solution contains DMSO and methylcellulose and is referred to as Cryo-SFM® .

A ml of suspension was then transferred to sterile cryovials and frozen at -80°C freezer at a rate of -1°C/minute using a Nalgene cryo freezing container. Cells were recovered from longterm storage by rapid thawing at 37°C and resuspension in 5ml of growth medium followed by centrifugation at 700g for 5 min. 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 PBS and fresh culture media added.

2.3.3 Cyclic Strain

For cyclic strain studies, cells were seeded into 6-well Bioflex plates (Dunn Labortechnik GmBH - Asbach, Germany) at a density of 10^5 cells/cm^2 to reach confluency in 24 h. For sub-confluent studies, cells were seeded at a density of 10^4 cells/cm^2. When the cells had reached full confluency, the cells were exposed to cyclic strain. For experiments requiring sub-confluence, cells were allowed to reach 50% confluency. Bioflex™plates contain a pronectin-coated

silicon membrane bottom that enables precise deformation of cultured cells by microprocessor controlled vacuum (Figure 2.1). The plates were also coated with fibronectin at 50 μg/ml for all cyclic strain experiments, unless otherwise stated (Liu *et al.*, 2007). A Flexercell Tension Plus™FX-4000T™system (Flexcell International Corp., Hillsborough, NC) was employed to apply different cyclic strain regimen to each plate (0-10% strain, 60 cycles/min; 0-7.5% strain, 60 cycles/min; 0-4% strain, 60 cycles/min; 8-10% strain, 60 cycles/min) providing equibiaxial tension using the Heartbeat™simulation protocol. The strain was employed for different time frames specified for each experiment. For cyclic strain stasis, cells were strained for 4 h after which it was maintained in stasis for 6 h. Cells were also acutely stretched to 20% non cyclically and equibiaxially, the stretch was induced slowly in 3 s. This reduced the flow velocity to less than 2.5 mm/s (Chiu *et al.*, 2008). Th Flexercell system used for stretching and biaxially straining cells is shown in (Figure 2.1).

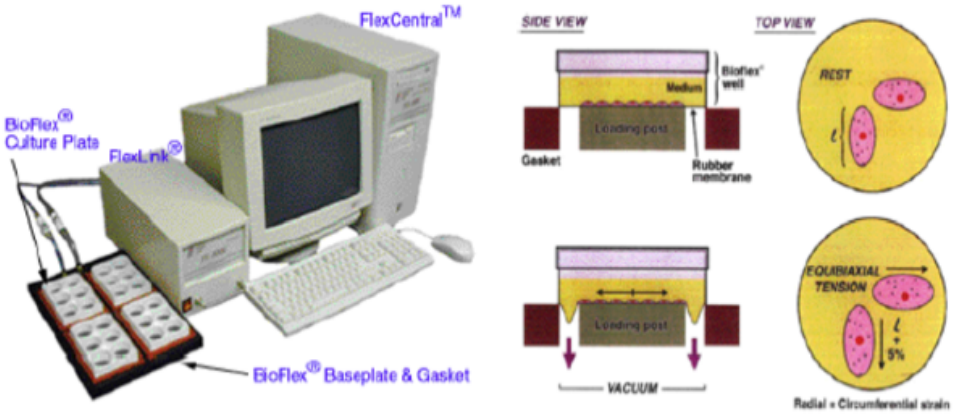

Figure 2.1: Flexercell system used for cyclic strain and stretching vascular cells

Control cells remained unstrained or unstretched. Following strain/stretch, the cells were washed twice in I x PBS, and protein, RNA or microRNA samples were isolated. For cell fractionation studies cells were harvested according to either Biovision™or Calbiochem™methodology. Where the experiments looked into cell cycle, cells previously stained with Edu (commercial nucleoside stain) at 10μM were harvested by trypsinization for monitoring by FACS. For proliferation studies cells previously stained with CFSE was trypsinized and monitored by FACS. For chemotaxis studies, cells were harvested by versene ® to avoid cleavage of extracellular domains. These were then monitored for migration over a boyden chamber to full serum media. Detailed experimental methodology of all experiments from this paragraph is described under respective sections.

2.3.4 Shear Stress

2.3.4.1 Orbital shaker methodology

HAECs were seeded at 1 x 10^5 cells/cm^2 in 6 well plates and allowed to come to confluency. Following this, media was removed and replaced with 4 ml of fresh media. Cells were then sheared at 10 dyne/cm^2 for 24 h on an orbital shaker (Stuart Scientific Mini Orbital Shaker) set to the appropriate RPM as determined by the following equation. Control cells were not sheared. Post shearing the cells were harvested for their protein after washing in 1x PBS twice.

shear stress=$\alpha\sqrt{\eta(2\pi f)\text{^}3}$

Where α = radius of rotation (cm), = density of liquid (g/l), η = 7.5 x 10^{-3} (dynes/cm^2 at 37^{o}C), f = rotation per second

Control cells were not sheared. Post shearing the cells were harvested for their protein after washing in 1x PBS twice. The method of shearing is illustrated in (Figure 2.2). As well, as per requirement of the experiment images were obtained using microscope (Olympus BX50).

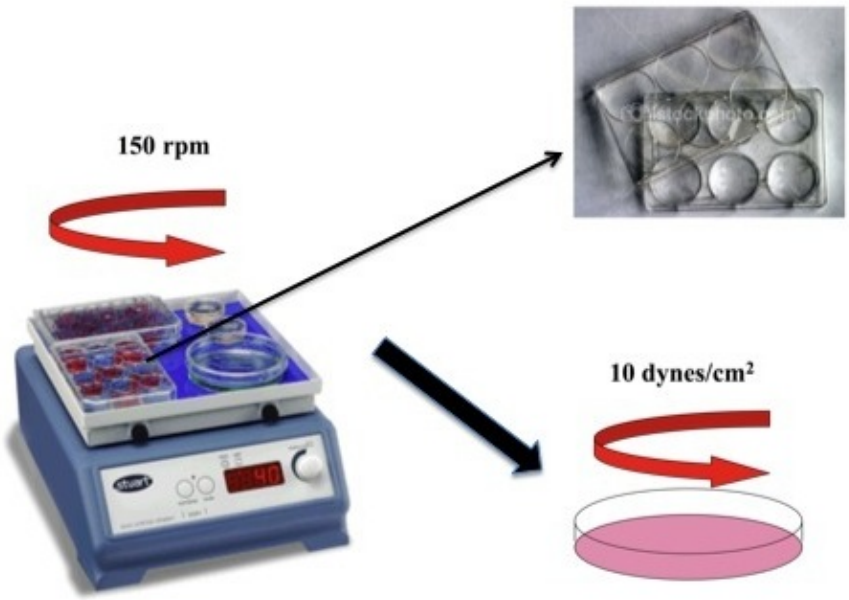

Figure 2.2: Orbital shaker used for shear stress
The figure shows the orbital shaker used for shear stress resulting in the cells experiencing 10dynes/cm^2. Six well plates with 4 ml of media was used at 150 rpm to generate the particiular shear stress according to the equation shear stress=$\alpha\sqrt{\eta(2\pi f)\text{^}3}$.

2.3.4.2 ibidi®

This is an innovative flow system that continuously flows media at precisely controlled shear stress over the flow surface. This allows for cultivation of cells under flow conditions, inside an incubator in long term assays. The system also provided microscope friendly system which allows for live imaging. Initially optimization was required to grow cells without coating with adhesive extracellular matrix. Matrix free enviroment was sought, as our current shear stress parameters used non-matrix coated system. Coating with extracellular matrix would introduce other variables in the system. Also, all our western blotting conducted for shear stress was conducted in the non-matrix coated system. The optimization provided the optimum shear stress that could be ap-

plied without affecting cell adhesiveness. This was found to be 8.2 dynes/cm^2 both unidirectionally as well as oscillating for HAECs. The system is capable of applying high shear stress of 40 dynes/cm^2, which is useful for acute studies without application of extracellular matrix.

The air pump component of the system is controlled by means of a computer. The pump is capable of generating pressure ranging from -100 to 100 mbar with $+/-$ 1 mbar accuracy. The system was controlled by means of software, which allows for precise control of the shear stress taking into account the density of media used.

There are two reservoirs for the cell media, a slide holder which holds the μ-slide. Additionally there are tubes that connect the reservoirs and μ-Slides. ibidi® , refers these reservoirs and the connecting tubes as perfusion sets which come in different diameters and lengths.

With optimization μ-Slide VI $^{0.4}$, was found to be the most user friendly. The specific slide μ-Slide y-shaped also had the same ease of use, and was useful to understand flow mediated responses in areas of arteries that are bifurcated. μ-Slide I was not very easy to use, and had leakage issues with media. Recently, the company has discontnued this slide for flow based system. Thus for the experimental setup μ-Slide VI $^{0.4}$ was used. The slides used are shown in (Figure 2.3)

Figure 2.3: ibidi® slides
The figure shows the slides that were used for various shear stress studies. According to manufacturer's protocol, slides were seeded with HAECs. Further to full confluence the slides were connected by means of tubes to the perfusion system. Using software, shear stress regime was selected taking into account the type of slide, perfusion system used. The manufacuturer's website is $http://www.ibidi.de$

Due to the design, perfusion sets allowed for varying the flow rates as well as placing the fluidic unit on the microscope stage. With flow, there was slight disruption of the stage which blurred the video capture with higher shear stress. Due to these reasons, with respect to video capture with flow, shear stress of 5.1 dynes/cm^2 was applied acutely for 10 min. As the shear applied is acute, it would show protein translocation (Hahn and Schwartz, 2009). For video capture studies, μ-Slide VI $^{0.4}$ was used. For extraction of total RNA after 24 h of shear stress (8.2dynes/cm^2), μ-Slide VI $^{0.4}$ was used. RNA was extracted with RNAeasy® (Qiagen) according to the manufacturer's protocol. The red perfusion set was used in all experiments.The control unit is connected to the fluidic unit by a thin, low-voltage electric cable and a tube which shifts the air pressure, driving the flow. The air tube and the electric cable was mounted into the incubator through a hole in the black wall. The principle of ibidi® based flow is shown in (Figure 2.4)

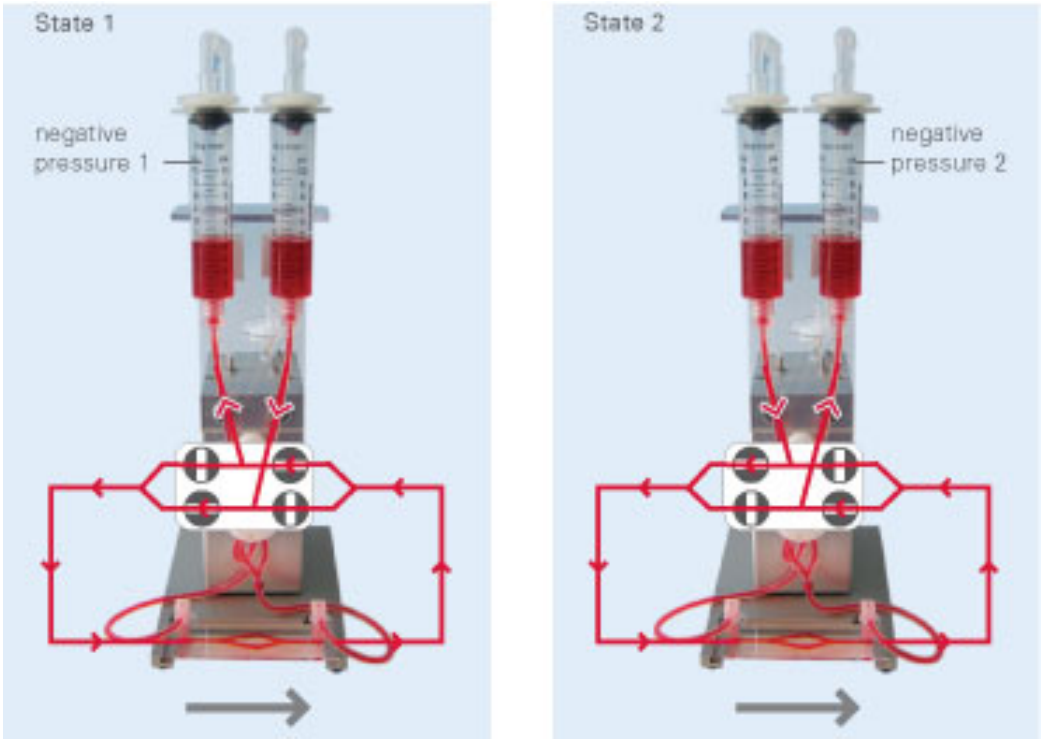

Figure 2.4: Principle of ibidi® flow system
The pressure generator utilises negative pressure. State 1 supplies negative pressure at reservoir 1. This causes media to be sucked from reservoir 2 into reservoir 1. When all of reservoir 1 is filled, all valves switch and the liquid gets sucked from reservoir 1 in the same way and thus in the same direction filling reservoir 2. The manufacturer's website is *http://www.ibidi.de*

2.3.5 Transformations

DH5α chemically competent cells were purchased from invitrogen. A volume of 50μl of the competent cells were placed in a sterile eppendorf. Following quantification of plasmid DNA, 50ng of DNA was added to the cells and the contents of the tube were mixed gently. The tube was then placed on ice for 30 min, before being heat shocked at 42^{o}C for 90 s. The tube was then placed on ice for 2 min. 1ml of sterile SOC medium (20% tryptone, 5% yeast extract, 0.5% NaCl, 1% 0.25M KCl, 2% 1 M glucose) was then added to the tube, and the tube was incubated at 37^{o}C for 1 h. The tube was then centrifuged for 2 min at 100 x g and the pellet then resuspended in 100μl of SOC medium. The culture was then plated out on LB agar plates containing 100μg/ml ampicillin and incubated overnight at 37^{o}C. Non-transformed competent cells were also plated out as a negative control. Only transformed colonies contain the ampicillin resistance gene, and therefore grow on the ampicillin-containing agar plates. A single colony, therefore, was transferred to a 50ml sterile tube containing 5mls of LB broth (1% tryptone, 0.5% yeast extract, 1.0% NaCl). This primary culture was then incubated at 37^{o}C with agitation of 200 rpm for 8h. 400μl of the culture was transferred to a 250ml sterile conical flask containing 50mls broth and incubated overnight at 37^{o}C with agitation of 200 x g. DNA minipreparation were then carried out on this secondary culture as described in.The remainder of the 5ml primary culture was used for glycerol stocks.

2.3.6 Plasmid purification protocol

Plasmid DNA was purified using the QIAGEN-tip HiSpeed® system from Qiagen, according to the manufacturer's instructions for plasmid purification of animal cells. DNA was then quantified by spectrophotometric analyzis.

2.3.7 DNA Quantitation and Storage

To determine the amount of DNA in a sample obtained from the Qiagen plasmid midi kit, the sample was analyzed using a NANODROP 1000 Spectrophotometer (Thermo Scientific) blanked with TE. The sample was read at wavelengths of 260 and 280 nm, and the concentration of the DNA in the sample was calculated out as follows;

Abs_{260nm} x dilution factor x 50 = concentration of DNA (μg/ml)

The purity of the DNA was determined by calculating the ratio of absorbance at 260 nm to 280 nm, the value of which should be greater than 1.6. All samples were tested in triplicate and were kept on ice at all times during the experiment. DNA samples were then stored at -20^{o}C for use in transient transfections.

2.3.8 Transient Electroporation (Transfections)

2.3.8.1 Nucleofection™

Nucleofection™is a transfection method based on the physical method of electroporation. Nucleofection™uses a combination of optimized electrical parameters, generated by a special device called Nucleofector® , with cell-type specific reagents. Cells were transiently electroporated using a Basic Nucleofector® Kit for endothelial cells. Cells were electroporated with plasmid DNA at no greater then 80% confluency. Cells were trypsinized as previously described in section and counted to determine the cell density. A volume containing 1 x 10^6 cells was used per transfection. The cells were centrifuged at 700g for 5 min, The pellet was resuspended in room temperature in the Basic Solution to a final concentration of 1 x 10^6 cells/100μl. 100μl of cell suspension was mixed with a total of 3μg DNA. The nucleofection sample was then transferred to an amaxa cuvette. The cuvette was inserted into the cuvette holder of

the Nucleofector and the appropriate Nucleofector program selected.After the programme was finished, the cells were transferred from the cuvette, into an eppendorf tube and 500μl of pre-warmed culture medium containing serum and supplements was added. The eppendorf tube was then incubated at 37^{o}C, until all transfections were completed. The samples were then transferred from the eppendorf tubes into 6-well plates containing 1ml of culture medium with serum and supplements (3 wells/sample). The cells were then incubated at 37^{o}C and the medium changed 16-18h post nucleofection.

2.3.8.2 Microporator™

Cells were also electroporated with Microporator™, based on electroporation method. Similar to Nucleofection™, Microporator™uses a combination of optimized electrical parameters, generated by a special device called Microporator™. Cells were transiently electroporated using Microporator™kit. 1 x 10^6 cells were electroporated in 95μl solution R. The solution was made upto 100μl solution with DNA (3μg).

Importantly, stock volume of all DNA used in an experiment was equalised before addition. Solution E2 (3ml) was used as per the manufacturer's protocol. The cells were electroporated as per the manufacturer's protocol. This was optimized for HAECs (voltage: 1000V, pulse width: 30ms, pulse number: 3) and BAECs (voltage: 1300V, pulse width: 20ms, pulse number: 2).

Prior to transfection the media was pre-warmed and acidity regulated by equilibriating in the incubator under the same conditions used for cell culture. Post transfection, cells were plated in this media whereby a third was added per well of a 6-well plate (9.6 cm^6).

Both Nucleofection™and Microporator™programs were optimised to yield approximately 95% transfection efficiency with respect to RNA and greater than 65% for the final protein. This was assessed by RT PCR and FACS.

Cells were routinely co-electroporated with green fluorescent protein (GFP) encoding plasmid as a means to determine approximate levels of transfection.

2.3.9 Rho A Inhibition

C3 Transferase (catalog number CTO4 from Cytoskeleton, Inc) was obtained. This is a highly purified C3 Transferase which is covalently linked to the manufacturer's proprietary cell penetrating moiety via a disulfide bond. The moeity allows for C3 Transferase to move into plasma membrane rapidly. Inside the cell only the C3 Transferase is released, discarding the moeity. C3 Transferase inactivates RhoA, RhoB, and RhoC, but not related GTPases such as Cdc42 or Rac1.

The exoenzyme C3 Transferase is derived from *Clostridum bolulinium*. It is commonly used to selectivley inactivate the GTPases RhoA, RhoB, and RhoC, both *in-vivo* and *in-vitro*. The inhibition involves ADP-ribosylation on asparagine 41 in the effector binding domain of the GTPase. Traditional usage of this protein required expensively high concentrations of 100μg/ml. The incubation time was also too long with overnight incubations required. This was fraught with difficulty when studying with transiently electroporated genetic material. Researchers had to often use less specific inhibitors.

The inhibitor was prepared according to the manufacturer's directions. The inhibitor was dissolved in sterile water to obtain 100μg/ml. As per their direction, 1μg/ml was added into each well of the 6-well fully confluent bioflex® plates. This was fulfilled as follows.

HAECs were electroporated with siRNA against Dicer and scrambled and seeded on bioflex® plates previously coated with fibronectin. The seeding density was 10^5 cells/cm^2 to reach confluency in 24 h Post transfection, cells were allowed to recover over 12 h after which the cells were fed with full serum media and left undisturbed for a further 12 h. The cells were serum starved for 12 h, after which they were treated with C3 transferase at 1μg/ml for 6 h to obtain maximal robust inhibition according to the manufacturer's scale of Rho activity in 1.2 ml serum free media. Further to this the cells were acutely stretched to 20% non cyclically and equibiaxially, the stretch was induced slowly in 3 s. This reduced the flow velocity to less than 2.5 mm/s (Chiu *et al.*, 2008). The

protein lysate was harvested.

2.3.10 Small-interfering RNA

All siRNA were obtained from Applied Biosystems. Non-specific sequence referred to as scrambled was used as control and also obtained from Applied Biosystems. Different siRNA against the same target was checked. This was verified by RT-PCR (only for GAPDH and moesin) further confirmed by westernblotting (for all siRNAs). The canditate siRNA that produced most knock down of target proteins was used for experiments. 4μg of siRNA was utilized for 10^6 cells. Transfection efficiency for each cell type was determined and optimised with siRNA against GAPDH.

The following siRNA IDs (used by Applied biosystems) correspond to the target proteins in humans. They were chosen for their high transfection efficiency. These were used for downstream experiments.

GAPDH	AM4624
Moesin	11593
Dicer	s23754
Scrambled	4390825

2.4 Analytical Methods

2.4.1 SDS-PAGE and Western Blot Analyzis

2.4.1.1 Preparation of Whole Cell Lysates

Cells were washed in 1 x PBS and then 100μl/cm^2 1X RIPA 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) supplemented with protease-phosphatase inhibitor cocktails (1/100 dilution of stock, Sigma Aldrich)was added to the cells. The

cells were then scraped into the lysis buffer using a cell scraper and transferred to an eppendorf. The lysates were centrifuged for 1hr or o/n at 4°C and centrifuged at 10,000rpm for 20min. Samples were stored at -20°C for short-term storage or -80°C for long-term storage.

2.4.1.2 Bicinchoninic Acid (BCA) Protein Microassay

The bicinchoninic acid protein microassay utilizes the biuret reaction, the reduction of Cu^{++} to the cuprous cation (Cu^{+}) by protein under alkaline conditions, with the selective colourimetric detection of Cu^{+}) using a reagent containing bicinchoninic acid. Bicinchoninic acid, sodium salt, is a stable, water-soluble compound capable of forming an intense purple complex with cuprous ion (Cu^{+}) in an alkaline environment. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations over a broad working range of 20-2000 μg/ml. The two separate reagents used were supplied in the commercially available assay kit (Pierce Chemicals): A, an alkaline bicarbonate solution and B, a copper sulphate solution. A working solution was prepared by mixing 1 part reagent B with 50 parts reagent A. On a microtitre plate 200μl of the working solution was added to 10 μl of the whole cell lysate or bovine serum albumin (BSA) protein standard. The plate was then incubated at 37°C for 30 min. The absorbance of each well was then read at 560 nm using a Tecan Spectra plate reader. All samples and standards were tested in triplicate. Quantification was carried out by interpolation from a BSA standard curve (0-2 mg/ml).

2.4.1.3 Western Blotting

SDS-PAGE was performed as described by Laemmli using 10% polyacrylamide gels (Laemmli *et al.*, 1970). 10% resolving and 5% stacking gels were prepared as follows:

Resolving Gel:

2.5	Buffer A (1.5M Tris pH8.8)
3.3	30% acrylamide stock
4.0	distilled water
100μl	10% (w/v) SDS
100μl	10% (w/v) ammonium persulphate
4μl	TEMED

Stacking Gel:

1.26ml	Buffer B(0.5M Tris pH6.8)
0.83ml	30%acrylamide stock
2.77ml	distilled water
50μl	10%(w/v) SDS
50μl	10%(w/v) ammonium persulphate
5μl	TEMED

For analyzis 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 min, then immediately placed on ice. The gel was electrophoresed in reservoir buffer (0.025M Tris pH 8.3; 0.192M Glycine; 0.1% (w/v) SDS) at 100V per gel using bio-rad ® gel electrophoresis system until the dye front reached the bottom of the gel.

Following electrophoresis the gel was soaked for 15 min in cold transfer buffer (0.025M Tris pH8.3; 0.192M Glycine; 15% v/v methanol). Nitrocelluose membrane and 12 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 overnight at 50V in a Bio-rad transfer cell with magnetic agitation. 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. Ponceau stains all transfered bands of proteins red. Membranes were blocked for a minimum of 1h in blocking solution (5% BSA- PBS-1%Tween). Membranes were then incubated overnight at 4ºC with the appropriate dilution of primary antibody in blocking solution (Table 2.3). The blots were then vigorously washed in three changes of PBST and then incubated for 2 h at room temperature with a suitable HRP linked secondary antibody diluted in PBST. Following incubation in secondary antibody, the blots were again washed in three changes of PBST. Antibody-antigen complexes were detected by incubation in West Pico Supersignal reagent (Pierce Chemicals).

The blot was previously cut precisely isolating the GAPDH area. This was subjected to similar procedure to detect GAPDH antibody-antigen complex. Briefly, an equal volume of solution A and B were mixed and the blot was incubated for 5 min 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).

Alternatively the blot was not exposed to autoradiographic film, rather was exposed to a high sensitivity camera G:Box ® from Syngene® . In certain experiments that required detection of phosphorylated moesin, initially total moesin was detected. This was then subjected to stripping over 10 min at 37ºC to remove primary antibody against moesin including the secondary antibody attached. This was achieved using commercial stripping buffer from pierce ®. The blot was then subjected to incubation with secondary antibody against total moesin and developed after the prescribed washes (explained earlier) . This

was to control for removal of primary antibody against total moesin. Further to this confirmation, primary antibody against phosphorylated moesin was incubated with the blot for 2 h. The blot was subjected to washes described earlier, and then incubated with the secondary antibody against phosphorylated moesin. The blot was subjected to washes and developed as described earlier.

Bands of interest were identified by use of molecular weight markers. Exposure times varied depending on the antibody being used and the developer used. The photograph was then processed for the value of the bands using Image J software. (Abramoff *et al.*, 2004).

2.4.2 Subcellular Fractionation

Biovision® Method

BAECs were exposed to cyclic strain of 7.5%, the cells were then fractionated to cytosolic and nuclear fractions according to the manufacturer prescribed protocol K266-100 (from Biovision ®). The protocol derived nuclear and cytosolic fractions. From the total volume of each fractions, half was used for western blotting using the standard procedure previously described. This allowed us to control for proportionate equal loading.

This was immunoblotted for moesin, similarly, the other half of the fraction was utilized to detect phosphorylated moesin. The studies were conducted to five independent experiments.

Acetone precipitation of proteins

Acetone precipitation is conducted to remove interfering contaminants from protein samples for downstream processing. It is also useful to concentrate the proteins. The procedure was applied in our experimental methodology for concentrating the protein. The disadvantage was the phosphorylation status of the proteins could be lost.

Pre-chilled acetone (at -20^{o}) was utilized for this procedure. Four times the sample volume of the cold acetone was added. The mixture was vortexed very

well and incubated at -20^{o}. The mixture was vortexed after every 10 minute incubation at -20^{o} for a total of 60 minutes. The vortexing was conducted at maximal speed vigorously ensuring robust mixing.

After the incubation and vortexing, the suprernatant was decanted and disposed without disturbing the pellet. The acetone was allowed to evaporate from the uncapped tube for 30 min on ice. Care was taken not to overdry the pellet, as it will render it not dissolvable. The pellet was then dissolved with 20μl sample buffer. Further to which, it was separated using western blotting procedure described earlier.

Calbiochem® Method

BAECs were exposed to cyclic strain of 7.5%, the cells were then fractionated to cytosolic, nuclear fractions, membrane and cytoskeleton according to the manufacturer prescribed protocol The entire volume of each fraction was precipated using Further to this, it was reconstituted in laemmli buffer 15 μl. This was used to confirm moesin's presence in nucleus using standard western blotting described previously.

This was immunoblotted for moesin, similarly, the other half of the fraction was utilized to detect phosphorylated moesin. This method was used to confirm biovision derived results, and was repeated to one independent experiment.

2.4.3 Polymerase Chain Reaction (PCR)

2.4.3.1 Preparation of Total RNA

The reagents were obtained from Qiagen® and was commercially referred to as RNeasy mini kit® . Total RNA was isolated using the manufacturer's methodology. Whereby, after washing the adherent cells with 1x PBS, the total RNA was isolated using RLT buffer. This was stored at -80^{o}C after lysing the cells or processed immediately. Post processing the RNA was stored at -80^{o}C.

Alternatively, total RNA was isolated from cells using Trizol® reagent according to the method of (Chomczynski and Sacchi, 1987). The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, maintains the integrity of RNA while disrupting cells and dissolving cell components.

Growth media was removed and cells were washed with 1XPBS twice. Cells were then lysed directly by adding trizol reagent to the flask, 1 ml per 10 cm^2. The lysate was transferred to a sterile falcone tube and incubated for 5 min at 15-30ºC to permit the complete dissociation of nucleoprotein complexes. Chloroform was added at a concentration of 0.2 ml of chloroform per 1 ml of trizol, the tube was then shaken vigorously for 15 s and then centrifuged down at 12,000 x g for 15 min at 2 to 8ºC. The resulting aqueous phase was then transferred to a fresh tube. RNA was then precipitated by mixing with isopropyl alcohol, 0.5 ml per 1 ml of trizol. The samples were then incubated at 15ºC to 30ºC for 10 min and centrifuged down at 12,000 x g for 10 min at 2 to 8ºC. The RNA was then visible as a gel like pellet on the side and bottom of the tube. The supernatant was then removed and the pellet washed with at least 1ml of 75% ethanol per 1 ml of trizol used. The sample was then mixed by vortexing and centrifuged down at 7,500 x g for 5 min at 2 to 8ºC. The supernatant was removed and the pellet washed again in ethanol. After washing the pellet, it was air-dried and the RNA re-suspended in 30-50μl of RNase free water and incubated at 60ºC for 10 min. All total RNA preparations were stored at -80ºC.

2.4.3.2 *in-silico* analyzis of microRNA targets

Target analyzis was performed using the bioinformatic software tool available in the internet at $http://microrna.sanger.ac.uk$

2.4.3.3 Preparation of microRNA in Samples

Preparation of microRNA was performed using the manufacturer's protocol utilizing the ABI based mirVANA® kit. There is a significant loss of microRNA if harvested using commercially available kit for total RNA. It has to be noted

that Trizol® based extraction method does harvest microRNAs. The processing to microRNA was performed immediately from cells using mirVANA® kit. Further processing included assessing the quality of the microRNA by means of Nanodrop 1000 spectrprophotometer (Thermo Scientific) and Agilent Microfluidic Bio-analyzer (Agilent technologies). This included synthesizing cDNA with 20μg of extracted microRNA. Towards this purpose thermal cycler was set to 9600 Emulation mode. Program according to the manufacurer was 30 min at 16^0C, 30 min at 42^0C, 5 min at 85^0C for a total volume of 10μl. The cDNA was diluted 62.5 fold and 50μl of this was mixed to 50μl of TaqMan Universal PCR master mix (2X). This 100μl was loaded onto the side port of TLDA v1.0 chip. After brief centrifugation according to the manufacturer, the card was run on the thermocycler (Applied Biosystems 7900HT Fast Real-Time PCR system). Loading controls included RNU44 and RNU48 while the primers were proprietary stem-loop (Chen *et al.*, 2005). The chip is shown in (Figure 2.5). The actual list of microRNAs are given with the results in the microRNA chapter. Data was analyzed

Figure 2.5: TLDA v1.0 Chip cDNA is loaded onto the right side of the chip. The cDNA runs over the proprietary stem-loop primers (Chen *et al.*, 2005), after which it is sealed and RT-PCR conducted. The card is a representation and the actual microRNAs analyzed are listed in the results section in the microRNA chapter.

2.4.3.4 Quantification of Total RNA in Samples

To determine the amount of total RNA in samples, the sample was analyzed using a Nanodop 1000 spectrophotomoter (Thermo Scientific) blanked with RNase free water. 1.5μl of sample was measured at wavelengths of 260 and 280 nm, and the concentration of the RNA in the sample was determined as follows;

Abs_{260nm} x dilution factor x 40 = concentration of RNA (μg/ml)

The purity of the RNA was determined by calculating the ratio of absorbance at 260nm to 280nm. A ratio of 1.9 to 2.0 was indicative of a highly purified preparation of RNA. A ratio lower than this was indicative of protein contamination. Absorbance at 230 nm reflected contamination of the sample by phenol, while absorbance at 325 nm suggests contamination by particulates. All samples were tested in triplicate and were kept on ice at all times during the experiment. RNA samples were then stored at -80^{o}C.

2.4.3.5 Design of PCR Primer Sets

A number of web based programs, "Primer 3 Output" and "NCBI/BLAST" were utilized to design the primer sets used in this study. The Primer 3 program picks primers from the given sequence, and the BLAST program allows multiple sequence alignment, which allows primers to be designed from highly conserved areas. Primers were designed with 50% GC content so the annealing temperature for all sets was 55-60^{o}C. The primers also spanned high length intron to avoid genomic DNA contamination prone products in qRT PCR.

2.4.3.6 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

All total RNA samples were prepared by the Trizol method as previously. RNA preparations were then quantified by absorbance spectroscopy as described in

Section 2.6.2. Reverse transcriptase was carried out using an iScript™cDNA Synthesis Kit according to the manufacturer's instructions. Briefly, 2μg of RNA was added to 1μl of reverse transcriptase enzyme and 4μl of 5x buffer in a PCR tube. RNase and DNase free H_2O was then added to make a total of 20μl sample volume. The tubes were centrifuged briefly, and placed in a PCR Sprint thermocycler (Thermo Hybaid) and the following programme was run: 25^{o}C for 5 min, 42^{o}C for 30 min, 85^{o}C for 5 min. The samples were then stored at -20^{o}C and used for real-time PCR.

2.4.3.7 Real-Time PCR

Quantitative PCR was carried out using a Real-time PCR. The principle of real-time PCR amplification detection is that the amount of fluorescence is proportional to the concentration of product in a reaction. Higher fluorescence indicates a higher concentration of a product. Each PCR reaction was set up as follows:

SYBR-Green	12.5μl
RNAse free water	8.5μl
cDNA	2.0μl
Forward primer(10μM)	1.0μl
Reverse primer(10μM)	1.0μl

Each sample was assayed in triplicate, and the program used for the different primer sets was as follows;

Denaturing Phase	95^{o}C - 20s
Annealing Phase	55-60^{o}C - 30s (55 cycles)
Elongation Phase	72^{o}C - 30s

The Comparative Ct method was used for quantitative analyzis while Melt

Curve analyzis was carried out for qualitative analyzis.

2.4.3.8 Agarose Gel Electrophoresis

All DNA gel electrophoresis was carried out utilizing GibcoBRL Horizon 20.25 Gel Electrophoresis Apparatus. Before use the gel box was cleaned with ethanol and the gel cast was set up as described in the manufacturers instruction manual. A 2.5% agarose gel stock was made up by dissolving 2.5 g of agarose in 100 ml of 1x Tris Acetate EDTA (TAE) (40 mM Tris-Acetate, 1 mM EDTA). The agarose was dissolved by heating in a microwave (700 mHz) at full power for 5 min. The agarose was then poured into the cast, the comb put into place and the gel allowed to set. Once it solidified, the comb was removed and the apparatus filled with 1x TAE buffer. Loading dye was added to the sample (17μ of loading dye to 6μl of sample). 10μl was then loaded to each well in duplicate. The gel was run at 80 V, 110 mA and 150 W until the dye front had migrated the length of the gel. The gel was stained in a 2mg/l ethidium bromide staining bath for 10-15 min, and then placed on an Ultra Violet Products UV transilluminator for visualization. A picture was taken using a Kodak DC290 digital camera. The gel was then disposed of in the appropriate EtBr waste container.

2.4.4 Immunocytochemistry

Cells cultured as described previously were washed in PBS and fixed with 0.3% formaldehyde for 15 min at room temperature. Cells were washed twice in PBS and permeabilised with 0.025% Triton-X-PBS. Cells were washed twice in PBS and blocked in 5% BSA- PBS-1% Tween for 1 h at room temperature and incubated overnight with the appropriate primary antibody at 4°C. Cells were washed twice for 15 min in PBS-1%Tween and incubated in the appropriate secondary antibody for 30mins in the dark. Following, two 15 min washes in PBS, cells were visualised using the Olympus DP-50 fluorescent microscope.

Constructs	Charachteristic	Vector	Antibiotic
Ezrin-GFP	wild type ezrin	pEGFP-N1	Kanamycin
Moesin-GFP	wild type moesin	pEGFP-N1	Kanamycin
plc-δ-RFP	wild type plc-δ	pEGFP M6BP	Kanamycin
Ezrin-FLAG	wild type ezrin	pEGFP-N1	Kanamycin
Moesin-FLAG	wild type moesin	pEGFP-N1	Kanamycin
T558D-GFP	phosphomimetic moesin	pEGFP-N1	Kanamycin
T558A-GFP	Moesin that cannot be phophorylated	pEGFP-N1	Kanamycin
GFP-Moesin	Moesin unable to bind to PIP_2	pEGFP-N1	Kanamycin
N1-GFP	Control for contructs with pEGFP-N1	pEGFP-N1	Kanamycin

Table 2.1: Plasmid Constructs

Constructs	Source	Verification
Ezrin-GFP	Richard Lamb, Cancer Research UK	(Lamb *et al.*, 1997)
Moesin-GFP	Ronan Murphy, DCU	Sequenced
plc-δ-RFP	Tamas Balla, NICHD,	(Varnai and Balla, 1998)
Ezrin-FLAG	Made the construct	Sequenced
Moesin-FLAG	Made the construct	Sequenced
T558D-GFP	Ronan Murphy, DCU	Sequenced
T558A-GFP	Ronan Murphy, DCU	Sequenced
GFP-Moesin	Ronan Murphy, DCU	Sequenced

Table 2.2: Source & Verification of the Plasmid Constructs

2.4.4.1 GFP protein visualization and FLAG tag plasmids used

Transient transfections were utilized as detailed before with the plasmids given in the table below. The concentration and volume of the construct was kept same for all constructs between each set of experiments. Each plasmid was electroporated with 3μg for 10^6 cells. The various constructs used are illustrated in the following figure (Figure 2.6). The constructs have been previously verified for its expression and localization.

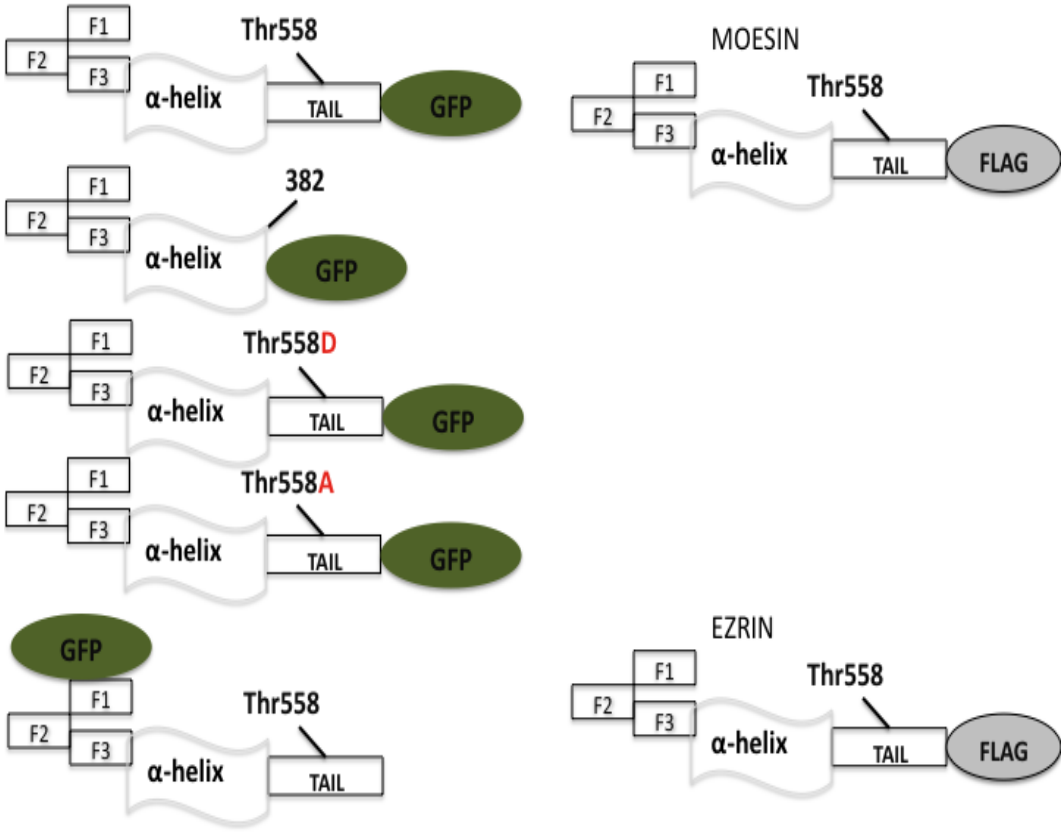

Figure 2.6: Constructs used in experiments Representation of all constructs used in the experiments. The illustration shows where the GFP/FLAG was cloned into. T558D is a constitutively phopho-mimetic form of moesin (Threonine 558 to Aspartic Acid, T558D). T558A is constitutively nonphopho-mimetic form of moesin (Threonine 558 to Alanine). Inactive moesin has GFP cloned into N-terminus, at the F1 domain, while FERM domain alone is created by removal of the C terminus tail at position 382 (GFP is cloned here). FLAG tag has been cloned into the C-terminus for both moesin and ezrin, availing functional full length. Similarly GFP is cloned into moesin C-terminus for functional full length.

2.4.5 Endothelial Microparticle Assay

2.4.5.1 Isolation of Endothelial Microparticles for Western Blotting

BAECs were grown in five 175^2 tissue culture flasks to confluency. These were then stiumulated/non-stimulated with TNF-α overnight. The supernatant was centrifuged at 2000xg for 10 min to clear debri. The supernatant was then collected undisturbed and ultra-centrifuged at 70,000g for 1 h. The pellet was then acetone precipated. This was immuonblotted for moesin after seperation of the proteins by gel electrophoresis

2.4.5.2 Calcein AM Staining of Microparticles

For ionophore activation C5149-5MG (A23187) from Sigma was prepared according to the manufacturer's guidelines. According to this, 15.9mM stock solution was prepared by addition of 5mg in 600μl of DMSO and stored in aliquots of 10μl at -20°C.

Fully confluent cells were in 6 well plates were stained with 2μl (in 1ml media) in each well for 20 min with calcein AM (Falati *et al.*, 2003). Calcein AM stock solution was previously prepared by dissolving 50μg of the same to 100μl DMSO (Falati *et al.*, 2003).

The cells were then incubated with fresh media (1.1ml media and 0.7μl of 15.9mM ionophore) and ionophore to obtain 10μM ionophore solution for a period of 4 h. Cells were also left untreated with ionophore for the same period. The supernatant was harvested or the cells utilized for visualizing under the microscope. The harvested supernatant was centrifuged at 2000xg for 10 min. 800μl of the supernatant was then obtained and the rest including cell debri discarded. This was assayed utilizing FACS for microparticles (Falati *et al.*, 2003).

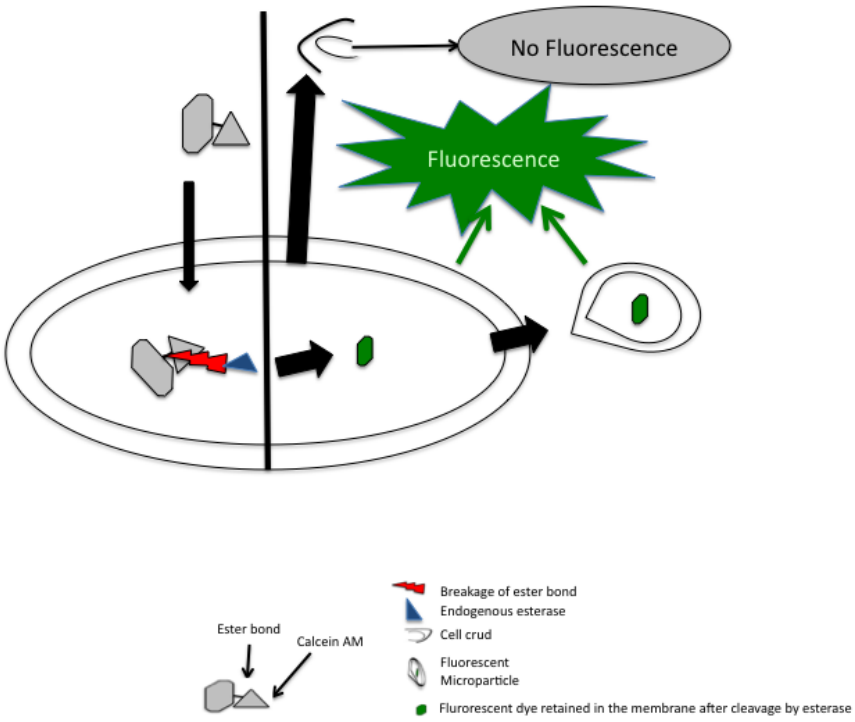

Figure 2.7: Fluorescence of Calcein AM, when membrane bound Calcein AM is a non fluorescent dye and readily diffuses across the plasma membrane of cells. This dye fluoresces on cleavage of its calcein AM (cleaved at the ester bonds), by endogenous esterases inside the cell. With this transformation, it becomes highly hydrophilic and intensely fluorescent but is retained inside the membranes. Microparticles then formed, contain membrane and thus encapsulates the now fluorescent dye. After removal of cells, cell debri by centrifugation microparticles (under 1 micron) can be gated using FACS. Particles and cell debri under 1 micron that are still present won't fluoresce due lack of intact membrane.

Instead of ionophore, microparticles were also induced by thrombin. For this purpose initially thrombin stock solution was prepared. Thrombin (lot number 087k7570) was obtained from Sigma. From Sigma, the amount of thrombin units were found to be 195 units. As per the manufacturer's directions thrombin was dissolved in de-ionoised water to obtain 1 unit per μl.

According to literature, confluent cells were serum starved for 12 h. The cells were then induced for microparticles with thrombin (1 IU/ml) and without (Sap, 2006). Prior to this the cells were stained with calcein AM in serum free media, otherwise as stated before. Microparticles were also harvested from the supernatant as stated before for analyzis with FACS.

For studies that utilized calpeptin (which is a a cell-permeable calpain inhibitor) final concentration according to literature was kept at 50μM. Stock solution was prepared to 27.5 mM by addition of 500 μl DMSO. Calpeptin was added together with media and ionohphore/thrombin towards obtaining final concentration of 50μM. If added together with thrombin the media was maintained without serum.

2.4.6 Microparticles analysis *in-vivo* model

Blood was drawn from participants using a 19C needle into 0.32% final concentration citrate. Anti-coagulant was utilized, after which samples were centrifuged at 1550g for 20min. Plasma was subjected to 18890g centrifugation for 30min at 20^o. 225μl from the supernatant was mixed with 225μl 1xPBS/citrate. This was centrifuged for 30min at 18890g at -20^o. 225μl was taken, mixed with 75μl 1XPBS/citrate. After incubation in dark for 15min it was subjected to FACS

2.4.7 Permeability assay

Following transfection and recovery of cells in fresh media, cells were serum starved for 12 h. The cells were then trypsinized and replated into Transwell-Clear plates at high density of 10^6cells/cm^2. After 24 h when cells were con-

fluent, transendothelial permeability was measured. At t=0, 4 ml of media was added to the subluminal chamber. Following this at t=0, fluorescein isothiocyanate (FITC)-labelled dextran (40 kDa, Sigma-Aldrich) was added to the abluminal chamber (to give a final concentration of 250 μg/mL) and diffusion of dextran across the monolayer allowed to proceed at 37^{o}C for 2 h. 10nM uPA in final concentration was added to the abluminal chamber at t=0 for experiments that monitored acute effect of uPA on barrier integrity.

Media samples (28 μl) were collected every 30 min from the subluminal compartment. This was diluted with 372μl media and monitored in triplicate for FITC-dextran fluorescence at excitation and emission wavelengths of 490 and 520 nm, respectively (Perkin-Elmer Luminescence Spectrometer LS50B with microplate reader attachment). 100μl was loaded per well of a 96 well plate for this purpose. Trans Endothelial Exchange (%TEE) of FITC-dextran 40 kDa is expressed as the total subluminal fluorescence at a given time point (from 0-180 min) expressed as a percentage of total abluminal fluorescence at t=0 min.

2.4.8 Chemotaxis

A filter (8 μm pore size) for microchamber unit was treated with 100 μg/ml Collagen I (rat tail, Roche) for 2h at 37^{o}C. For this purpose initially the filter was washed in serum-free cell culture medium for a few seconds (10 ml of medium in a 10 cm Petri dish). After drying the filter it was immersed completely in 1.5 ml of the 100 μg/ml collagen I solution in a 10 cm Petri dish. Further to this 1.5 ml of collagen I solution was applied on the top of the filter in order to have both side treated. The Petri dish was placed at 37^{o}C for 2 h.

The solutions at appropriate final concentration of reagents (to be tested for their efficacy in enticing chemotaxis in cells) in serum-free media was prepared. The final concentration of the reagents used is given below.

uPA	10nM
SRSRY	1pM
D2A	1pM
D2A-Ala	100pM
VN	1μg/ml

After the 2 h incubation, the filter was dried. Meanwhile the solutions prepared was added in bottom well at a volume of 25μl. The filter was centered correctly onto the apparatus containg the lower wells. The upper part of the microchamber unit was immediately positioned and tightened with screws whilst holding tightly. The cells were then prepared by washing the cell monolayer with 1x PBS after removal of media. The cells were trypsinzed. Critically after trypsinization cells were subjected to two washes with 14 ml of serum-free medium. This was to remove any factors that might interfere with chemotaxis. Further to centrifugation cells were resuspended in 1 ml of serum-free medium. 5 x 10^5 cells were then seeded at 50 μl volume on the upper well. This entire procedure was performed expediently to avoid evaporation in the lower well. The cells were then allowed to migrate overnight in a tissue culture incubator. At the end of the migration period, the filter was fixed in 20% methanol (w/v). The cells were then stained in 10% (w/v) crystal violet in 20% methanol (w/v) for 10 min at room temperature. Further to this the filter was washed in distilled water and mounted on glass slide. Cells were counted under the microscope using 20X lens. For each well five fields were taken into account for counting. Each independent experiment conducted had at least three duplicates for each condition.

2.4.9 Wound Healing

Wound healing measures the ability of the cell to migrate and close introduced gap between cells. Ibidi® provide a 35mm dish (μ-slide$^{35\text{mm, high wall}}$) with high walls that enable to grow cells in precise chambers. These chambers are inserts,

which when removed introduce defined gaps. Cells were seeded according to the manufacturer's protocol. Post seeding, cells were allowed to achieve full confluence in full media. The inserts were washed with 1 x PBS twice and serum starved overnight. Following this, the insert was removed introducing the gap. Immediately following this petridish was washed twice with serum free media and image captured (t=0). The dish was incubated in the tissue culture incubator. Every 1 h image was captured upto 8 h, image was captured after overnight incubation. Due to the lack of serum, proliferation was avoided but migration occurred, resulting in closure of the gap was measured. The images were measured by means of a simple ruler using CELL software to quantitate. The mean measurements was plotted against control to generate fold difference.

2.4.10 Angiogenesis

2.4.10.1 Angiogenesis in Collagen I

Acetic acid 0.1M was used to prepare Collagen I 1.5 mg/ ml (rat tail, Sigma Aldrich). This preparation was mixed in the following preparation.

Collagen I 1.5 mg/ml	84.5%(w/v)
full serum media	8.45%(w/v)
1M NaOH	7.04%

The media for uPA based study in collagen gel was titrated to yield the most stringent result. This was 1.5% serum for HAECs in collagen gel. Less serum was detrimental to invasion while too much serum drowned the effects of uPA. The other area where stringency was evident was the number of cells to be seeded. Collagen gels typically required more cells, while Matrigel required much less cells. The cells required for stringency was even less, if the experimental situation required full serum media. Collagen solidifies at neutral pH and when in solution with acetic acid, it remains in liquid due to its

low pH. NaOH is utilized to neutralize the pH allowing collagen to solidify. It is also possible to use NaOH vapours to solidify collagen. Studies in collagen provided a one dimensional system to analyze with stringency, effects of overexperssion of various moesin plasmids.

100μl of the above mix was added per well of 96 well plate, which then solidified in 1 h. The above plate was then incubated with 100μl of treatment media in tissue culture incubator. The following day 20,000 cells were seeded onto this plate with fresh media. The following day, the matrix was observed for tube formation/elongation. Images were taken with 10x objective, four different fields were obtained per duplicate conditions. Each independent experiment constituted at least three different duplicates. As well, at least three independent experiments were conducted (Von Offenberg Sweeney *et al.*, 2005). Further to image capture, tube length was measured using CELL software, which calculated tube length of each tube. Graphs were plotted as fold difference in mean tube length.

2.4.10.2 Angiogenesis in Matrigel™

Studies in Matrigel™is much easier to conduct compared to collagen gel. Matrigel™, (BD Biosciences) is derived from Engelbreth-Holm-Swarm Mouse Tumor. Matrigel is widely used to study angiogenesis (Suarez *et al.*, 2007). This provides a mechanism to study angiogenesis, simulating tumour like conditions. An array of titrations were carried out to gain optimum serum concentrations to use with this batch of Matrigel™. Titration also took into account the optimum serum concentration that enabled to discern differences between various conditions used. Further to this, optimum incubation period that enable discerning differences between various conditions was validated. Gel was prepared according to the manufacturer's protocol. Prior to solidifying the gel, the plates were centrifuged at maximum speed for 10 s to even the meniscus and remove any air bubbles. Further to this, 8000 cells in media (0.5% serum) media was seeded onto the Matrigel. Cells were adjusted to equal stock vol-

ume for each condition prior to addition. In case addition of uPA was required, media added with the cells had uPA in final concentration of 10nM.

Otherwise the experiment was conducted according to manufacturer's protocol. Further to which, images were taken using 4x objective. The images were qualitative in nature. For discerning the difference, images were looked for web like formation which constitute angiogenesis. As well the ability of the webs to interconnect each other (Suarez *et al.*, 2007).

2.4.11 Cell Count

To analyze HAEC proliferation post transfection, cells were seeded at equal density of 10^3 cells per well after serum deprivation for 24 h. Cells were then counted using ADAM automatic cell counter® 24 h after. The technology uses propidium iodide based dye to intercalate nuclear DNA. This is then measured and processed by the machine giving a more precise count (Herrmann *et al.*, 1994). Similarly for routine cell count the same methodology was used.

2.4.12 Alamar Blue® methodology for proliferation

Alamar Blue® is a non toxic dye used to measure cell proliferation and health. The dye consists of resazurin (a non-fluorescent and blue in colour) which is converted to resorufin (fluorescent and bright red in colour) by the cells. The change is directly proportional to the number of metabolically active cells. Cells are plated at equal density in 96-well tissue culture plates post serum starvation. The media added with the cells contain 10% of the dye. As a control the same preparation is taken into account without cells. The cells are incubated for the period that the cells are monitored for cell proliferation. Following this, 200μl of the media is loaded onto a 96 well plate and absorbance of alamarBlue measured at 600nm and 570nm. The measurement is conducted using a spectrophotometer. Post measurement data were analyzed using $www.abdserotec.com/antibodies/\_705.html$ (Ahmed *et al.*, 1994).

2.4.13 Click-iT ® EdU Cell Proliferation Assays

This method is an alternative to the commonly used BrdU based cell cycle analyzis. BrdU uses nucleoside analog bromo-deoxyuridine (BrdU) while EdU uses 5-ethynyl-2´-deoxyuridine which is a nucleoside analog to thymidine (Lee *et al.*, 2008; Yu *et al.*, 2009; Buck *et al.*, 2008). This is then incorporated into DNA during the synthesis of DNA. For detection a copper catalyzed covalent reaction between an azide (Alexa Fluor® 488 dye) and an alkyne (which is the Edu) is utilized. The main difference in contrast to BrdU based method is the non necessity of DNA denaturation. Edu based method utilizes standard aldehyde-based fixation and detergent (saponin) permeabilisation. Further to this 7-AAD (7-Aminoactinomycin D) was incubated according to Click-iT ® protocol. 7-AAD binds with high affinity to GC region in DNA and is useful for monitoring cell cycle.

Due to their high sensitivity, EdU based method is useful for detection of cell cycle progress at an early stage (Buck *et al.*, 2008). The cells further to processing according to the manufacturer's protocol was monitored by FACS. For the detection of EdU with Alexa Fluor® 488 azide we used 488 nm excitation. Prior to starting the experimental procedures, compensation with various controls were done and a template created, validated for accurate measuerment of the cell cycle.

Cell synchronization to G_o for various experiments

For cell cycle analyzis for static cells, HAECs were seeded equally at 10^5 cells/cm^2 and allowed to reach confluence over 24 h. The cells were then synchronized by serum starvation for 24 h to G_o phase. The cells were labelled with EdU containing the full media 12 h prior to harvestation by trypsin. For cell cycle analyzis of cells subjected to cyclic strain followed by stasis, HAECs were seeded equally at 10^5 cells/cm^2 and allowed to reach confluence over 24 h. The cells were then synchronized by serum starvation for 24 h to G_o phase. The cells were labelled with EdU containing the full media prior to straining.

The cells were then strained for 4 h. The strain was stopped and the cells maintained in stasis for 6h following which the cells were trypsinized. HAECs were seeded equally at 10^5 cells/cm^2 and allowed to reach confluence over 24 h. The cells were then synchronized by serum starvation for 24 h to G_o phase. The cells were labelled with EdU containing the full media prior to straining. The cells were then strained for 12 h. The cells were harvested post strain by trypsinization. Following trypsinzation all cells were monitored by FACS post processing according to the Click-iT® .

2.4.14 Carboxy-fluorescein diacetate succinimidyl ester method

Carboxy-fluorescein diacetate succinimidyl ester (CFDA-SE) is a colorless and nonfluorescent dye which passively diffuses into cells. Its acetate groups are cleaved by intracellular esterases to yield highly fluorescent, amine-reactive carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well-retained and can be fixed with aldehyde fixatives. Excess unconjugated reagent and by-products passively diffuse to the extracellular medium, where they can be washed away. The dye - protein adducts that form in labeled cells are retained by the cells throughout development, mitosis, and *in-vivo* tracing. The label is inherited by daughter cells after cell division or cell fusion, and is not transferred to adjacent cells in a population (Hodgkin *et al.*, 1996).

For proliferation analyzis, 12 h post transfection HAECs were fed. A further 12 h later the cells were seeded equally at 10^4/cm^2 to bioflex plates. 8 h post seeding, they were stained with CFDA-SE. For staining the cells were washed once with PBS and 1 ml of 5 μM CFDA-SE, prepared in PBS, was added to each well for 5 min at 37^{o}C. Following incubation, CFDA-SE was replaced with fresh media and the cells allowed to recover for 1 h. Cyclic strain was applied after this for 24 h. Cells were harvested after, by trypsinization and centrifugation and washed twice with 1 ml ice-cold PBS (containing 0.1% BSA). Cells were then placed on ice and immediately analyzed using flow cytometry.

The concentration of the dye in the cells, which is inversely proportional to the rate of proliferation, was measured by flow cytometry (Becton Dickinson FACSCaliber) at an excitation peak of 492nm and emission peak of 517nm.

2.4.15 Flow Cytometry

Flow cytometry is a method for counting, and sorting microscopic particles suspended in a stream of fluid. FACS™(Fluorescent Activated Cell Sorting) is a specialised type of flow cytometry that measures the amount of light emitted by fluorescent molecules associated with individual cells. Lasers are used to excite the fluorescent molecules, which are excited at one range of wavelengths and emit at a second range. Filters in front of each of a series of detectors restrict the light that reaches the detector to only a small range of wavelengths (Tung *et al.*, 2007). Examples of the fluorescent molecules that can be detected are calcein AM, used with microparticle studies. This is used to measure the number of microparticles in supernatant suspension and carboxy-fluorescein diacetate succinimidyl ester (CFDA-SE), which can be used to measure proliferation. This is also used to measure Click-iT ® EdU based cell cycle analyzis.

Chapter 3

Haemodynamic regulation in the vasculature; the need for moesin

3.1 Introduction

Vascular cells are subjected to various stimuli such as the mechanical forces of cyclic strain and shear stress. This induces numerous molecular responses, which are still not fully characterized. The vasculature fine tuned by evolution has been purpose built, for the flow of blood, thus carrying vital nutrients to the far reaches of the body. The flow of blood and the associated haemodynamic forces induce the necessary functional responses, allowing self repair and remodeling according to the specific needs and challenges.

Functionally, it has been demonstrated that proliferation is increased by cyclic strain in endothelial cells. Research labs (including ours) have previously shown enhanced migration of endothelial cells previously subjected to cyclic strain. Cyclic strain is transduced across the vasculature and thus smooth muscle cells also experience cyclic strain. Proliferation in smooth muscle cells show

the opposite effect from endothelial cells and thus is decreased with cyclic strain. Inflammatory signals such as in areas with irregular cyclic strain (such as turbulent, oscillatory, low flow) would induce a differential proliferation response.

The *in-vitro* model that we use to subject cells to cyclic strain has been characterized extensively and is well published in the literature. The model has been extensively characterized with respect to migration, proliferation, angiogenesis and eNOS production, induced with cyclic strain. As endothelial nitric oxide synthase (eNOS) is increased by cyclic strain, the model is frequently assessed for this response. These functional responses due to cyclic strain, detailed in the introduction, has been published extensively.

It has been shown that integrins and their associated adapter proteins, including the FERM domain containing protein talin, have roles associated with cyclic strain. The forces are transmitted and modulated by these cytoskeletal proteins. Other FERM domain containing proteins such as, moesin, ezrin, radixin have not been studied to date. Though difficult to study due to high level of redundancy in mammalian cell model, the ERM proteins still might have roles specifically suited to the adaptive nature of the vasculature.

Moesin is highly expressed in endothelial cells compared to ezrin, while radixin is expressed at the lowest level of all three ERMs. Thus, moesin might have specific and key regulatory roles suited to the function of the vasculature. Rho kinase is activated in smooth muscle cells experiencing stretch, while conversely Rac is activated in endothelial cells. Rho kinase activates moesin by phosphorylation while Rac might dephosphorylate moesin.Furthermore, other research groups (including our lab) have recently noticed the presence of moesin in the nucleus.

This chapter looks into biochemically elucidating moesin with cyclic strain and shear stress mainly in endothelial cells and also in smooth muscle cells (cyclic strain). It is investigated whether moesin is translocated to the nucleus with cyclic strain. Further more, we look into mRNA, protein and also look into their correlation.We characterize if fibronectin coated plates or the widely

used synthetic derivative pronectin, coated plates are a better inflammatory model for cyclic strain. Functionally, we profile cell cycle soon after induction of cylic strain in endothelial cells. We see if our cyclic strain model induces migration as seen in literature. As well, moesin and actin is checked to see if indeed it does co-localise in the flexible membrane.

3.2 Results

3.2.1 Biochemical profile of moesin under cyclic strain mRNA, protein, phosphorylation status (endothelial cells).

In order to investigate sensitivity of moesin to mechanical stimuli, bovine aortic endothelial cells (BAECs) were subjected to cyclic strain (7.5%, 24 h) following which the cell's RNA and protein was harvested. The cDNA was analyzed using RT-PCR which showed increased mRNA of moesin with cyclic strain (60% fold) (Figure 3.1). Moesin protein though increased at a much higher level post cyclic strain (2.5 fold). The protein levels were confirmed to be similar in HAECs with moesin increasing hugely with cyclic strain. The phosphorylation state of moesin is upregulated with chronic strain over 24h (370% difference compared to static).

Similar investigation into sub-confluent cells, show an increase of moesin/ phosphorylated moesin with cylic strain in BAECs (50% difference for total moesin and 70% difference for phosphorylated moesin), albeit not to huge levels seen in confluent cells. The actual phosphorylation levels were also higher in static cells in sub-confluent cells.

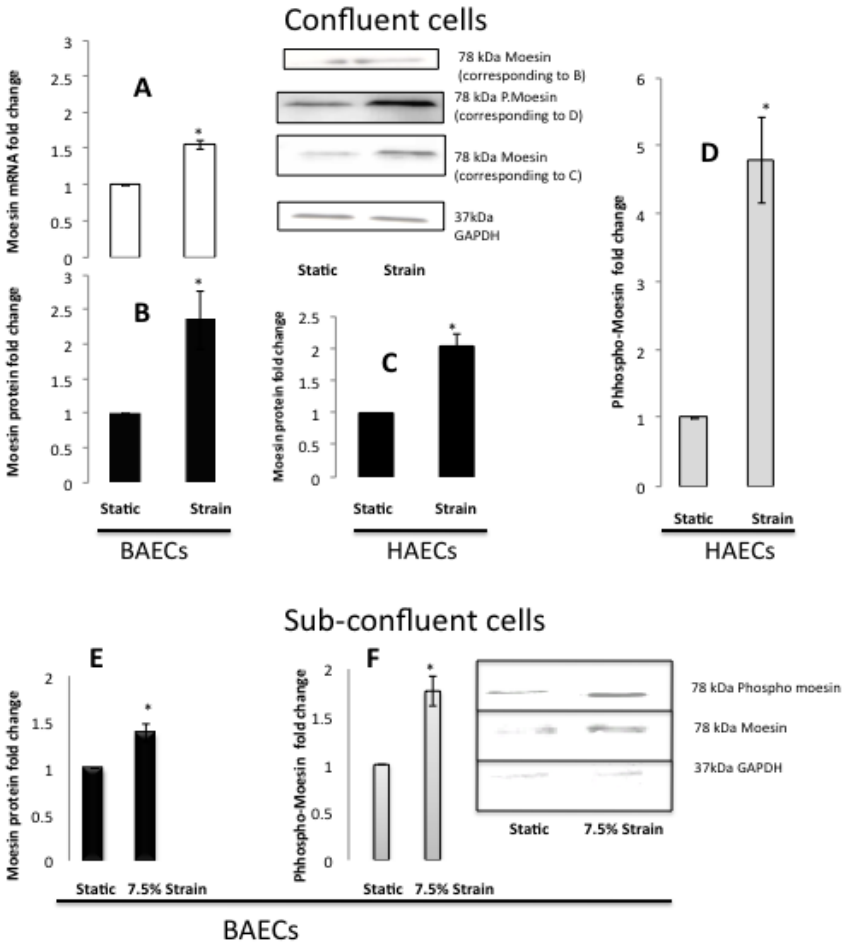

Figure 3.1: Effect of cyclic strain on Moesin in endothelial cells
Endothelial cells were exposed to cyclic strain (7.5% for 24 h) in (50 μg/ml) fibronectin coated bioflex® plates (HAECs) (Liu *et al.*, 2007). BAECs were grown on pronectin coated bioflex® plates. mRNA change was monitored by RT-PCR, while protein change was monitored by western blotting. Studies A to D were conducted on fully confluent cells seeded at 10^5 cells/cm^2. Studies E and F were conducted on subconfluent cells seeded at 10^4 cells/cm^2 (Liu *et al.*, 2007). A) Moesin mRNA change in BAECs. B) Moesin protein change in BAECs. C) Moesin protein change in HAECs. D) Phosphorylated Moesin change in HAECs. E) Moesin protein change in subconfluent BAECs. F) Phosphorylated Moesin change in subconfluent BAECs. Histograms represent fold change in band intensity relative to unstrained controls and are averaged from three independent experiments $\pm$ SEM; All values were controlled for equal loading by equalising for corresponding GAPDH. Representative blots are shown. $*P<0.05$ Statistically significant compared to static control.

3.2.2 Moesin translocated to the nucleus with cyclic strain

BAECs exposed to cyclic strain (24h) showed increase in moesin expression previously. The flexible membrane used for cyclic strain proved to be difficult in viewing with fluorescence microscopy. This was due to the silicone lubricant applied on the base. The material was not also very conductive to viewing with microscopy. We thus employed two different biochemical kits to fractionate the cells post cyclic strain (7.5%, 24 h) (Figure 3.2) (Cattaruzza *et al.*, 2004; Batchelor *et al.*, 2004).

Using Biovision based extraction method the cells were harvested into subcellular fractions. Simlarly to be conclusive, another kit from Calbiochem was utilized. The cells were fractionated post cyclic strain. There was clear translocation using both methods to the nucleus. The cells were fully confluent prior to the application of cyclic strain. We found no bands for moesin in the nucleus when no strain was applied at this concentration of protein.

The cytosolic fraction using Biovision based method yielded, increase (35% difference) in cytosolic fraction due to cyclic strain.

The group that published moesin's presence in nucleus used calyculin A at 300 nM for 30 min before harvesting the cells. This was to preserve the phosphorylation status of moesin with downstream processing. We followed the same protocol in terms of addition of 300nM calyculinA for 30 minutes prior to harvesting the cells. Thus cyclic strain was stopped very briefly for addition of calyculin A. Otherwise same method as was utilized for fractionation post cyclic strain (7.5%, 24 h, BAECs).

The results also clearly indicate moesin translocated to the nucleus, is phosphorylated. While there was no moesin present from these in confluent cells in the nucleus. Calbiochem method couldn't be used as acetone precipitation was found to affect the phosphorylation state of moesin.

The results from cytosolic fraction found moesin increased (25% difference) with cyclic strain to be phosphorylated.

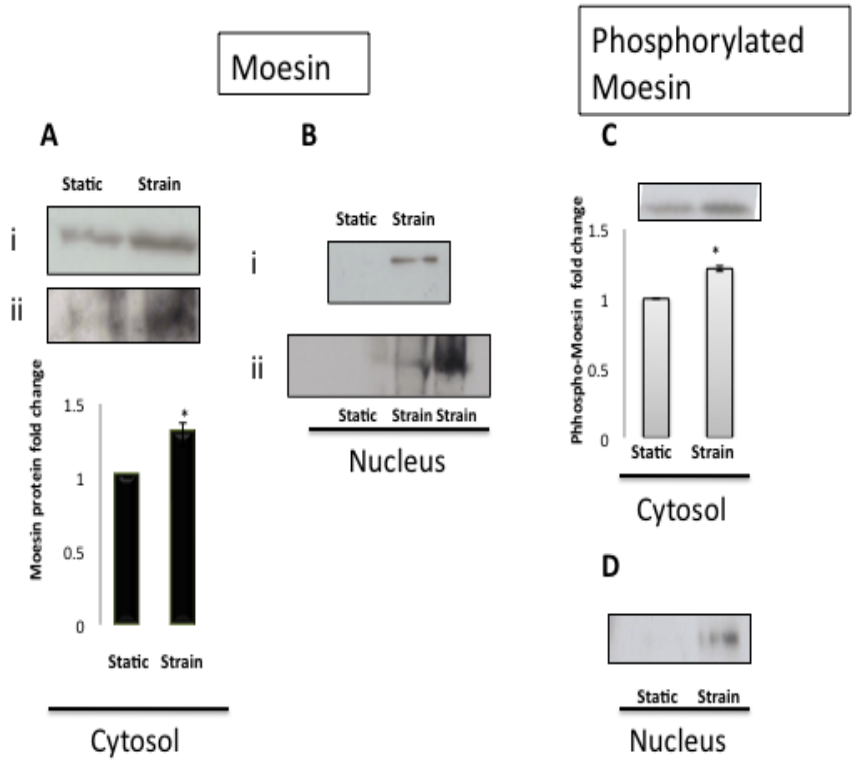

Figure 3.2: Moesin is translocated to nucleus with cyclic strain BAECs were exposed to cyclic strain (7.5% for 24 h) in pronectin coated bioflex® plates, the cells were then fractionated to cytosolic and nuclear fractions. Two different methods were employed for this purpose. Thirty minutes prior to harvesting the cells, it was treated with 300 nM calyculin (Batchelor *et al.*, 2004). This was to preserve the phosphorylation status of moesin with downstream processing. When, (representative blots labelled i, or not labelled) Biovision® based extraction method was utilized equal volume of each fraction was monitored by western blotting for Moesin and Phosphorylated Moesin. Further to (represetnative blots labelled ii) Calbiochem® based extraction method, each fraction was acetone precipitated, mixed in equal volume of sample buffer. Further to which, the fractions were monitored for Moesin by western blotting. A) Cytosolic fraction monitored for Moesin. B) Nuclear fraction monitored for Moesin. C) Cytosolic fraction monitored for Phosphorylated Moesin. D) Nuclear fraction monitored for Phosphorylated Moesin. Equal loading was monitored by ponceou staining. The fractions were verified for nuclear or cytosolic fraction by means of antibodies against GAPDH (positive for cytosolic fraction, negative for nuclear fraction); TATA binding protein (postive for nuclear fraction, negative for cytosolic fraction). Histograms represent fold change in band intensity relative to unstrained controls and are averaged from at least three independent experiments $\pm$ SEM; representative blots are shown $*P<0.05$ Statistically significant to static

3.2.3 Moesin protein change associated with cyclic strain in smooth muscle cells.

Smooth muscle cells were also investigated to understand moesin expression pattern with mechanical stimuli. Cyclic strain (7.5%, 24h) on bovine aortic smooth muscle cells reduced moesin protein expression (50%). There was no disparity between mRNA change and protein change (Figure 3.3). Protein change thus reflected the mRNA change in smooth muscle cells.

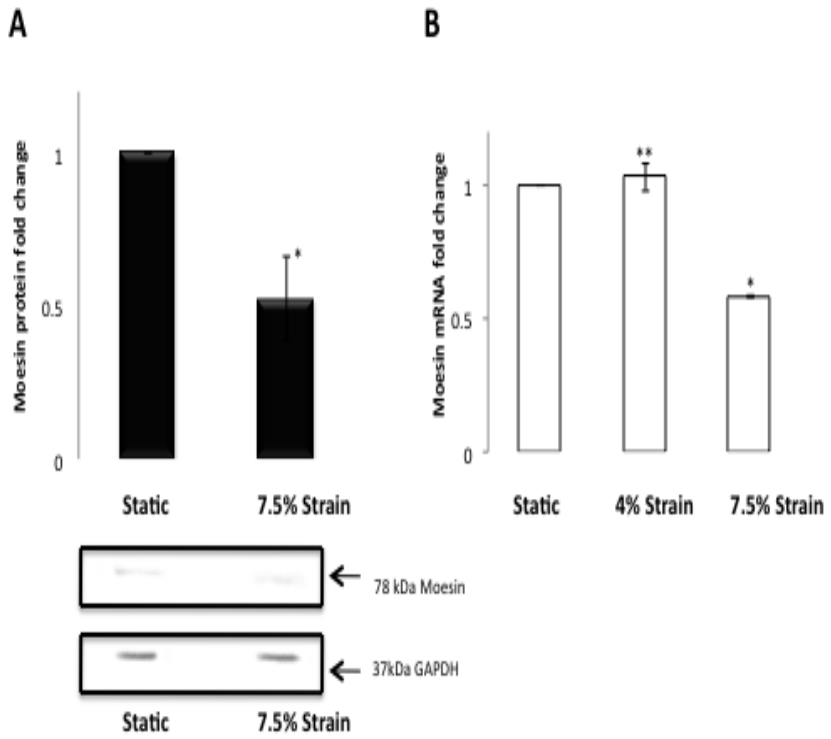

Figure 3.3: Effect of cyclic strain on Moesin in BASMCs
BASMCs were exposed to cyclic strain (7.5% and 4% for 24 h) and monitored for A) Moesin Protein expression by western blotting and B) Moesin mRNA change using Real-Time PCR. Histograms represent fold change in band intensity relative to unstrained controls and are averaged from three independent experiments $\pm$ SEM; All values were controlled for equal loading by equalising for corresponding GAPDH. Representative blots are shown. $*P<0.05$ Statistically significant, $**$ not statistically significant

3.2.4 Effect of shear on moesin expression

Moesin expression was also investigated with respect to shear stress in endothelial cells. Shear stress measured in dyne/cm^2 was subjected on HAECs at 10dynes/cm^2. This resulted in reduced moesin protein expression (60%) (Figure 3.4). The reduced expression pattern was in contrast to the previously studied cyclic strain, wherein moesin expression increased. There was a similar paradigm with respect to mRNA. Where there was a considerable increase in moesin mRNA (140%).

mRNA expression profile of the highly redundant ERM proteins provide interesting insights. Moesin mRNA expression was substantially increased under shear stress (10 dynes, 24h) using microfludic ibidi® system. Meanwhile ezrin mRNA did not change significantly with shear. This was in contrast to the huge shift in moesin and radixin (300%).

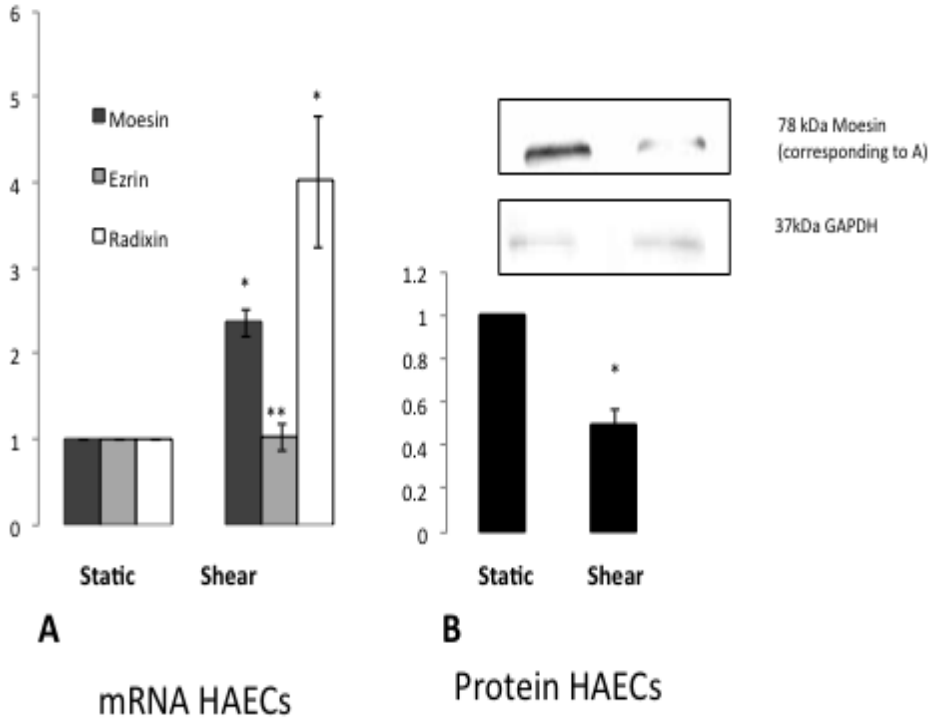

Figure 3.4: Moesin protein change associated with shear stress
HAECs were exposed to 10 dynes/cm^2 shear stress for 24 h using ibidi® microslide vi for mRNA and orbital methodology of shear for protein. Following this it was monitored for A) (moesin, ezrin, radixin) mRNA and B) (moesin) protein by RT-PCR and western blotting respectively. Histograms represent fold change in band intensity relative to unsheared controls and are averaged from three independent experiments $\pm$ SEM ; All values were controlled for equal loading by equalising for corresponding GAPDH. All values are controlled for equal loading by equalising for GAPDH under same conditions. Representative blots are shown. $*P<0.05$ Statistically significant.

3.2.5 Moesin-GFP distribution under shear stress

In order to understand shear stress induced moesin translocation, using standard fluorescence microscopy Moesin-GFP arrangement post shear (10dynes/cm^2, 24 h) was monitored in BAECs. Moesin-GFP and GFP (on control cells) was transfected in using lipofectamine. Under static conditions there is an uneven distribution adhering to no particular pattern (Figure 3.5). There is a higher localization of moesin across the cell. Compared to this, there is a an even distribution of moesin post shear. Moesin takes on a brushed distribution aligning in the direction of the flow. GFP control cells meanwhile show no change between static and shear.

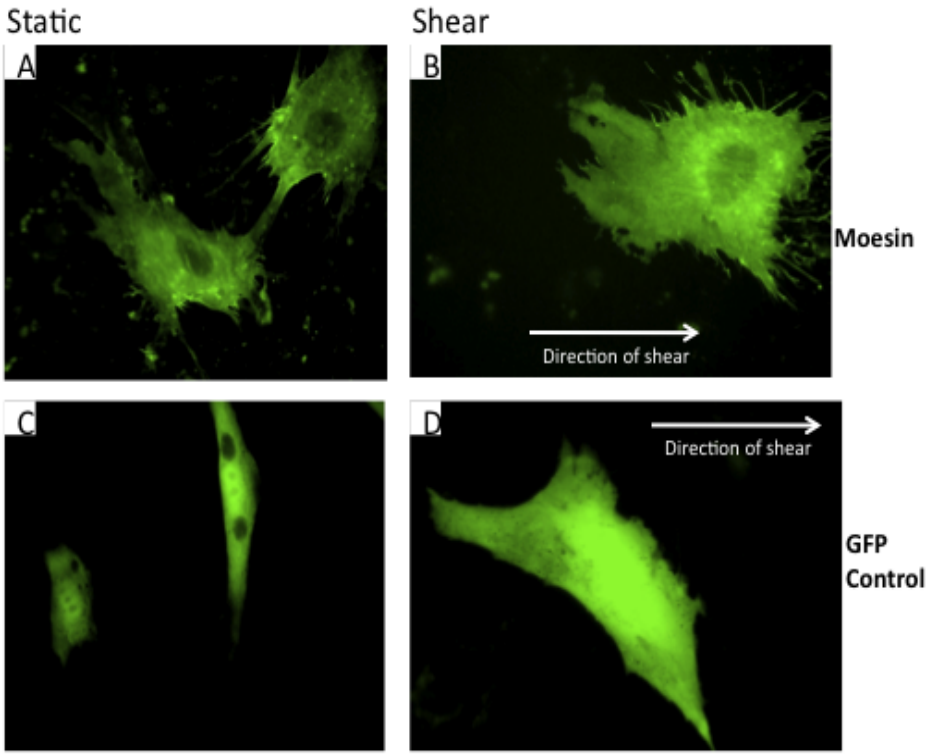

Figure 3.5: Moesin-GFP showing peripheral alignment with shear
BAECs were exposed to constant laminar shear of 10 dynes/cm^2 for 24 h further to transfecting in Moesin-GFP and GFP control with lipofectamine A) MoesinGFP (no shear, 1000X magnification). B) MoesinGFP (sheared, 1000X magnification). C) GFP control (no shear, 1000X magnification). D) GFP control (sheared, 1000X magnification). Images are representatives of three independent experiments.

3.2.6 Moesin-GFP redistribution under shear stress

Using microfluidic ibidi® flow system HAECs were subjected to shear at 5.1 dynes/ cm^2. Prior to this, Moesin-GFP was electroporated in, utilizing the high efficiency electroporator amaxa. The cells were subjected to shear stress for 10 minutes. With fastprocessing of the videograph, redistribution of moesin due to shear stress can be clearly noted. The force of 5.1 dynes/cm^2 was used to reduce the distortion on the microscope. The video can be viewed at (www.youtube.com/watch?v=BcOC7PZrK1U). Alternately, the video can be viewed at $http://www.youtube.com/user/mishanv$. The figure representing this, is Figure 3.6.

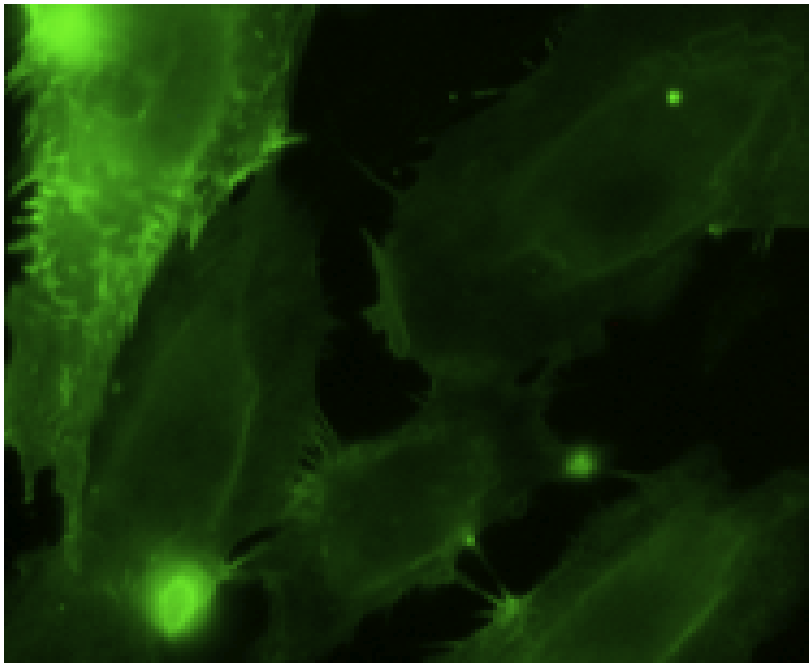

Figure 3.6: Live visualization of Moesin-GFP peripheral alignment with shear
HAECs were exposed to constant laminar shear of 5.1 dynes/cm^2 for 24 h further to electroporating in Moesin-GFP and grown on ibidi® microslide Y. The region of the slide that undergoes laminar shear was subjected to videography. Moesin-GFP rearrangement in cells were monitored by live microscopy for 10 minutes. The magnification is 1000X. The video in actual time can be viewed at (www.youtube.com/watch?v=BcOC7PZrK1U). Alternately, the video can be viewed at $www.youtube.com/user/mishanv$

3.2.7 Comparison of cell cycle profile Fibronectin versus Pronectin coated flexible plates

This study compares the cell cycle profile between cells grown on fibronectin and pronectin. Changes in cell cycle in HAECs was compared when grown on different matrices fibronectin and pronectin (Figure 3.7). The coated plates were made of the silicone membrane, referred to as Bioflex® plates . An improved method to Brdu referred to as Click it® was used to monitor this with FACS.

The data is expressed as fold difference compared to cells grown on pronectin coated plates. This showed no significant difference in apoptosis, G_0/G_1, S phase between cells on fibronectin and pronectin. There was a significant difference in the G_2/M phase between the two.

The effect of a pathogenic cyclic strain was also monitored, wherein we introduced cyclic strain for 4h followed by stasis (Shown in the same figure; (Figure 3.7). Six hours after stasis, the cells were analyzed with FACS. The effect of a denuded cyclic strain was used as a model to compare cell cycle differences between fibronectin and pronectin coated plates.

The cell cycle trend of cells grown on fibronectin when compared to pronectin is similar with exception of apoptosis. The differences are more profound when pronectin plates are used, except for G_2/M phase. With cells grown on fibrnoectin plates, effect on G_2/M phase of cell cycle is profound.

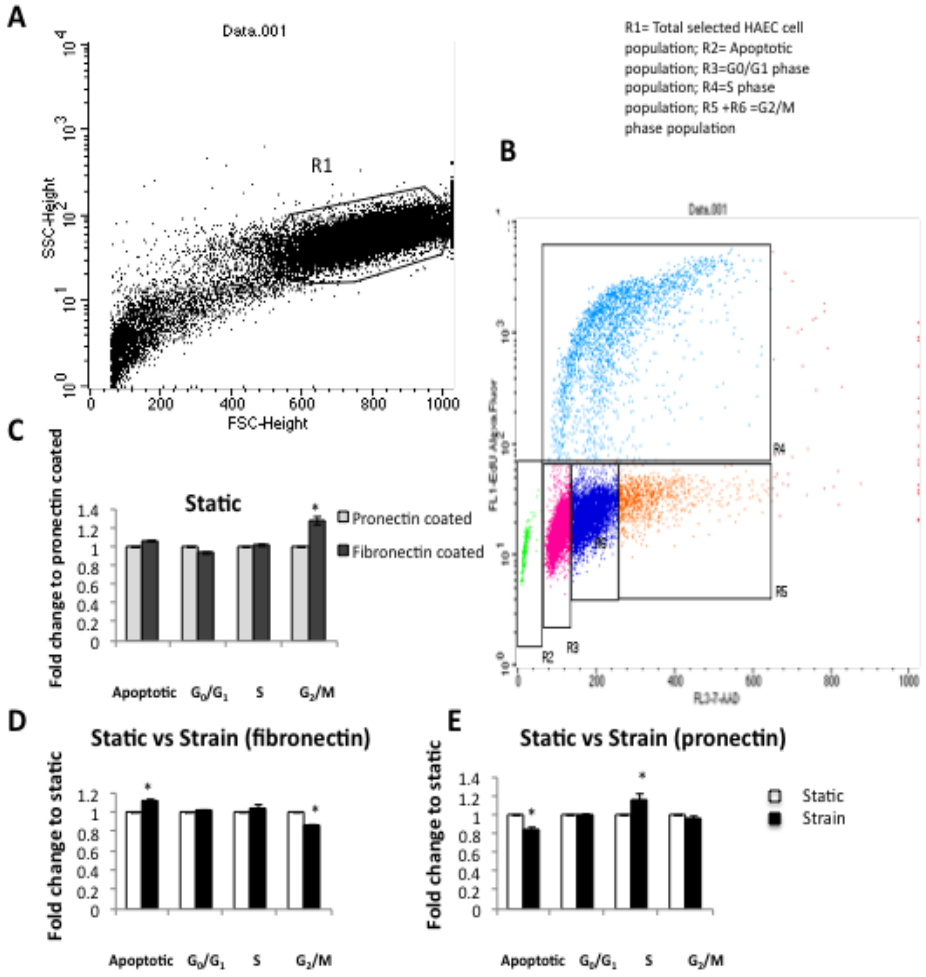

Figure 3.7: Cell cycle progression due to fibronectin and pronectin
HAECs were seeded equally at 10^5 cells/cm^2 and allowed to reach confluence over 24 h on either pronectin coated bioflex® plates or fibronectin(50 μg/ml) coated bioflex® plates. The cells were then synchronized by serum starvation for 12 h to G_0 phase. The cells were labeled with EdU® (according to the "Click-iT® EdU" manufacturer's guidelines) containing the full media 24 h prior to harvestation by trypsin. For experiments requiring cyclic strain, labeling was done prior to the application of strain for 4 h. The strain was stopped and the cells maintained in stasis for 6h. The cells were then monitored for cell cycle change by FACS. A) Representative of cell population selected. B) Representative FACS analysis. C) 12 h cell cycle change due to fibronection coated plates controlled to pronectin coated plates. D) Strain versus Static in fibronectin coated plates E) Strain versus Static in pronectin coated plates Histograms represent fold change and are averaged from three independent experiments $\pm$ SEM $*$ P<0.05 Statistically significant compared to pronectin coated

3.2.8 Effect of cyclic strain on cell migration

This study was conducted to understand the effect of cyclic strain on the migratory apparatus of a cell. HAECs grown to confluency on fibronectin coated plates were subjected to cyclic strains of 5% and 10% for 24h. These cells post harvestation, was monitored over night across membrane in boyden chamber. The data is expressed as fold change compared to static.

Cyclic strain increased the migration of cells (Figure 3.8). As well, it showed a dose based increase in cell migration. Thus, 10% cyclic strain regime compared to 5% (3 fold change) moulded the cells to migrate at a much higher rate (17 fold change).

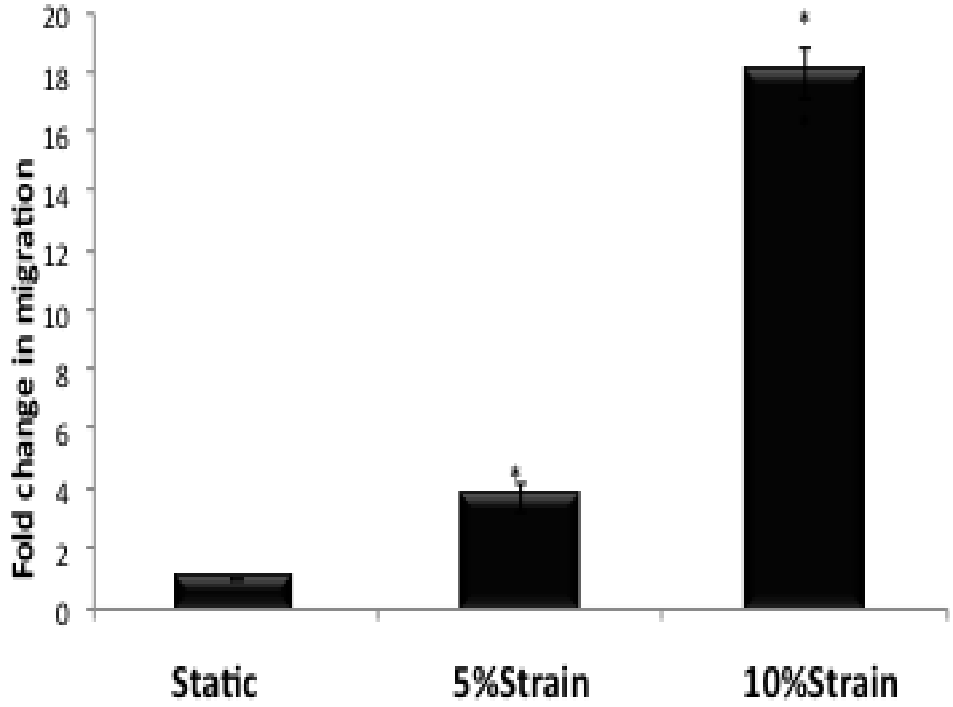

Figure 3.8: Cyclic strain increase migration of HAECs
HAECs were seeded equally at 10^5 cells/cm^2 and allowed to reach confluence over 24 h in (50 μg/ml) fibronectin coated bioflex® plates. Cyclic strain was then applied for 24 h after which cells were harvested with versene ®, washed and seeded in serum free media. The cells were then allowed to migrate over boyden chamber to full serum media. Histograms represent fold change in mean migration measured by counting number of endothelial cells that has migrated. Atleast five different fields were counted, and the experiment repeated at least three times. The cells were visualized by means of crystal violet. $*P<0.05$ Statistically significant compared to static

3.2.9 Effect of acute cyclic strain on cell cycle

In this study different parameters of acute cyclic strain (12 h) are compared. HAECs were grown to confluency on fibronectin plates. The flexible membranes were subjected to different cyclic strain regimes. The study profiled the effect of 10% and 8-10% regimes, on cell cycle with induction of cyclic strain (Figure 3.9). The effect of cyclic strain on proliferation in endothelial cells grown on fibronectin was analyzed.

8-10% showed no significant differences when compared to static cells. 10% cyclic strain induces a very different profile compared to static cells immediately on induction of cyclic strain.

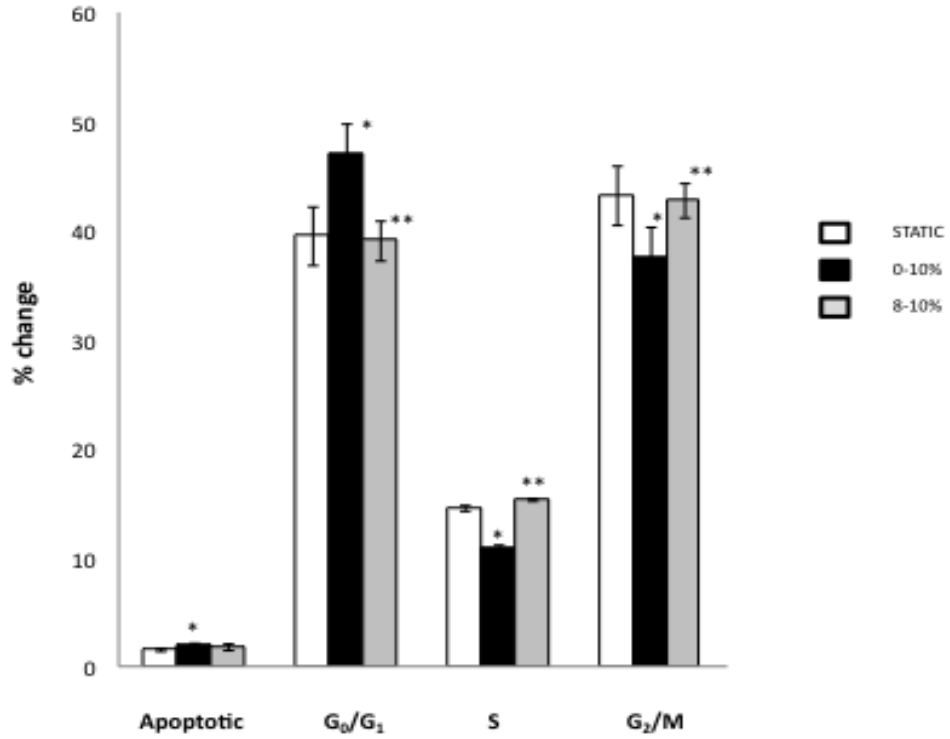

Figure 3.9: Effect of acute cyclic strain (12 h) on cell cycle HAECs were seeded equally at 10^5 cells/cm^2 and allowed to reach confluence over 24 h in (50 μg/ml) fibronectin coated bioflex® plates. The cells were then synchronized by serum starvation for 24 h to G_0 phase. The cells were labelled with EdU® (according to the "Click-iT® EdU" manufacturer's guidelines) containing the full media prior to straining. The cells were then strained for 12 h. The cells were harvested post strain and monitored for cell cycle by FACS. Histograms represent percentage change and are averaged from three independent experiments $\pm$ SEM $*$ P$<$0.05 Statistically significant compared to static control $**$P$<$0.05statistically significant compared to 0-10%

3.2.10 Conclusion

In this chapter the studies clearly demonstrate that moesin is sensitive to mechanical stimuli. In addition, there is a differential regulation of moesin with shear stress and cyclic strain.

Furthermore, similar mechanical stimuli (cyclic strain) regulates moesin differentially in different vascular beds. Moesin is also translocated to the nucleus with cyclic strain. We also characterize proliferation, migration induced by various cyclic strain.

Chapter 4

microRNA regulation of moesin under mechanotransduction

4.1 Introduction

In the previous chapter, we found a disparity between moesin expression at the mRNA and protein levels in cells that were mechanotransduced via cyclic strain and shear stress. There could be many different reasons for this lack of correlation between the mRNA and proteins. These could involve either post transcriptional or post translational modifications. As explained in depth in the introduction, moesin is a likely candidate for both scenarios.

We nevertheless decided to investigate the possibility that microRNA might interfere with moesin expression. It is known that at least a third of human genes could be under the conrol of microRNA (Selbach *et al.*, 2008). Suarez *et al*'s classic study into Dicer down regulation has unearthed diverse functions affected with respect to vasculature (Suarez *et al.*, 2007).

In-silico analysis was conducted to generate microRNAs that shared the 7

nucleotide seed region with moesin. As explained in the introduction unlike conventional importance for nucleotide paring, microRNAs need only a 7 nucleotide complimetary region to repress (or even activate) (Vasudevan *et al.*, 2007). This is the widely held view, though increasingly the proximity of the microRNA is found to be of importance as well. Further to this we exploited a proprietary new technology by Applied Biosystems ®. This lets you analyze an array of microRNA relevant to vascular cells and profile their expression using RT-PCR.

We wanted to see if moesin expression changes with Dicer downregulation. We also wanted to investigate whether RhoGTPases control moesin dephosphorylation with cyclic strain. Further to this there will be interesting implications if we find dephosphorylation status of moesin was under microRNA control. With respect to proliferation, Suarez *et al* has shown endothelial cell proliferation reduced with dicer down regulation(Suarez *et al.*, 2007). We wanted to elucidate if proliferation and also endothelial microparticle release, with mechanotransduction is under microRNA regulation. Detailed investigation into endothelial microparticles and moesin's role in EMP release is detailed in the next chapter.

Aim:

To profile micrRNAs affected and their change with shear stress and cyclic strain in HAECs. To investigate whether moesin expression is affected with chronic cyclic strain. To investigate moesin's phosphorylation status and regulation with acute cyclic strain. Similarly confirm moesin's expression pattern with chronic shear stress. See if endothelum is able to sense shear stress, and take on the charachteristic physiological arrangement i.e, in the direction of flow. To investigate what other functions were under the control of microRNA regulation.

4.2 Results

4.2.1 Insilico analyzis of microRNAs for determination of moesin as a target

Here we explore the microRNAs that potentially regulate moesin. Using in-silico analyzis using the bioinformatic tool at $http://microrna.sanger.ac.uk$ provided the microRNAs hsa miR 489, hsa miR 221, hsa miR 215, hsa miR-542-3p, hsa miR-767-5p. Their seed region's alignment and p value are significant towards moesin mRNA. This is shown in (Figure 4.1).

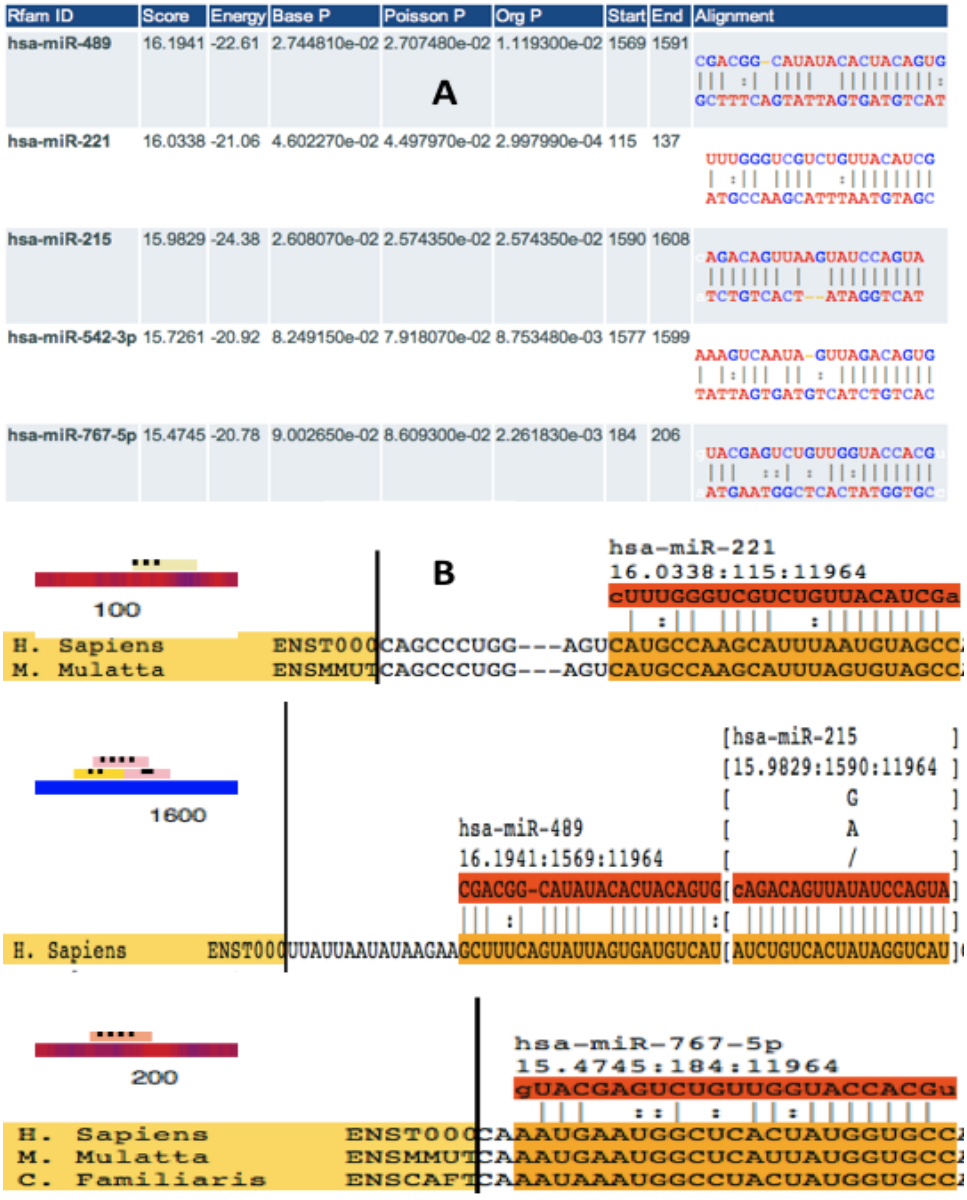

Figure 4.1: MicroRNAs with moesin as probable target
In silico analyzis was carried out using data from http://microrna.sanger.ac.uk A) MicroRNAs predicted to target moesin with high probablity, their alignment to moesin mRNA (note that microRNAs don't align fully but, rather targets the mRNA utilizing a few complimentary seed region). B) Their alignment with respect to moesin and evaluation of their interspecies conservation.

4.2.2 microRNAs of interest regulated with mechanotrasduction

Here we investigate the mechano-regulation of the microRNAs complimentary to moesin mRNA. Previously identified microRNAs that possibly target moesin were found to be regulated with mechanotransduction. There are other microRNAs listed that might be of interest, which is also regulated with mechanotransduction. We find here that hsa-miR-221 (4.4 fold) and hsa-miR-489 (2.9) are both increased with cyclic strain. With shear stress hsa-miR-221 increase (1.5 fold), while hsa-miR-489 is not present anymore. The figure also shows the methodology used for isolation of microRNA, verification and expression analyzis. This is shown in (Figure 4.2).

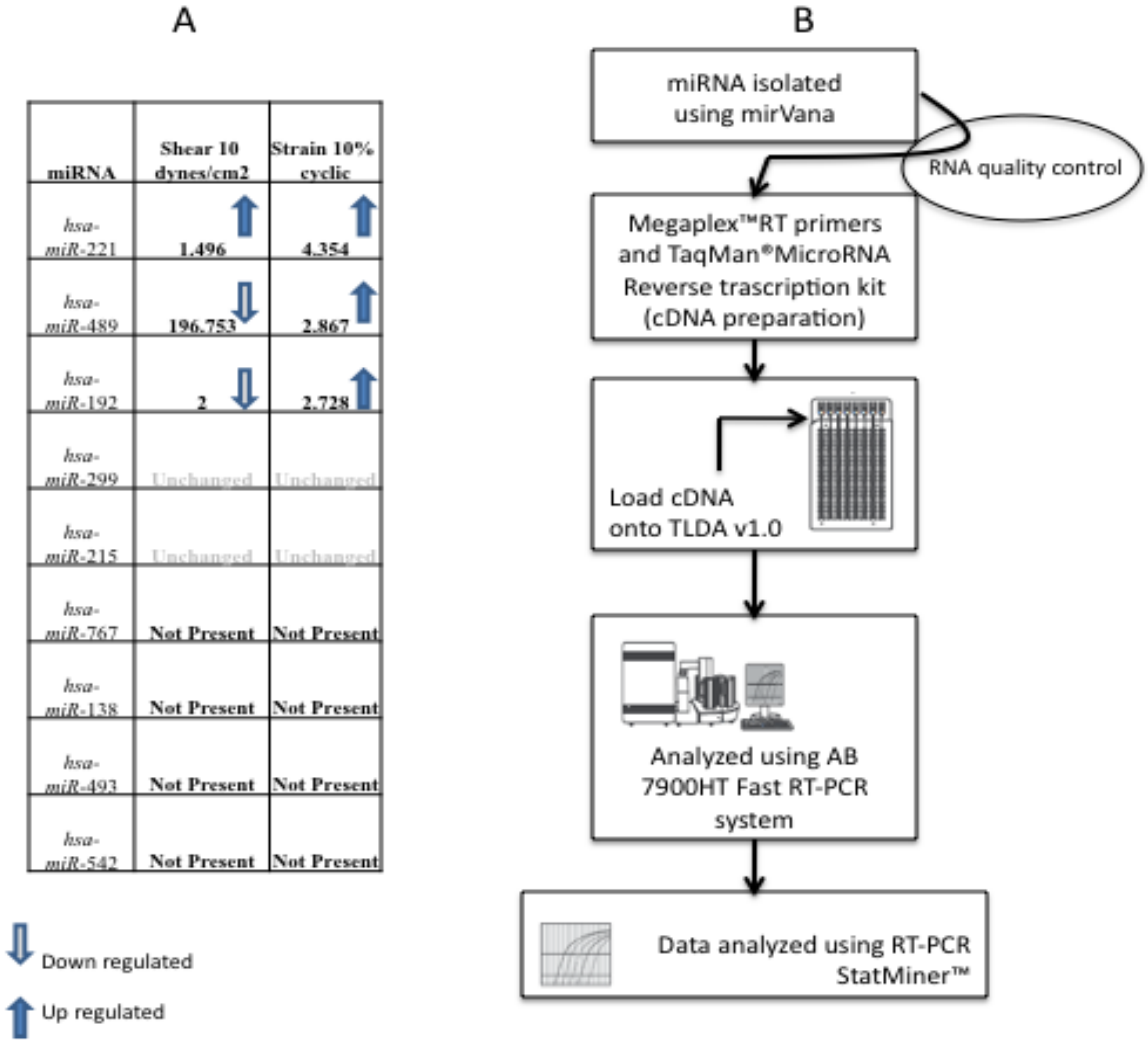

Figure 4.2: MicroRNA change with shear stress and cyclic strain
HAECs were exposed to laminar shear of 10 dynes/cm^2 and cyclic strain (10%) for 24 h. Cyclic strain was carried out using pronectin coated bioflex plates. Further to microRNA being extracted it was subjected to quality control using a Nano drop 1000 spectrprophotometer and Agilent Microfluidic Bio-analyzer 2100 (Agilent technologies). TLDA v1.0 card was loaded with prepared cDNA and analyzed utilizing RT-PCR using AB 7900HT Fast RT-PCR system. In silico analyzis was carried out using http://microrna.sanger.ac.uk. Data was analyzed using RT-PCR StatMiner. A) Change in a selection of microRNAs(including the previously identified microRNAs with moesin as possible target) with shear and cyclic strain (fold difference to static). B) Outline of the process utilizing TLDA v1.0 card.

4.2.3 microRNAs upregulated with cyclic strain

Our investigation also led to the identification of an array of microRNAs up-regulated with cyclic strain. This is shown in (Figure 4.3).

Static vs 10% Strain

microRNA	Fold Difference	microRNA	Fold Difference	microRNA	Fold Difference
hsa-miR-519e-4373267	345.1099	hsa-miR-26a-4373070	2.6305428	hsa-miR-551a-4380929	10.771076
hsa-miR-519d-4373266	329.69852	hsa-miR-517c-4373264	2.61268	hsa-miR-626-4380966	10.66952
hsa-miR-521-4373259	86.47126	hsa-miR-328-4373049	2.4800103	hsa-miR-600-4380963	10.641705
hsa-miR-485-5p-4373212	82.878105	hsa-miR-19b-4373098	2.4009106	hsa-miR-29a-4373065	8.665357
hsa-miR-96-4373010	43.449364	hsa-miR-24-4373072	1.9780768	hsa-miR-210-4373089	5.9375515
hsa-miR-149-4373128	43.389435	hsa-miR-200a-4373273	1.5824093	hsa-miR-19a-4373099	5.4044394
hsa-miR-103-4373158	43.371197	hsa-miR-31-4373190	1.579103	hsa-miR-601-4380965	4.124224
hsa-miR-30a-3p-4373062	43.359615	hsa-miR-221-4373077	1.4967916	hsa-miR-99b-4373007	3.922831
hsa-miR-140-4373138	43.347237	hsa-miR-518b-4373246	1.4410692	hsa-miR-15b-4373122	3.5242774
hsa-miR-381-4373020	21.495382	hsa-miR-296-4373066	1.3015991	hsa-miR-106b-4373155	3.3021252
hsa-miR-519c-4373251	21.420092	hsa-miR-515-3p-4373241	1.2245839	hsa-miR-575-4381020	3.1993234
hsa-miR-184-4373113	10.852667	hsa-miR-222-4373076	1.2183377	hsa-miR-21-4373090	2.993132
hsa-miR-544-4380919	10.797082	hsa-miR-224-4373187	22.004553	hsa-miR-99a-4373008	2.8302479
hsa-miR-520g-4373257	1.1844572	hsa-miR-301-4373064	21.955618		
hsa-miR-125a-4373149	1.1068379	hsa-miR-342-4373040	21.88965		
hsa-miR-16-4373121	1.1054837	hsa-miR-518c-4378082	21.882938		
hsa-miR-650-4381006	1.081578	hsa-miR-148b-4373129	21.813152		
hsa-miR-518d-4373248	21.406786	hsa-miR-553-4380931	21.791649		
hsa-miR-362-4378092	21.188906	hsa-miR-193b-4373185	21.7225		
hsa-miR-449-4373207	21.125803	hsa-miR-194-4373106	21.6105		
hsa-miR-500-4373225	20.881224	hsa-miR-345-4373039	21.556055		
hsa-miR-324-3p-4373053	20.839909	hsa-miR-376a-4378104	21.498053		
hsa-miR-614-4380990	20.49742				
hsa-miR-20a-4373286	13.927899				
hsa-miR-93-4373012	12.650005				
hsa-miR-193a-4373107	12.628182				
hsa-miR-302c-4378072	11.017218				
hsa-miR-152-4373126	11.015317				

Figure 4.3: Upregulated microRNAs under cyclic strain.
HAECs were exposed to cyclic strain (10%). Cyclic strain was carried out using pronectin coated bioflex plates. Further to microRNA being extracted it was subjected to quality control using a Nano drop 1000 spectrprophotometer and Agilent Microfluidic Bio-analyzer 2100 (Agilent technologies). TLDA v1.0 card was loaded with prepared cDNA and analyzed utilizing RT-PCR using AB 7900HT Fast RT-PCR system. In silico analyzis was carried out using http://microrna.sanger.ac.uk. Data was analyzed using RT-PCR StatMiner. Values are expressed as positive fold change compared to static control. This corresponds to upregulation of microRNAs.

4.2.4 microRNAs upregulated with shear stress

The investigation also led to the identificatin of an array of microRNAs upregulated with shear stress. This is shown in (Figure 4.4).

Static vs 10 dynes/ cm^2

microRNA	Fold Difference	microRNA	Fold Difference	microRNA	Fold Difference
hsa-miR-196a-4373104	1.9311714	hsa-miR-526b-4378080	23.452225	hsa-miR-657-4380922	696.03406
hsa-miR-126-4378064	1.9191885	hsa-miR-642-4380995	22.593906	hsa-miR-149-4373128	373.98755
hsa-miR-486-4378096	1.836432	hsa-miR-487b-4378102	22.304123	hsa-miR-324-3p-4373053	349.69495
hsa-miR-30e-5p-4373058	1.8328658	hsa-miR-19a-4373099	15.115925	hsa-miR-223-4373075	181.99768
hsa-miR-594-4380958	1.79502	hsa-miR-591-4380955	11.760793	hsa-miR-518d-4373248	177.12056
hsa-miR-646-4381002	1.6921682	hsa-miR-99b-4373007	9.779538	hsa-miR-152-4373126	93.22238
hsa-miR-100-4373160	1.6579003	hsa-miR-575-4381020	9.721761	hsa-miR-30a-3p-4373062	91.97808
hsa-miR-515-5p-4373242	1.6554067	hsa-miR-193a-4373107	8.17428	hsa-miR-301-4373064	91.38536
hsa-miR-181b-4373116	1.5946747	hsa-miR-210-4373089	7.84533	hsa-miR-339-4373042	90.93895
hsa-miR-24-4373072	1.5745689	hsa-miR-93-4373012	6.115616	hsa-miR-520f-4373256	50.261654
hsa-miR-484-4381032	1.4844468	hsa-miR-146a-4373132	6.0524087	hsa-miR-96-4373010	45.82008
hsa-miR-572-4381017	1.4494225	hsa-miR-644-4380999	5.8623605	hsa-miR-34c-4373036	45.81257
hsa-miR-222-4373076	1.3844278	hsa-miR-21-4373090	5.1502576	hsa-miR-374-4373028	45.689056
hsa-let-7c-4373167	1.3705213	hsa-miR-133b-4373172	4.7533255	hsa-miR-381-4373020	45.18041
hsa-miR-197-4373102	1.2987561	hsa-miR-221-4373077	4.3541074	hsa-miR-586-4380949	44.7312
hsa-miR-202-4378075	1.2127199	hsa-miR-99a-4373008	4.2037134	hsa-miR-432-4373280	44.175476
hsa-let-7b-4373168	1.1784751	hsa-miR-496-4373221	4.047563	hsa-miR-345-4373039	23.890219
hsa-miR-659-4380924	1.1558864	hsa-miR-145-4373133	2.4997413		
hsa-let-7g-4373163	1.1412042	hsa-miR-630-4380970	2.3500931		
hsa-miR-30e-3p-4373057	1.0863243	hsa-miR-26a-4373070	2.2864676		
hsa-miR-565-4380942	1.0617158	hsa-miR-650-4381006	2.2815523		
hsa-miR-31-4373190	1.0484308	hsa-miR-125a-4373149	2.1741154		
hsa-miR-331-4373046	1.0369698	hsa-miR-422b-4373016	2.165498		
hsa-miR-423-4373015	3.943463	hsa-miR-19b-4373098	2.0408816		
hsa-miR-545-4380918	3.881376	hsa-miR-517b-4373244	2.028293		
hsa-miR-328-4373049	3.802022				
hsa-miR-518b-4373246	3.7978134				
hsa-miR-296-4373066	3.5837991				
hsa-miR-30d-4373059	2.9198005				
hsa-miR-181c-4373115	2.8312826				
hsa-miR-192-4373108	2.7286916				
hsa-miR-216-4373083	2.6057336				

Figure 4.4: Upregulated microRNAs under laminar shear
HAECs were exposed to laminar shear at 10 dynes/cm^2. Further to microRNA being extracted it was subjected to quality control using a Nano drop 1000 spectrprophotometer and Agilent Microfluidic Bio-analyzer 2100 (Agilent technologies). TLDA v1.0 card was loaded with prepared cDNA and analyzed utilizing RT-PCR using AB 7900HT Fast RT-PCR system. In silico analyzis was carried out using http://microrna.sanger.ac.uk. Data was analyzed using RT-PCR StatMiner™. Values are expressed as positive fold change compared to static control. This corresponds to upregulation of microRNAs.

4.2.5 microRNAs downregulated with cyclic stain

Majority of microRNAs investigated meanwhile was found to be downregulated with cyclic strain. This is shown in (Figure 4.5) and (Figure 4.6).

Static vs 10% Strain

microRNA	Fold Difference	microRNA	Fold Difference	microRNA	Fold Difference
hsa-miR-517b-4373244	1.063888075	hsa-miR-524-4378087	6.127199059	hsa-miR-549-4380921	12.59096437
hsa-miR-518e-4373265	1.075970165	hsa-miR-142-5p-4373135	6.194249432	hsa-miR-202-4373274	12.59326255
hsa-miR-214-4373085	1.120302571	hsa-miR-105-4373157	6.194679576	hsa-miR-383-4373018	12.67081847
hsa-miR-18a-4373118	1.130249679	hsa-miR-218-4373081	6.207167441	hsa-miR-137-4373174	12.73328897
hsa-miR-631-4380971	1.133011788	hsa-miR-653-4381012	6.260522373	hsa-miR-378-4373024	14.85826478
hsa-miR-20b-4373263	1.139202711	hsa-miR-622-4380961	6.279755049	hsa-miR-196b-4373103	16.82085673
hsa-let-7a-4373169	1.142080398	hsa-miR-143-4373134	6.282262559	hsa-miR-216-4373083	1.30787415
hsa-miR-30a-5p-4373061	1.19982461	hsa-miR-146a-4373132	6.290789875	hsa-miR-565-4380942	1.333843858
hsa-miR-26b-4373069	1.240630295	hsa-miR-199a-4378068	6.350974138	hsa-miR-646-4381002	1.37378534
hsa-miR-423-4373015	1.241409293	hsa-miR-135b-4373139	6.372471738	hsa-miR-594-4380958	1.392801035
hsa-miR-126-4378064	1.261423292	hsa-miR-30e-5p-4373058	7.791356814	hsa-miR-486-4378096	1.461498787
hsa-miR-331-4373046	1.276333146	hsa-miR-30e-3p-4373057	8.635322432	hsa-miR-181d-4373180	1.607581871
hsa-miR-145-4373133	2.479040088	hsa-miR-630-4380970	9.386705515	hsa-miR-186-4373112	1.624490138
hsa-miR-30b-4373290	2.530346445	hsa-miR-564-4380941	3.042082342	hsa-let-7b-4373168	1.768058954
hsa-miR-100-4373160	2.551678702	hsa-miR-125b-4373148	3.146790244	hsa-miR-572-4381017	1.770034749
hsa-miR-484-4381032	2.598312344	hsa-miR-365-4373194	3.329219971	hsa-miR-496-4373221	1.813395443
hsa-miR-520a-4373268	2.605584607	hsa-miR-432-4378076	3.629305627	hsa-miR-491-4373216	1.937594949
hsa-miR-516-5p-4378099	5.996746166	hsa-miR-425-5p-4380926	3.721207852	hsa-miR-517a-4373243	2.052050128
hsa-miR-548d-4381008	6.073582546	hsa-miR-197-4373102	4.08079584	hsa-miR-192-4373108	2.102295778
hsa-miR-373-4373279	11.31622908	hsa-miR-133b-4373172	4.152665264	hsa-miR-30c-4373060	2.163872581
hsa-miR-199a-4373272	11.76072661	hsa-miR-659-4380924	4.475693738		
hsa-miR-141-4373137	11.96390919	hsa-miR-181c-4373115	4.729379915		
hsa-miR-196a-4373104	12.10288835	hsa-miR-155-4373124	4.913779146		
hsa-miR-485-3p-4378095	12.14883489	hsa-miR-130b-4373144	4.979696036		
hsa-miR-520f-4373256	12.15818627	hsa-miR-433-4373205	5.274777953		
hsa-miR-323-4373054	12.16175573	hsa-miR-126-4373269	5.527880223		
hsa-let-7f-4373164	12.16780227	hsa-miR-563-4380940	5.78617112		
hsa-miR-373-4378073	12.2008236	hsa-miR-520c-4373253	5.886258993		
hsa-miR-337-4373044	12.2112818				
hsa-miR-502-4373227	12.2434185				
hsa-miR-488-4373213	12.25025107				
hsa-miR-613-4380989	12.25112603				
hsa-miR-429-4373203	12.27921058				

Figure 4.5: Down regulated microRNAs under cyclic strain
HAECs were exposed to cyclic strain (10%) for 24 h. Cyclic strain was carried out using pronectin coated bioflex® plates. Further to microRNA being extracted it was subjected to quality control using a Nano drop 1000 spectrprophotometer and Agilent Microfluidic Bio-analyzer 2100 (Agilent technologies). TLDA v1.0 card was loaded with prepared cDNA and analyzed utilizing RT-PCR using AB 7900HT Fast RT-PCR system. In silico analyzis was carried out using http://microrna.sanger.ac.uk. Data was analyzed using RT-PCR StatMiner™. Values are expressed as negative fold change compared to static control. This corresponds to downregulation of microRNAs.

Static vs 10% Strain

microRNA	Fold Difference	microRNA	Fold Difference
hsa-miR-518a-4373186	24.87118838	hsa-miR-92-4373013	18.72480905
hsa-miR-569-4380946	24.91660723	hsa-let-7c-4373167	20.24648475
hsa-miR-591-4380955	24.95608353	hsa-miR-425-4373202	23.21935956
hsa-miR-545-4380918	25.35270493	hsa-miR-185-4373181	23.60669731
hsa-miR-151-4373179	25.54709748	hsa-miR-644-4380999	24.43329417
hsa-miR-191-4373109	28.24292303	hsa-miR-618-4380996	24.60905138
hsa-miR-501-4373226	48.33738489	hsa-miR-422a-4373200	24.71416829
hsa-miR-452-4378077	49.52400007	hsa-miR-606-4380974	24.74563528
hsa-miR-649-4381005	49.63359488	hsa-miR-195-4373105	24.75262229
hsa-miR-520d-4373254	49.84833644	hsa-miR-515-5p-4373242	24.84297563
hsa-miR-130a-4373145	67.84498479		
hsa-miR-566-4380943	92.08603414		
hsa-miR-320-4373055	93.75869026		
hsa-miR-422b-4373016	95.12244899		
hsa-miR-490-4373215	98.26957112		
hsa-miR-532-4380928	99.33985685		
hsa-miR-181b-4373116	100.1546287		
hsa-miR-489-4373214	196.7537597		
hsa-miR-633-4380979	200.623055		
hsa-miR-206-4373092	251.9466469		
hsa-miR-204-4373094	1534.775244		
hsa-miR-211-4373088	1559.318503		

Figure 4.6: Down regulated microRNAs under cyclic strain
HAECs were exposed to cyclic strain (10%) for 24 h. Cyclic strain was carried out using pronectin coated bioflex plates. Further to microRNA being extracted it was subjected to quality control using a Nano drop 1000 spectrprophotometer and Agilent Microfluidic Bio-analyzer 2100 (Agilent technologies). TLDA v1.0 card was loaded with prepared cDNA and analyzed utilizing RT-PCR using AB 7900HT Fast RT-PCR system. In silico analyzis was carried out using http://microrna.sanger.ac.uk. Data was analyzed using RT-PCR StatMiner. Values are expressed as negative fold change compared to static control. This corresponds to downregulation of microRNAs.

4.2.6 microRNAs downregulated with shear stress

Simiarly, majority of microRNAs investigated was found to be downregulated with shear stress. This is shown in (Figure 4.7).

Static vs 10 dynes/ cm^2

microRNA	Fold Difference	microRNA	Fold Difference	microRNA	Fold Difference
hsa-miR-26b-4373069	3.026152158	hsa-miR-196b-4373103	23.22547608	hsa-miR-517c-4373264	1.468977113
hsa-miR-501-4373226	3.455404719	hsa-miR-433-4373205	23.25669338	hsa-miR-30c-4373060	1.527089887
hsa-miR-320-4373055	3.520175074	hsa-let-7a-4373169	23.37926736	hsa-miR-16-4373121	1.552752596
hsa-miR-633-4380979	3.625128901	hsa-miR-452-4378077	23.43100266	hsa-miR-106b-4373155	1.580540487
hsa-miR-425-4373202	4.027364978	hsa-miR-649-4381005	23.48285493	hsa-miR-155-4373124	1.630295481
hsa-miR-181d-4373180	4.049735121	hsa-miR-130a-4373145	23.61960531	hsa-miR-520a-4373268	1.72137542
hsa-miR-618-4380996	4.926976542	hsa-miR-211-4373088	42.19327389	hsa-miR-518e-4373265	1.771037759
hsa-miR-29a-4373065	5.073511632	hsa-miR-566-4380943	43.5681913	hsa-miR-532-4380928	1.823387856
hsa-miR-373-4373279	5.353985726	hsa-miR-490-4373215	46.49371377	hsa-miR-569-4380946	1.963320462
hsa-miR-199a-4373272	5.564288397	hsa-miR-214-4373085	47.30836682	hsa-miR-378-4373024	2.02140637
hsa-miR-141-4373137	5.660418904	hsa-miR-425-5p-4380926	92.683199	hsa-miR-125b-4373148	2.045410778
hsa-miR-200a-4373273	5.704774063	hsa-miR-204-4373094	726.1398435	hsa-miR-195-4373105	2.060427221
hsa-miR-15b-4373122	5.70995072	hsa-miR-520g-4373257	1.103355466	hsa-miR-520d-4373254	2.123823227
hsa-miR-365-4373194	5.742473383	hsa-miR-518c-4373247	1.105101447	hsa-miR-564-4380941	2.317168135
hsa-miR-323-4373054	5.754024653	hsa-miR-622-4380961	1.127509484	hsa-miR-185-4373181	2.531007371
hsa-let-7f-4373164	5.756885681	hsa-miR-92-4373013	1.305059208	hsa-miR-186-4373112	2.56030904
hsa-miR-142-5p-4373135	5.771700946	hsa-miR-485-3p-4378095	1.314750648	hsa-miR-206-4373092	2.720867953
hsa-miR-515-3p-4373241	5.771731261	hsa-miR-126-4373269	1.378770818	hsa-miR-563-4380940	2.737579465
hsa-miR-373-4378073	5.772509555	hsa-miR-105-4373157	1.43192348	hsa-miR-520c-4373253	2.784933665
hsa-miR-337-4373044	5.777456757	hsa-miR-601-4380965	1.432581429	hsa-miR-516-5p-4378099	2.837207663
hsa-miR-20a-4373286	5.790716555	hsa-miR-432-4378076	1.433008104	hsa-miR-142-3p-4373136	5.919551402
hsa-miR-18a-4373118	5.792186988	hsa-miR-489-4373214	2.86707728	hsa-miR-376a-4373026	5.927131724
hsa-miR-502-4373227	5.792661416	hsa-miR-548d-4381008	2.873560741	hsa-miR-549-4380921	5.957094149
hsa-miR-488-4373213	5.795894223	hsa-miR-524-4378087	2.898928005	hsa-miR-202-4373274	5.958180958
hsa-miR-491-4373216	5.850477817	hsa-miR-30b-4373290	2.925671958	hsa-miR-383-4373018	5.994875341
hsa-miR-517a-4373243	5.855832564	hsa-miR-218-4373081	2.936763034	hsa-miR-137-4373174	6.024431358
hsa-miR-550-4380954	5.865533352	hsa-miR-653-4381012	2.962006959	hsa-miR-30a-5p-4373061	7.527249018
hsa-miR-449b-4381011	5.885434832	hsa-miR-143-4373134	2.972292439	hsa-miR-422a-4373200	11.6928868
hsa-miR-1-4373161	5.887006447	hsa-miR-199a-4378068	3.004801733	hsa-miR-606-4380974	11.70776071
hsa-miR-134-4373141	5.903826199	hsa-miR-135b-4373139	3.014972898	hsa-miR-191-4373109	11.7623714
hsa-miR-627-4380967	5.91592127	hsa-miR-613-4380989	5.796308447	hsa-miR-518a-4373186	11.76716176
hsa-miR-511-4373236	5.822560045	hsa-miR-130b-4373144	5.801904754	hsa-miR-151-4373179	12.08696718
hsa-miR-190-4373110	5.829970315	hsa-miR-20b-4373263	5.804436912	hsa-miR-631-4380971	5.846967096
hsa-miR-554-4380932	5.830447893			hsa-miR-429-4373203	5.809596291

Figure 4.7: Downregulated microRNAs under laminar shear
HAECs were exposed to laminar shear at 10 dynes/cm^2. Further to microRNA being extracted it was subjected to quality control using a Nano drop 1000 spectrprophotometer and Agilent Microfluidic Bio-analyzer 2100 (Agilent technologies). TLDA v1.0 card was loaded with prepared cDNA and analyzed utilizing RT-PCR using AB 7900HT Fast RT-PCR system. In silico analyzis was carried out using http://microrna.sanger.ac.uk. Data was analyzed using RT-PCR StatMiner™. Values are expressed as negative fold change compared to static control. This corresponds to upregulation of microRNAs.

4.2.7 Moesin and dicer are downregulated robustly with siRNA

Further to optimization with different siRNAs against Dicer and Moesin, two of them were chosen that provided robust downregulation of both proteins. In comparison to the control (scrambled sequence) siRNA, they both provide upto 60% downregulation. We find levels of moesin and dicer remain dowregulated even after 48 h. (Figure 4.8).

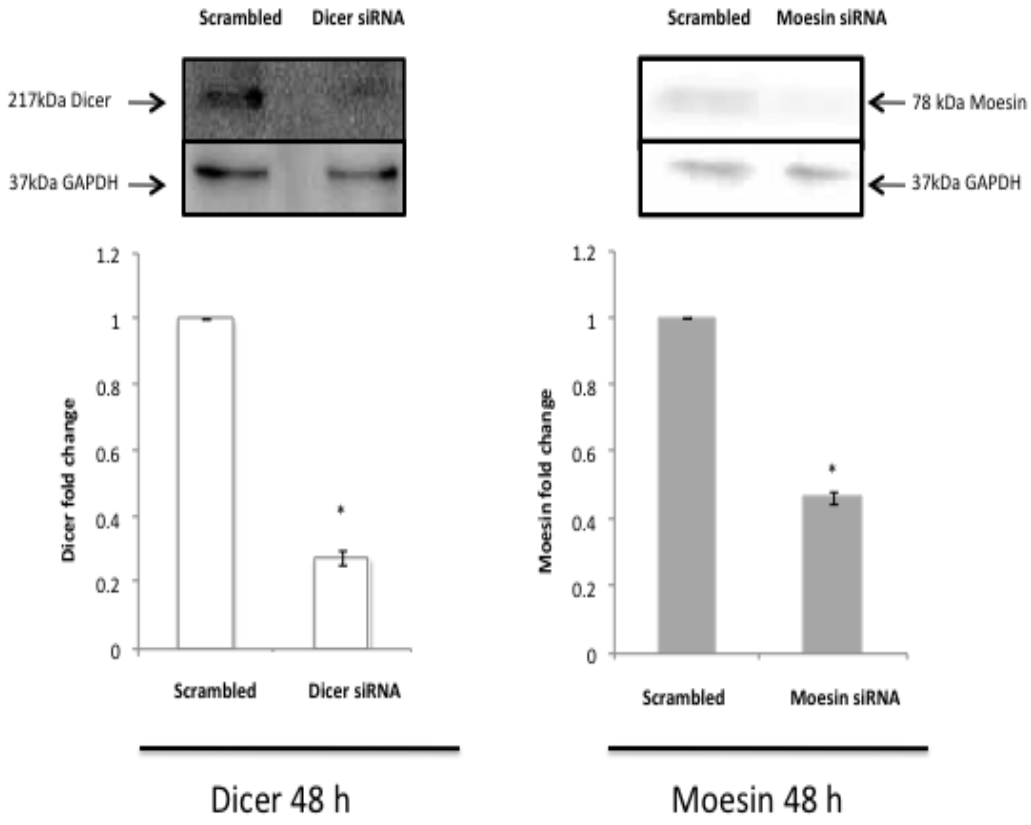

Figure 4.8: Moesin and dicer are downregulated robustly with siRNA
HAECs at 10^6 cells were electroporated with siRNA against dicer and moesin and controlled with scrambled. This was seeded onto three wells of a six well plate. Cells were fed after 24 h, and further grown to 48 h for dicer and 72 h for moesin. Protein was then harvested and monitored for both dicer and moesin utilizing western blotting.
Histograms represent fold change in band intensity relative to scrambled controls and are averaged from three independent experiments $\pm$ SEM; All values are controlled for equal loading by equalising for GAPDH under same conditions. Prior to this it was also controlled for poncoue staining immediately after protein transfer. Representative blots are shown.$*P<0.05$ Statistically significant compared to static

4.2.8 Moesin protein expression with cyclic strain is regulated by microRNA system

Here we investigate whether moesin is regulated by microRNAs (directly or indirectly) during cyclic strain. Moesin's protein expression previously found to be regulated by cyclic strain was reflected in this study. Whereby we see an increase in moesin protein expression with cyclic strain (200%). As well, the investigation led to conclusive evidence of microRNA impacting moesin's actual protein expression (50% decrease in moesin protein compared to static control). Protein as well as phosphorylated moesin (50% reduction compared to static) was found unable to reach the four fold increase with cyclic strain when dicer was knocked out. This is shown in (Figure 4.9).

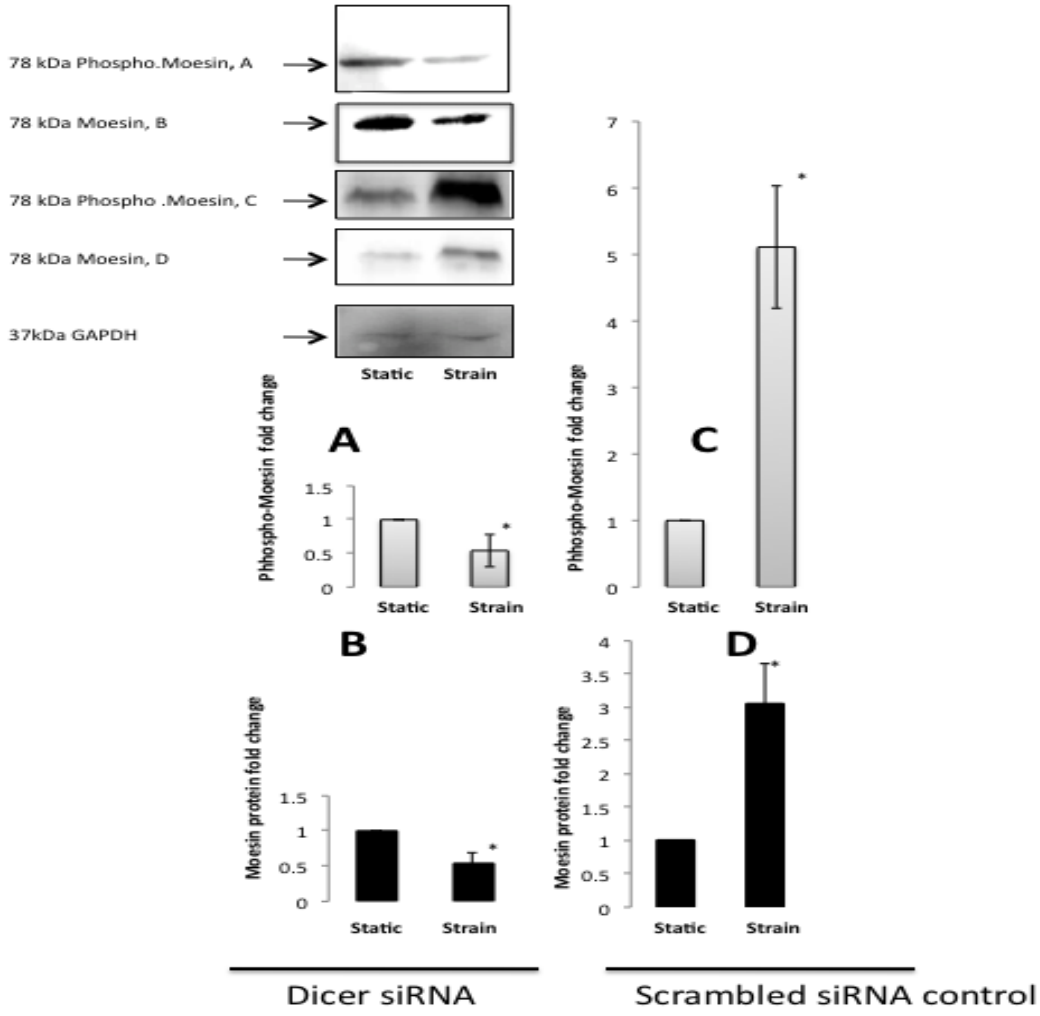

Figure 4.9: Moesin protein change with dicer downregulation in HAECs with cyclic strain

HAECs at 10^6 cells were electroporated with siRNA against dicer/scrambled. This was seeded onto three wells of a pronectin coated bioflex plate. Cells were fed after 24 h, after which a further 12 h was past before starting the cyclic strain (7.5%). The cells were monitored 24 h post cyclic strain for the following. A) Phospho moesin change in dicer siRNA treated sample. B) Total moesin change in dicer siRNA treated sample. C) Phospho Moeisn change in scrambled siRNA treated sample. D) Total moesin in scrambled treated sample Histograms represent fold change in band intensity relative to unstrained controls and are averaged from three independent experiments $\pm$ SEM; All values are controlled for equal loading by equalising for GAPDH under same conditions. Prior to this it was also controlled for poncoue staining immediately after protein transfer. Representative blots are shown.$*P<0.05$ Statistically significant compared to static

4.2.9 Moesin protein expression with shear stress is regulated by microRNA system

Here we investigate whether moesin is regulated by microRNAs (directly or indirectly) during shear stress. Regulation of moesin with microRNA was found to be not just regulated by cyclic strain. Mechanotransduction in general, profoundly is regulated by microRNAs when it comes to moesin. This study shows shear stress, similarly having an opposing effect when dicer is knocked out, with moesin protein expression similar to static. This is shown in (Figure 4.10).

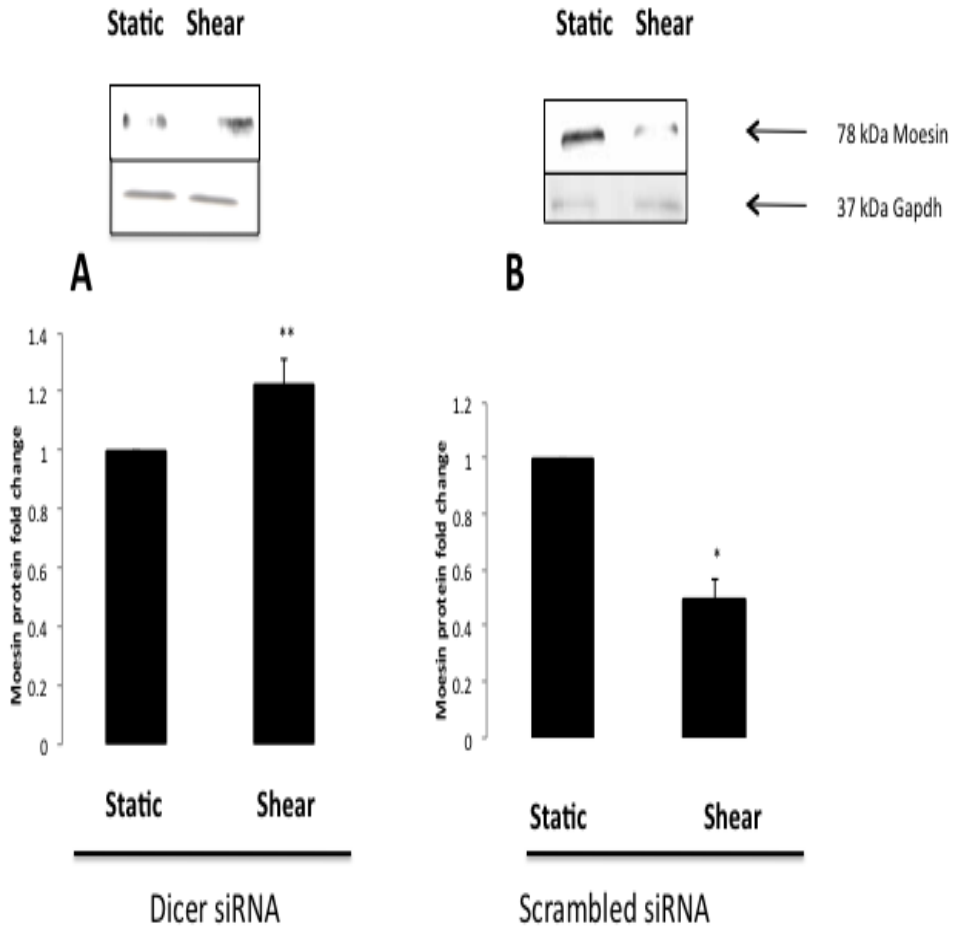

Figure 4.10: Moesin protein change with Dicer downregulation with shear stress in HAECs HAECs at 10^6 cells were electroporated with siRNA against dicer/scrambled. This was seeded per three wells of a 6-well plate. After feeding the cells after 24 h a further 12 h was past before starting the shear stress regime of 10dynes/cm^2. The cells were monitored for the moesin protein change 24 h post shear stress. A) Dicer downregulation B) Scrambled downregulation. Histograms represent fold change in band intensity relative to static controls and are averaged from three independent experiments $\pm$SEM; All values are controlled for equal loading by equalising for GAPDH under same conditions. Prior to this equal loading was also controlled for poncoue staining immediately after protein transfer. Representative blots are shown. $**$ $P<0.07$ not statistically significant compared to static, $*P<0.05$ Statistically significant compared to static

4.2.10 Phospho Moesin with acute stretch is regulated by Rho A and microRNA

Here we investigate the phosphorylation status of moesin with acute stretch (2min-10min). Further to this we also investigate whether the signaling molecules regulating moesin is effected by microRNAs.

Acute stretch induces dephosphorylation of moesin in minutes. Two minutes of stretch decreases moesin phosphorylation by 40%. This we find is regulated by Rho A and microRNAs. Dicer knockdown and Rho inhibition both increase moesin phosphorylation 1.5 fold. The similar effect also calls into question whether Rho A is regulated by microRNAs. And whether, Rho A is the reason for moesin not dephosphorylating with stretch when microRNA machinery is down. This is shown in (Figure 4.11).

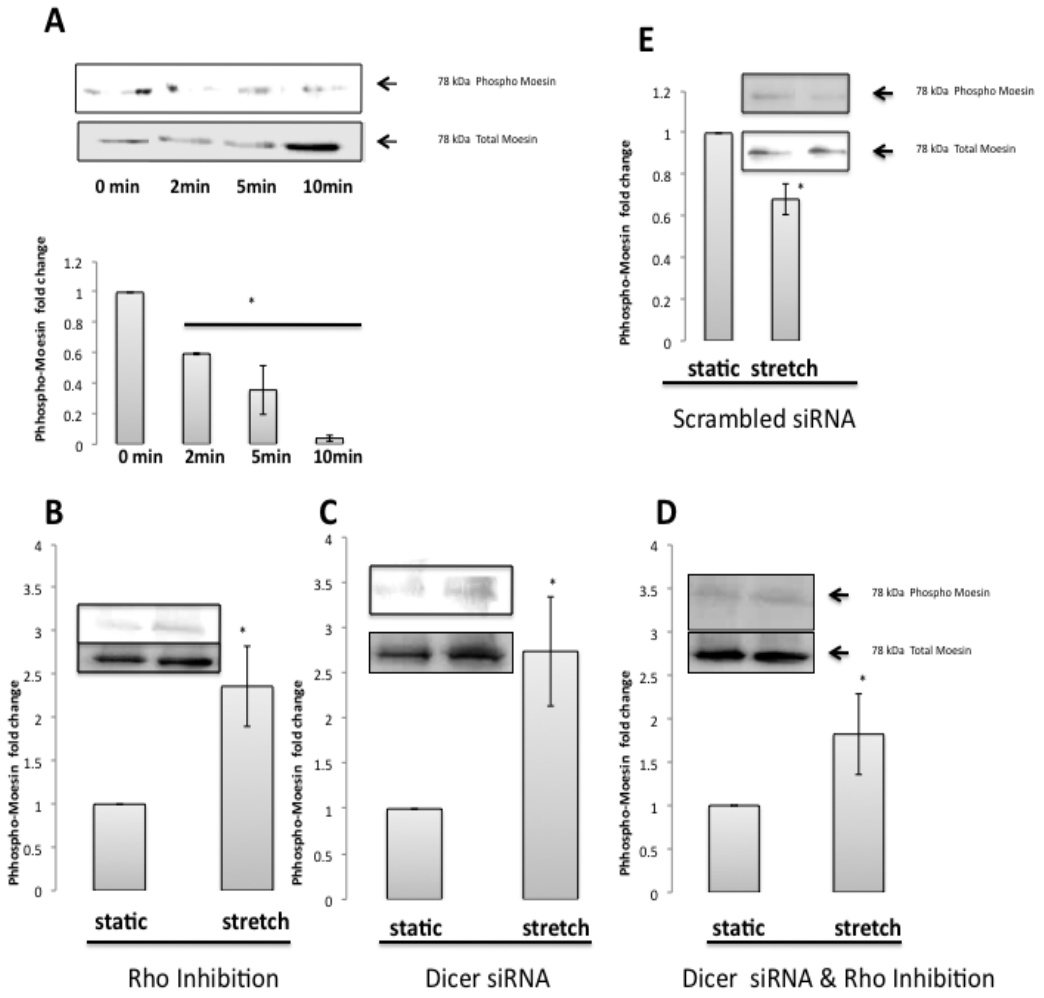

Figure 4.11: Rho and dicer regulation of phospho moesin with acute stretch in HAECs

A) Confluent HAECs was serum starved for 24 h. These were subjected to non cyclical static stretch biaxially (20%). This was applied acutely over 0min, 2min, 5min and 10min. B) Cells treated with highly cell permeable C3 transferase (Rho A inhibitor) and simlarly stretched for 2 min. C) Cells with siRNA against Dicer and was similarly stretched for 2 min. D) Cells with siRNA against Dicer was treated with highly cell permeable C3 transferase (Rho A inhibitor) and was similarly stretched for 2min. E) Cells with siRNA against scrambled and similarly stretched for 2 min. The cell lysate was monitored for phospho moesin and total moesin by immunoblotting. Histograms represent fold change in band intensity relative to non-stretched cells and are averaged from three independent experiments $\pm$SEM; All values of phospho moesin was equalised for total moesin to control for equal loading. Representative blots are shown. $*$ $P<0.05$ Statistically significant compared to static

4.2.11 Endothelial cell alignment is disrupted with flow without microRNA regulation

Here we investigate if the characteristic pattern of endothelial cells under flow is maintained. The normal physiological alignment seen of endothelial cells subject to shear stress is lost with Dicer knockdown. This is shown in (Figure 4.12).

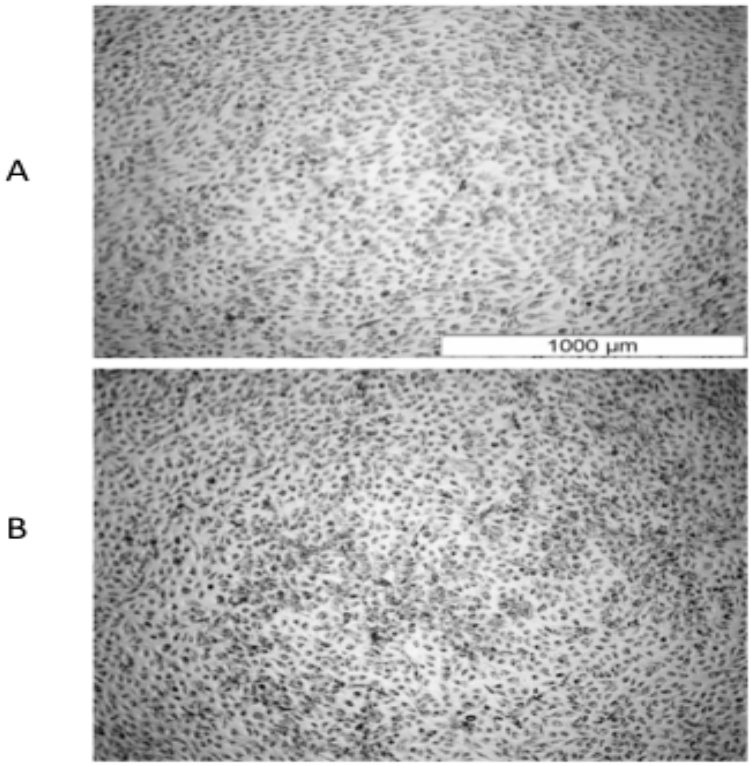

Figure 4.12: Endothelial cells don't align with shear when Dicer is downregulated
HAECs at 10^6 cells were electroporated with siRNA against dicer, while scrambled siRNA was used as a control. Cells were recovered by feeding them 12 h post electroporation. A further 12 h was past before starting the shear stress regime of 10dynes/cm^2. The cells were monitored for endothelial cell alignment 48 h after shear stress. A) Scrambled siRNA treated cells. B)Dicer siRNA treated cells.

4.2.12 MicroRNA system regulates uncontrolled microparticle release during flow and most agonists

We found earlier that microRNAs are needed for a physiologically functioning endothelium. Here we investigate whether microRNAs are essential to functions regulated by endothelium, such as the microparticle release.

Shear stress induced microparticle release (23,000 MPs in dicer knocked down cells, compared to 15,000 MPs in scrambled control) accumulated over 48h. This show microRNAs regulating microparticle release with flow. Similarly ionophore induction has the same effect (30,000 MPs in dicer knocked down cells, compared to 20,000 MPs in scrambled control). This is shown in (Figure 4.13).

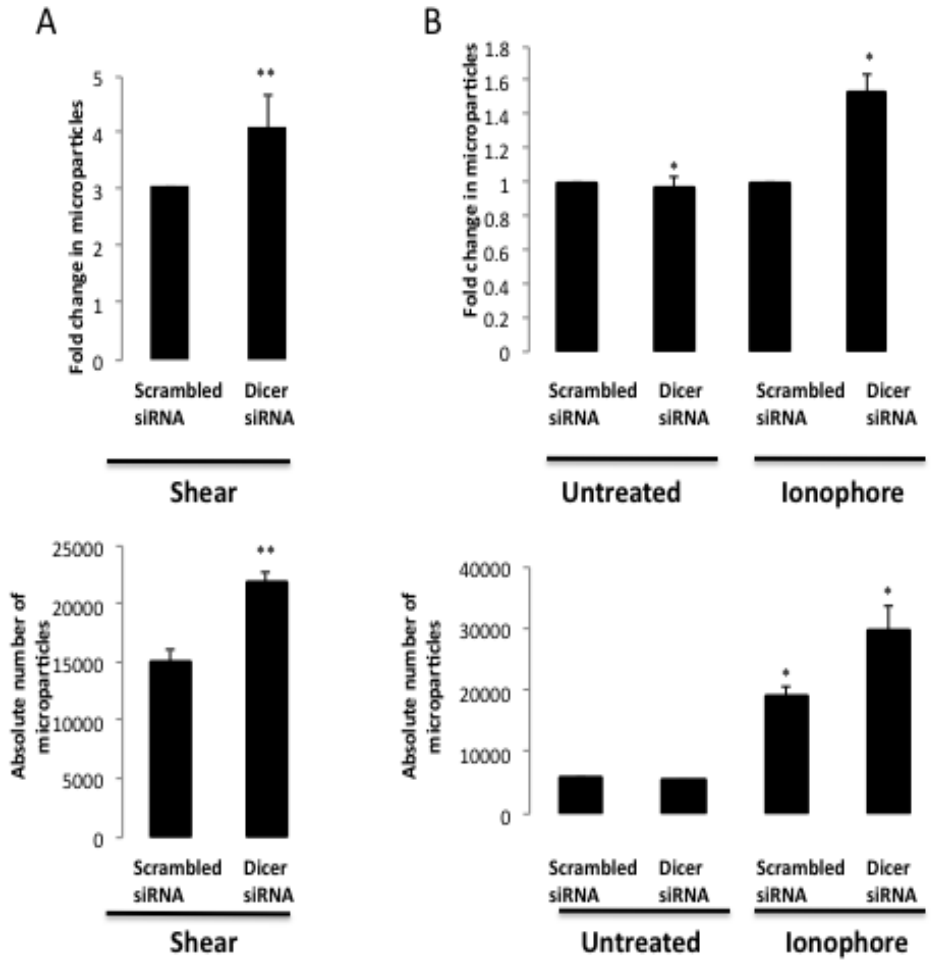

Figure 4.13: Endothelial microparticle increase with Dicer downregulation with shear and ionophore
HAECs at 10^6 cells were electroporated with siRNA against dicer, while scrambled siRNA was used as a control. Cells were recovered by feeding them 12 h post electroporation. A) A further 12 h was past before starting the shear stress regime of 10dynes/cm^2. The microparicles were allowed to accumulate from the onset of acute shear over 48 h. B) 24 h post electroporation cells were induced for microparticles with 10μM ionophore in full media and without. Prior to agonist induction cells were stained by Calcein AM. The MPs stained for calcein AM was collected from the supernatant and monitored by FACS. Histograms represent fold change, absolute numbers and are averaged from at least three independent experiments $\pm$SEM.
$**$P$<$0.05 Statistically significant compared to control for the same treatment.
$*$P$<$0.05 Statistically significant across all samples.

4.2.13 Proliferation of endothelial cells with cyclic strain is under microRNA regulation

Previous findings show that microRNAs are essential for physiological functioning of the endothelium. The study here investigates proliferation. microRNA we find regulates mechanotransduction mediated proliferation as well. The level of change between static control and cyclic strained cells are decreased with dicer knockdown. This is shown in (Figure 4.14).

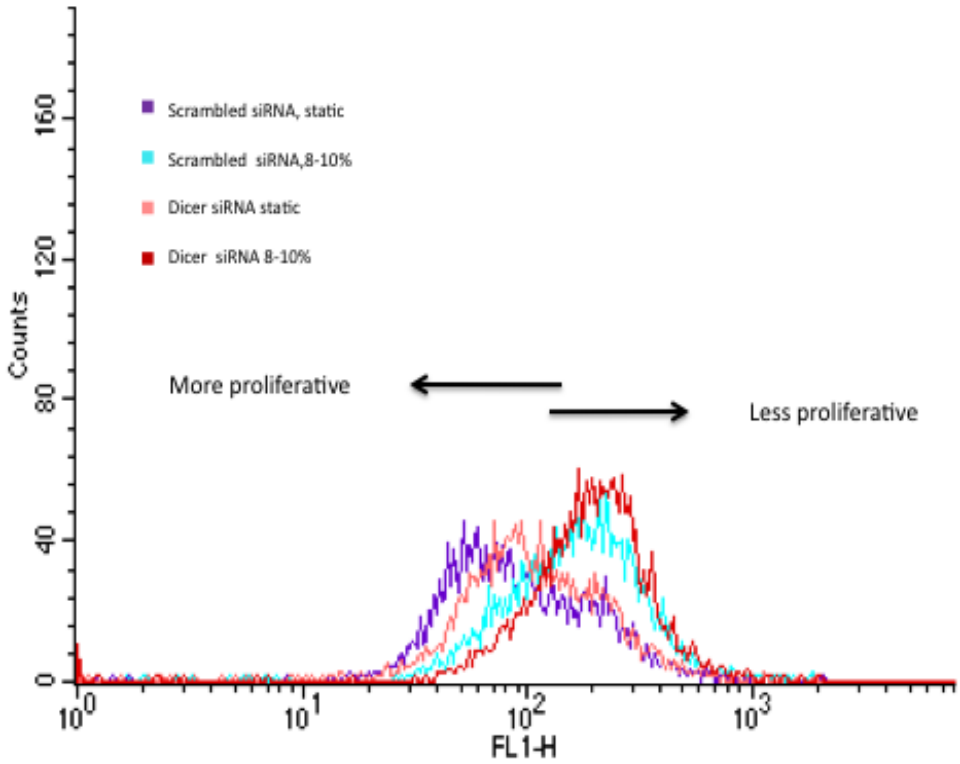

Figure 4.14: Endothelial cell proliferation: Disruption with dicer Downregulation
HAECs at 10^6 cells were electroporated with siRNA against dicer, while scrambled siRNA was used as a control. Cells were recovered by feeding them 12 h post electroporation. After a further 12 h the cells were seeded equally at 10^4 /cm^2 to pronectin coated bioflex plates. 8 h post seeding, they were stained with 5 μM CFDA-SE (5 min), prepared in PBS. Cells were fed full media(recovered for 1h) and strained for 24 h. The cells were then harvested and monitored for proliferation change using FACS. Representative of three independent experiments $\pm$SEM

4.2.14 Conclusion

In this chapter we find that moesin regulation is impacted by microRNAs. This is evidenced by our studies with dicer knockdown. Furthermore, this regulation is present with shear stress and cyclic strain. We characterize cell functions such as cell proliferation, endothelial microparticle release and physiology of the endothelium with blood flow.

Early in the chapter, we identify plausible moesin targeting microRNAs. They are analyzed to identify their expression pattern with shear stress and cyclic strain. Moreover, we identify a series of microRNAs regulated by shear stress and cyclic strain.

Chapter 5

Endothelial derived microparticles: Implicating moesin

5.1 Introduction

Utilizing blood flow, microparticles relay information throughout the organism. It They present antigens, provide slice of information of cells afar. It acts like a "news broadcaster" and with pathogenesis and inflammation they are considerably increased. We suspected moesin the prominent ERM protien in endothelial cells to have a role in microparticle formation. It is extensively shown how PIP_2 is implicated in microparticle release in the introduction. Cleavage of proteins by calpain is one of the distal factors associated with most microparticle formation (Geo, 2008). The process of EMP release and PIP_2 implication is extensively covered in the introduction.

PIP_2 binds to ERM proteins, also to talin via its FERM domain (Flaumenhaft, 2006). The significance of PIP_2 in microparticle formation in platelets has already been demonstrated(O'Connell *et al.*, 2005). Moesin is the only ERM

protein in the platelets. Induction with thrombin has shown dynamic transient change associated with moesin (Shcherbina *et al.*, 1999b). With its pivotal importance in binding to PIP_2, we suspect moesin to have a role in endothelial microparticle formation. This as discussed in the introduction, is important with respect to cytoskeleton-membrane adhesion.

We have previously shown how moesin is regulated extensively both chronically and acutely with cyclic strain. We have demonstrated how it is moesin and not ezrin that is regulated by shear stress. Meanwhile ezrin is also cleavable by calpain. Moesin meanwhile, is insensitive to calpain cleavage (Shcherbina *et al.*, 1999a). We hypothesise that the reason moesin is expressed highly in endothelial cells compared to its other ERM counterparts, is because moesin is suited to this environment. Moesin is highly sensitive to mechanotransduction and possibly is atheroprotective, bufferring against unnecessary microparticle induction.

Moreover in the last chapter we find endothelial cells lacking dicer, to be more sensitive towards agonist induced microparticle formation. Dicer downregulation affects a lot of proteins as we were knocking down the microRNA machinery. Nevertheless, we have demonstrated that moesin is under microRNA regulation.

5.2 Results

5.2.1 Determination of moesin in EMPs

This study investigates if moesin is present in the endothelial microparticles by western blotting. The induction of microparticles is conducted by TNF-α. Further to this the cell crud is removed, and EMPs are pelleted by ultracentrifuge. Here we find unequivocal evidence of moesin in EMPs using western blotting. The induction was by TNF-α. This is shown in Figure 5.1

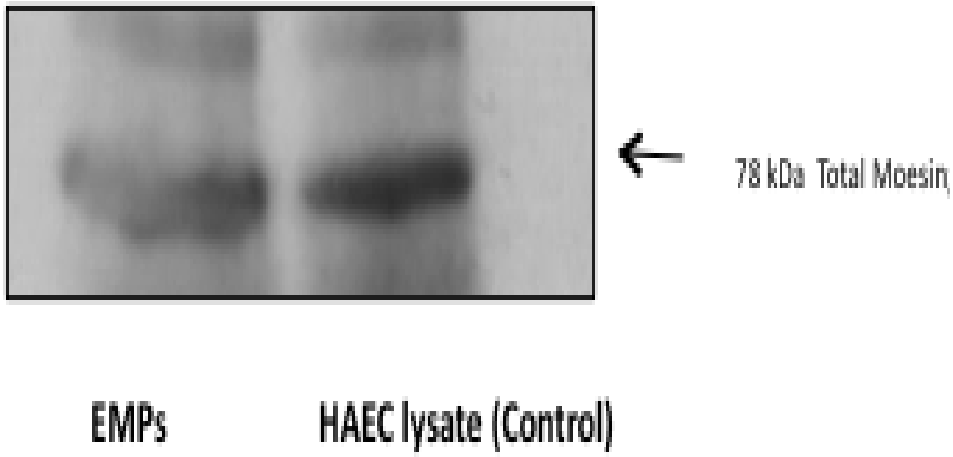

Figure 5.1: Moesin is in endothelial derived microparticles
BAECs were grown in five T175 plates to confluency. These were then stiumulated/non-stimulated with TNF-α overnight. The supernatant was centrifuged at 2000xg for 5 min to clear debri. The supernatant was then collected undisturbed and ultra-centrifuged at 70,000 g for 1 h. The pellet was then acetone precipated. This was immuonblotted for moesin after seperation of the proteins by gel electrophoresis.

5.2.2 Microparticles are increased post exercise and with shear stress

As we discovered earlier that microparticles are released due to shear stress, for perspective, *in-vivo* was conducted. Acute shear clearly accumulates MPs considerably. In *in-vitro* studies we find shear stress increasing EMPs to 16000 compared to 5000 in static control. In *in-vivo* studies we find shear stress increasing EMPs to 10000 compared to 2000 in non-exercised control. Further to the exercise the MPs clear after two hours decreasing to 5000 MPs.

Ethical approval was granted for the exercise study and was conducted together with Ronan Murphy. We find post exercise that there is an increase in MPs.

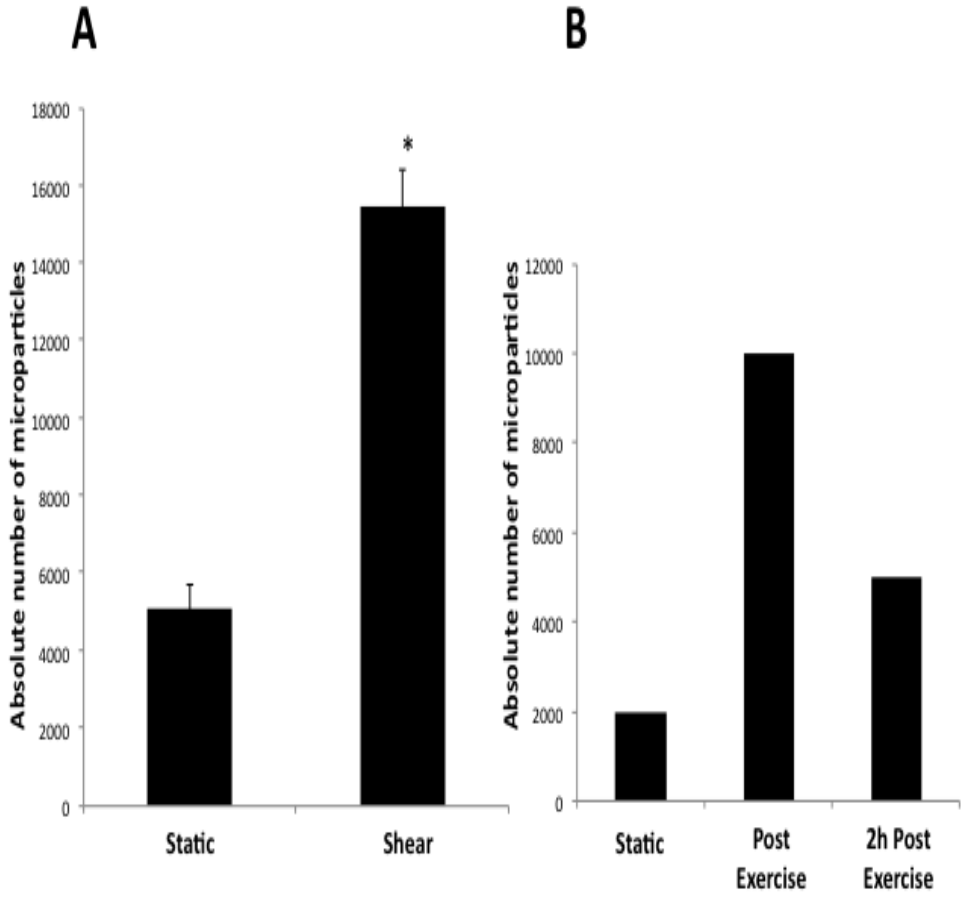

Figure 5.2: Microparticles are increased post excersice and with shear stress
A) HAECs were grown to confluence, further to which, they were subjected to shear stress regime of 10dynes /cm^2. The microparicles were allowed to accumulate from the onset of acute shear over 48 h. Prior to shear induction, cells were stained by calcein AM. The MPs stained for calcein AM was collected from the supernatant and monitored by FACS. B) Blood was drawn from participants using a 19C needle into 0.32% final concentration citrate. Anti-coagulant was utilized, after which samples were centrifuged at 1550g for 20 min. Plasma was subjected to 18890g centrifugation for 30min at 20^o. 225μl from the supernatant was mixed with 225μl 1xPBS/citrate. This was centrifuged for 30min at 18890g at -20^o. 225μl was taken, mixed with 75μl 1XPBS/citrate. After incubation in dark for 15min it was subjected to FACS
Histograms represent fold change of absolute numbers and are averaged from at least three independent experiments $\pm$SEM.
$*P<0.05$ Statistically significant compared to control.

5.2.3 EMPs: Moesin versus Ezrin and the microRNA regulation

Here we compare EMP formation due to ezrin and moesin overexpression. We also look into the effect of dicer knock down. Meanwhile all parameters are investigated for calpain activation by utilizing calpeptin to inhibit calpain. For induction of microparticles we utilize ionophore.

Induction of microparticles with ionophore we find lead to considerably higher EMPs when ezrin is overexpressed (3000 EMPs). Moesin overexpression meanwhile leads to a reduction in EMPs (2000 EMPs). This status quo is maintained in cells releasing microparticles where they are untreated (1500 with ezrin overexpression and 1000 with moesin overexpression). The reduction of microparticles with calpeptin is slightly higher when ezrin is overexpressed compared to moesin overexpression (1800 and 1000 EMPs respectively). 4 h of induction of microparticles we find doesn't lead to more microparticles than in control (such as in 12 h induction). The sensitivity to ionophore induction is viable and so is the sensitivity to calpeptin. Cells with dicer knocked out is also sensitive to calpain induced EMP release (ionophore induced EMPs are 5000 compared to 1500 EMPs). It is similarly sensitive to calpain inhibition as seen from calpeptin treated cells that reduce to 2200EMPs. Thus fold differences with induction is suggestive of its sensitivity. This is shown in Figure 5.3

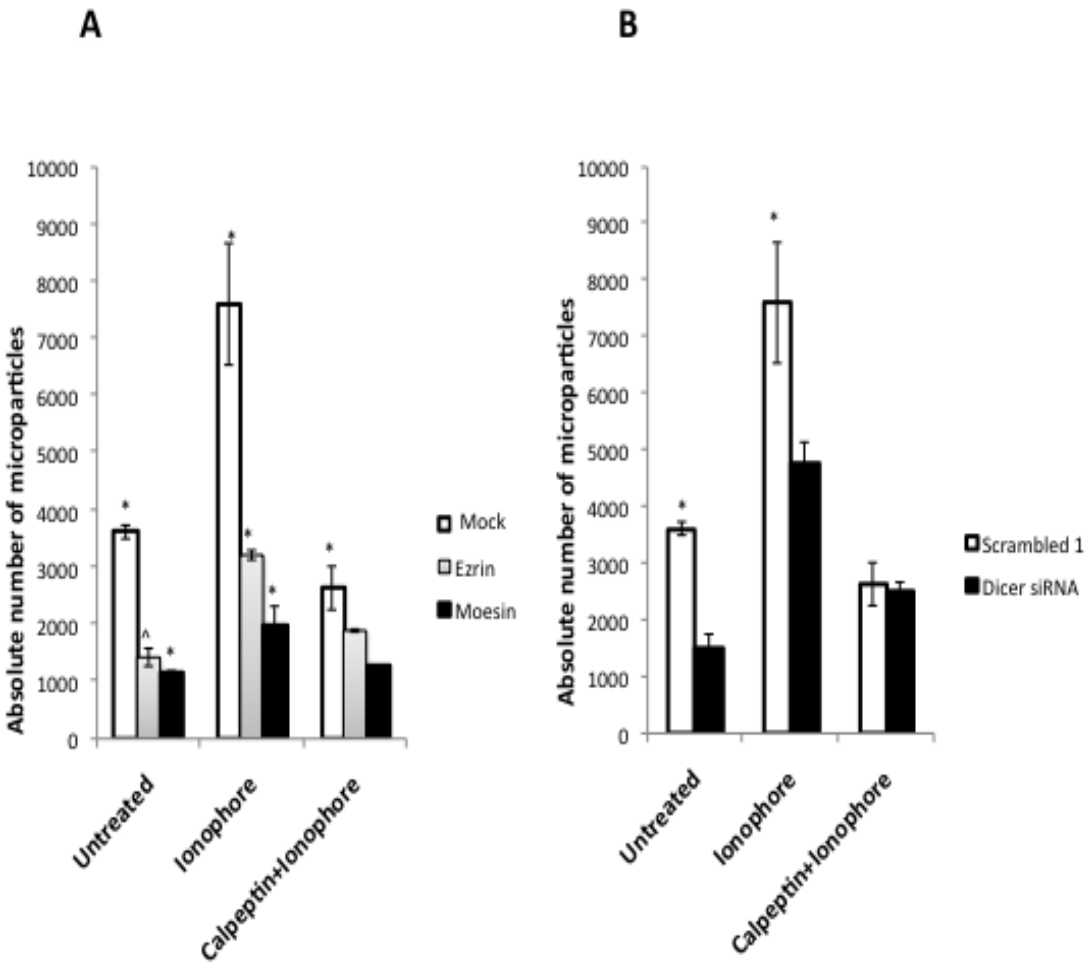

Figure 5.3: Endothelial microparticle regulation by Ezrin, Moesin overexpression and dicer downregulation mediated by ionophore
Endothelial cells were either overexpressed with FLAG tag constructs of ezrin or moesin. Also, dicer was down regulated by means of siRNA. Scrambled was utilized as control and all constructs were electroporated using microporator. 12 h post electroporation cells were recovered in full media. 24 h post electroporation cells induced for microparticles with 10μM ionophore in full media and without. Prior to this the cells were stained with calcein AM in full media.The MPs were monitored by FACS for effect of moesin, ezrin overexpression and dicer downregulation post 4h. Histograms are averaged as absolute microparticle numbers from at least three different experiments. $*P<0.05$ Statistically significant across all samples for the same treatment. $P<0.05$ Statistically significant against control for the same treatment. $**P<0.05$ Statistically significant across all samples for the same treatment except the control.

5.2.4 Thrombin mediated EMPs: Moesin versus Ezrin and the microRNA regulation

In this study we utilize the same parameters as the previous study, using thrombin to induce EMP formation. Here we compare EMP formation due to ezrin and moesin overexpression. We also look into the effect of dicer knock down. Meanwhile all parameters are investigated for calpain activation by utilizing calpeptin to inhibit calpain.

Induction with thrombin we find cells overexpressed with moesin to be quite sensitive when fold change (3000 EMPs compared to 1800 EMPs in untreated) is taken into account. Meanwhile, interestingly cells overexpressed with ezrin leads to lack of sensitivity to thrombin in terms of microparticle release. There is also an apparent lack of sensitivity to microparticle release with thrombin compared to ionophore induction. Dicer knockout renders the cell sensitive to calpeptin when thrombin is used for induction. Dicer knockout is also sensitive to EMP release (4800 EMPs compared to untreated having 2800 EMPs) This is shown in Figure 5.4

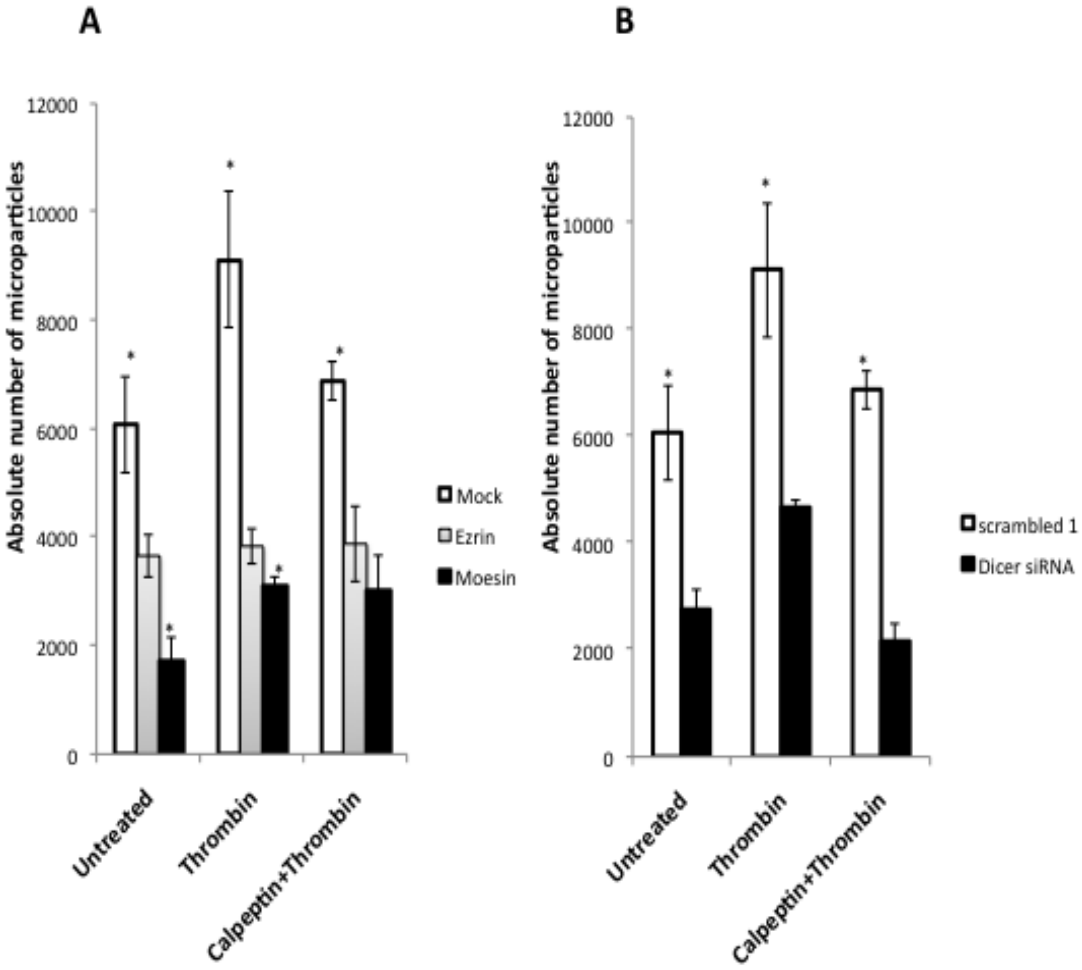

Figure 5.4: Endothelial microparticle regulation by Ezrin, Moesin overexpression and dicer downregulation mediated by Thrombin
Endothelial cells were either overexpressed with FLAG tag constructs of ezrin or moesin. Also, dicer was down regulated by means of siRNA. Scrambled was utilized as control and all constructs were electroporated using microporator. 12 h post electroporation cells were recovered in full media. 24 h post electroporation cells were subjected to serum starvation for 12 h. The cells were then induced for microparticles with thrombin (1 IU/ml) and without. Prior to this the cells were stained with calcein AM in serum free media.The MPs were monitored by FACS for the effect of moesin, ezrin overexpression and dicer downregulation. Histograms are averaged as absolute microparticle numbers from at least three different experiments. $*P<0.05$ Statistically significant across all samples for the same treatment. $P<0.05$ Statistically significant against control for the same treatment. $P<0.05$ Statistically significant across all samples except against moesin for the same treatment.

5.2.5 Moesin and Ezrin GFP expression pattern

Overexpression of moesin and ezrin leads to its localization in certain areas of a cell. We investigate in this study this aspect utilizing microscopy. The images show the different expression pattern of moesin and ezrin when they are overexpressed. This is shown in Figure 5.5

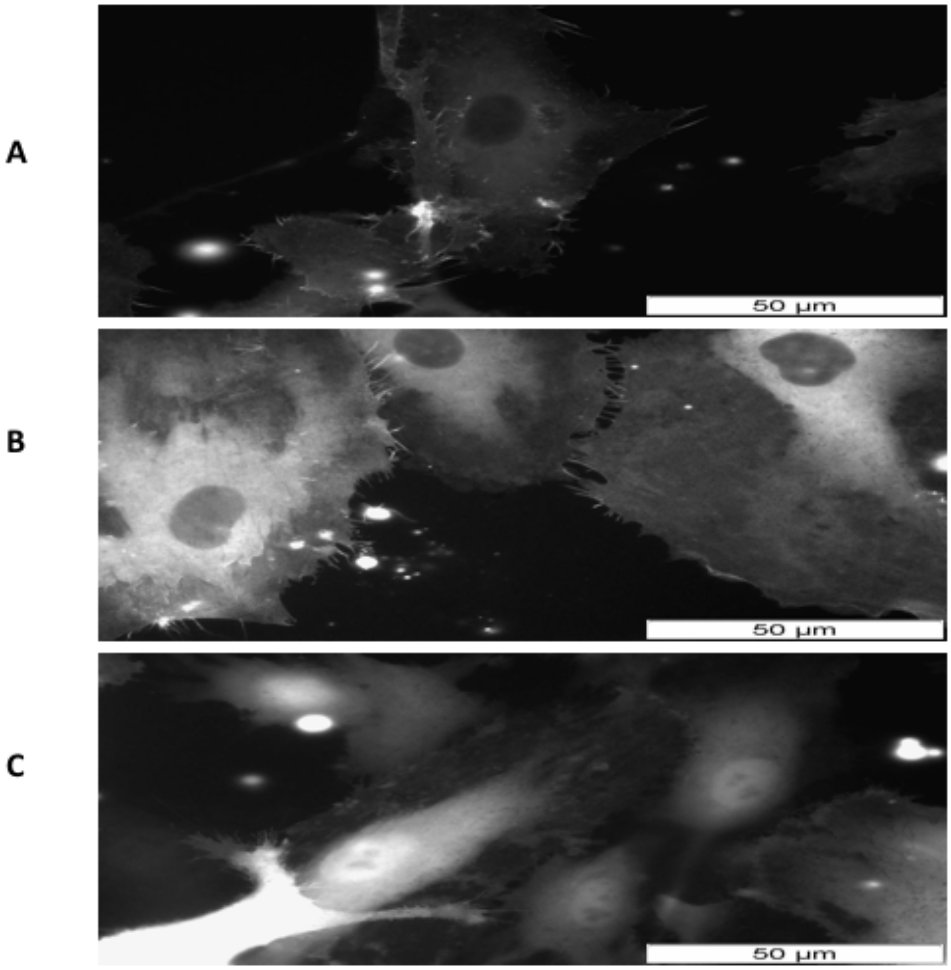

Figure 5.5: Differential expression of ezrin and moesin in HAECs
Moesin GFP and ezrin GFP constructs was overexpressed by electroporation into HAECs. The cells were then visualised by fluorescence micropscope.
A) Moesin-GFP. B) Ezrin-GFP. C) GFP control. Images are representative of at least three individual sets of experiments.

5.2.6 Further confirmation of moesin and ezrin's differential expression in HAECs

In this study we investigate how moesin and ezrin is differentially localized with the unbound PIP_2. This study has significance in PIP_2 explaining the membrane-cytoskeletal adhesion. We find moesin, ezrin is differentially localized when overexpressed with phospholipase C (which is a marker of unbound PIP_2). This is shown in Figure 5.6.

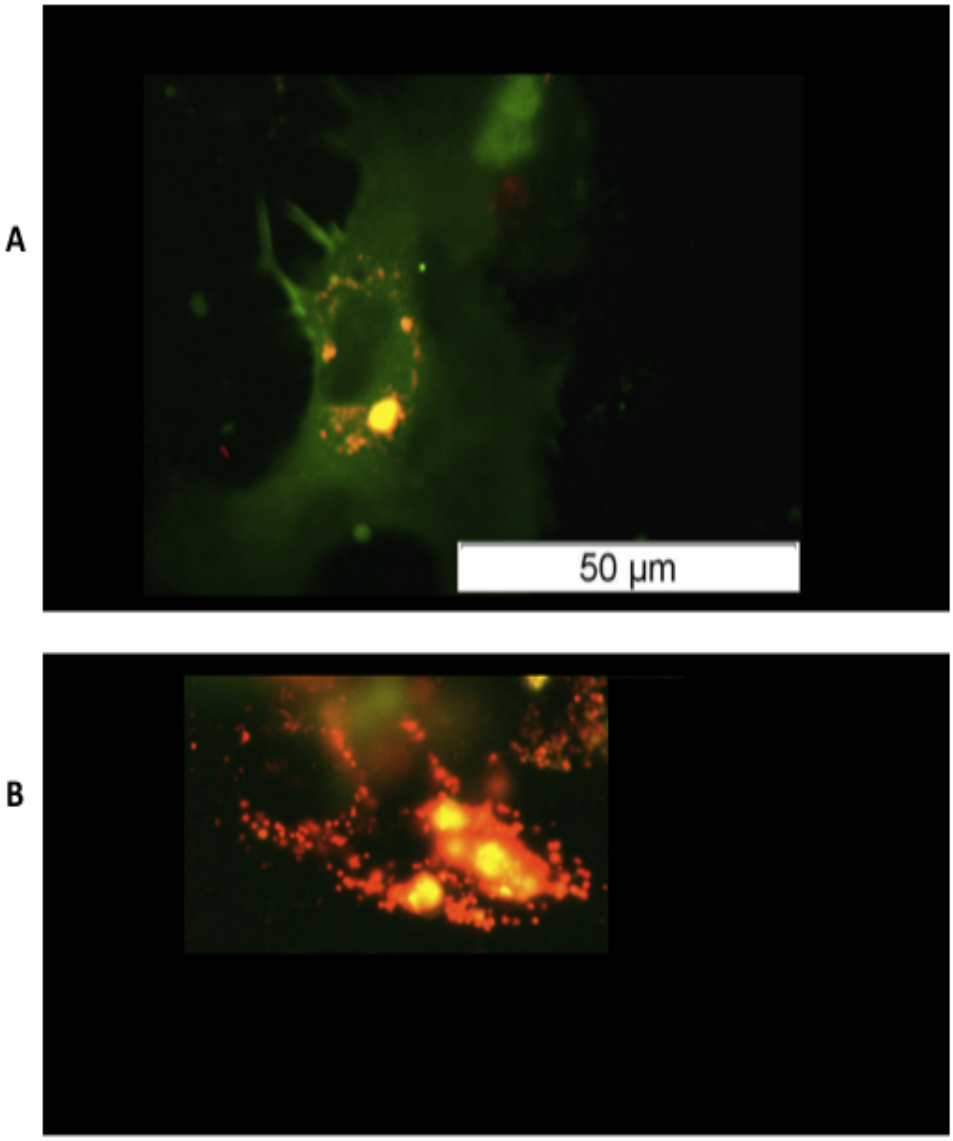

Figure 5.6: Differential expression of ezrin and moesin in HAECs
Moesin and ezrin GFP constructs was overexpressed by transfection into HAECs in combination with plc$-\delta-$RFP. The cells were then visualised by fluorescence microscope. A) Moesin-GFP. B) Ezrin-GFP; note differential expression of ezrin compared to moesin. Images are representative of at least three individual sets of experiments.

5.2.7 Visualisation of EMPs

As previous studies showed that moesin and ezrin have differential roles in EMP induction, we investigate utilizing microscopy to observe EMP formation.

Visualisation of EMPs reflect the sensitivity to the respectively overexpressed cytoskeletal proteins (moesin and ezrin) found in the previous study. We thus find increased EMP formation with ezrin overexpression compared. This is shown in Figure 5.7.

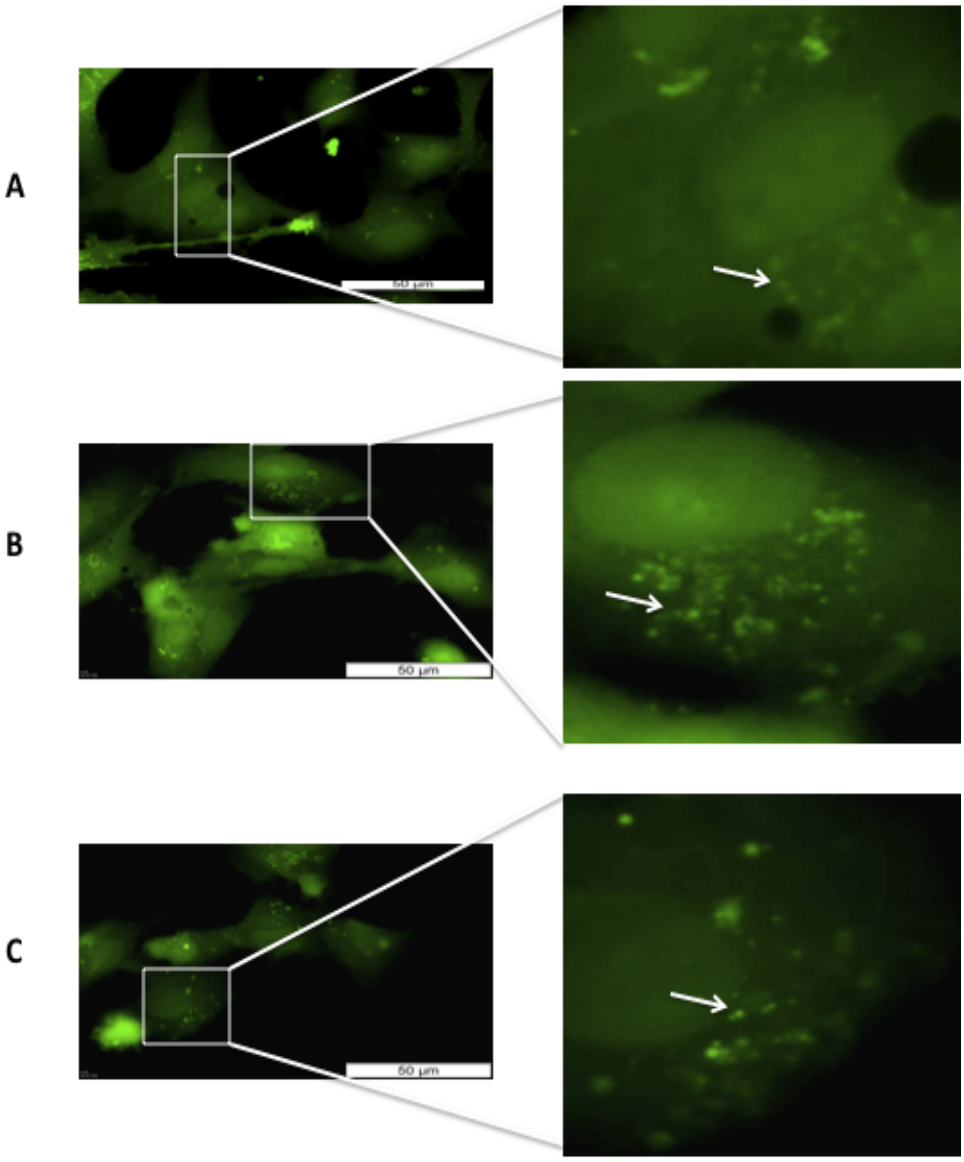

Figure 5.7: Visualisation of microparticles
The cells were calcein AM stained after which MPs were induced by ionophore(10μM in full media). The cells were then visualised with a fluorescent microscope after 4h. Notice the granulated MPS on the right pane magnified from the left pane. White arrows in the right pane point to an example of microparticle. A)Moesin-Flag. B)Ezrin-Flag. C) Mock.
Images are representative of at least three individual sets of experiments.

5.2.8 Lack of moesin is pathogenic with respect to an increase in EMPs

Further to our understanding of EMP release by overexpression studies, we investigate here using moesin knock down.

The knock out study of moesin, shows a huge surge in microparticle release with moesin knockdown in ionophore treated cells (2.5 fold increase). There is also an increase in microparticles in untreated cells (1.5 fold increase). This is shown in Figure 5.8.

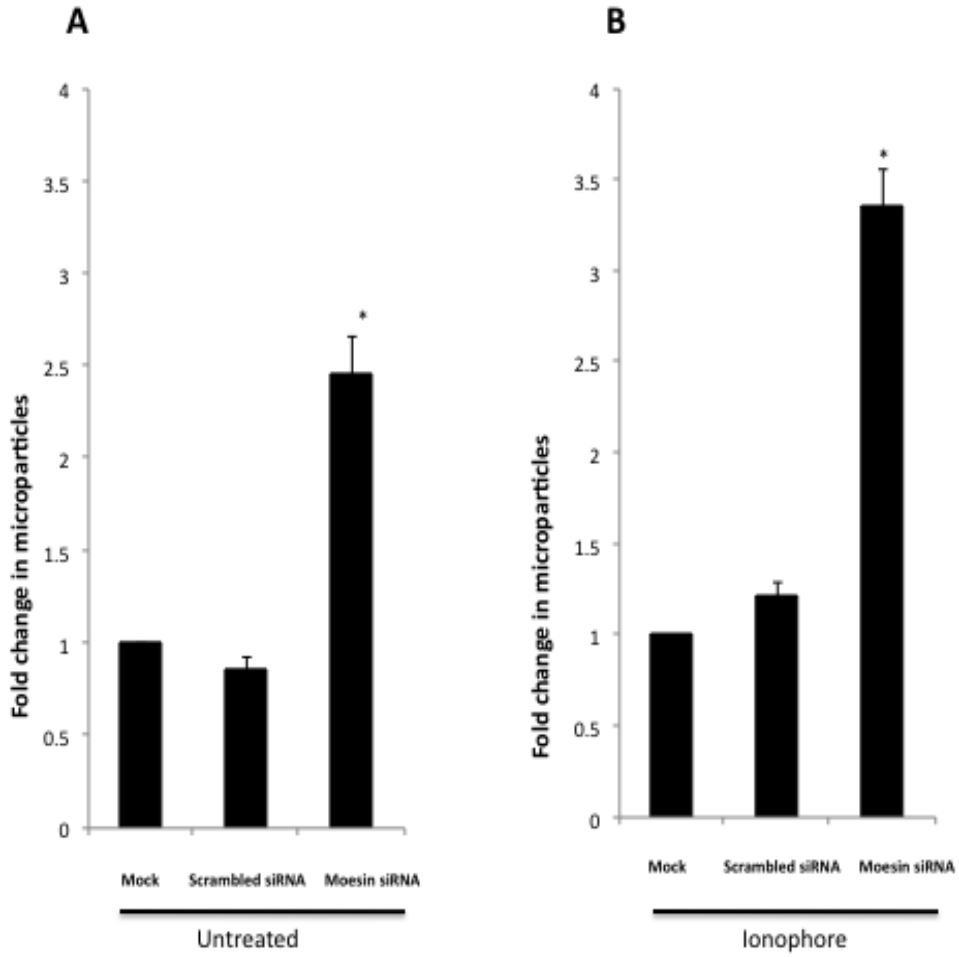

Figure 5.8: Endothelial microprticles: Effect of moesin downregulation
Endothelial cells had moesin down regulated by electroporation utilizing moesin siRNA. For control, both mock transfection and a scrambled siRNA was employed. Following calcein AM staining the cells were left either untreated or subjected to ionophore treatment. Ionophore was made up in full media at a conc of 10μM. Following 4h the MPs stained for calcein AM were monitored by FACS. A) Untreated. B) Ionophore induction. Histograms are averaged absolute microparticle numbers from at least three different experiments. P<0.05 Statistically significant compared to mock and scrambled controls

5.2.9 Conclusion

In the earlier chapter we found moesin expression is impacted by microRNAs. Moreover we found microRNAs regulation various cell functions with mechanotransduction including microparticle release. Thus in this chapter we look into whether moesin regulates microparticle release, owing to its significance in the cell cortex.

We find moesin and ezrin having differential roles in microparticle release owing to non-similar calpain sensitivity. Furthermore, we identify differential EMP release owing to thrombin and ionophore induction. The results are discussed in the final chapter. We also discover that shear stress induced acutely during exercise increase microparticle formation, which is cleared later. The importance of this is due to the correlation of our earlier finding of moesin's presence in EMPs.

Chapter 6

The urokinase moesin interaction

6.1 Introduction

Our lab has characterized uPA's critical role in migration and tube formation post cyclic strain, in endotheilial cells (Von Offenberg Sweeney *et al.*, 2005). Moreover Sweeney *et al* found uPA involved only in strain-induced tube formation. Previous chapters have shown that moesin is highly mechanosensitive under both shear stress and cyclic strain. We also found moesin increased with cyclic strain in the first results chapter (chapter 3).

As discussed in the introduction, uPA and uPAR interaction needs other transducers to interact inwards to the cell and its cellular architecture. These like integrins (Figure 1.28), recruit cytoskeletal adapter proteins that push, pull and rearrange the cellular architecture to meet the new needs. These might require transient phosphorylation or transient dephosphorylation of different linker proteins. This is found to be the biochemical change required for the reshuffle. There could be as well, inside out signaling whereby these transient changes work outwards to the same receptors.

We wanted to see whether moesin, the actin binding cytoskeletal protein, is involved in migration and tube formation. We also wanted to see if these functions recruit uPA/uPAR. In the results elucidate the biochemical needs and implications of these interactions using different constructs.

6.2 Results

6.2.1 uPA and SRSRY dephosphorylates moesin acutely

We investigate whether moesin phosphorylation is effected by uPA. Biochemically, moesin is dephosphorylated in concert with the effect of uPA. This can be seen 2 min after uPA induction (60% decrease in phosphorylation). Moesin is similarly dephosphorylated when SRSRY peptide is used (60% decrease in phosphorylation after 2 min). The study is shown in Figure 6.1.

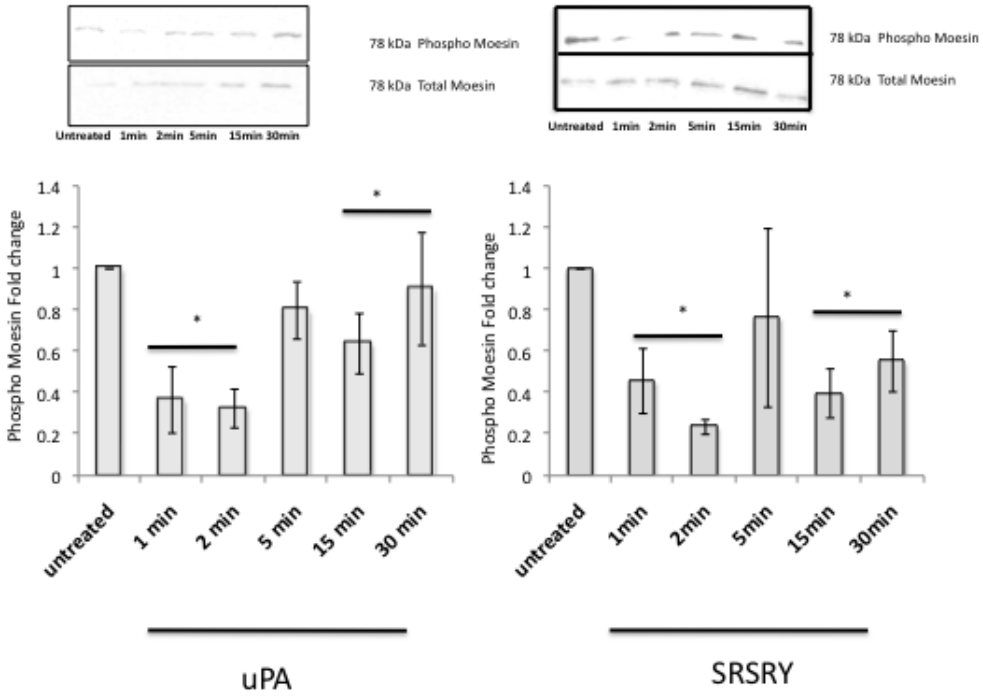

Figure 6.1: uPA and SRSRY dephosphorylates moesin acutely
HAECs were exposed to A) (10nM) uPA B) (1pM) SRSRY acutely for the times stated above. This was then monitored for phospho moesin protein expression by western blotting. Histograms represent fold change in band intensity relative to untreated control and are averaged from three independent experiments $\pm$ SEM; All values are controlled for equal loading by equalising for total moesin under same conditions. Representative blots are shown. $*P<0.05$ Statistically significant compared to untreated.

6.2.2 Constructs used

Constructs used

1. Parental EC: (HAECs) Endothelial cells at passage 7
2. Mock: Mock electroporated.

All constructs below have GFP at their C terminus, unless specified.

3. N1-GFP: Control GFP vector.
4. Moesin wt GFP/Moesin GFP: Full length Moesin.
5. Inactive Moesin: Full length Moesin with GFP at N terminus (FERM domain) due to which it is unable to bind to membrane, and thus to PIP_2.
6. T558A: Moesin that cannot be phosphorylated.
7. T558D: Phosphomimetic Moesin.

6.2.3 uPAR peptides and their effect

To explain how peptides used in subsequent study act, the following figure (Figure 6.2 is used for illustration. The peptides have been extensively utilized and their efficacy published (Degryse *et al.*, 2001; Degryse, 2008; Degryse *et al.*, 2005; Dass *et al.*, 2008). Detailed explanation of the various receptors, how they act following uPA induction is given in the introduction (Blasi and Carmeliet, 2002)

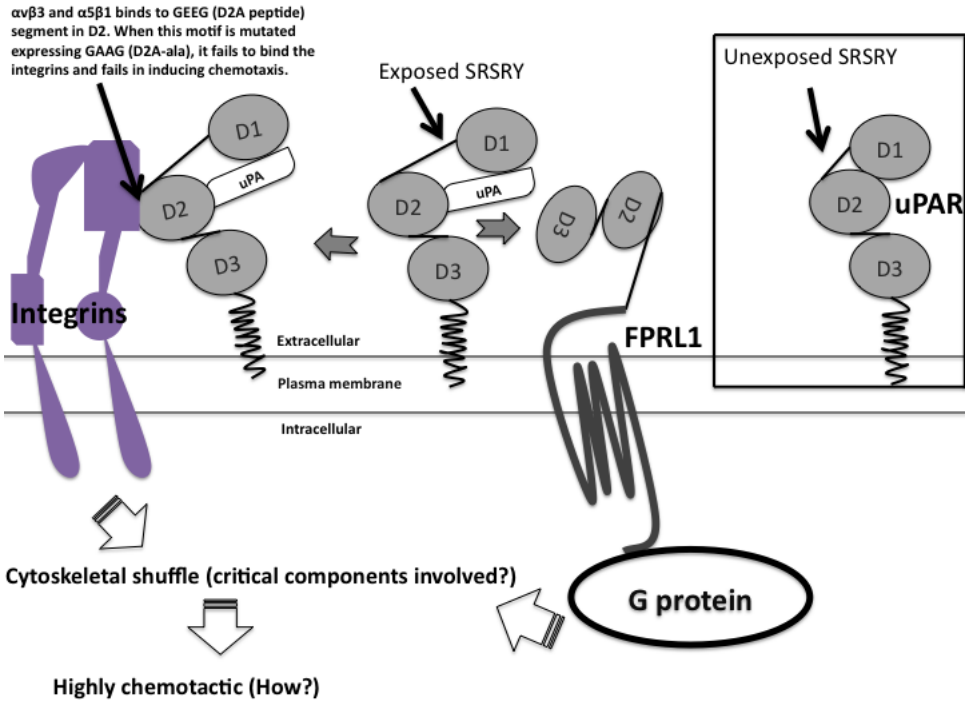

Figure 6.2: Different uPA peptides used in the studies and their downstream effectors

uPAR receptor do not have a cytoplasmic domain, thus any effect it has on cytoskeletal proteins require molecules that span both extracellularly and intracellularly (eg:-FPRL1, $\alpha v\beta 3$ / $\alpha 5\beta 1$ integrins). With uPA addition, SRSRY amino acid sequence is exposed which binds to FPRL1. This peptide is used to decipher, if FRPL1 is recruited towards moesin dephosphorylation. Meanwhile $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins interact with uPAR post uPA addition. They also interact with uPAR in concert with vitronectin addition. Their binding to uPAR either way utilize the GEEG (D2A) sequence on the D2 domain of uPAR. This peptide has been shown to be highly chemotactic. The mutated form GAAG (D2A-ala) meanwhile inhibits such binding and is found to reduce chemotaxis substantially (Degryse *et al.*, 2001; Degryse, 2008; Degryse *et al.*, 2005).

6.2.4 Determination of how moesin interacts with uPA in migrating cells

With addition of uPA, moesin overexpression (Moesin wt GFP) does not seem to overly increase migration of HAECs. Phosphomimetic construct (T558D) or dominant negative for phosphorylation (T558A) meanwhile, decrease migration substantially (50% decrease compared to control treated with uPA). Similarly substantial reduction in migration can be seen when FERM domain (1-382) or when inactive moesin (GFP cloned into the FERM domain, rendering it unable to phosphorylate due to its inability to bind to PIP_2) is overexpressed.

Without uPA, the inactive moesin or T558D when overexpressed reduce endothelial cell migration (50% decrease compared to control untreated with uPA). The study is shown in Figure 6.3.

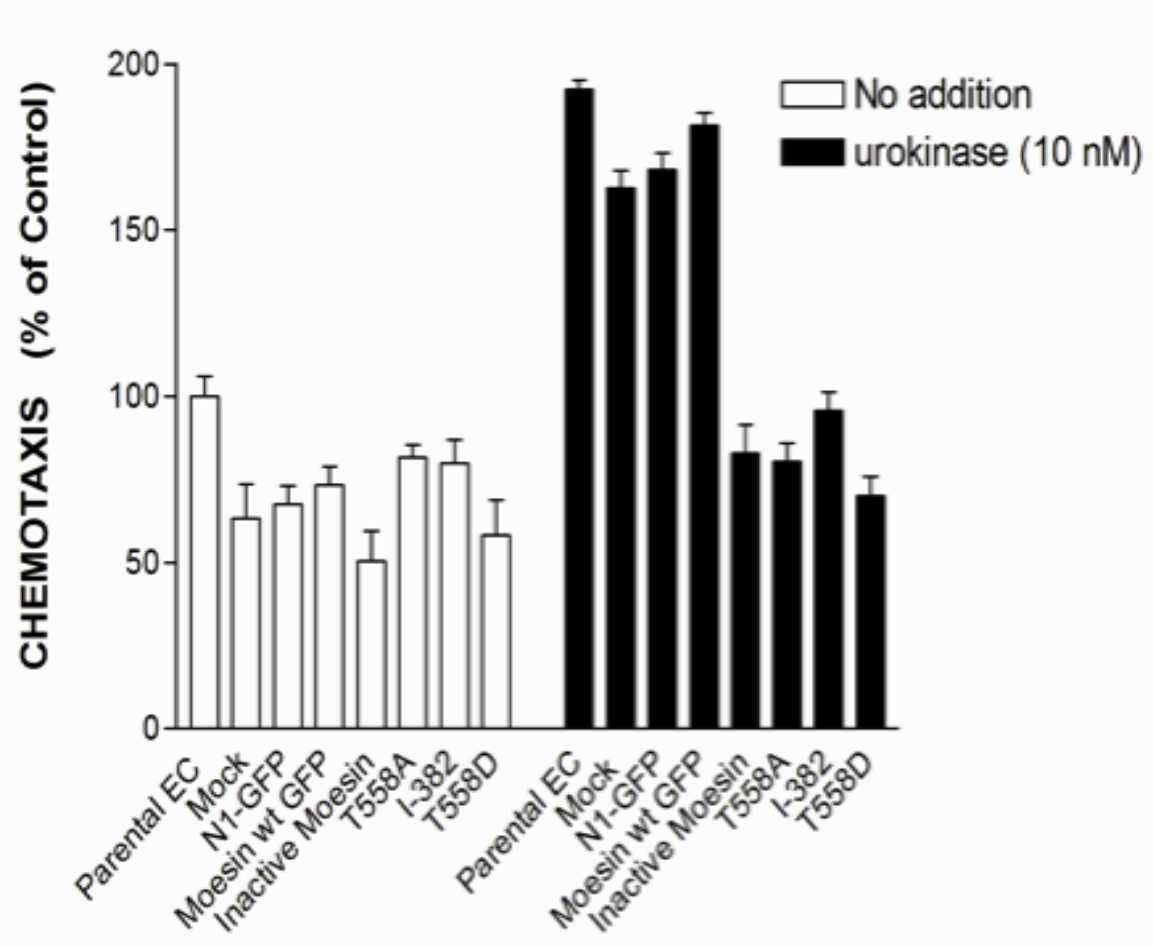

Figure 6.3: Determination of how moesin interacts with uPA in migrating cells

Respectively transfected and untransfected HAECs were monitored for migration to (10nM) uPA containing wells. This was conducted using boyden chamber overnight. Histograms represent fold change in mean migration measured by counting number of migrated endothelial cells. Cells were visualized by crystal violet staining. This is averaged from at least three independent experiments $\pm$ SEM. $P<0.05$ All histograms statistically significant to respective parental EC control.

6.2.5 Moesin downregulation stops uPA mediated migration

To investigate if there is a critical need for moesin in uPA mediated migration, down regulation of moesin is necessary. Moesin is highly expressed in migrating cells, hence overexpression of moesin will provide very little evidence regarding its migratory potential. The study (Figure 6.4), clearly finds moesin is required to induce migration downstream of uPA. uPA induced migration is abolished with moesin knocked out.

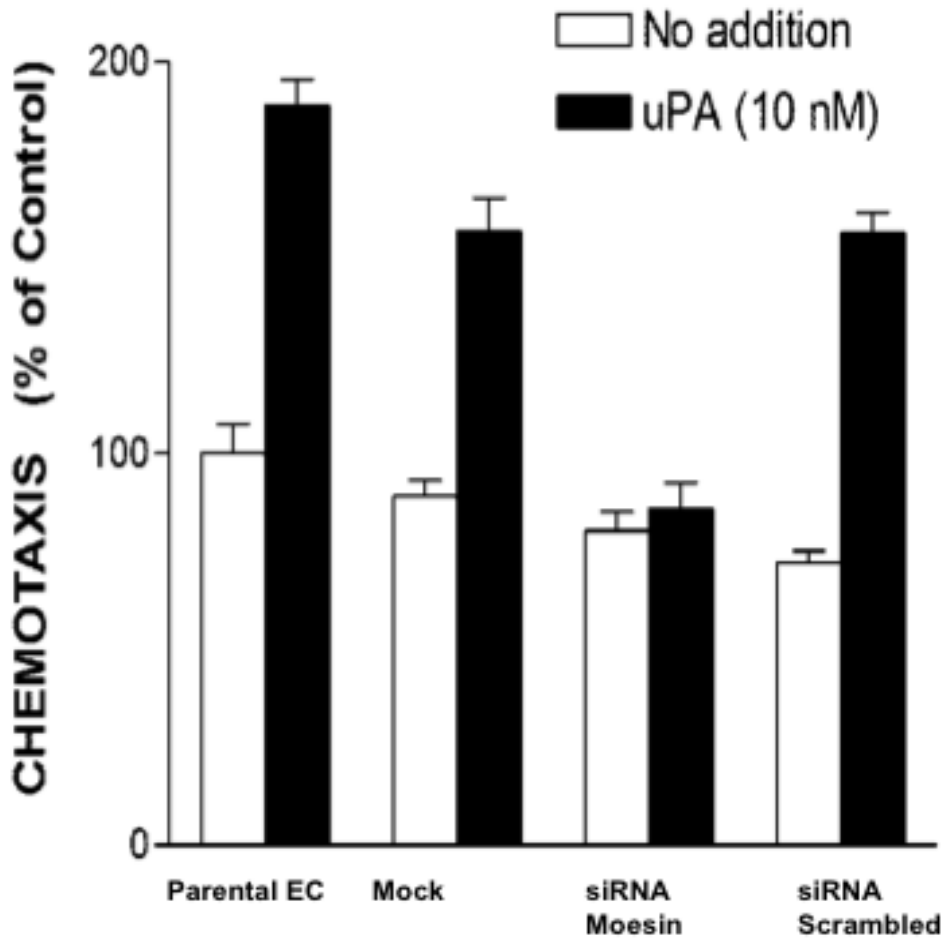

Figure 6.4: Moesin downregulation stops uPA mediated migration
Respectively transfected and untransfected HAECs were monitored for migration to (10nM) uPA containing wells. This was conducted using boyden chamber overnight. Histograms represent fold change in mean migration measured by counting number of migrated endothelial cells. Cells were visualized by crystal violet staining. This is averaged from at least three independent experiments $\pm$ SEM. $P<0.05$ All histograms statistically significant to respective parental EC control.

6.2.6 Determining whether SRSRY is the modus operandi of uPA-Moesin migration

Previous biochemical study showed both SRSRY and uPA inducing dephosphorylation of moesin. Hence, in this study (Figure 6.5) migration is investigated downstream of SRSRY.

The study shows predisposition of moesin constructs with impaired phosphorylation in reducing migration downstream (50% decrease compared to moesin wild type control). The constructs T558D, T558A, inactive moesin and FERM domain (1-382, lacks the segment that could phosphorylate). The results reflect similar earlier study with uPA.

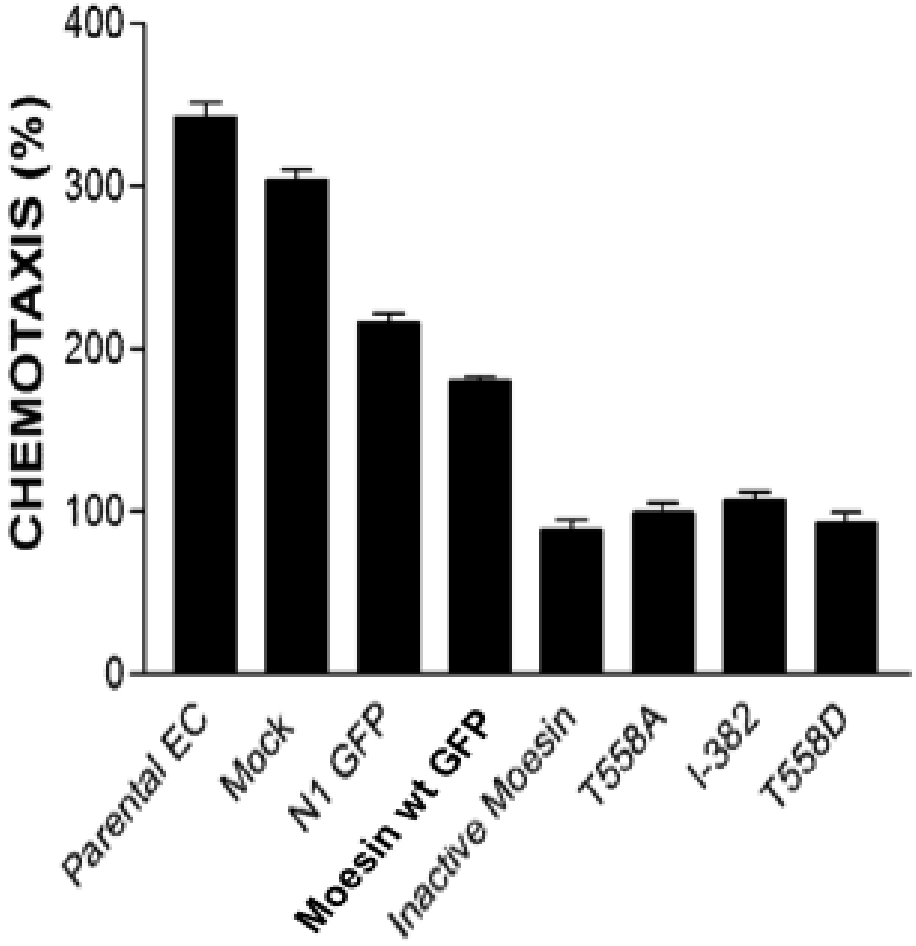

Figure 6.5: Determining whether SRSRY is the modus operandi of uPA-Moesin migration
Respectively transfected and untransfected HAECs were monitored for migration to (1pM) SRSRY containing wells. This was conducted using boyden chamber overnight. Histograms represent fold change in mean migration measured by counting number of endothelial cells that has migrated. The cells were stained with crystal violet for visualisation. This is averaged from at least three independent experiments $\pm$ SEM. $P<0.05$ All histograms statistically significant to parental EC control.

6.2.7 How does D2A peptide affect migration with respect to moesin

This study (Figure 6.6, was devised to investigate how integrins, downstream of uPA-uPAR interaction results in migration mediated by moesin. The study reflected previously shown studies here, regarding requirement for intact phosphorylation site (3 fold decrease compared to moesin wild type control). Interestingly, integrin mediated migration was substantially enhanced (1 fold increase compared to GFP control) when moesin (Moesin wt GFP) was overexpressed.

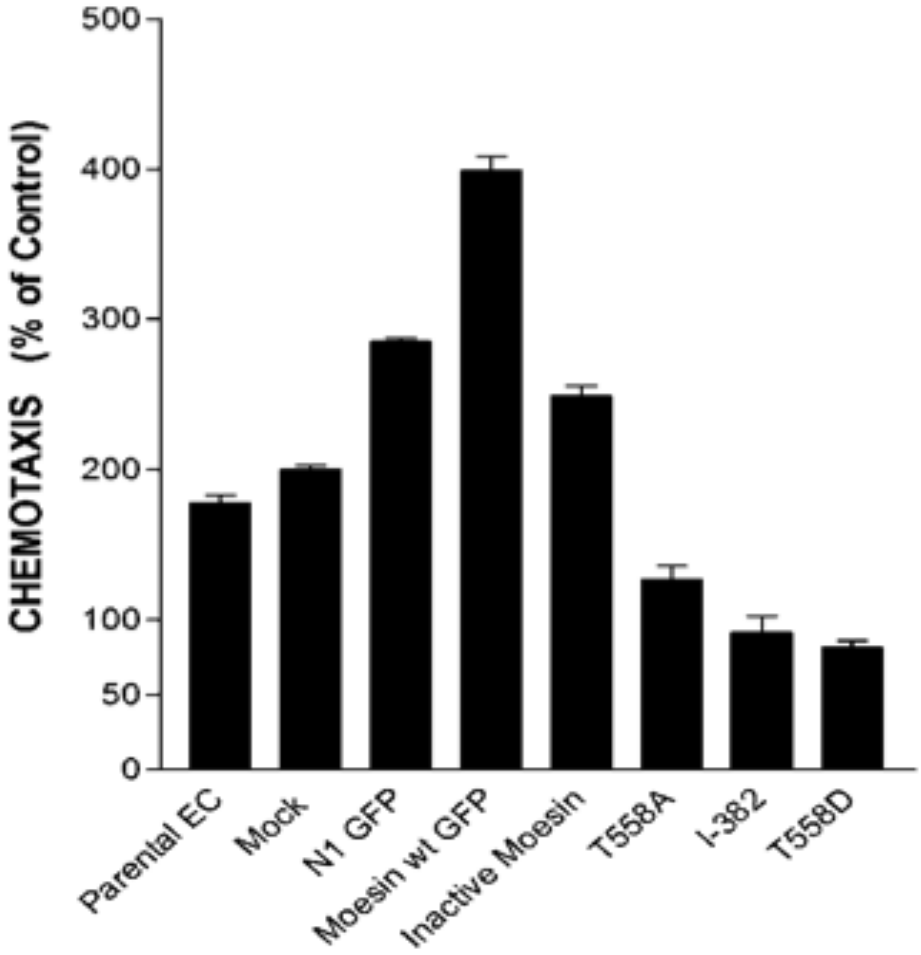

Figure 6.6: How does D2A peptide affect migration with respect to moesin Respectively transfected and untransfected HAECs were monitored for migration to (1pM) D2A containing wells. This was conducted using boyden chamber overnight. Histograms represent fold change in mean migration measured by counting number of endothelial cells that has migrated. The cells were stained with crystal violet for visualisation. This is averaged from at least three independent experiments $\pm$ SEM. $P<0.05$ All histograms statistically significant to respective parental EC control.

6.2.8 D2A-ala inhibits migration with respect to moesin

Peptide studies with D2A showed interesting migration pattern when moesin was overexpressed downstream of integrins.

To further check this, D2A-ala peptide was utilized. This peptide should, inhibit integrin binding to uPAR at the D2 region, thus stopping the migration with respect to integrins. The study clearly shows moesin overexpression (moesin wt GFP) to be further detrimental for migration whilst integrins are inhibited.

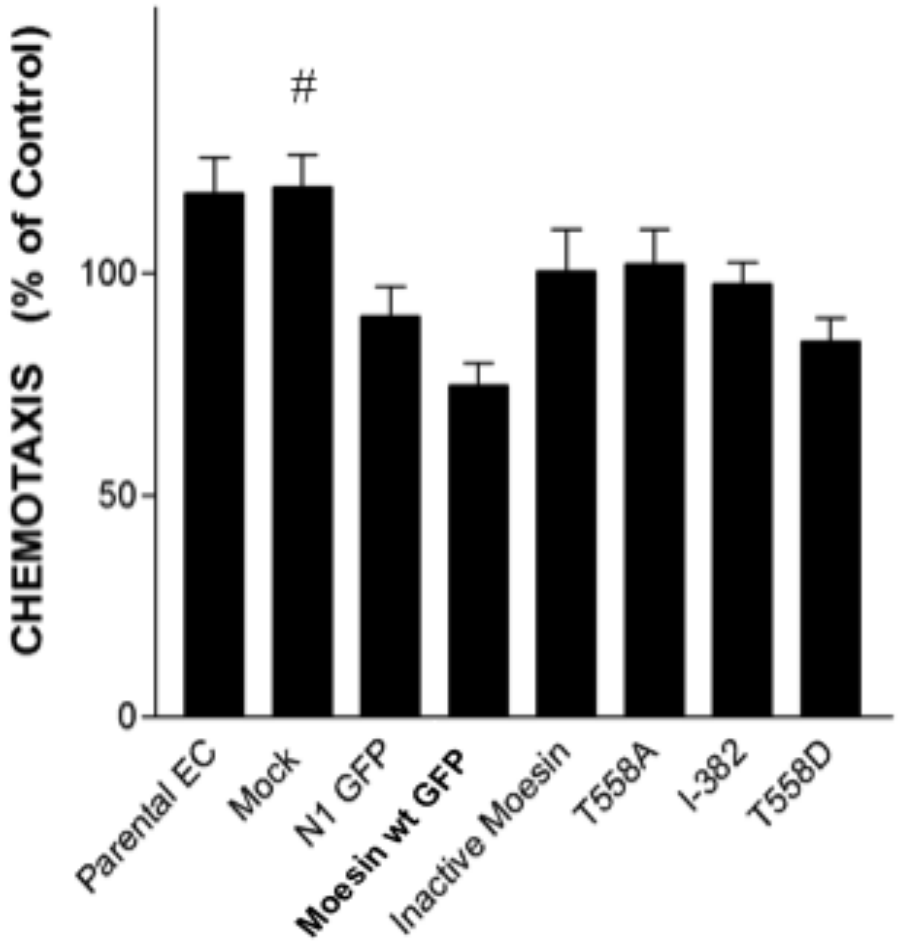

Figure 6.7: D2A-ala inhibits migration with respect to moesin
Respectively transfected and untransfected HAECs were monitored for migration to (100pM) D2A-Ala containing wells. This was conducted using boyden chamber overnight. Histograms represent fold change in mean migration measured by counting number of migrated endothelial cells. Cells were visualized by crystal violet staining. This is averaged from at least three independent experiments $\pm$ SEM. $P<0.05$ All histograms statistically significant to parental EC control. $P>0.05$ Statistically not significant to parental EC control.

6.2.9 Vitronecin mediated migration needs moesin's phosphorylation machinery intact

Vitronectin (VN) as discussed in the introduction, can bind both integrins and uPAR. The study (Figure 6.8), shows that extracellular matrix (VN in this case) mediated migration requires moesin's transient phosphorylation apparatus intact (50% decrease in migration compared to moesin wild type control). VN recruits both integrins and uPAR towards migration.

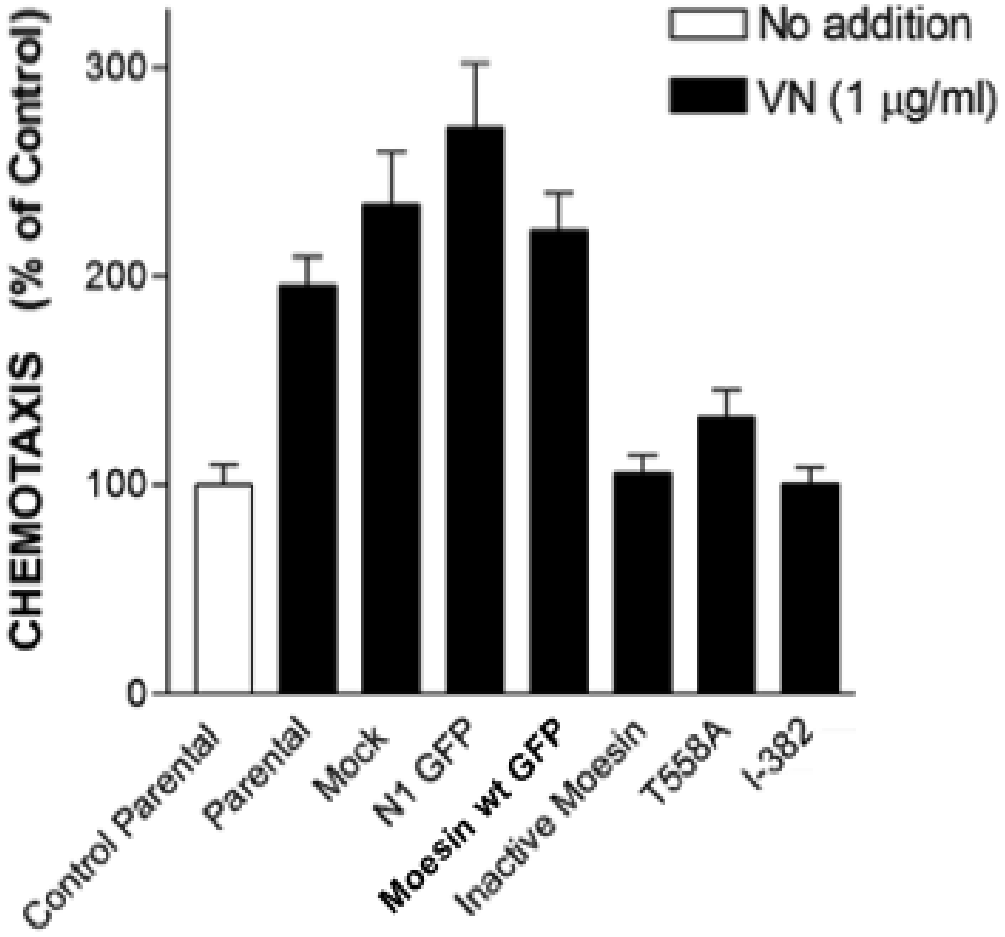

Figure 6.8: Vitronecin mediated migration needs moesin's phosphorylation apparatus intact
Respectively transfected and untransfected HAECs were monitored for migration to (1μg/ml) VN containing wells. This was conducted using boyden chamber overnight. Histograms represent fold change in mean migration measured by counting number of migrated endothelial cells. Cells were visualized by crystal violet staining. This is averaged from at least three independent experiments $\pm$ SEM. $P<0.05$ All histograms statistically significant to parental control.

6.2.10 Moesin increase angiogenic tube length

Studies with collagen gel, show moesin overexpression stimulating angiogenic growth compared to control. The phopho-mimetic construct (T558D), increase tube length the most (1 fold increase compared to GFP control). Moesin overexpression also increase tube length (0.5 fold increase compared to GFP control).The experimental setup provides one dimensional approach towards deciphering the angiogenic potential. This is because collagen gel, has no other components that might interfere with the study.

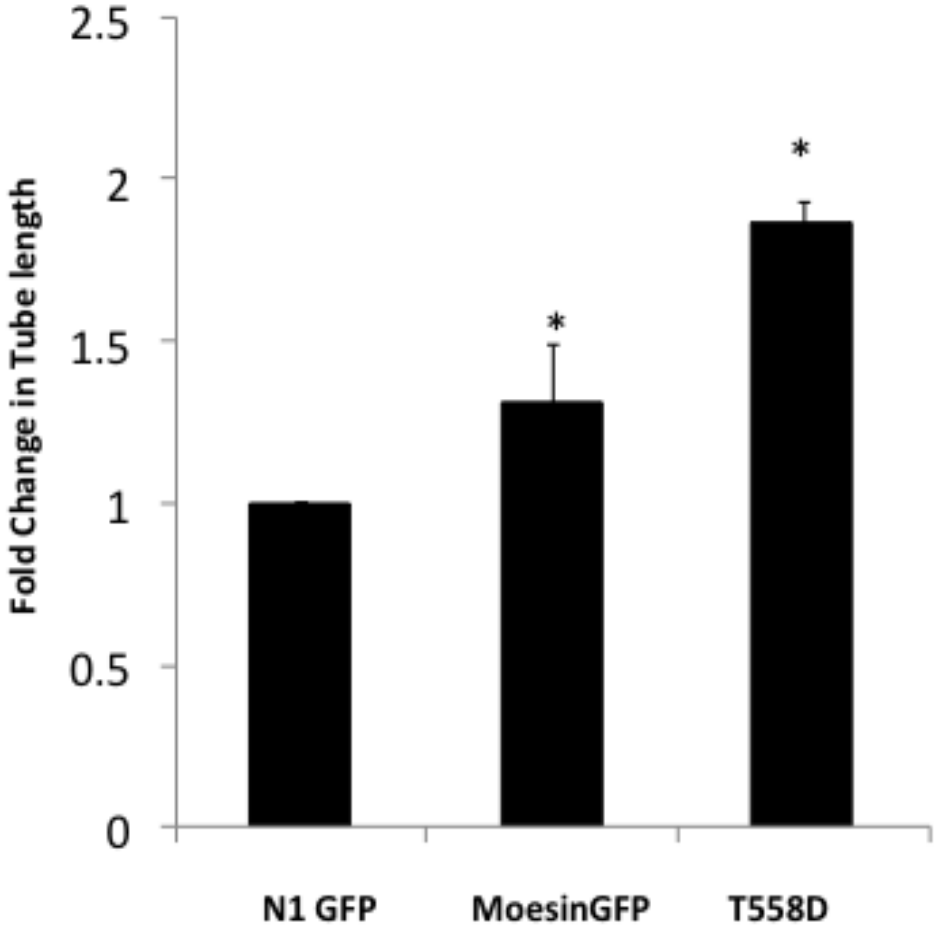

Figure 6.9: Moesin increase angiogenic tube length
HAECs were transfected with the plasmids listed on the graph and seeded equally on collagen gel. The cells were allowed to invade the matrix overnight and images taken with a microscope. The tube length was then measured using CellF® software. Histograms represent fold change in mean tube length compared to GFP control and are averaged from five independent experiments $\pm$ SEM. $*P<0.05$ Statistically significant to each other.

6.2.11 Transient de-phosphorylaton of moesin is required for uPA mediated angiogenesis

As moesin and uPA interact, the next study was developed to elucidate if the synergy exists in a multifunctional process such as angiogenesis. Angiogenesis require digestion of ECM, migration and proliferation to generate new capillary sprouts (Rao *et al.*, 2005). There exists a prerequisite for uPAR receptors even with the classical VEGF mediated angiogenesis mechanisms (Prager *et al.*, 2004).

Moesin we find from studies in this chapter is critical in downstream cytoskeletal rearrangements leading to migration. The angiogenesis studies show uPA increasing angiogenesis (B compared to A). uPA and moesin (overexpression) interact to further the angiogenesis (D compared to C). Moesin overexpression alone increase angiogenesis (A compared to C). We also see the requirement of an intact dephosphorylatory mechanism (F compared to D).

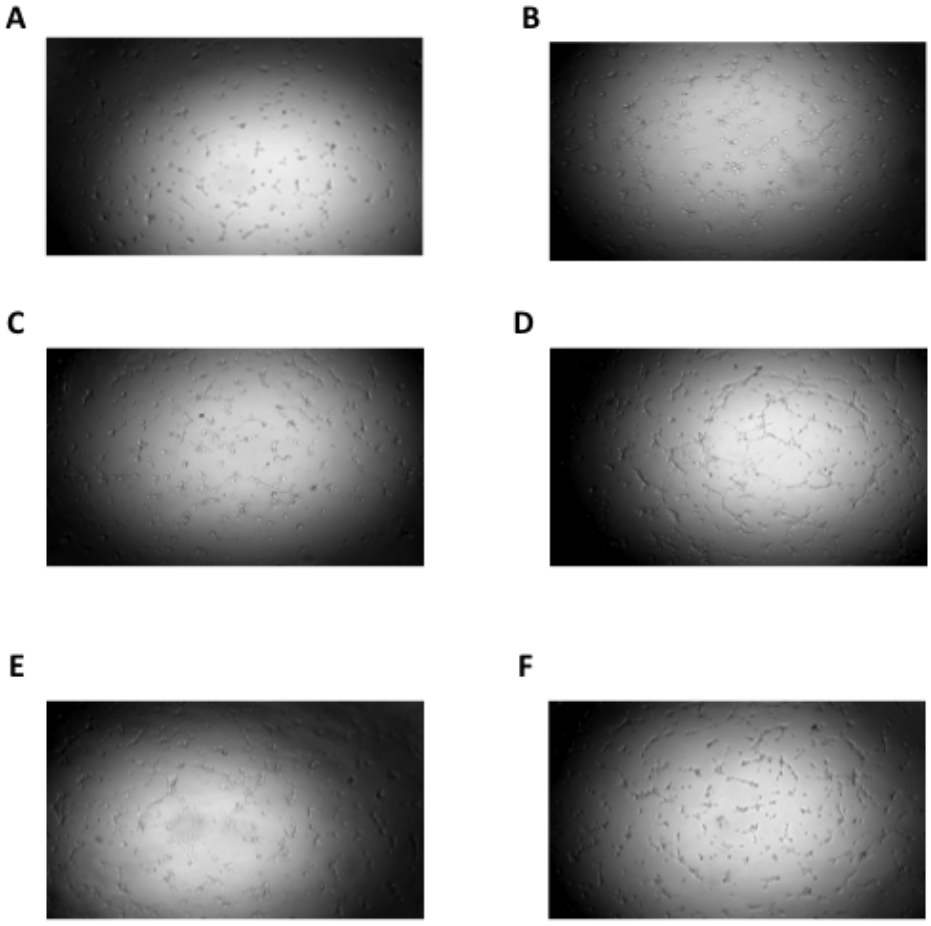

Figure 6.10: Transient de-phosphorylaton of moesin is required for uPA mediated angiogenesis
HAECs were transfected with the plasmids listed below and seeded equally on matrigel. The cells were allowed to invade the matrix overnight after which images were taken with microscope. The matrix contained 10nM uPA/ no uPA. The media added to the matrix contained 0.5% serum. A) GFP control (no uPA). B) GFP control (uPA). C) Msn GFP (no uPA). D) Msn GFP (uPA). E) T558D GFP (no uPA). F) T558D GFP (uPA). Images are representative of at least six independent experiments.

6.2.12 Moesin and uPA can modulate endothelial barrier

Further to our understanding of moesin-uPA interaction in migration and angiogenesis, we decided to investigate cell permeability. This experiment also provides us insight into the cytoskeletal modulation with acute induction of urokinase.

Moesin and uPA independently, tighten endothelial barrier acutely (Figure 6.11A). The effect starts immediately with moesin overexpression, whilst uPA starts affecting the barrier post 30 min (Figure 6.11B). Moesin overexpression leads to reduced permeability. uPA progressively tightens the barrier acutely, showing a marked shift compared to moesin overexpression.

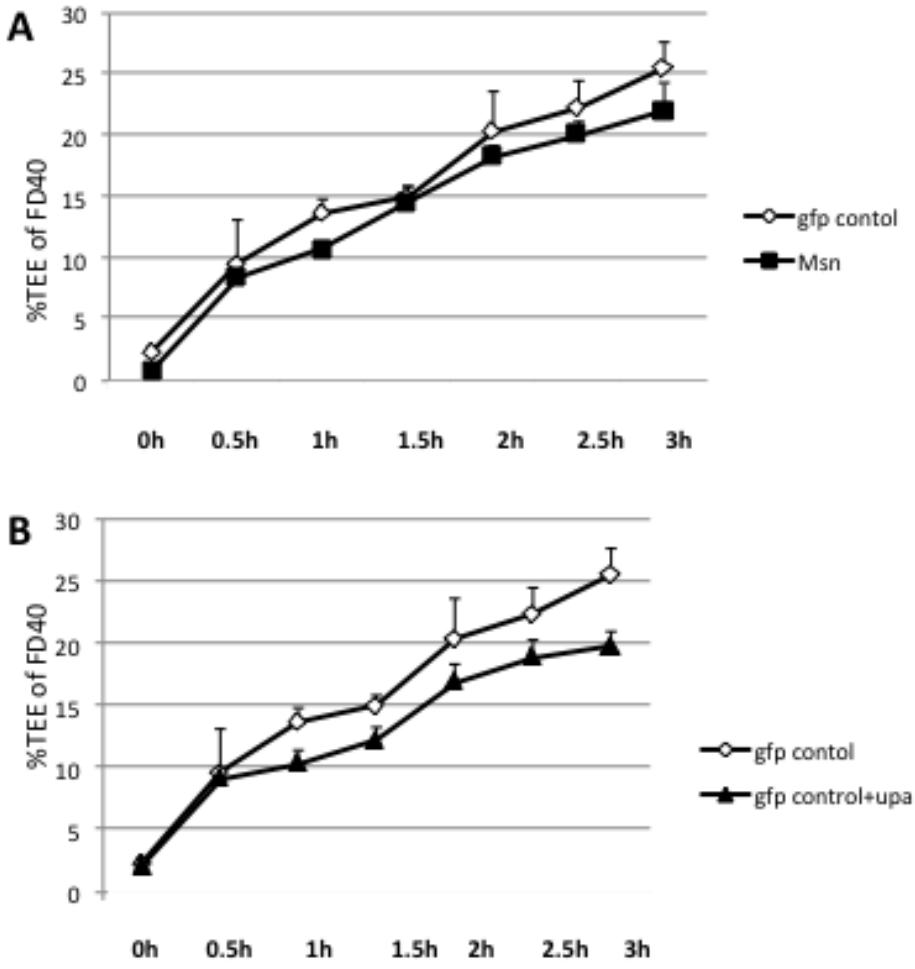

Figure 6.11: Effect of moesin and uPA on endothelial cell barrier
HAECs were transfected with the plasmids for moesin GFP and GFP control. Following trypsinization was re-plated into Transwell® -Clear plates and monitored for permeability to 40 kDa FITC-dextran. Data points are shown as total subluminal fluorescence at a given time point (from 0-180 min) expressed as a percentage of total abluminal fluorescence at t=0 min (i.e. %TEE of FD40 or % Trans Endothelial Exchange, at t=0 uPA was added/not added). A)Effect of moesin. B) Effect of uPA. The results are averaged from three independent experiments $\pm$ SEM.

6.2.13 Moesin and upA interact while modulating endothelial barrier

Earlier study, showed that moesin and uPA individually reduces cell permeability acutely. Previous findings had shown the synergetic interaction of moesin and uPA. Thus here, we investigate moesin-uPA interaction effecting endothelial cell permeability. This study moreover, provide us the significance of the acute moesin-uPA interaction on the cell cortex.

When moesin is overexpressed, acute uPA induction results in loss of barrier integrity and an increase in cell permeability (Figure 6.12A). This reduction in barrier is seen instantaneously, on induction with uPA with a marked shift at 30 min.

When compared to moesin overexpression alone, the barrier loss is higher with uPA addition (Figure 6.12B). The effect though, is transient lasting only to 1 h significantly. This provide insight into moesin-uPA interaction, and the time scale of its effect on the cell cortex.

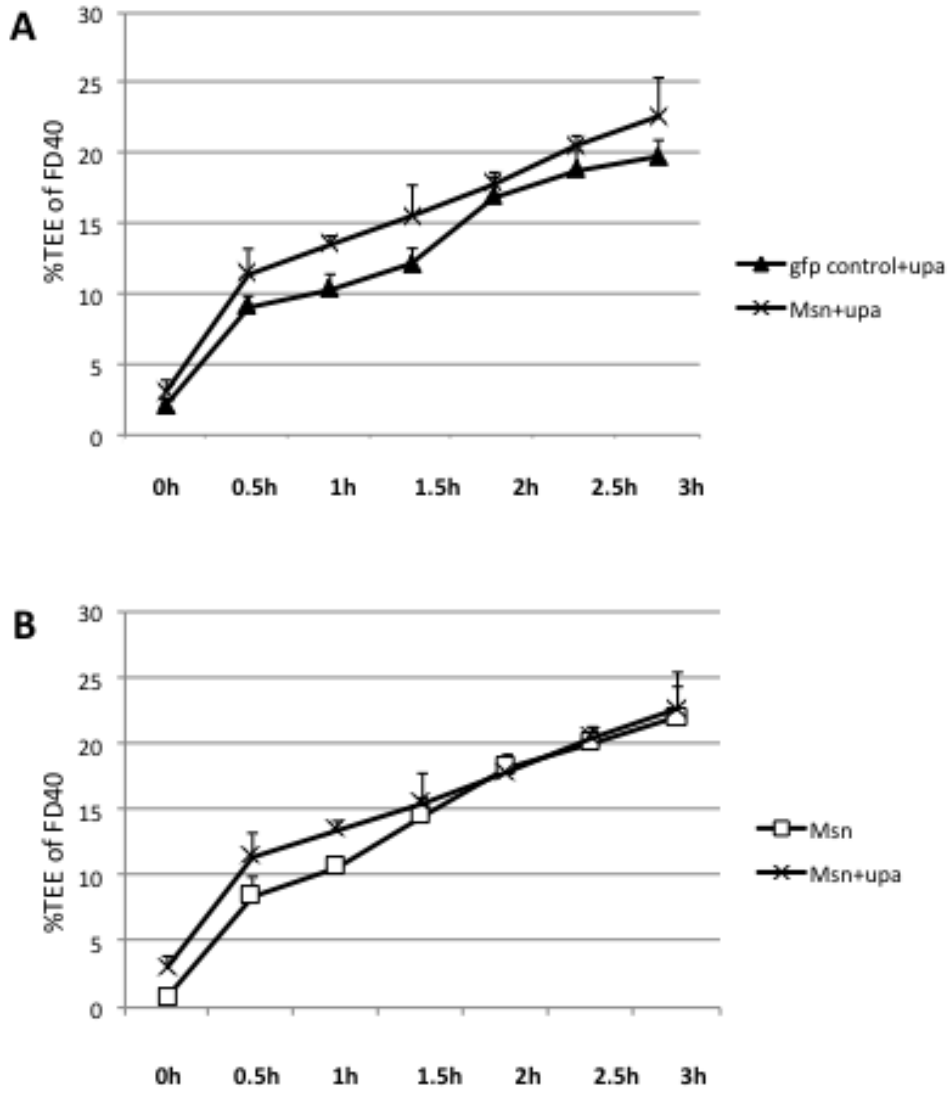

Figure 6.12: Moesin-uPA interaction and endothelial cell barrier
HAECs were transfected with the plasmids for moesin GFP and GFP control. Following trypsinization was re-plated into Transwell® -Clear plates and monitored for permeability to 40 kDa FITC-dextran. Data points are shown as total subluminal fluorescence at a given time point (from 0-180 min) expressed as a percentage of total abluminal fluorescence at t=0 min (i.e. %TEE of FD40 or % Trans Endothelial Exchange, t=0 uPA was added/not added). A) Effect of moesin and uPA interaction.B) Effect of uPA on moesin. The results are averaged from three independent experiments ± SEM.

6.2.14 Visualization of the effect of uPA on moesin

Biochemically, we know that acute induction of uPA results in moesin dephosphorylation. Towards understanding this better, we have visualized overexpressed moesin (GFP).

plc$-\delta-$RFP was also overexpressed, to understand membrane unbound PIP_2 localization under uPA induction. Moesin can be seen co-localising with plc$-\delta-$RFP on uPA induction. This suggests uPA effects loss of membrane bound moesin acutely.

Moreover, the effect is profound, and explains the barrier loss evidenced in earlier studies. There is considerable shrinkage of the cells and modulation of the associated cytoskeletal system.

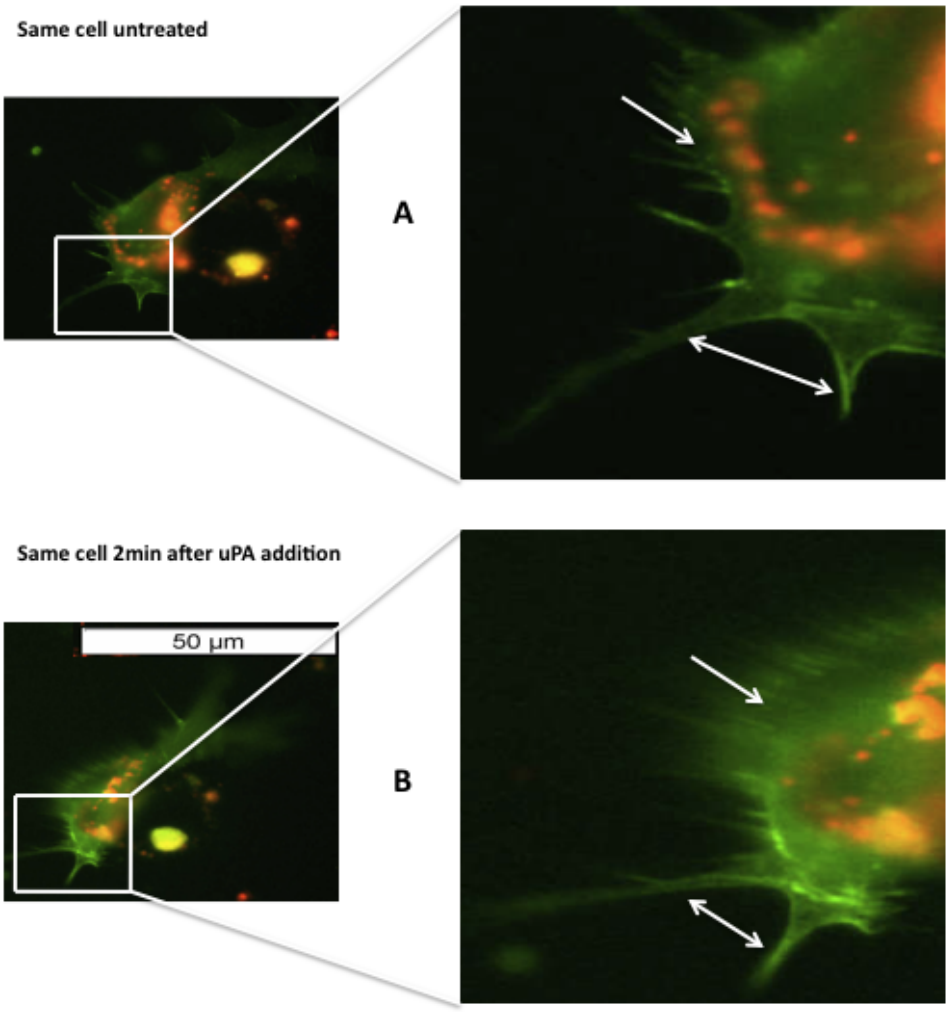

Figure 6.13: Visualisation of barrier loss with acute induction by uPA Moesin-GFP construct was overexpressed by transfection into HAECs in combination with plc$-\delta-$RFP. The same cell was visualized by fluorescence microscope following addition of uPA. Right pane has magnified area of interest in the cell. A) Same cell 0 min. B) 2min after addition; note the cells contracting (bottom arrow) and Moesin GFP losing membrane adhesion (with plc$-\delta-$RFP; upper arrow) Images are representative of at least three individual sets of experiments.

6.2.15 Conclusion

This chapter details evidence regarding moesin-uPA interaction. Furthermore, utilizing various constructs, peptides, we delineate the framework of this interaction. During the course of the study we investigate cell function including angiogenesis, migration and cell permeability. The results and its implication is discussed in the next chapter.

Chapter 7

Discussion

7.1 Haemodynamic regulation in the vasculature; the need for moesin

7.1.1 Characterization of pronectin and fibronectin as an inflammatory matrix

This chapter describes the temporal and spatial regulation of moesin, at both the molecular and cellular levels, with respect to mechanotransduction. In addition, we also endeavored to investigate the role of moesin in various endothelial cell functions. Among the cell fate decisions studied were proliferation, migration, angiogenesis, barrier function and endothelial cell microparticle release.

7.1.1.1 Elucidation of cell growth on pronectin and fibronectin

The standard extracelluar matrix employed during mechanotransduction studies is generally pronectin. This is a synthetic RGD containing peptide, used to facilitate cell adhesion before cyclic strain is applied. This model has been well characterised in the literature (Kakisis *et al.*, 2004; Sweeney *et al.*, 2004; Hunter,

2009; Pyle *et al.*, 2008). Pronectin is widely used, due to its high purity (no contamination) and uniform deposition on hydrophobic plate membranes. We undertook basic preliminary studies to investigate subtle differences between cells mechanotransduced on both pronectin and fibronectin coated plates.

At a technical level we discovered the plate membrane to be highly hydrophobic, an unfavorable platform for cell adhesion and growth. Moreover, this renders it difficult to coat with experimental extracellular matrix. The Flexcell International Corp, meanwhile utilize a proprietary method for coating the membranes. They provide, as standard, pronectin coated plates as an alternative to fibronectin. We overcame the technical difficulties with respect to this hydrophobicity, by exploiting pronectin coated plates for fibronectin deposition.

Experimentally, we devised studies on both matrices in order to characterize the specific cellular response elicited. As pronectin is mainly an RGD containing peptide, its principle binding sites are on specific integrins (see Figure 7.1 (Takagi *et al.*, 2003). Integrin mediated adhesion involves, mainly the RGD binding domain of these heterodimeric receptors. As seen (Figure 3.7), we characterized the cell cycle profile of cells grown on both matrices.

The results obtained indicate no major differences in the cell cycle profile between pronectin and fibronectin. Previous studies have shown that integrins provide support for cells undergoing mechanotransduction (Hirayama and Sumpio, 2007; Lehoux and Tedgui, 1998; Wernig *et al.*, 2003; Sweeney *et al.*, 2004). This is true for both VSMCs and endothelial cells. As oultined in the introduction, different integrins are engaged with specific matrices, irrespective of the agonist. Resulting cell fate decisions are also mediated by specific integrin-matrix engagement. Members of the integrin family containing subunit $\beta 1$, play an important role in cyclic strain mediated mechanotrasduction. Specifically $\alpha 5 \beta 1$ has been found to facilitate endothelial cell polarization on fibronectin via cyclic strain. In addition $\alpha 2 \beta 1$ has been shown to synergistically facilitate endothelial cell realignment on collagen matrix.

The endothelial cell realignment perpendicular to cyclic strain, on both fi-

bronectin and collagen, does not involve β3 integrin subfamily (Yano *et al.*, 1997b; Wernig *et al.*, 2003; Hirayama and Sumpio, 2007). Cytoskeletal remodeling via β1 integrin mediated signaling utilize a unique subset of cytoskeletal proteins. Moesin potentially could play a role in differential integrin signaling depending on the matrix or stimulus employed.

The lack of differences, thus possibly indicate the key importance of RGD peptide. In this study investigating the cellular growth pattern i.e., focussing on cell cycle progression to endothelial cells grown on either pronectin or fibronectin, we identified an increased progression to the G_2/M phase. Potentially various biochemical factors could be involved, as fibronectin unlike pronectin is highly complex. Pronectin an RGD containing peptide, engages the majority of integrins to the exclusion of other receptors. Conversely, fibronectin due to its complex nature engages both directly and laterally a wide range of receptors.

7.1.1.2 What is pronectin?

"ProNectin® F is a protein polymer, produced from a synthetic gene via bacterial fermentation. This presents multiple copies of the RGD cell attachment sequence from human fibronectin (*http://www.sanyo-chemical.co.jp*). While fibronectin a glycoprotein is more complex. It has 4-9% carbohydrate moieties including the integrin engaging RGD amino acid sequence, it also binds to diverse biological molecules. These include matrices generated in response to cyclic strain such as collagen (Wang and Thampatty, 2006). It also binds to heparin, fibrin and a whole array of other molecules, engaging various moeities of its structure (Pankov and Yamada, 2002; Chiang *et al.*, 2009a).

7.1.1.3 Relevance of pronectin and fibronectin as matrices under mechnotransduction

The pronectin coated plates still provide information, but if the relevant question is poised. These studies would include investigating RGD-integrin me-

diated outside-in signaling (Figure 7.1). Fibronectin deposition, meanwhile is increased with inflammation, and thus provides a physiologically relevant inflammatory matrix (Gayer and Basson, 2009). Pronectin is also often used as an alternative towards this purpose. Areas in the vasculature with pathogphysiological shear or cyclic strain leads to inflammation (Hahn and Schwartz, 2009). When stasis of blood flow occurs, such as during heart failure, the patients are predisposed to accelerated thromboembolic events resulting in stroke (Kapur *et al.*, 2007). In such a context delineation of differential signaling between pronectin and fibronectin matrix could be bio-mechanically relevant. In the second part of the study as outlined in (Figure 3.7), we investigate the role of both pronectin and fibronectin on cyclic strain mediated cell cycle.

These experiments were carried out under acute conditions for 4h (Yano *et al.*, 1997b). The study show less apoptotic cells with acute strain, when grown on pronectin relative to fibronectin. Apoptosis rate was upregulated for fibronectin, while downregulated with pronectin under strain stasis. Cell cycle profile meanwhile, show statistical significance in S and G_2/M phase for cells on pronectin.

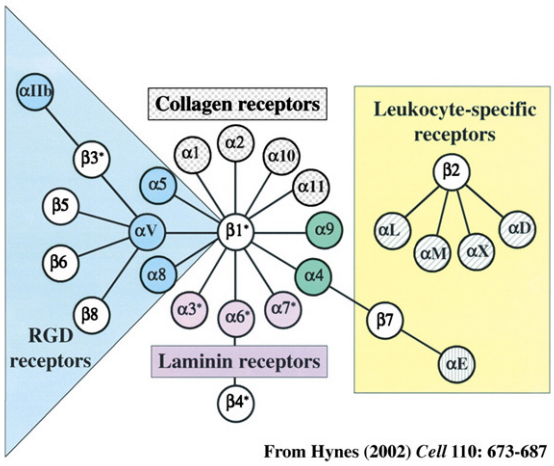

Figure 7.1: Integrins adhesive to RGD

Biochemically in such a model there are different extracellular receptors acting on the cell. Not taking into account other cryptic sites and fragments, such as the fibronectin self-assembly sites whose exposure induce fibronectin fibrillogenesis. There is also the FnIII1that localize to lipid rafts (Sechler *et al.*, 2001). This interaction has importance in cell contraction and cell growth, proliferation (Hocking and Kowalski, 2002). There are sites with enzymatic activity that include FnCol-ase, which is a metalloprotease (Vogel, 2006).

7.1.1.4 Fibronectin is a complex matrix

Fibronectin also has integrin activating sites other than the RGD segment. These include the synergy site Pro-His-Ser-Arg-Asn (PHSRN),which is also recognized by $\alpha 5\beta 1$ integrins (Vogel, 2006).There is also the Ile-Asp-Ala-Pro-Ser (IDAPS) sequence at the Fibronectin Type III domain (FnIII)$13-14$ junction in the heparin binding site II (HepII) region of fibronectin which supports $\alpha 4\beta 1$. This particular interaction plays a role in cell adhesion via mechanical stimulus (Pierschbacher and Ruoslahti, 1984; Redick *et al.*, 2000; Takagi *et al.*, 2003; Mould and Humphries, 1991).

Nevertheless, $\alpha 5\beta 1$ and $\alpha \mathrm{v}\beta 3$ among other integrins, recognize the RGD motif (Hynes, 2002a). We find RGD-integrin (grown on pronectin) specific interactions i.e., induce apoptosis with inflammatory cyclic strain. Similarly they increase proliferation and positively support cell survival. In terms of confirming which matrix to use, towards understanding inflammation with cyclic strain, the complexities laid out are vast with fibronectin. Pronectin is not a holistic matrix that will allow deciphering of signal transduction. As appreciated earlier, matrix has to be chosen to answer the relevant questions.

7.1.2 Cyclic strain can mould the cytoskeleton to generate a highly tactile cell

In (Figure 3.8), we show how cyclic strain increase migration of endothelial cells. Cylic strain induced migration has been found to engage not integrins,

rather the G-coupled proteins (Von Offenberg Sweeney *et al.*, 2005). We were able to reflect the results obtained by other groups, including the 2005 publication from our lab by Nicholas Von Offenberg Sweeney *et al*. Where as their study found increase in migration with 5%cyclic strain, we also find a dose dependent increase in migration with increasing cyclic strain (Von Offenberg Sweeney *et al.*, 2005). The increase in migration rate with increase in cylic strain was not a linear progression, but was quite dramatic. This difference shows the profound latent tensile strength on the migratory apparatus, including the underlying cytoskeleton due to cyclic strain.

7.1.2.1 Outline of relevant molecular determinants that regulate cell migration post cyclic strain

The study by Sweeney *et al*, had also shown that uPA is upregulated by 6.7−fold. Furthermore they had shown G-coupled proteins to be critical in cyclic strain induced migration and angiogenesis. They found uPA and integrins not to play a role in this migration induced by cyclic strain. All three though had roles in increased angiogenesis induced by cyclic strain.

This leads to the question, which particular pathways that engage cytoskeleton might be regulated by uPA/uPAR? That is, cytoskeletal elements that are regulated by hemodynamic forces. We were interested in moesin, an ERM protein discussed in the introduction. Moesin has been found to be dephosphorylated with a ligand against FPRL-1 in a proteomics based study (Boldt *et al.*, 2006b). uPAR is known to interact with FPRL-1. There is however, no known association of uPA/uPAR with moesin via FPRL-1. FPRL-1 meanwhile, is also a G-coupled protein. There is also no evidence of moesin's role in hemodynamic force in the literature. We thus wanted to investigate whether moesin was regulated with cyclic strain. Furthermore, we endeavored to decipher any association between moesin and uPA/uPAR. This would have implications in angiogenesis, migration (due to moesin's known association with G-coupled proteins).

Angiogenesis is a multifactorial process also involving proliferation. There is enough evidence in the literature that show the effect of cyclic strain on proliferation. Our previous study also showed different receptors regulating proliferation with cyclic strain (Figure 3.7). We now wanted to see how an acute cyclic strain, that is the induction of cyclic strain, might change the cell cycle profile. This early phase of cell cycle was studied using fibronectin coated flex plates. As appreciated earlier, fibronectin provides a holistic matrix to study signal transduction with inflammation. Firstly, the results show differences in cell cycle progression with cyclic strain.

7.1.3 Understanding different cyclic strain regimes

There is as well two different regimes involved (Figure 3.9). The 0-10% cyclic strain model and the 8-10% cyclic strain model. During 8-10% cyclic strain, the elastic surface on which human aortic endothelial cells are grown are strained equibiaxially to 8%. Here the membrane is held at 8% while it strains upto 10%. This model is reflective of a more subtle change in cyclic strain.The 0-10% cyclic strain meanwhile induce the maximum equibiaxial strain at 1hz with the machine.

Mechanical stimuli is the fastest agonist to reach the nucleus compared to diffused chemical messengers (Wang *et al.*, 2009b). This allows the cell to shift its cell cycle status in real time. From our results we see differing results for different cyclic strain regimes. Low impact cyclic strain of 8-10% does not show any significant difference when compared to static cells, though both static and 8-10% does progess into cell cycle. High impact cyclic strain meanwhile, holds the cell from progressing into cell cycle. This being the case with acute induction of cyclic strain.

7.1.3.1 Relevance to other cyclic strain studies

Meanwhile others have indicated deformation of adherent cells to be a contributory factor in cell cycle progression (Orr *et al.*, 2006; Huang and Ingber, 1999).

Studies have demonstrated increase in proliferation with mechanical stretch. The regimes they have applied was much higher than what we had applied, 40% stretch and 20% cyclic strain (Liu *et al.*, 2007).

Also, none of these studies investigated cell cycle progression acutely (Liu *et al.*, 2007). The particular study from Christopher Chen's lab, also reflected our study at lower cyclic strain regimes (7%), where they noted no significant difference between the static. Acute sudden onset of hemodynamic force induce an inflammatory response from the cells (Hahn and Schwartz, 2009). This is illustrated in (Figure 1.2) in the introduction. Thus a transduction of high acute force, reflective of a pathogenic stimuli, the cells don't progress fully into cell cycle. Meanwhile the low impact cyclic strain is more conductive of a physiological model. And it reflects other studies at lower strain conducted on fibronectin coated plates (Liu *et al.*, 2007).

Our primary interest in cytoskeletal dynamics involved the FERM domain containing protein moesin. We concurred with others that this actin binding protein is able co-localise with actin on the flex membrane (Figure **??**).

7.1.4 Moesin is highly sensitive to mechanical stimuli

Further to this, we were interested in investigating if this protein is responsive to mechanical stimuli. Endothelial cells subjected to cyclic strain was deciphered for moesin's mRNA, protein and phosphorylation status. We clearly find moesin responsive to cyclic strain (Figure 3.1). The phosphorylation state had also increased with cyclic strain.

7.1.4.1 Moesin protein expression is post-transcriptionally regulated

Interestingly, mRNA levels with cyclic strain did not correspond to moesin protein increase. This could be because moesin protein had a longer half life. This is quite possible, especially with moesin's F1 lobe containing an ubiquitin similar domain. Since its discovery in 1987 there has not been significant work gone into understanding more about this aspect (Vijay-Kumar *et al.*, 1987). It

could be that moesin is targeted less to the 26S proteosome with cyclic strain or otherwise for degradation. This ubiquitin like domain refered to as UBX, is implicated in protein targeting (Buchberger *et al.*, 2001). Recently discovered role of moesin in endocytosis reaffirms the need to fill these gaps in knowledge (Barroso-Gonzalez *et al.*, 2009).

Techniques reviewed in Science (2008) provide powerful high-throughput method exploiting FACS, to elucidate the time delay of multiple proteins being targeted to the proteosome (Grabbe and Dikic, 2008). We also speculated that there could be a role for microRNA machinery, discovered only recently.

7.1.4.2 Relevance of moesin phosphorylation status with cyclic strain

The increase in phosphorylation state suggests an unmasked active moesin, bound to the actin. This is demonstrated in 24 hours, where moesin is bound to actin. As well, there is an increase in moesin after 24 hours, thus it definitely plays a huge role in mechanotransduction. An unmasked moesin thus tugs at F-actin with its C-terminus while bound to PIP_2 at the membrane. This model seems to be the case over 24 h, what about on the induction of cyclic strain?

7.1.4.3 Moesin can also mediate mechanical stimuli in smooth muscle cells

What about in smooth muscle cells over 24 hours (Figure 3.3)? Moesin seems to be a key player in VSMCs as well. The change though is quite the opposite to endothelial cells. Opposing roles of same protein in endothelial cells compared to smooth muscle cells is well characterized. The differential role of moesin in smooth muscle cells would be interesting to decipher and understand. Pivotally, here there was no change between mRNA and protein levels. This was indeed intriguing, is there contrasting protein degradation pathways of moesin associated with smooth muscle to endothelial cells? Smooth muscle cells need paracrine signaling with endothelial cells? And does this lead to probable microRNA regulation or vice versa?

7.1.5 Shear stress also regulate moesin expression

Shear stress is the other hemodynamic force resulting from friction of blood flowing on the endothelium. We find that moesin is regulated by shear stress as well as cyclic strain. 24 hours of shear stress induced a reduction in moesin protein expression (Figure 3.4).

Meanwhile, the shear applied was physiological. It will be interesting to see how shear stress affects a denuded endothelium such as after balloon angioplasty. In such a situation, we would also see smooth muscle cells exposed. Replication of a denuded shear model would also require non confluent HAECs exposed to shear. A clear gap in the study is our knowledge of moesin's phosphorylation status. This is something to be investigated in future, together with their status under cyclic strain in smooth muscle cells.

What about on induction of shear stress, the acute phase rather than the 24 hour chronic phase? Shear stress, we find induced moesin change and has quite a different response to cyclic strain. We find moesin also peripherally translocating, aligning itself in in the direction of flow. It is well known that actin aligns itself in such a manner (Hahn and Schwartz, 2009). Moesin in an F-actin binding protein, takes on a brushed alignment in response to shear. Meanwhile static cells are punctuated by moesin (Figure 3.5).

We wanted to visualize this shift, i.e, from moesin peripherally realigning in response to flow (please refer the video $www.example.com$, (Figure 3.6)). This response was found to start immediately on induction of flow. Moesin can been seen realigning, especially in the vicinity of cortical actin and membrane. This plausibly in conjunction with actin, stablilises the cell adapting it to shear.

7.1.5.1 Moesin is the major ERM protein in endothelial cells capable of mediating mechanical stimuli

Radixin and ezrin are not found to the same levels as moesin in cells of hamatopoetic lineage and endothelial cells (Berryman *et al.*, 1993; Pataky *et al.*, 2004; Ivetic and Ridley, 2004). On checking the response to shear stress, ERM mRNA

levels generated two interesting results (Figure 3.4).

1. Moesin mRNA levels were quite high compared to down regulation of moesin protein levels in (Figure 3.4).

2. Interestingly this study shows ezrin mRNA not significantly changing with shear stress.

7.1.5.2 Appropriate mechanical response off ERM proteins, is suited to respective molecular niche

Radixin mRNA changes substantially. Radixin though, is not highly expressed in endothelial cells. Moesin's expression dwarfs radixin and ezrin in endothelial cells (Schwartz-Albiez *et al.*, 1995). As well, there might be specialised roles for these proteins in transducing different mechanical stimuli. Touch, hearing, flow of urine and flow of blood require sensitive receptors to trasduce mechanical force. The receptors and their associated molecules are specialized to the organ (Orr *et al.*, 2006). Arterial shear stress generates 1-3 N/m^2 (Davies, 1995). Meanwhile outer ear cell stereocilia needs to be sensitive to 0.001 N/m during compression stiffness and generates 0.1-20 pN/mV as force/Δ membrane potential. Radixin in contrast to moesin and ezrin, is a component of sterocilia (Pataky *et al.*, 2004).

Thus in areas of very low shear and low oscillatory shear, radixin might have a certain "molecular environmental niche" role to play. Cells might be able to differentiate a pathogenic low flow, due to plausible insensitivity of moesin versus the high sensitivity of radixin, though radixin is expressed at low levels. Radixin might be able to take up the function of moesin during a potential X chromosome linked disorder, albeit with certain functional loss. This is because moesin in contrast to radixin has the locus on X chromosome (Wilgenbus *et al.*, 1994). Radixin is found at comparatively higher levels in sinuosoidal endothelial cells, though all studies show that moesin is the dominant ERM protein in endothelium (Doi *et al.*, 1999). Studies into hearing disorders,

might also provide insightful signaling information associated with such high sensitivity of radixin.

Nevertheless, radixin due to its hypersensitivity is possibly not suited to engage in endothelial cells. This hypersensitivity might not be suited to normal physiological flow. This could thus be the reason for radixin's scant expression in endothelial cells. If radixin was heavily expressed instead of moesin, this could result in unalleviated downstream signaling, leading to chronic inflammation. Moesin as we discover later is atheroprotective and necessarily sensitive (chapter relating to microparticle release and thrombin induction). Expression of radixin is justified in specific organs such as the ear canaliculi for sound transduction, not for the cardiovascular system regularly exposed to blood flow (and the various molecules associated).

7.1.6 Moesin in nucleus

7.1.6.1 Moesin is translocated to the nucleus with cyclic strain

Moesin is the major ERM protein in endothelium. It is also regulated by hemodynamic forces in endothelial cells and smooth muscle cells. Earlier we noted cell cycle regulation by cyclic strain. We now find that moesin is as well, translocated to the nucleus with cyclic strain (Figure 3.2). We have demonstrated this using two different biochemical seperation methods.

7.1.6.2 Moesin in sub-confluent cells and its relevance

Moesin has been found to translocate to the nucleus in subconfluent cells. In this stage, the cells are highly proliferative and migratory, two functions proven to increase with cyclic strain. Moesin meanwhile is not present in the nucleus of static confluent cells (Batchelor *et al.*, 2004). This group has shown undetectable level of moesin in the nucleus when cells are confluent.

Meanwhile, in an injured endothelium such as with balloon angioplasty, endothelial cells are scraped. This results in sub-confluent monolayer of endothelium which is subject to migration and proliferation. Moesin thus in an injured

endothelium, i.e., subject to cyclic strain but with a subconfluent phenotype would be an interesting model. Smooth muscle cells also need to be elucidated in this context. This is because VSMCs are also exposed under such conditions. Blindt *et al* has already shown moesin overexpressed post restenosis in migrating smooth mucle cells *in-vivo*. This group has thus also clearly demonstrated moesin overexpression to increase migration substantially (Blindt *et al.*, 2002).

Thus moesin's role in an injured endothelium, especially with regard to its requirement towards the migratory apparatus is intriguing. Our lab has previously shown uPA involved in migration and post cyclic strain in angiogenesis (Von Offenberg Sweeney *et al.*, 2005). As we discussed earlier in section i of this chapter, is moesin an adapter protein regulated by upA/uPAR complex in migration and angiogenesis?

The persistent increase of moesin with cyclic strain in sub−confluent cells denote the significance of integrins in mediating mechanical stimuli via moesin. The RGD-matrix pronectin, and fibronectin matrix show similar increase in moesin further strengthening integrins in this particular role.

7.1.6.3 Moesin increases with mechanotransduction in the cytosol

There is an increase of moesin in cytosol with cyclic strain (Figure 3.2). As stated earlier, cyclic strain neverthless increase moesin. It would be interesting to elucidate moesin's expression in the membrane and cytoskeletal fractions. A role for moesin in cell cycle is an obvious hypothesis due to its presence in the nucleus.

7.1.6.4 Phosphorylation of moesin found with translocation to various compartments of the cell

We also find moesin phosphorylated in the nucleus with cyclic strain (Figure 3.2). The moesin in cytosolic fraction with strain we find is also phosphorylated. This is intriguing, as it could potentially have roles in transport of molecules. The ability of moesin to bind filamentous actin, PIP_2 (in the nuclear

membrane) with phosphorylation possibly facilitates this. There could also be other unidentified cytosolic proteins that moesin is able to link. Identification of these proteins utilizing pull-down studies might lead to insightful information.

In the nucleus, moesin could have a role in tethering F-actin. In such instance what binds to the FERM domain of moesin? PIP_2 as mentioned earlier has been demonstrated to be on the nuclear envelope, as well as inside the nucleus (Mazzotti *et al.*, 1995). Also, PIP_2 is well charachtersised to stabilize moesin near to the cell membrane, at the FERM domain for phosphorylation (Niggli and Rossy, 2008). Thus, it is an obvious FERM domain binding partner for moesin at the nuclear envelope.

Moesin is also phosphorylated in the cytosol. This implies moesin is bound to actin, and bound to an unknown FERM domain binding partner. It could be that moesin's N−ERMAD, might be bound to another ERM protein's C−ERMAD. While this particular ERM protein might be bound to PIP_2 on the membrane. There could also be other interactions, with other adapter proteins being part of a larger complex. Which might then be stabilising actin bound moesin in the cytosol. The presence of oligomers are known for ERM proteins, but unlike heterodimers, their functional significance is unknown (Bretscher *et al.*, 2002). The increase in cytosolic phosphorylated moesin with cyclic strain is thus of significance.

7.1.7 Is moesin a determinant for cell proliferation in the vasculature?

Our studies with respect to moesin's role in proliferation has been inconclusive (not shown). As previously explained Blindt *et al* have proven moesin overexpression to increase proliferation of coronoary smooth muscle cells. Though VSMCs and ECs have contrasting roles with respect to protein expression and proliferation.

7.1.7.1 Plausible regulators of moesin derived proliferation in vascular cells

Meanwhile, Raj Kishore *et al* have proven ezrin the ERM protein, regulating endothelial cell proliferation. They have shown that ezrin translocates to nucleus post phosphorylation, mediated by Rho kinase downstream of TNF-α (Kishore *et al.*, 2005). Importantly, we find ezrin expression not changing with mechanotransduction (Figure 3.6). They also note cyclin A directly mediating ezrin regulation and show implications in angiogenesis.

A recent publication sheds light on the plausible regulatory elements affected by moesin's overexpression, pertinent to proliferation. They noted down-regulation of p16, cyclin D1 and cdk4 with moesin overexpression in human dermal microvascular endothelial cells (HDMEC) (Lee *et al.*, 2009). Though they concluded that lentiviral application must have rendered the increase in proliferation paradoxical.

7.1.7.2 Matrix mediation in proliferative response

Our studies further show moesin downstream of RGD mediated integrins. In section i we compared proliferation of endothelial cells, with respect to pronectin and fibronectin. Meanwhile in the study conducted with cyclic strain (Figure 3.1), we also discovered that moesin expression with cyclic strain is regulated mainly by integrins. This is because, we conducted the studies with both fibronectin and the pronectin (containing only integrin specific RGD). As shown in the figure, both resulted in similar moesin expression. There was also no statistical significance attributed to the difference.

Furthermore, translocation of moesin to the nucleus (under cyclic strain) results from integrins (owing to pronectin), (Figure 3.2). Sub-confluent cells indicative of an injured endothelium, also show integrin mediating increase in moesin with cyclic strain (Figure 3.1). Conversely, any role moesin might have in proliferation with respect to cyclic strain can be attributed to integrin signaling. Further investigation into this should yield other interesting results. Moesin expression is also influenced by confluency under mechanotransduc-

tion.

7.1.8 Conclusion

Thus there are various roles moesin, the major ERM protein might have in endothelium. These possibly include proliferation, migration and angiogeneis. Meanwhile we are also interested to see how uPA modulates these cell fate decisions. Also, moesin expression with mechanotransduction, did not differ when either fibronectin or pronectin was used (Figure 3.1). This demonstrates that integrins are major transducers of moesin under cyclic strain.

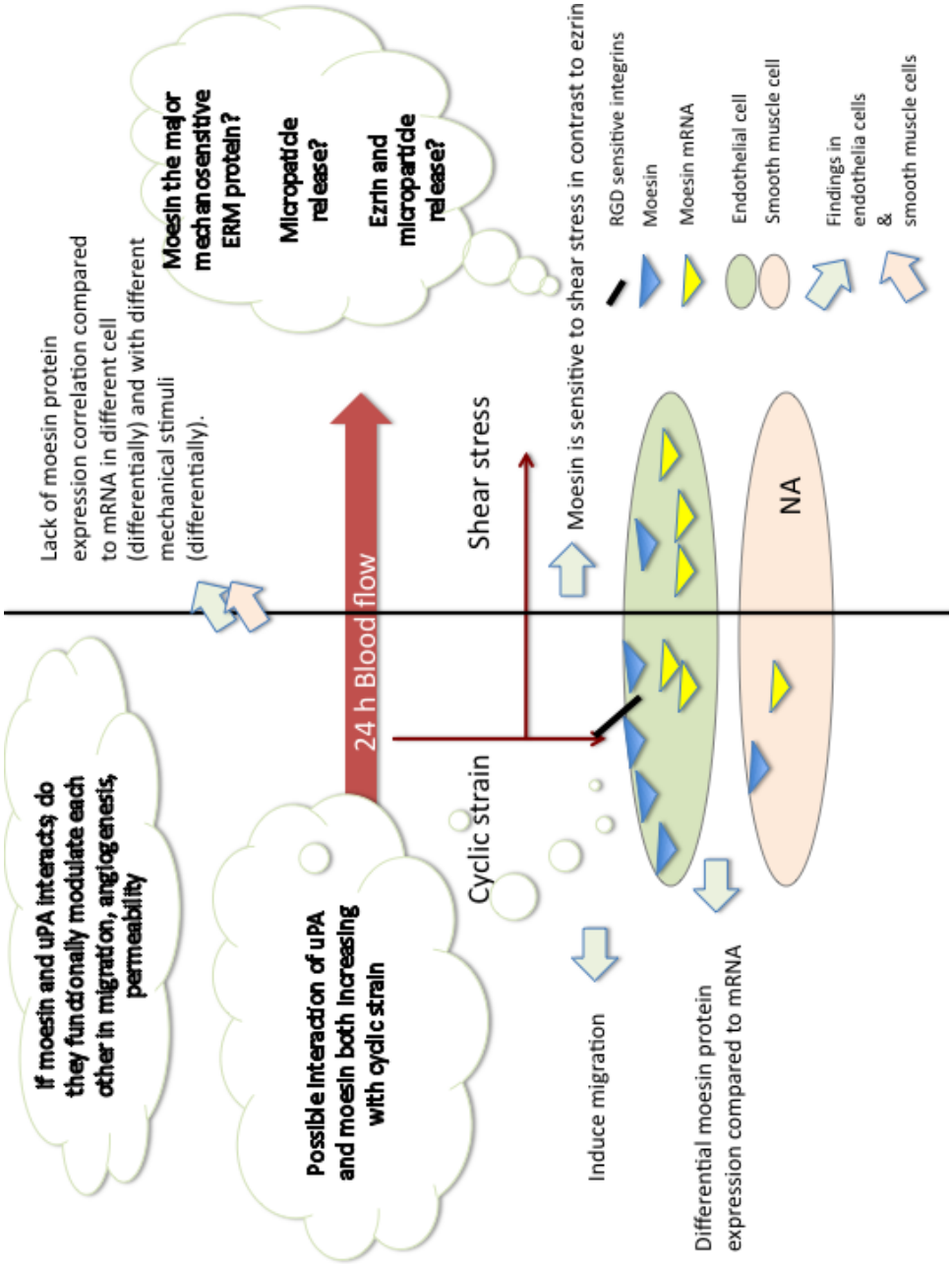

Figure 7.2: Moesin and mechanotransduction

We also have the added knowledge of its pivotal importance with mechanotransduction contrasting other ERM proteins (Figure 3.4). In the following chapters we investigate why mRNA and protein levels of moesin do not correlate. We also see if moesin has a role in microparticle release. We also investigate if moesin is an eligible candidate that link uPA/uPAR signaling towards angiogeneis and migration.

7.2 microRNA regulation of moesin under mechanotransduction

7.2.1 microRNAs regulated by shear stress and cyclic strain

7.2.1.1 Identification of microRNAs that could potentially target moesin

We analyzed microRNAs using *in-silico* bioinformatic studies. This generated microRNAs that share homology to moesin mRNA (Figure 4.1). When homology is considered with respect to microRNA, the importance is in the seed region (Lewis *et al.*, 2005). As illustrated in (Figure 4.1) this was found in hsa-miR-221, hsa-miR-489, hsa-miR- 215 and hsa-miR- 767-5p .

7.2.1.2 Current knowledge of identified microRNAs in the vasculature

miR-221 has been found to inhibit normal erythropoiesis, while miR-489 is implicated in breast cancer metastasis (Felli *et al.*, 2005; Foekens *et al.*, 2008). They found miR-489 in estrogen receptors (ER) positive cells, primarily driven by these receptors. Estrogen receptor α is known to drive actin remodeling and endothelial cell migration. Furthermore, the mechanism discovered, was via Rho A-Rho kinase-Moesin pathway (Simoncini *et al.*, 2006; Sanchez *et al.*, 2009). With regard to erytropoeisis, moesin's expression decreased as erythroid cells progressively matured to erythrocyte (Masumoto *et al.*, 1998; Winckler *et al.*, 1994).

miR-215 has been implicated in cell cycle arrest owing to its upregulation

via p53(Braun *et al.*, 2008). Thus in addition to complimentary seed region these microRNAs have complimentary roles to moesin. The study by Harvey *et al* meanwhile, implicated moesin and ezrin regulation by microRNA system (Harvey *et al.*, 2008). Additionally they found moesin and ezrin downregulation with dicer knock out in podocytes. Generation of mice dicer knockout mice is not feasible, due to fatality in 7.5 embryonic week. There is a lack of knowledge in endothelial cells, particularly with mechanotransduction. It was interesting to note that gelsolin (distally involved in microparticle formation) was also under microRNA regulation. This has implications in endothelial microparticles (Figure 1.24) (Piccin *et al.*, 2007).

7.2.1.3 microRNAs that potentially targets moesin are sensitive to mechanical stimuli

Further to this, we have shown how mechanotraduction affects the microRNAs identified in the earlier study (Figure 4.2) . The microRNA responsible for sharp shifts in cell proliferation hsa-miR-215, did not change with mechanotransduction and neither did hsa-miR-767-5p. Meanwhile we find variations in hsa-miR-221 and hsa-miR-489.

The full list of microRNAs analyzed and their change is given in (Figure 4.3), (Figure 4.4), (Figure 4.5), (Figure 4.6), (Figure 4.7). Since presenting this profile at three international conferences, there are now few reported microRNAs confirmed in mechanotrasduction (Kuang *et al.*, 2009).

It is clear from these studies that there exists a large difference between hsa-miR-489 with shear and cyclic strain. The shift is in opposing directions, when compared between shear and cyclic strain. This interestingly reflects differing moesin expression with shear stress and cyclic strain. With shear stress meanwhile, we see a bigger shift in moesin protein expression when compared to its mRNA. In future, it will be easier to tease out the specific microRNAs involved, with respect to moesin. This is because our studies found only two microRNAs (that plausibly targets moesin mRNA), hsa-miR-221 and hsa-miR-

489 under mechano-regulation. Thus overexpression studies will provide information whether moesin is impacted by these microRNAs. Knocking out these microRNAs concurrent to induction of mechanical stimuli (shear stress, cyclic strain) will provide more conclusive evidence. This study will further confirm if the effects due to dicer knockdown is specifically due to these microRNAs. Furthermore, reporter based study (commercially available from Applied Biosystems®) will confirm the specificity of microRNAs to moesin mRNA.

7.2.1.4 Moesin is regulated by microRNAs

Further to this study we wanted to demonstrate how actual moesin protein expression, is impacted by microRNA regulation (Figure 4.9). For this we looked for inspiration to Suarez *et al*'s study (Suarez *et al.*, 2007). Their studies utilizing dicer downregulation were elegant. They showed role of microRNA regulation in diverse functions, such as angiogenesis, proliferation and in other functional aspects.

7.2.1.5 Appreciation of Dicer knockdown

In the introduction it is explained how Dicer, drosha and Ago proteins generate microRNA. Dicer down-regulation thus is a better target than drosha or Ago proteins, for understanding microRNA system. This is because, all microRNAs equally pass through dicer processing whilst, there are drosha independent mechanisms for microRNA generation. There are some microRNAs also differentially processed by many Ago proteins (Ghildiyal and Zamore, 2009). Our experiments involved investigating if moesin was under microRNA regulation. Thus, Dicer down-regulation would be an ideal solution towards this purpose.

7.2.1.6 Molecular elements responsible for moesin phosphorylation are regulated by microRNA

There was a marked change in moesin protein levels with dicer downregulation (Figure 4.9). This demonstrated that moesin is regulated by the microRNA machinery, when subjected to cyclic strain. The phosphorylation of moesin had also decreased considerably. This suggests the regulatory mechanism required for moesin phosphorylation is also under microRNA regulation.

7.2.2 microRNA can activate as well as repress protein expression

It is a widely held view that microRNAs repress proteins. Our experiments meanwhile show that with microRNA expression, moesin protein was in fact up regulated. It has to be noted that the only other research that studied moesin with respect to microRNAs (on kidney podocytes, mentioned earlier), also demonstrated that moesin downregulation with dicer knock down. Thus, they too observe increase in moesin expression with intact microRNA machinery (Harvey *et al.*, 2008).

The study by Vasudevan *et al* in Science has confirmed that specific microRNAs can in fact activate protein translation (Vasudevan *et al.*, 2007). Initially they found microRNA miR369-3 oscillating between its repressive and activating role. There was a dependence on cell cycle, with repression in proliferating cells. While in a quiescent state (physiologically, endothelium exists in a quiescent state unless injured), the microRNA initiated activation of the mRNA (Dejana *et al.*, 2009). They found these factors holding true for the well studied Let-7 and miRcxcr4 microRNAs.

Direct identification of specific microRNAs utilizing reporter studies is needed to fully clarify the issue. There could be other answers such as interaction of other proteins, that could repress or activate the protein studied.

7.2.2.1 Agonists can differentially regulate same microRNA

Their studies led to identification of TNF-α RNA activation/repression. TNF-α incidentally phosphorylates moesin (Koss *et al.*, 2006). Furthermore, we has discussed previously that moesin expression is regulated by cell-cell contact.

Mechanotransduction with respect to moesin as demonstrated previously, was increased irrespective of cell-cell contact (Figure 3.1). This suggests integrin to be the primary transducer of mechanical stimuli with respect to moesin. Plausibly, same microRNA could differentially activate or repress proteins downstream of RGD specific integrins. We identified cyclic strain mediated increase in moesin during sub-confluence and confluence. Mechanical stimuli engages integrins irrespective of cell cell contact. Please refer Figure 1.19 for the various receptors involved with mechanical stimuli. Thus, integrins could potentially clarify whether microRNA repress or activate proteins.

Activation of proteins by microRNAs was discovered only recently. This was discovered as stated earlier by Vasudevan *et al*. They found miR369-3 recruiting proteins Argonaute (Ago) and fragile X mental retardation–related protein 1 (FXR1). This activated the translation by binding to the TNF-α RNA at its AU-rich elements (AREs). This clearly has relevance to moesin phoshorylation, as TNF-α can phosphorylate moesin (Koss *et al.*, 2006). Science also published work by Jopling *et al*, who discovered activation of the viral RNA (Hepatitis C) by miR-122(Jopling *et al.*, 2005). Widely held view has been that microRNAs repress protein expression. Mechanical regulation as we discovered, down regulates majority of microRNAs. What significance this will have on protein expression, require further study. Our research, thus is one of the only few studies that actually confirm activation of a protein with an intact microRNA system.

The agonist dependence on microRNA repression or activation is intriguing. It would also be of interest, if shear induced a differential response. Mechanotransduction, might similar to Vasudevan *et al*'s discovery be agonist/context dependent. We confirmed repression of moesin translation by microRNA ma-

chinery under shear stress. Meanwhile down regulation of dicer under shear stress, spurred moesin protein expression (Figure 4.10). Here we find different agonists of hemodynamic forces inducing differential microRNA regulation.

7.2.3 Acute stretch induced dephosphorylation, and are regulated by microRNAs

Change in phosphorylation status of moesin with mechanotransduction, as discussed previously, suggest regulators of moesin might be under microRNA regulation. We thus devised acute studies to understand the phosphorylation change of moesin. Biaxial stretch was gently applied according to the study by Yi-Jen Chiu *et al*, whereby shear stress was kept minimal (Chiu *et al.*, 2008). Furthermore, it was interesting to observe how application of acute stretch dephosphorylated moesin. This we discovered to be maximal at 10 minutes (Figure 4.11), while chronic (24 hour) application of cyclic strain produced a hyperphosphorylated moesin (Figure 4.11).

7.2.4 Rho A mediates stretch induced de-phsophorylation

We have a list of possible suspects that might dephosphorylate moesin. In the introduction we pointed out the unappreciated role of dephosphorylation, with regard to moesin. We wonder, about the structural change associated with moesin dephosphorylation.

Dynamic dissociation is a charachteristic of actin binding adapter proteins like moesin. We hypothesised that the disruption of cortical actin and the ensuing turnover will lead to translocation of proteins. This requires moesin to lose affinity for actin in one area for another. As well this might be a transient mechanism, universal for agonists that require the same. The stretch applied on the cell tugs at the actin, which tries to stabilise itself to this mechanical stimuli (as visible in (Figure 3.6)). This possibly require cytoskeletal remodeling. Dephosphorylation might be a required conformational change to effect this process.

7.2.4.1 Rho A and moesin

Rho mediated pathways in BAECs are deemed critical for stress fibers to orient perpendicularly to stretch (Chien, 2007; Kaunas *et al.*, 2005). Rho Kinase phosphorylates moesin, but without the stabilisation by PIP_2, it is unable to achieve this. Further to this, PIP_2 is seen as a critical factor in moesin's phosphorylation (Matsui *et al.*, 1999; Niggli and Rossy, 2008). Another study meanwhile has challenged moesin and Rho GTPases, in its ability to regulate each other in a directly proportional manner. They discovered moesin phosphorylation and Rho pathway being antagonistic to each other (Speck *et al.*, 2003). Meanwhile we were using fibronectin coated plates, which stimulate inflammatory pathways. In this context Rho, not Rac is shown to be activated (Gu *et al.*, 2001). Our study meanwhile found moesin unable to dephosphorylate with acute stretch, with Rho abrogation whilst grown on fibronectin matrix (Figure 4.11). We were thus able to confirm the negative feedback between moesin and Rho, found initially by speck *et al* (Speck *et al.*, 2003).

7.2.4.2 Dephosphorylatory mechanisms with respect to moesin

From the literature there is the Calyculin-A (phosphatase inhibitor) insensitive threonine phosphatase type 2 protein phosphatase (PP2C), that can dephophorylate moesin. The study though was conducted with highly purified moesin and PP2C. This study first proposed a phophatase and is nevertheless important (Hishiya *et al.*, 1999). From our observation and the observation of others, the calyculin sensitive phophatase seems to rapidly dephosphorylate moesin. This is also possibly functionally related, such as mechanotransduction (shear stress, cyclic strain) might involve a different phophatase to some other agonist.

We do find moesin phosphorylation preserved with Calyculin A as shown in (Figure 3.2). Functionally we appreciated earlier how, phosphorylation of moesin is pertinent to its ability to translocate to various cell compartments.

Phosphorylation − dephosphorylation mechanisms could also vary between

shear stress and cyclic strain. It has been shown chemokine stromal cell-derived factor 1α (SDF-1α) is able to dephosphorylate via calyculin-A sensitive phosphatase, and thus is not dephosphorylated by PP2C (Ivetic and Ridley, 2004).

With respect to SDF-1α, it has been shown that it is Rac1 and not Rac2, Rho, or Cdc42, that is able to block the functional event of microvillar loss. They went on to prove that Rac-1 is able to do this via dephosphorylating the ERM proteins (Nijhara *et al.*, 2004b). Structurally the C terminal, especially the basic residues held there, is responsible for Rac-1 based dephosphorylation of moesin.

The dephosphorylation event was found by us and others to be very transient, as this is required to re-arrange the actin cytoskeleton towards the new agonist's signaling route. Also, further in the discussion with respect to the study in urokinase, we note the dephosphorylation as an absolute requirement to effect chemotaxis.

This is also the case with Rac-1 activation, where it is transiently activated by the chemokine SDF-1α. This was one of the reasons why others had previously not observed Rac-1 activation with respect to this chemokine (Nijhara *et al.*, 2004a; Shaw *et al.*, 2001).

One deficiency in this study, is the lack of insight into merlin's whereabouts when Rac-1 is activated. Merlin is phosphorylated by p21-activated kinase (Pak) in response to activated Rac (and to lesser extent due to cdc42) (Daniels and Bokoch, 1999). As explained earlier merlin has more affinity towards the unbound monomer of ERM proteins than to its own unbound monomer. Thus, a plausible mechanism is whereby recently unbound merlin(activated/phosphorylated) clutches to the nearby moesin. Moesin is more expressed than its couterparts in endothelial cells and leukocytes and thus has higher probability to be targeted (Berryman *et al.*, 1993; Pataky *et al.*, 2004). Localising the phophatases to its substrate obviously makes it a target. Thus, there are other possible phophatases that might be involved, such as the myosin phosphatase, whose subunit myosin binding subunit (MBS) interacts with moesin. There is also positive loop system with respect to ROCK, one of the well known

phosphorylator of moesin. ROCK also phosphorylates myosin phosphatase, which inactivates its phosphatase function (Fabian *et al.*, 2007).

Protein tyrosine phosphatase (PTPL1) is a cytoplasmic protein and a tyrosine phosphatase, that also contains the FERM domain. This makes it a possible target for the C-ERMAD of the ERM proteins. There are no known associations so far with the ERM proteins. Even though this FERM domain exists, PTPL1 is a tyrosine phophatase and thus not relevant to moesin which is only phophorylated at the threonine residue. Ezrin on the other hand is phosphorylated at the tyrosine residue, and this might be a route of inhibition with active moesin. Incidentally, PTPL1 also targets PIP_2 (major stabilizer of ERM proteins, for their phosphorylation) on the plasma membrane (Bompard *et al.*, 2003; Abaan and Toretsky, 2008). This might cause ezrin to encourage forming heterodimer formation with actin bound active moesin. If heterodimer does form, this will result in dephosphorylation of moesin. Further, it will lead to a decoupling of actin from the membrane and PIP_2 disassociation from the cytoskeleton. Thus, all phosphatases that interfere with the ERM protein self associated monomers, active actin bound monomers, oligomers (with other FERM domain proteins, such as merlin and probably talin) will have profound effect on the cytoskeletal architecture. This could result in sudden loss of membrane adhesiveness to the cytoskeleton, leading to increased microparticle formation with role in cell-cell adhesion thus resulting in spurt of permeability loss. This is also true with respect to cleavage of ezrin with calpain, the only calpain cleavable ERM protein (Shcherbina *et al.*, 1999a). This might be also a mechanism of transient change, required for plasticity of the cytoskeleton towards any agonist.

7.2.4.3 Rho A is potentially regulated by microRNAs

We also find moesin dephosphorylation with stretch regulated by microRNAs. The results (Figure 4.11), have an uncanny reflection of Rho abrogation study. There was also no statistical significance in any perceived change. We thus find moesin phosphorylation / dephosphorylation and its expression, is con-

trolled substantially by the microRNA system. We also provide evidence to the possibility that activity of Rho is regulated by microRNAs.

Rnd proteins reviewed in the introduction, provide example of GTPases that are regulated by their expression. These atypical GTPases and moesin, does seem to be the usual suspects. They are both implicated with respect to rounding of cells (Chardin, 2006; Rosenblatt, 2008). Phosphorylation of moesin is critical to rounding of cells. Researchers are finding it hard to pin the entire phosphorylation event on Rho A, with respect to cell rounding. Our findings with respect to microRNA regulation with respect to Rho A, moesin and the speculation regarding Rnd proteins, might be significant here.

Our findings here thus endeavor to answer questions raised earlier in this section, regarding differences in moesin protein expression to its mRNA. There are other plausible mechanisms which could equally contribute. There are many open questions regarding the mechanism by which Rho controls dephosphorylation. Does sudden change in stretch protect myosin phosphatase from Rho kinase (Kimura *et al.*, 1996)? Speck *et al*, meanwhile has showed phenotypic change associated with overexpression, similar to abrogation of the other. Though, what are the intricate mechanisms of Rho antagonism with respect to moesin (Speck *et al.*, 2003)?

7.2.5 microRNA regulation is critical to physiological functioning of the endothelium

7.2.6 microRNAs regulate cell alignment with shear stress

Mechanical stimuli on endothelial cells is impacted by a whole array of microRNA (Figure 4.3),(Figure 4.4), (Figure 4.5), (Figure 4.6), (Figure 4.7). We were also able to demonstrate that endothelial cells are dysfunctional and don't align in the direction of flow when Dicer is downregulated (Figure 4.12). Harvey *et al* has shown how cytoskeletal proteins including moesin is downregulated with dicer knockdown (Harvey *et al.*, 2008). Our own studies have also

shown moesin's role in dynamically associating with cortical flow (Figure 3.6). Here we demonstrate holistic architectural change in HAEC with dicer knockdown. This demonstrates that microRNAs play a role in maintaining normal cell alignment with hemodynamic flow. Obviosly, lack of mechano sensitive proteins such as moesin and others mould the cells in this manner. The impact on the cells, we show to be profound with dicer down regulation.

7.2.6.1 Endothelial cells are hypersensitive to microparticle release when lacking microRNA regulation

We find HAECs more prone to endothelial derived microparticles when an agonist is introduced (ionophore and chronic shear stress) (Figure 4.13). Interestingly microRNA machinery knockdown do not constitutively activate microparticle formation. This could be due to reduction in gelsolin, which has been demonstrated (Harvey *et al.*, 2008). Gelsolin, together with calpain are majorly involved in most microparticle formation as a distal event (Figure 1.24) (Piccin *et al.*, 2007).

7.2.6.2 microRNAs regulate endothelial cell proliferation under cyclic strain

We were able to reflect the studies by Suarez *et al*, where with dicer down regulation decreased proliferation of endothelial cells occur (Figure 4.14) (Suarez *et al.*, 2007). As this study was conducted with subconfluent cells, we discover cell-cell contact does not change the *status quo* in terms of proliferation (as discovered by Suarez *et al*).

We also discovered that dicer downregulation affected the shift in proliferation with mechanotransduction. When microRNA system is intact, the shift being higher. The results also show cyclic strain to reduce proliferation in endothelial cells. Furthermore, Liu *et al* has already shown that endothelial cells proliferate maximally at suconfluence even when stretched. They found requirement for cell-cell contact and identified cadherin's role (Liu *et al.*, 2007).

7.2.7 Conclusion

microRNAs that have the seed region complimentary to moesin are implicated with shear stress and cyclic strain. We have also discovered a list of microRNAs, and their associated change.

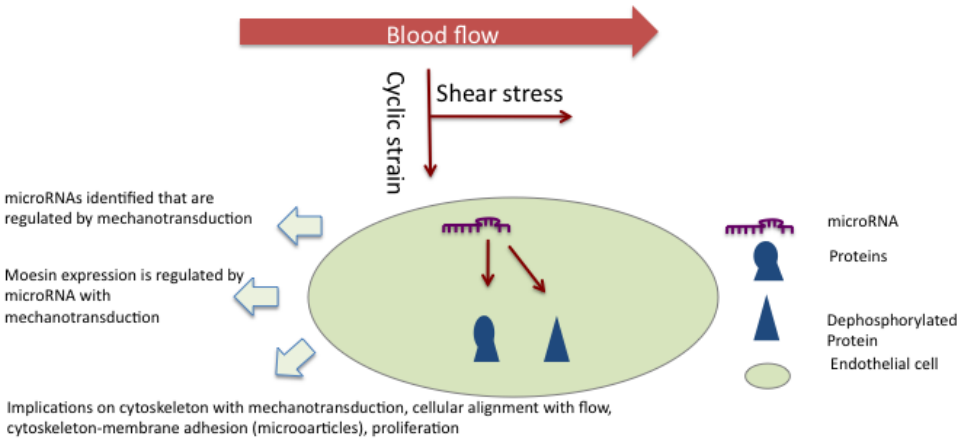

Figure 7.3: microRNA regulation of mechanotransduction

We see that moesin protein levels are controlled by shear stress cyclic strain and regulated by microRNAs. Furthermore we find moesin de−phosphorylation with stretch controlled by microRNAs. The identification of Rho associated dephosphorylation was intriguing, so was its regulation by microRNAs.

Further, we demonstrate the profound implications of these regulatory mechanisms in terms of lack of cellular alignment with shear stress (flow). We also show the significance of microRNA machinery in cytoskeleton−plasma membrane adhesion, in terms of microparticle formation. Here we see, both shear and ionophore agonist inducing blebs in cells that lack microRNA regulation. With respect to cell proliferation, lack of microRNA machinery curtails the proliferative response of the cell. This reflects the study by Suarez *et al* (Suarez *et al.*, 2007). Furthermore, we show mechanotransduction induced proliferative responses are also sabotaged by lack of microRNA machinery.

7.3 Endothelial derived microparticles: Implicating moesin

7.3.1 Implication of cytoskeletal protein moesin, in microparticles

TNF-α was able to induce microparticles which contained moesin as demonstrated by western blotting (Figure 5.1). Moesin as an actin binding protein, is structurally important. Microparticles meanwhile being an actin derived process (Flaumenhaft *et al.*, 2009), could involve moesin structurally. It has been shown recently, what was speculated over time, that microparticles presents proteins based on the their mode of induction. The microparticles as appreciated in the introduction, are thus also representative of cells from which they are derived (Peterson *et al.*, 2008). Thus, TNF-α owing to its phosphorylation of moesin, plausibly shift moesin to the microparticle (Koss *et al.*, 2006). Inherent structural and cytoskeletal changes with moesin phosphorylation, is also

obvious (McClatchey and Fehon, 2009).

The mechanisms that underlie moesin's significance to microparticle release is unclear. As an actin binding protein with intricate linkage to the membrane, independent of the agonist, moesin plausibly plays key role in the release of microparticles. Moreover, moesin is highly expressed in endothelial cells. Also it is mechanically sensitive unlike ezrin. Could there be thus a difference in terms of microparticle release, between ezrin and moesin?

7.3.2 Microparticles increase with variation in shear stress

Mechanically, we have shown that microRNA regulation protects endothelial cells from undue microparticle release (Figure 4.13). We have also shown moesin to be under microRNA regulation. Following it up, acutely, shear accumulated EMPs over 48 h (Figure 5.2). Exercise acutely, also produce EMPs to similar levels (Figure 5.2). Exercise increases both cyclic strain and shear stress substantially. Thus, this is an *in-vivo* confirmation of a phenomenon we discover *in-vitro* (Birukov, 2009a; Kojda and Hambrecht, 2005). Chronically the effect of exercise may also lead to decrease in EMPs. The trend towards this can be seen 2 h post exercise. Thus studies, conducted possibly a day after exercise might see a substantial reduction in EMPs. This though, needs experimental verification. Exercise even moderately leads to NO production and is well characterized to be atheroprotective (Hahn and Schwartz, 2009). There are gaps in our knowledge about mechanosensitive proteins, that archtietecturally mould the cells imparting atheroprotectiveness.

7.3.3 Moesin has the ability to buffer microparticle release

7.3.3.1 Moesin and ezrin differs in its ability to regulate microparticle release

We decided to compare microparticle induction with moesin overexpression. We utilized the FLAG-tag construct of moesin and ezrin (for comparison) at-

tached to the C- terminus. This FERM domain is able to bind via its PH domain to PIP_2. Interestingly we found clear evidence of moesin overexpression substantially decreasing endothelial microparticle formation (Figure 5.3). Ezrin overexpression rendered endothelial cells sensitive to microparticle production. This clearly illustrates that moesin expression is constitutively atheroprotective. Moreover, agonist (Ca^{+}) induced EMP production is buffered when moesin is overexpressed. Calcium ionophore has been shown to induce calpain signaling downstream with the associated cleavage of proteins (Chua *et al.*, 2000). Detailed in the introduction calpain induces microparticle formation, while ionohore is used to study this mechanism.

The study by Falati *et al* was the basis of our study with respect to staining the microparticles with calcein AM (Falati *et al.*, 2003). They have recently published in Blood, actin rather than microtubules to be involved in microparticle formation (Flaumenhaft *et al.*, 2009). We used calpeptin to block calpain formation with ionophore induction (O'Connell *et al.*, 2005). With calpeptin, moesin and ezrin overexpression derived microparticles both were reduced (Figure 5.3).

Clearly from these studies a few factors are pertinent. Moesin seems to downregulate microparticle formation in endothelial cells. Overexpression of a calpain cleavable protein ezrin (due to its redundancy), takes on moesin's role, but is unable to stop the blebbing and microparticle formation. Moesin and ezrin interestingly differ in their sensitivity to calpain (Shcherbina *et al.*, 1999a).

7.3.3.2 Moesin is sensitive to thrombin, unlike ezrin with regard to microparticle release

Meanwhile studies with thrombin (Figure 5.4), that directly activate moesin show how moesin is significant to vasculature (Shcherbina *et al.*, 1999b). Ezrin like in the previous study constitutively increase EMPs, compared to when moesin is overexpressed. But, when induced with thrombin, moesin overex-

pressing cells increase EMPs, while cells overexpressing ezrin remain insensitive. The EMPs are still significantly less compared to ezrin overexpression. This has implication in thrombosis and haemostasis. This shows that moesin is able to buffer endothelium against blebbing, loss of plasma membrane− cytoskeleton adhesion and thus microparticle formation. Moesin though, still retains the subtle sensitivity required of the endothelial cells with respect to thrombin induction.

7.3.3.3 Ezrin overexpression leads to constitutive microparticle release

The images of ionohphore induced microparticle formation in HAECs compliments our biochemical studies (Figure 5.7). Here we can visualize endothelial microparticles. Ezrin and moesin both bind to the PIP_2 via their PH domain. Meanwhile as discussed in the introduction, we know the significance of PIP_2 towards cytoskeleton−plasma membrane adhesion (Raucher *et al.*, 2000). We have also appreciated how, phosphatidylserine's localization is a defining process of microparticle formation (Yeung *et al.*, 2008, 2009). Plc$-\delta-$rfp binds to only the free PIP_2 and not when PIP_2 is bound to a binding partner (Raucher *et al.*, 2000). Thus plc$-\delta-$rfp is an inverse indicator of protein bound PIP_2 , whereby, more red (rfp) expression, less protein bound PIP_2 is available in the cell. Utilization of this technique, shows that moesin and plc$-\delta-$rfp co-overexpression results in less rfp expression compared to similarly co-overexpressed ezrin and plc$-\delta-$rfp (Figure 5.6). More unbound PIP_2 meanwhile, is sequestered away over time by plc$-\delta-$rfp, decreasing the cytoskeleton−plasma membrane adhesion. This experiment visually demonstrates the cleavage of ezrin and the insensitivity of moesin to calpain, resulting in microparticle formation (Shcherbina *et al.*, 1999a). Meanwhile, we also show how moesin and ezrin localize in endothelial cells, with its overexpression (Figure 5.5).

7.3.3.4 Moesin is critical to membrane-cytoskeletal integrity with regard to microparticle release

We demonstrate this further with our study utilizing moesin siRNA. The study show endothelial microparticles production dramatically affected. There is a massive increase in EMPs with and without ionophore treatment. Moesin loss has been shown to induce blebbing in cells previously (Rosenblatt, 2008). Our study shows how lack of moesin generates microparticles with cells unable to focus the curvature of its membrane. This, as appreciated in the introduction results from loss of membrane-cytoskeletal adhesion.

Lack of moesin has also been shown to affect the membrane integrity with respect to HIV infection. This is through its ability to regulate microtubules, but the role of actin has not been excluded (Naghavi *et al.*, 2007). There are as well, differences in signal transduction with respect to viral entry between moesin and ezrin. Such as the CagA-mediated Src inactivation leading to ezrin dephosphorylation (Selbach *et al.*, 2004). Moesin meanwhile, is immune to this process, due to the lack of tyrosine phoshorylation residue. Essentially an actin driven process (Flaumenhaft *et al.*, 2009), microparticle production, we find require moesin critically towards its regulation.

7.3.3.5 Microparticle release is globally mediated by microRNA machinery

In the previous chapter we note how microRNAs are also involved in the microparticle formation. As well, moesin was shown to be under microRNA regulation. With dicer downregulation, moesin is reduced in static cells (Harvey *et al.*, 2008). Further we wanted to repeat one of the earlier studies (Figure 4.13), conducted with ionophore induction (of dicer knockdown HAECs). This was to see if calpeptin can rein in the micropartilces (Figure 5.3).

In this study, we find lack of microRNA regulation renders the cell sensitive to calpeptin. In such scenario, calpain thus seems to be the most distal event in EMP formation. Further evidence, we see with EMPs generated by thrombin (Figure 5.4). Thus from our studies in the earlier chapter and here, we find

microRNA regulation protects endothelium against agonist induced microparticle formation. It will be interesting to understand using proteomics, the many different proteins associated with microRNA regulation and microparticle release (Peterson *et al.*, 2008). Usage of large scale arrays, such as what we used in the previous section, will elucidate microRNAs associated with microparticle release.

7.3.4 Conclusion

Thus here, we find moesin as a mechanosensitive cytoskeletal protein able to buffer microparticle formation (Figure 7.4). Meanwhile moesin is also able to maintain the necessary sensitivity to signals, associated with thrombosis and haemostasis. Furthermore, we demonstrate the functional significance of low levels of ezrin in endothelium compared to moesin. We show ezrin's inherent lack of ability to function as a major actin linker protein. In previous chapters we have also shown ezrin is insensitive to shear stress in contrast to moesin. We further demonstrate the global regulation of EMP release, and mechanotransduction by microRNA dynamics.

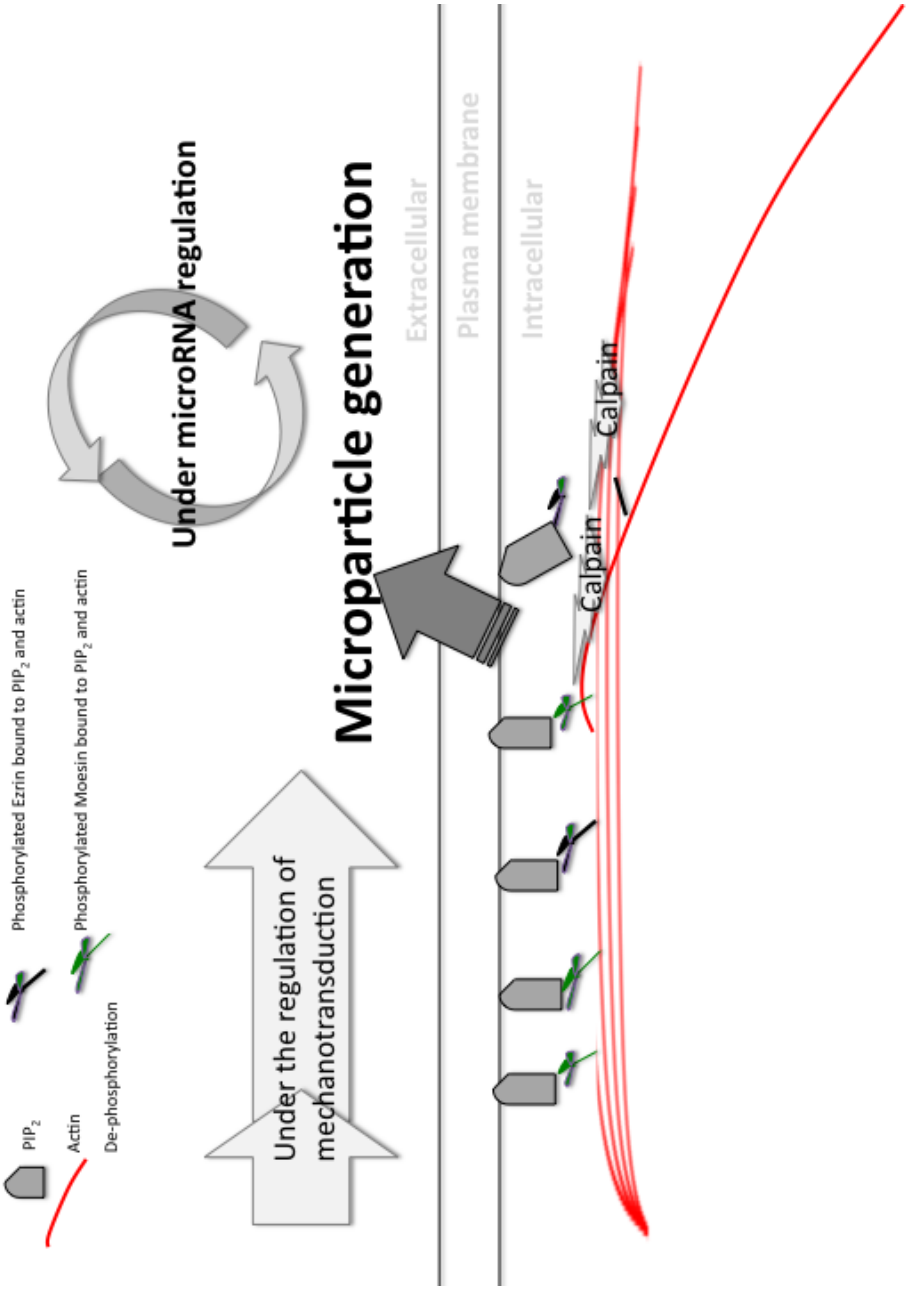

Figure 7.4: Moesin and microparticles Moesin we demonstrate, is a necessity for buffering endothelial microparticles. Also, we show how ezrin is cleavable by calpain thus causing intramembrane blebs. We also find that shear stress regulates microparticle release. All these process through out our study, demonstrate to be under microRNA regulation.

7.4 The urokinase moesin interaction

7.4.1 Mechanical stretch and uPA both induce moesin dephosphorylation to effect its downstream partners

Different agonists such as mechanical force (Figure 4.11) or urokinase (Figure 6.1), both effect same biochemical response in moesin. Moesin is dephosphorylated within minutes of introduction of either of them.

We discover from the biochemical study, that moesin is dephosphorylated acutely. This dephosphorylation event is transient, lasting up to 30 min. Further to the studies shown in HAECs, the results have been replicated in BAECs. BAECs showed similarly de-phsophorylation of moesin one minute post uPA induction.

As noted earlier, this response is similar with acute induction of stretch. We have also demonstrated in the previous chapter, how stretch mediated dephosphorylation is under Rho A regulation. Furthermore, we also showed this dephosphorylation event mediated by microRNAs. This is illustrated in the Figure 7.5.

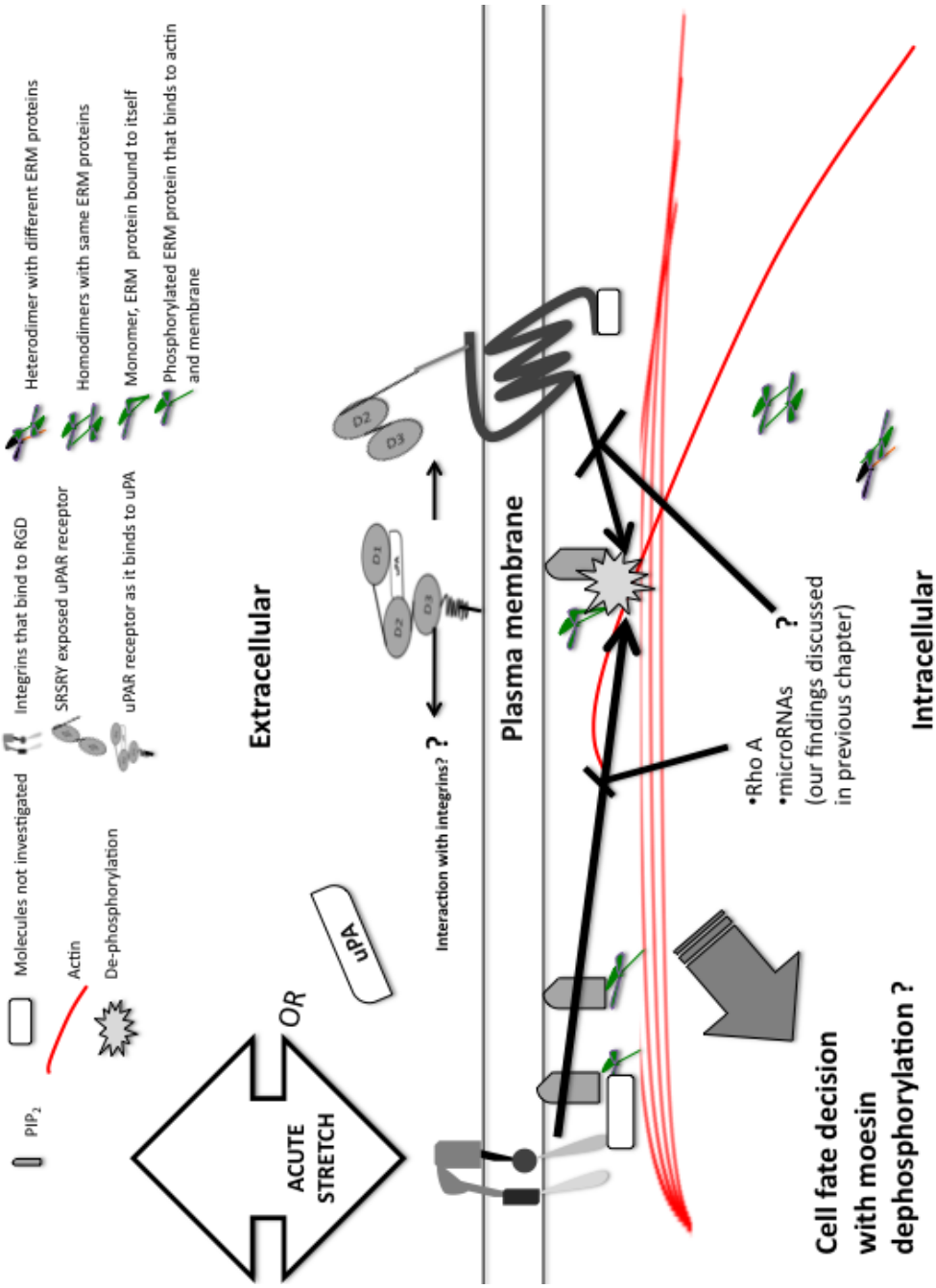

Figure 7.5: Stretch and uPA effect moesin de-phsophorylation

7.4.1.1 FPRL1 mediates uPA induced moesin dephosphorylation

Meanwhile we have also shown moesin dephosphorylation by the SRSRY sequence of amino acids (Figure 6.1). Exposed in the c-suPAR when cleaved, this sequence activates FPRL1. fMLP (formyl−methionyl−leucyl−phenylalanine), meanwhile is the major activator of FPRL1 (N-formyl-peptide receptor 1). Another study by Karsten Boldt *et al*, discovered FPRL-1 stimulation to de-phosphorylate moesin (Boldt *et al.*, 2006a). Thus, we find c-suPAR transducing the signals downstream via FPRL1 towards moesin. An open question is whether Rho mediates in this dephosphorylation (Alblas *et al.*, 2001). There is evidence towards this as a study has shown fMLP to activate Rho A in a highly transient manner i.e., in 30 seconds. Also we have shown that Rho A regulates dephosphorylation of moesin with stretch (Figure 4.11). Our lab has previously shown that migration and tube formation is modulated via G-coupled receptors with cyclic strain (Sweeney *et al.*, 2004; Von Offenberg Sweeney *et al.*, 2005).

7.4.2 Moesin and uPA interaction is chemotactic and is adjudicated by its ability to transiently de-phosphorylate

As described in the introduction, c-suPAR signaling via FPRL1 is highly chemotactic (Blasi and Carmeliet, 2002). Similarly Sweeney *et al*, has shown uPA mediated migration with cyclic strain in endothelial cells (Sweeney *et al.*, 2004; Von Offenberg Sweeney *et al.*, 2005). Meanwhile we find moesin and uPA interaction only provides a slight increase in migration (Figure 6.3). Interestingly we find full length moesin was a requisite for this chemotaxis. This seems quite logical as we have shown moesin dephosphorylating immediately on induction with SRSRY and uPA. The non-full length moesin such as the T558D stifles this migration.

As this study looks into multiple parameters, we also see the FERM (1-382) domain on its own quite migratory, compared to other mutated moesin constructs. This is especially true when induced with uPA. FERM domain, because it lacks the C terminus, is originally unbound to actin, thus immune to

the acute requirement of dephopshorylation. Without the locked C terminus such as the T558A / T558D, FERM domain is denied the ability to hinder the migratory response to a similar fashion. This is as stated earlier is because of the lack of C-terminus, where phosphorylation / dephosphorylation occurs. The other constructs meanwhile are all halted by uPA due to their non de-phosphorylatory segments. The importance of the C terminus of moesin is still evident in the study, when you compared to the migration rate with moesin full length protein.

7.4.2.1 Membrane-cytoskeletal adhesion mediated by moesin is required for chemotaxis

The "inactive-moesin" is a construct which is unable to bind cell membrane due GFP cloned onto the N-terminus. PIP_2 is the main binding partner of moesin to the membrane. PIP_2 interaction also is a requirement for moesin to be activated, i.e, phosphorylated. Thus purely overexpressing inactive moesin do not increase chemotaxis with uPA interaction. Thus there seems to be a requirement for rather a reversible nature, a possible transient de-phopshorylatory mechanism involved. This in conclusion reflects our biochemical study. A locked construct such as the T558D / T558A, might have therapeutic values in treating metastasis.

7.4.3 Moesin is critically required for uPA induced chemotaxis to engage

We found in the same study, moesin overexpression do not increase migration dramatically. Interestingly, thus moesin dephosphorylation is the major critical requirement for uPA mediated migration. We cannot entirely absolve moesin and shift all migratory ability of moesin-uPA interaction to moesin's biochemical status. Though, we find dephosphorylation is important, down regulation of moesin also had a dramatic decrease in uPA mediated migration (Figure 6.4). Thus we find without moesin uPA mediated migration is stopped.

We also find the same reversible de-phosphorylatory requirement found with uPA mediated migration holding true with our studies with SRSRY (Figure 6.5). Thus though there might be other transducers, FPRL1 is a transducer of uPA signaling towards moesin, critically requiring the reversible dephosphorylation of moesin in effecting the migratory apparatus.

7.4.4 Other inducers

7.4.5 Appreciation of integrin as another transducer of moesin-uPA interaction

7.4.5.1 αvβ3/α5β1 integrins are engaged with moesin-uPA interaction

There are other modes of uPAR mediated migration whereby it can increase the migratory response due to vitronectin-αvβ3 interaction (Figure 6.2), (Figure 1.28). uPAR is known to induce migration by laterally interacting with αvβ3 integrin. D2A is a synthetic peptide from the D2 region of the uPAR receptor shown to induce migration via specific interaction with αvβ3 and α5β1 integrins. It has been shown that the D2 region of the uPAR is the lateral latch that increase the VN-αvβ3 migration (Degryse *et al.*, 2001; Degryse, 2008; Degryse *et al.*, 2005; Dass *et al.*, 2008).

The D2A peptide has the sequence GEEG. This peptide specfically activate integrins and are reflective of the uPAR mediated cytoskeletal overhaul. Meanwhile the other peptide "D2A-ala" is a mutated version of this peptide and is GAAG, where the two glutamic acids have been replaced by alanine residues. This peptide is found to inhibit migratory response through these mechanisms (Figure 6.2).

7.4.5.2 uPAR-integrin-moesin association mediated migration is uniquely different to uPA-moesin migratory apparatus

We wanted to see if moesin downstream of uPAR-integrin-VN interaction is similarly migratory. The experiment utilized D2A peptide for this purpose.

The results (Figure 6.6) clearly show moesin is very pertinent to these signals with increase in migration. The increase was considerably higher relative to moesin-uPA mediated migration (Figure 6.3) (when wt type moesin is over-expressed). Similarly we see previously unseen migration increase with the "inactive moesin". This construct has the GFP on the FERM domain disabling its binding ability to membrane (hence plausibly to PIP_2). As previosly stated, PIP_2 stabilizes moesin for phosphorylation. While T558A (constitutively de-phosphorylated) and T558 D (constitutively phosphorylated) overexpression didn't increase migration. This showed that the reversible dephosphorylation of moesin is still a necessity for migration. Thus there is possibly a mechanism that is able to reversibly phosphorylate moesin without PIP_2 attachment, unique to uPAR-integrin-VN interaction.

7.4.5.3 Other plausible intracellular mechanisms

The intracellular mechanisms involved with migration through this pathway seems very different from migration induced by uPA alone. Though the reversiblity of the phosphorylation is important, we don't know if dephosphorylation is still the initial change induced on moesin. If it is, this could initially rescue "inactive moesin" from dephosphorylation, but moving it into a niche enviroment capable of PIP_2 independent phosphorylation. This is because it exits in de-phophorylated state normally, while still retaining all other faculties for re-phosphorylation except for the inability to bind to the PH domain of PIP_2. Moesin overexpression clearly is very chemotactic when integrin is activated. It will be interesting if uPA based migration allows moesin to induce inside out signaling via integrinβ3 orβ1.

7.4.5.4 Moesin mediated migration involves the D2 domain of uPAR binding to integrin

Our next study (Figure 6.7), with the synthetic peptide "D2A-ala" reduce migration of moesin overexpressed cells. This proves that moesin is involved in

signaling mechanisms emanating from the D2 region of the uPAR with respect to this interaction. As moesin response was quite robust in the earlier study (Figure 6.6), moesin is involved in integrin mediated migration with respect to VN.

7.4.6 Vitronectin matrix further confirm moesin requirement for migrating cells involving integrins

We found this to be true when VN alone (Figure 6.8) was used. Meanwhile we also find moesin's reversible dephosphorylation necessarry to this response from T558A. Interestingly here we don't see the increase in migration when inactive moesin is overexpressed. Thus it seems that particular response is unique to uPA-integrin-VN based chemotaxis. The physiological response is that alternate mechanisms possibly exists to phosphorylating moesin without PIP_2 stabilisation. There could as well be interplay with merlin and other ERM proteins in the heterodimer formation. It has to be borne in mind that merlin has higher affinity to moesin's monomer than its own. These are open questions which will provide interesting insight to further workings.

7.4.7 Angiogenesis employs moesin mediated mechanisms

7.4.7.1 Moesin overexperssion increase angiogenic tube length

Functionally, studies into migration has implications in angiogenesis. Angiogenesis utilises invasive response of the cell, as well digestion of ECM and proliferation. We found T558D moesin having high angiogenic potential (tube length) (Figure 6.9), in a one dimensional environment of just collagen. We did not use Matrigel® in this particular study as it contains many different growth factors. By using such a one dimensional study we were able to tease out how moesin overexpression alone affects tube length. Interestingly we found T558D quite high in its angiogenic potential. This was interesting, as we found reversiblity of moesin's phosphorylation quite important in earlier studies with

respect to migration. Angiogenesis utilizes multiple functions of a cell to invade, migrate, proliferate and grow. We know from chapter 3 that moesin is phosphorylated in the nucleus when mechanically stimulated (Figure 3.2). It might be that phosphorylated moesin is proliferative. There are studies that corroborate the proliferative response of moesin (Lee *et al.*, 2009). Nevertheless there needs a better understanding of moesin's phosphorylation status with respect to angiogenesis. This is necessary for any targeted therapeutic application. The studies have shown moesin overexpression to be angiogenic.

7.4.7.2 Delineation of uPA-moesin interaction with respect to angiogenesis

In the following experiment (Figure 6.10), we investigate what responses from migration studies hold true. The studies clearly show moesin is highly angiogenic when interacting with uPA. T558D, when interacting with uPA is unable to proliferate similar to full length functional moesin when overexpressed. The change though not as dramatic as we see in migration independently, it is still significant. It is a substantial decrease compared to the previous study where there was no uPA (Figure 6.9).

It has to be also noted that matrigel on its own also contain some uPA. As previously stated, our lab has shown migration and tube formation to be modulated via G-coupled receptors with cyclic strain. The reason we chose matrigel in this experiment was to decipher the therapeutic potential of moesin-uPA interaction. Matrigel is derived from tumour, specifically Engelbreth-Holm-Swarm (EHS) mouse sarcoma. They are rich in extracellular matrix proteins and the major components are laminin, followed by collagen IV, heparan sulfate proteoglycans, entactin/nidogen *http://www.bdbiosciences.ca* (Adini *et al.*, 2009; Ribatti, 2009; Teklenburg and Macklon, 2009). Thus, they provide pathophysiological relevance in understanding metastasis, and angiogenesis in a tumour.

7.4.8 Barrier integrity with endothelial injury, recruit the uPA-moesin cytoskeletal remodeling apparatus

Further to this we decided use permeability assay as a means towards understanding the time scale of transient change in cytoskeletal architecture associated with uPA addition. It has been previously shown that with thermal injury both tPA and uPA levels are increased. Further to this the investigators found increased permeability *in-vivo* in the brain of mouse with respect to water retention (Patel *et al.*, 2008).

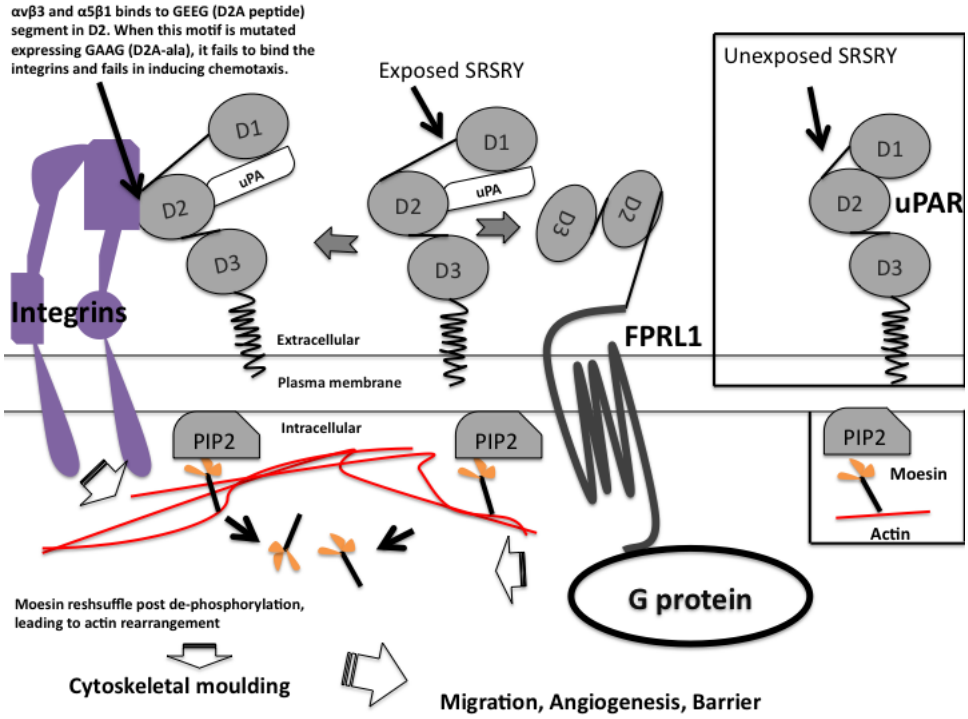

Figure 7.6: Moesin is a critical requirement for uPA mediated functions
Moesin is critical requirement for uPA mediated functions, such as migration, angiogenesis, endothelial barrier integrity. The main mode of transduction involve dephosphorylation of moesin. The transducers that co-act with uPAR are integrins (either $\alpha v\beta 3$ or $\alpha 5\beta 1$) and FPRL1. The resulting reshuffle of the cytoskeleton moulds the cell towards a migratory phenotype.

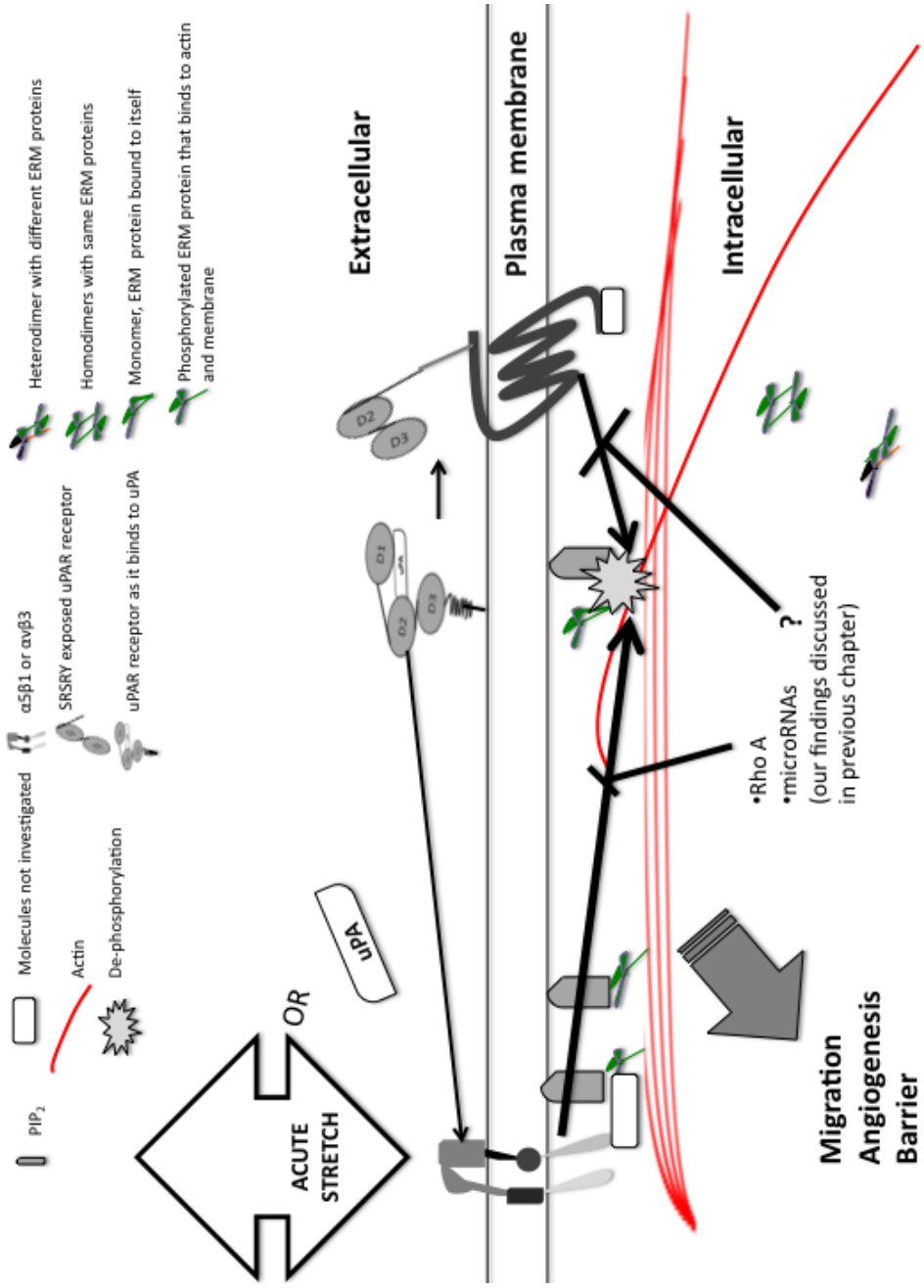

Figure 7.7: Answering the moesin-uPA questions
The results show how moesin dephosphorylation is effected, by both acute stretch and uPA. Strain we have shown utilize RGD-sensitive integrins to transduce their signals. Meanwhile, uPA we find utilize either $\alpha 5\beta 1$ integrins or $\alpha \mathrm{v}\beta 3$ integrins (both of whom are RGD-sensitive). uPA signaling we find is also transduced via FPRL1. Though the partners responsible for moesin dephosphorylation with respect to uPA is still lacking, we have shown Rho A and microRNA regulation with stretch.

7.4.8.1 Moesin and uPA both independently regulate endothelial cell's permeability

We find that on addition of uPA, the permeability in fact decrease in HAECs (Figure 6.11A). Interestingly moesin overexpression also tightens the endothelial barrier (Figure 6.11B).

As explained in the introduction, in a quiescent monolayer of endothelium, there is very little uPA or moesin (Reidy *et al.*, 1996; Batchelor *et al.*, 2004). In an injured model, concurrent to uPA increase cytoskeletal proteins also increase. We had previously discussed how moesin (Figure 3.1) and uPA both increase with onset of cyclic strain (Sweeney *et al.*, 2004; Von Offenberg Sweeney *et al.*, 2005). Moesin and uPA interaction is critical to migration. Thus in an injured model, wherein the endothelial cells would be sparse, both uPA and moesin would be increased. Also this rise in their expression will occur with mechanotransduction.

7.4.8.2 In injured endothelium and with cyclic strain, both uPA and moesin interact and regulate endothelial cell junctional integrity

Towards identifying uPA-moesin interaction in a confluent monolayer, moesin was overexpressed, further to which uPA was added. Here we see the transient effect of moesin uPA interaction, first identified to be important in migration. We deemed this transient phosphorylation critical in angiogenesis as well. The study (Figure 6.12)(Figure 6.13), show that they do interact with respect to permeability. Interestingly, we also have insight into the timescale of their interaction (change at 1.5h post uPA addition, reversal steadily starting at 2 h.

A study reflecting increase in one protein fail to reflect *in-vivo* models, as seen from our study where uPA alone actually tighten the permeability. The latter study, meanwhile show how in injured endothelium, where both moesin and uPA increase, transient dephosphorylation of moesin is critical to final loss of permeability as seen in the *in-vivo* study.

Further to this, we were able to use this assay to understand the time scale of cytoskeletal shuffle associated with uPA increase, in an injured model relative to a single variable.

7.4.9 Conclusion

Moesin and uPA modulate cell fate decisions, such as migration, angiogenesis, barrier integrity. Cytoskeletal remodeling is specifically required to mould the cells towards this purpose. Moesin we find, is critically involved in these processes.

There is also requirement for moesin dephosphorylation in effecting these functions. Furthermore, we have identified unique mechanisms involving FPRL1 and RGD-specific integrins as transducers. We have also delineated their differential requirement off moesin. Additionally we strongly speculate moesin involvement in integrin inside-out signaling.

7.4.10 Brief summary and future studies

Moesin, we find through investigation into different aspects of a vasculature to be an absolute necessity in its physiological functioning. Moesin is a mechanosensitive element and is regulated by microRNA. It is the atheroprotective ERM cousin and the major mechanically sensitive ERM protein in endothelial cells. Unlike ezrin, moesin is also subtlety sensitive to thrombosis and haemostasis with respect to EMPs. This is because of its sensitivity to thrombin unlike ezrin.

Moesin we find is important to EMP release with respect to microRNA regulation. The major transducers of stimuli to moesin via uPAR receptor are FPRL1 and either αvβ3 or α5β1 integrins. Moesin meanwhile is up regulated with mechanotransduction via integrins. This was proven with RGD containing pronectin relative to fibronectin matrix. Cell-cell contact is important for moesin, wherein, in static sub$-$confluent cells moesin is expressed highly. We still find moesin responsive in sub$-$confluent cells, implicating integrins.

Moesin we also demonstrate as a necessity in effecting migration, angiogenesis and barrier integrity of endothelial cells. Biochemically, moesin dephosphorylation is demonstrated as as a prerequisite to cell mechanics, including migration and mechanotransduction.

This biochemical response we find is regulated by Rho A antagonism. We find the dephosphorylation mechanism also regulated by microRNAs. Throughout the research we highlight the importance of microRNA in all facets of vascular functioning. We demonstrate its global regulation of mechanotransduction, endothelial microparticle generation and endothelial cell proliferation. As illustrated in the discussion, the research also endeavor throughout to showcase other lateral aspects of the study.

For translating the research for bench to bedside therapeutics, utilization of d2a-ala peptide (Degryse *et al.*, 2005) and phopho-mimetic moesin constructs are obvious candidates. Localized treatments such as utilization in stents to avoid restenosis are some immediately obvious low risk ventures. Moesin and uPA as appreciated earlier, is highly expressed in injured vasculature prone to restenosis (Blindt *et al.*, 2002). Our understanding of the synergistic uPA-moesin interaction could be thus exploited. Metastasis is also an obvious target.

Furthermore, we have shown EMP release is also an important tool in diagnostics. Characterization of ERM signature, i.e, the ratio of ezrin, radixin, moesin in EMPs physiologically could yield insightful information. Furthermore, this ERM signature could be translated into identifying eventual progress into arteriosclerosis.

HIV entry has appreciated earlier, has been shown to involve moesin. Moesin overexpression was found to downregulate the formation of stable microtubules, whereas knockdown of moesin increased stable microtubule formation (Naghavi *et al.*, 2007). Viral entry meanwhile, required the stabilization of the microtubules. Studies have noticed enhanced HIV infection with moesin knockdown. At the molecular level, we show FPRL1 transducing signals toward moesin via uPAR (SRSRY)-uPA . FPRL1 has been found to be a highly effi-

cient coreceptor for human immunodeficiency virus (Nobuaki *et al.*, 2008). As microtubule stabilization is favoured for HIV entry, understanding uPA mediated effects on microtubules via moesin is significant. This might even lead to an unprobable but possible drug eg:- either uPA or d2a-ala, depending on the effects. uPA has to be noted, is already a Food and Drug Administration (FDA) approved drug (*http://www.fda.gov*). This additionally mean, clinical studies and translative research borne from this research could be expedited.

More complex approaches possibly involve biomedical engineering for artificial grafts to utilize ERM proteins. Further understanding of the microRNAs involved could lead to novel therapeutics. Designer EMPs that might enable delivery, targeted antigen presentation, novel vaccination techniques. These though require thorough studies for understanding risks involved, and *in-vivo* studies will have to be undertaken. This will provide the holistic response to any mode of treatment. Moesin dynamics, nevertheless holds promise in many facets of cardiovascular and other pathogenesis. Our understanding of it has taken it that little forward towards this.

7.4.10.1 Further studies

The immediate step will be to translate our understanding of moesin into an *in-vivo* model. Further to which, understanding its clinical implications will be more obvious. There are as well some gaps in our understanding of the study. These include, specific knowledge of the integrins involved with our study. We did show $\alpha 5\beta 1$ integrins and $\alpha \mathrm{v}\beta 3$ involvement. Studies previously conducted by Ronan Murphy (personal communication) has ruled out a role for $\beta 3$ integrin subunit.

Meanwhile, involvement of $\alpha 5\beta 1$integrin could be verified by means of specific antibodies. These antibodies would recognize activated $\alpha 5\beta 1$integrins. Overexpression studies with various constructs of moesin will also lead elucidation of $\alpha 5\beta 1$integrins's activation (Michael *et al.*, 2009). Similarly, $\beta 3$ integrin subunit activation with respect to moesin biochemistry (with its various con-

structs) could be verified and confirmed by utilizing similar antibodies against β3 integrin subunit (Byron *et al.*, 2009).

Moesin constructs could be also utilized with respect to understanding G actin and F actin ratios. microRNA regulation with respect to their ratios, alignment with mechanotransduction will yield insightful data. We have also identified two microRNAs (with respect to moesin verified by *in-silico*) regulated by mechanotransduction. These could be verified by knock out-knock in studies (of the microRNAs).

Specific requirement of moesin in endothelial cell proliferation is lacking in our study. Similar lack of understanding could be seen in migration studies. siRNA studies with respect to moesin would yield this information.

We also speculate, moesin involvement in inside out signaling with respect to uPA-moesin interaction. Previously appreciated studies with respect to integrin activation, could demonstrate inside out signaling mediated by moesin.

The possibility for furthering these studies is limitless, with many interactions (Figure 1.10) occurring in the minute cell. Expanding this knowledge at the basic level, would at least appeal to our curiosity.

The list below, identify studies that would further expand our understanding of moesin dynamics, in the cardiovascular system with relevance to research here.

Cell Fractionation: Cytoskeleton, Membrane fraction in addition to cytosol and nuclear fractions

F actin and G actin ratio

This study gives important insight into the F actin/G actin ratio. Below is enumerated different parameters which will further compliment the study conducted so far. Towards this a luciferase reporter based system is currently being characterized.

1. Comparison with ezrin construct for differentiating microparticle formation with respect to actin change in contrast to moesin.
2. siRNA against moesin.
3. With moesin constructs.
4. Above studies with thrombin and ionophore.
5. uPA and SRSRY interaction (siRNA moesin).
6. Dicer siRNA.

HUTS4 and WOW1 studies

As discussed in the thesis, specific integrin activation due to moesin is still largely unknown. We have shown αvβ3 or α5β1 mechanistics involved with respect to uPA-Moesin interaction. Antibodies that target activated integrins β1 or β3 utilizing HUTS4 and WOW1 respectively this information. Given below are parameters that will compliment this information further to this thesis.

1. siRNA against moesin
2. With moesin constructs , Static versus Strain with Moesin GFP

3. uPA and SRSRY interaction (siRNA moesin)

Strain induced migration

As shown in chapter 3, the first results chapter, we find cyclic strain inducing dose dependent cell migration. Though, it compliments our study with urokinase, it lacks specific information with regard to moesin. Thus repeating the study utilizing moesin knocked out cells would yield interesting insights.

1. siRNA against moesin, cyclic strain induced migration

Dicer siRNA

We have discovered potential regulation of Rho A with microRNAs. This though obvious, needs information regarding active Rho A (and Rac1). We are currently utilizing Elisa based system to yield information to this regard.

1. RhoA and Rac1 Elisa

Human study

From our studies on microparticles from exercise induced subjects, shear stress show its induction. Further to exercise, we find clearance of microparticles post 2h. Thus understanding the proteins expressed in microparticles will help us understand the clearance mechanism.

Furthermore, we have already shown moesin in EMPs. Thus study on human subjects, further to isolation of the microparticles will help us elucidate this mechanism further. Ultimately, a proteomics based approach will help us deduct the diagnostic importance of specific proteins in MPs.

1. Endothelial microparticles, moesin western

Thrombin

From our studies we found moesin sensitive to thrombin, unlike ezrin towards microparticle release. This proposed study will help us understand the significance of thrombin-moesin interaction in MP release. We had discussed earlier that moesin has been shown to be acutely sensitive to thrombin (Shcherbina *et al.*, 1999b). Furthermore thrombin induces a differential signaling route in releasing MP release (Sap, 2006).

1. Moesin siRNA and its effect on endothelial microparticle release with thrombin

PhosphoERM Imaging

To compliment our studies that show moesin phosphorylation-dephosphorylation requirement, imaging utilizing specific antibodies could be conducted.

1. Cyclic strain
2. Microparticles via thrombin, ionophore
3. Effect of uPA, SRSRY

Immunoprecipitation

Towards understanding binding partners of moesin-uPA interaction, immunoprecipitation would be an important tool. This would involve pulling down moesin-GFP post induction of uPA using GFP specific antibody. Further to this approach mass spectrometry could be utilized towards identifying the binding partners compared to control.

1. Moesin constructs with uPA bound to dynal beads

Ibidi Y slide: disturbed and normal flow areas

Further to our study with Ibidi in chapter 3, more studies with Ibidi Y slide will provide a platform to investigate areas of the vasculature with bifurcation. Below are enumerated the parameters that will compliment the thesis.

1. Global actin change siRNA moesin

2. Ezrin, Moesin GFP or FLAG tag

3. Dicer siRNA

4. Elucidating signaling requirements using different moesin constructs towards global actin change with flow

Shear and cyclic strain

Our studies have mainly focussed on moesin. A derivative of this study would be to understand Ezrin and Merlin with mechanotransduction. We have opened up questions, that are discussed in the earlier chapter with regard to other FERM domain containing proteins. Importantly, the relevance of differential ERM regulation with mechanical stimuli would be interesting to know. A starting point would be to characterize using western blotting. Specifically to the thesis understanding Ezrin protein expression during mechanical stimuli alone would suffice.

1. Protein expression of Ezrin, Merlin

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