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**Studying the Role of Spatial Cell
Distribution and Substrate Stiffness in
Inflammatory and Fibrotic responses in
Human Lung Using Bioengineered
Platforms**

Submitted by

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Abstract

The extracellular matrix (ECM) has emerged as a major regulator of cell behaviours. Changes in extracellular matrix, especially its composition, organization/dimensionality, and rigidity have been implicated in various aspects of cellular functions including cell growth, migration, and differentiation. In my thesis, I have focused on the effect of two biophysical properties of the extracellular matrix namely dimensionality and rigidity in the inflammatory and fibrotic pathologies of human lung.

To study the role of matrix dimensionality, firstly electrospun scaffold based three-dimensional (3D) culture with similar architecture of human lung was developed. By applying this 3D model, inflammatory response was studied in an *in vivo* like environment by using NF- κ B transcription factor activation as a tool for probing inflammatory response in human lung fibroblasts. According to my observations, it was confirmed that the matrix dimensionality together with spatial organisation of cells is crucial in lung inflammatory response, evidenced by the observation of the differences in the level and pattern of inflammatory response between 2D and 3D culture systems.

To study the role of matrix rigidity in progression of lung fibrosis, we developed the ECM-based hydrogel platform with tuneable stiffness level relevant to normal and fibrotic lung. By using this disease relevant platform, I have shown that stiff matrix but not soft matrix can induce the myofibroblast differentiation and fibroblast proliferation, the two major features of lung fibrosis. To date, the molecular mechanisms underpinning this cellular mechanosensing process in response to matrix stiffening remains unknown. To achieve this, I further investigated the involvement of two potential mechanosensitive signalling pathways namely, Rho associated coiled

coil forming kinase (ROCK) signalling and talin- (focal adhesion adaptor) signalling in this process. Interestingly, my data show that ROCK signalling differentially regulated stiffness induced myofibroblast differentiation between soft normal and stiff fibrotic matrix. Moreover, both ROCK isoforms 1 and 2 are synergistically important in myofibroblast differentiation driven by rigid matrix and the absence of one ROCK isoform can exaggerate myofibroblast differentiation on stiff fibrotic matrix. Regarding talin signalling, my preliminary data confirms that talin1 can control both stiffness induced fibroblast proliferation and myofibroblasts differentiation on stiff matrix. In contrast to talin1, talin2 showed a protective role in controlling myofibroblast differentiation.

In conclusion, we have successfully developed two *in vitro* lung models for studying the effect of matrix dimensionality and rigidity in lung inflammation and fibrosis. Overall my PhD work has elucidated the significant contribution of biophysical cues of external cellular environment in lung inflammation and fibrosis.

Publications arising from this thesis

1. Htwe SS, Harrington H, Knox A, Rose F, Aylott J, Haycock JW, Ghaemmamghami AM: **Investigating NF- κ B signaling in lung fibroblasts in 2D and 3D culture systems**. Respiratory research 2015, 16:144.
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2. Htwe SS, Cha BH, Yue K, Khademhosseini A, Knox AJ, Ghaemmamghami AM. **Role of Rho associated coiled-coil forming kinase isoforms in regulation of stiffness-induced myofibroblast differentiation in lung fibrosis**. American Journal of Respiratory Cell and Molecular Biology 2017;56(6):772-783. <https://www.ncbi.nlm.nih.gov/pubmed/28225294>

Conferences and poster presentation

1. **A 3D culture system for investigating inflammatory responses in human lung**
S S Htwe, H Harrington, A J Knox, F R A J Rose, J W Aylott, J W Haycock and A M Ghaemmamghami
9th World Congress on Alternatives and Animal Use in the Life Sciences (August 2014), Prague, Czech Republic
2. **A Micro-engineered Biomimetic Hydrogel for Studying the Role of Extracellular Matrix Stiffness in Lung Fibrosis**
Su S Htwe, João Ribas, Alan J. Knox, Ali Khademhosseini , Amir Ghaemmamghami
TERMIS World Congress (2015), Boston, MA, USA
3. **Role of ROCK Isoforms in Regulation of Stiffness Induced Myofibroblast Differentiation in Lung Fibrosis**
Su Su Htwe, A J Knox, A M Ghaemmamghami
American society for cell biology meeting (December 2016), San Francisco, USA.
4. **Micro engineered hydrogel for 3D culture of human lung fibroblasts**
S S Htwe, A J Knox, A M Ghaemmamghami

M5 biomedical Imaging Conference, School of Veterinary Medicine and Science, University of Nottingham, UK (2014)

5. ***In vitro* lung Fibrosis model**

S S Htwe, A J Knox, A M Ghaemmaghami

Post Graduate Research Symposium, School of Life Science, and University of Nottingham, UK (2015)

Oral Presentation

1. **A 3D culture system for investigating inflammatory responses in human lung**

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2. **Differential NF- κ B activation in lung fibroblasts in 2D and 3D culture system**

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Abbreviations

18S	18 Svedberg units
2D	Two-dimensional
3D	Three-dimensional
ABS	Actin binding site
ANOVA	Analysis of variance
AP1	Activator protein 1
APCs	Antigen presenting cells
ARDS	Acute respiratory distress syndrome
ASM	Airway smooth muscle
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CAF	Cancer associated fibroblast
COPD	Chronic obstructive pulmonary disease
CSPG	chondroitin sulphate proteoglycan
CTGF	Connective tissue growth factor
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DCM	Dichloromethane
DD	Dimerisation motif
DDT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
Erk	Extracellular signal regulated kinase
F actin	Filamentous actin
FA	Focal adhesion
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FN	Fibronectin
FRET	Fluorescence Resonance Energy Transfer
G actin	Globular Actin
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GelMA	Gelatin Methacrylate
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPa	Gigapascal

HA	Hyaluronic acid
HGF	Hepatocyte growth factor
hMSC	human mesenchymal stem cell
hr	hour
HRP	Horseradish Peroxidase
HSPG	Heparin sulphate proteoglycan
IBS	Integrin binding Site
ICAM-1	Intercellular adhesion molecule -1
IFN- γ	Interferon- γ
IL	Interleukin
ILK	Integrin-linked kinase
IPF	Idiopathic pulmonary fibrosis
I κ B	Inhibitor Kappa beta
JNK	c-Jun N-terminal kinases
KANK	KN motif and ankyrin repeat domains
KD	Knock Down
KGF	Keratinocyte growth factor
kPa	kilopascal
LPS	Lipopolysaccharide
nM	Nano Molarity
MAPK	Mitogen-activating protein kinases
MCP	Monocyte chemoattractant protein
MEM	Modified Eagle's Medium
MFI	Mean fluorescence Intensity
MIP-1- α	Macrophage inflammatory protein-1-alpha
ml	Milliliter
MLC	Myosin light chain
MLCP	Myosin light chain phosphatase
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MRTF-A	Myocardin related transcription factor A
NF- κ B	Nuclear factor kappa B
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffer saline
PDGF	Platelet derived growth factor
PEG	Polyethylene glycol
PEGDA	Polyethylene glycol diacrylate
PET	Poly-ethylene terephthalate
PFA	Paraformaldehyde
PGE2	Prostaglandin E2

PI (4, 5) P2	Phosphatidylinositol 4, 5 bisphosphate
PI3K	Phosphoinositide 3-kinase
PLA	Poly lactic acid
PLGA	Poly (lactic-co-glycolic) acid
PLG	Polyactide -co-glycolide
PolyHEMA	Poly (2-hydroxyethyl methacrylate)
PRK	protein kinase C related kinase
PRRs	Pattern recognition receptors
PVA	polyvinyl alcohol
RANTES	Regulated on activation, normal T cell
RBC	Red Blood cells
RGD	Arginine-Glycine-Aspartic acid
Rho	Ras homolog family member
RhoA	Ras homolog family member A
RIAM	Rap1-GTP-interacting adapter molecule
RIPA	Radio immune precipitation assay
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT	Room temperature
RTK	Receptor tyrosine kinase
RT-PCR	Real time Polymerase Chain Reaction
SDF1	Stromal Derived Factor 1
SEM	Standard error of mean
SEM	Scanning electron microscopy
siRNA	Small-interfering RNA
Sulfo-SANPAH	Sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate
TBS	Tris buffer saline
TFA	Trifluoroacetic acid
TGF- β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinases
TMSPMA	(Trimethoxysilyl) propyl methacrylate
TNF- α	Tumour necrosis factor alpha
VBS	Vinculing binding site
VCAM1	Vascular cell adhesion molecule 1
WB	Western blotting
WBC	White blood cells
ul	Micro litre
α -SMA	Alpha-smooth muscle actin

Chapter 1

General Introduction

1 Chapter 1- General introduction

1.1 The extracellular microenvironment

In multicellular organisms, cells reside in complex microenvironments with diverse biochemical (soluble and insoluble factors) and biophysical properties (matrix texture, spatio-temporal orientation or dimensionality, topography). Depending on organ systems, cells are exposed to different types of microenvironment either in solid or liquid phase. Figure 1.1 summarises the general overview of the complex tissue microenvironment in our body. For instance, common structural cells such as epithelium and fibroblasts firmly attach to their ground substance through interactions with various ligands of extracellular matrix (ECM). Some hemopoietic cells (red blood cell, white blood cells and platelets) circulate in vascular system and contact a largely fluid microenvironment.

Such cells in circulation are continuously subjected to shear and hydrostatic stress resulting from changes in viscosity and flow rate in the microfluidic environment (1) whereas structural cells are mainly subjected to changes in biochemical and mechanical properties of ECM. In addition to these factors, some organs in motion are exposed to additional tension due to cyclical stretch and rhythmic contraction e.g., heart, lung, blood vessels and joints. Cells of the alveolar epithelium lining the alveolus, encounter multiple forces from different aspects; the tension at their apical side due to alveolar extension and forces from ECM at their basolateral side (2).

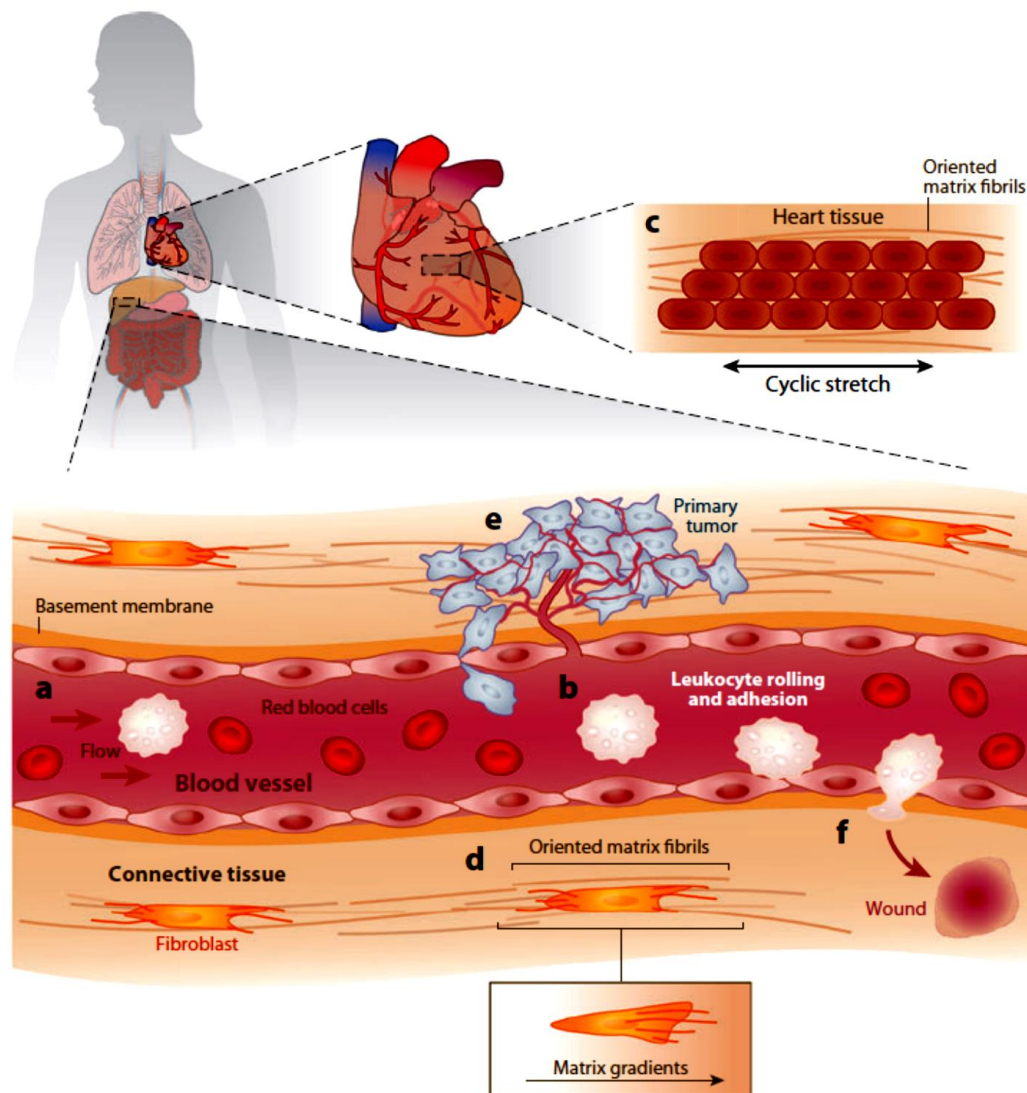


Figure 1.1 Overview of tissue microenvironment of human body in health and disease state. (A) Cells in circulation are consistently exposed to shear stress of dynamic flow. (B) Shear stress within the dynamic flow of blood have effect on the leucocytes rolling and adhesion function. (C) Heart and lungs are exposed to cyclical tension and stress under rhythmic movement. (D) Orientation of matrix fibrils induced by cyclical movement of heart and blood vessels also determines the direction of cardiocytes and fibroblasts. (E) Metastasis of cancer cells sense along collagen matrix fibres when they exit from the primary organ and invade the other organs. (F) Many immune cells are entered the tissue from circulation experiencing new biophysical interface during tissue injury [Kim *et al* 2009, (2)].

The ECM is a complex and highly dynamic biochemical structure made of a variety of proteins such as fibrous proteins (collagen, elastin), amorphous, high-molecular weight proteoglycans (glycosaminoglycans) and specialized proteins (fibronectin, laminin, growth factors) (Figure 1.2).

Among them, the most abundant one is collagen which can be organised into three groups based on their tissue distribution and function in the body: (1) fibril forming, (2) network forming and (3) fibril associated (Table 1)(3,4). Regarding collagen structure, it has central core with amino- and carboxy terminal ends, initially synthesised as a propeptide. The main structure of collagen is formed by the interaction of three α helix chains, namely α triple helix. This triple helix is assembled intracellularly and then secreted by the cells as procollagen into the extracellular space. By the action of different matrix metalloproteinases (MMP), procollagen is cleaved into collagen by removing the N and C terminal propeptide ends. Further crosslinking of collagen molecules is mediated by lysyl oxidase enzymes for the formation of more stable cross striated collagen (4,5) (Figure 1.3).

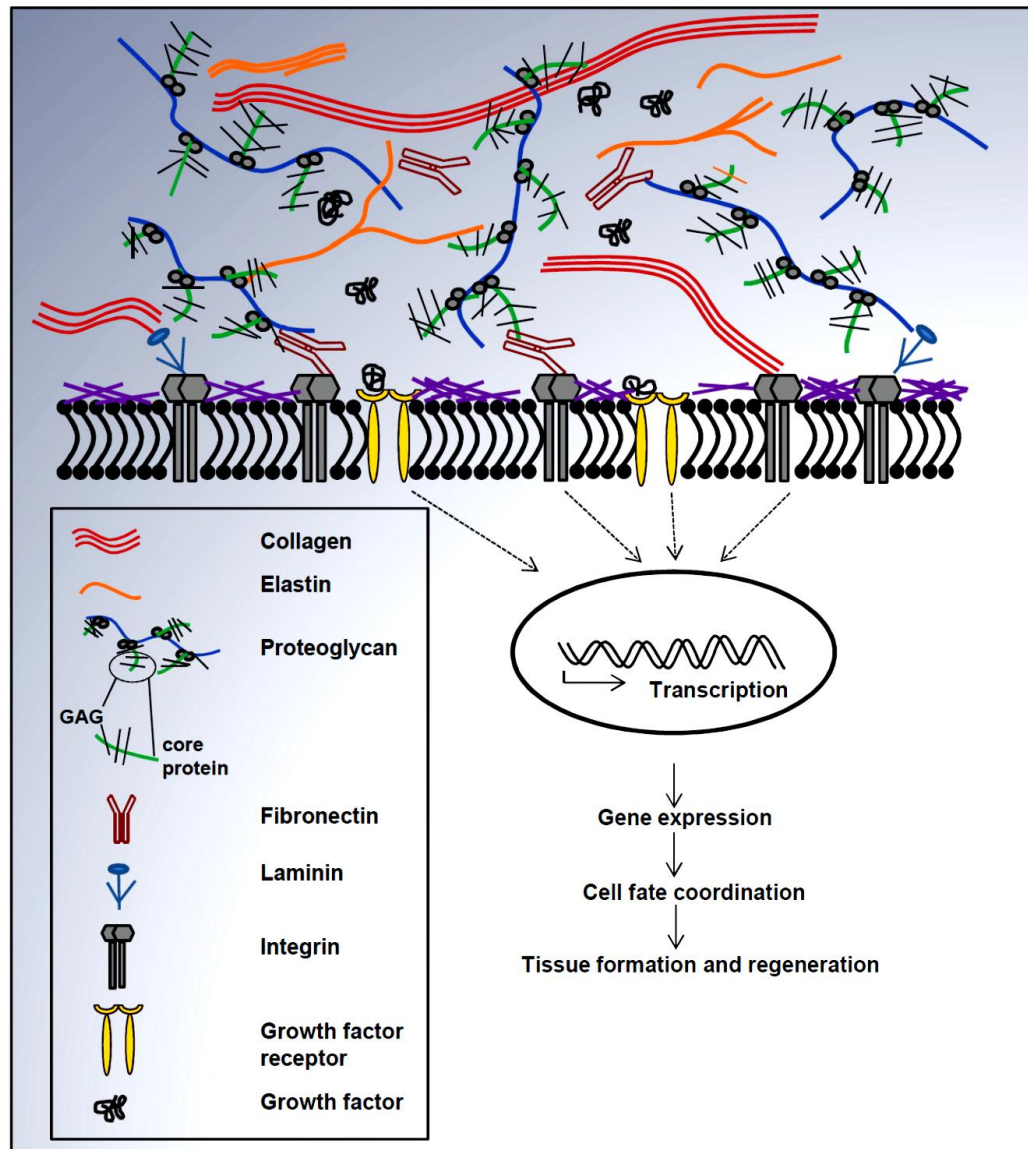


Figure 1.2 Schematic Illustration of key ECM components and their interactions with cells [Papavasiliou *et al* 2012, (6)].

Table 1. 1 Types of Collagen and Their Distribution in the Body

Collagen type	Tissue Distribution
Fibril Forming	
Type I	Most connective tissues like skin, bone, dermis, tendon, ligaments, cornea, blood vessels
Type II	Hyaline Cartilage, Intervertebral disc, vitreous body
Type III	Blood vessels, fetal skin
Network Forming	
Type IV	Basement membranes
Type VII	Beneath stratified squamous epithelia
Fibril Associated	
Type IX	Cartilage
Type XII	Tendon, ligament and some other tissue

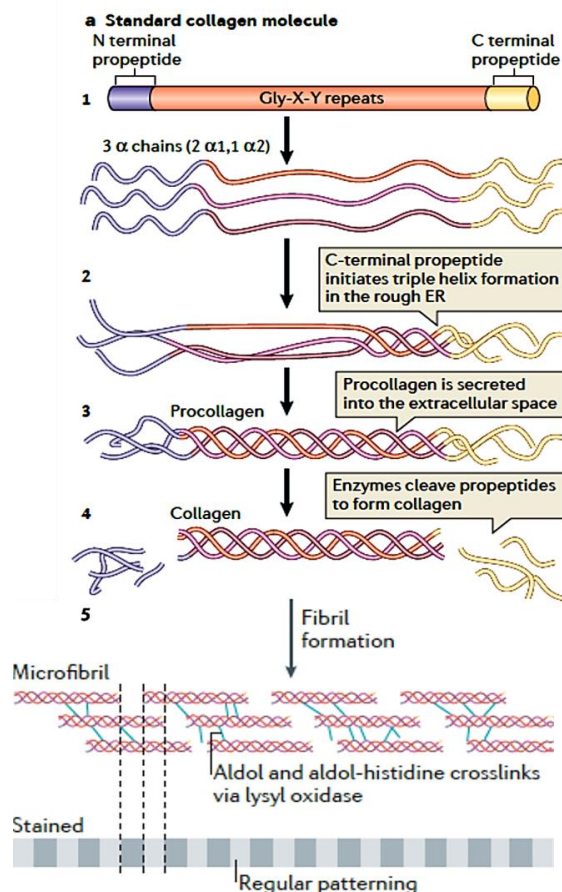


Figure 1.3 Schematic diagram of collagen fibre assembly. (1) Basic structure of collagen α chain. Four different stages are involved (2) Imports of α chains into the rough endoplasmic reticulum where triple helix of α chains formation was occurred initiating from C terminal end (3) Procollagen is secreted into the extracellular space (4) the procollagen molecules is cleaved to form the collagen in extracellular space (5) further crosslinking of collagen by lysyl oxidase enzyme.

[Mouw *et al* 2014, (5)]

In addition to collagen, proteoglycan is another type of important ECM molecules in patterning of ECM around the cells. Structurally proteoglycans consist of a main core protein which links many glycosaminoglycans (GAG) (Figure 1.2). Depending on the structure, density and chains of GAG attached to main core protein, proteoglycans are classified into many subtypes including heparin sulphate proteoglycan (HSPG), chondroitin sulphate proteoglycan (CSPG) and hyaluronic acid (hyaluronan) (5). Specifically, HSPG is mainly found in the basement membrane and also found as cell bound HSPG like syndecans. Being a negatively charged protein, HSPG can bind easily to cell surface receptors, many growth factors like fibroblast growth factor, chemokines and other ECM proteins like fibronectin and laminin. As such, they play an important role in many physiological functions like cell migration and cell proliferation (7). CSPG are mainly associated with brain and cartilage ECM. They are good affinity for water binding which is important for compressive resistance property of cartilage and neural tissue (5).

The other group of specialized ECM proteins includes laminin and fibronectin, which mainly function as connectors within ECM (8). They are important for interconnecting cells to ECM as well as strengthening the ECM framework by bridging between ECM molecules (5,9). Fibronectin consists of many binding motifs that can be recognised by the cell surface receptors called integrins and other ECM proteins like collagen (9,10). Structurally they are assembled as fibrillar adhesions around the cells and functionally, binding of fibronectin to integrins is the initial step of cell adhesion to ECM and provides the force transmission pathway between cell and ECM (11).

Through the unique physical, chemical and biological properties of ECM, the unique feature of an organ is determined. For examples, some organs become solid organs like bone and liver and some organs become porous/spongy organ like lungs. In addition to the physical support for cell anchorage, the three-dimensional (3D) ECM environment serves as not only a reservoir for soluble factors like growth factors and cytokines, but also delivers spatial and contextual cues for the regulation of cellular behaviours such as cell adhesion, migration, proliferation/survival and differentiation.

1.2 Biophysical cues in extracellular tissue microenvironment

Living cells face the diverse nature of biophysical cues in extracellular environment including substrate stiffness, shear stress, dimensionality and topographic cues (12-16). Cells sense the changes in biophysical cues of ECM via various transmembrane and intracellular mechanosensory organelles and convert these mechanical signals into biological responses by triggering rearrangement of cytoskeleton and activating intracellular signalling pathways, which in turn regulate many cell functions that are critical for the tissue homeostasis (Figure 1.4) (2).

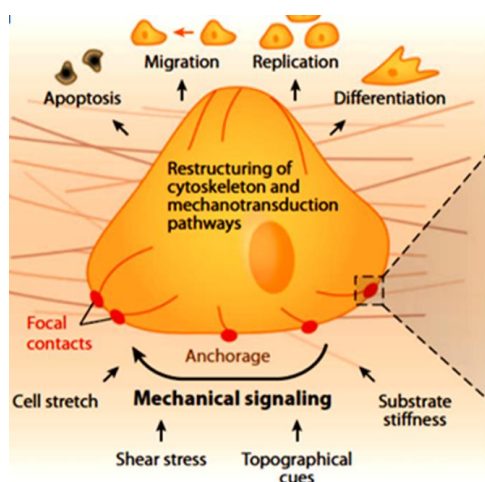


Figure 1.4 Overview of effect of biophysical cues of ECM on the regulations of cell functions. The biophysical cues of ECM include substrate stiffness, shear stress and topography from microenvironment. Cell senses such biophysical cues and convert into biological responses such as proliferation, differentiation and migration through the mechanosensitive signalling pathways [Kim *et al* 2009, (2)]

1.2.1 Role of matrix dimensionality in cell response

Matrix dimensionality is one of the important factors in cell and tissue particularly in the context of biophysical cues. A growing body of evidence has shown that cell cultures using conventional tissue culture plates cannot mimic most of the features that exist in natural microenvironment such as tissue specific 3D architecture, biochemical and mechanical cues. (17,18). This fact raises the question about their biological relevance in many studies.

With advances in tissue engineering approaches, particularly regarding the development of biomimetic scaffolds, there are now many available 3D culture systems and tissue models used in basic biological studies such as cell adhesion and migration (19,20), stem cell research (21,22), and tumour biology (23,24) as well as disease modelling and testing new drugs (18).

Generally 3D culture techniques can be classified into two groups namely scaffold free and scaffold dependent types (18). Scaffold independent 3D culture can be generated in media suspension by using hanging drop approach or forced agitation method - known as spheroid culture (25). However, the majority of 3D cultures are scaffold based where cells are seeded on or within 3D matrices with varying degree of porosity which is determined by the type tissue they are designed to mimic (26). Under these conditions, cells are allowed to re-establish natural cell-cell and cell-ECM interaction in 3D arrangement mimicking the specificity of real tissue compared to conventional 2D culture.

Moreover, common biomaterials used in 3D culture systems can be classified into mainly two groups **A) Synthetic materials** such as polyethylene glycol (PEG), polylactide-co-glycolide (PLG), polyvinyl alcohol (PVA) and

polyethylene terephthalate (PET) and **B) *Biologically derived materials*** such as matrigel (basement membrane matrix), crosslinked collagen/ gelatin /hyaluronic acid in single or combination (27,28).

Compared to the findings generated from the 3D culture, many downsides of 2D culture system relating with many cell biological functions have become uncovered. One of the prominent downsides of 2D culture system is distortion of the original cell morphology. For instance, chondrocytes lose their oval shape appearance and adopt the spindle shaped fibroblast like morphology in 2D culture but they maintain their oval morphology when cultured in 3D using appropriate scaffolds (29). Moreover, abnormally high level of $\beta 1$ integrin expression was observed on mammary epithelial cells when grown on 2D compared to 3D cultures on matrigel in which comparable level of $\beta 1$ integrin expression to normal breast tissue was observed (30).

Cell proliferation, the most important and widely studied cell function, has been shown to be slower in 3D cultures compared to 2D cultures in many normal and cancerous cell lines (24,31). Consistent with this, genes that induce the cell proliferation in response to soluble factors in culture medium are upregulated in 2D cultures. In addition, cells in 3D culture exist in different stages of cell proliferation such as proliferating, apoptosis, and necrosis which is in contrast with 2D cell culture where we can see only proliferating cells (32). Interestingly, many studies from different tissues (e.g. lung, breast, colon and prostate) has shown that 30% of the gene profile of cells cultured on 2D are inconsistent with the gene expression profile of freshly isolated cells from respective native tissues (18,32). The difference in gene expression between cells cultured in 2D vs. 3D is not limited to normal cells and also observed in cancer cells (33).

According to microarray analysis of many tumour cell types, the subsets of genes which display different expression profile between the 2D and 3D culture systems are mainly associated with tumour pathogenesis such as cell proliferation, migration, drug sensitivity and metastasis (19,25,34). A number of studies have confirmed the finding of exaggerated drug sensitivity in 2D culture compared to 3D (18). For example, paclitaxel treatment can reduce ovarian tumour cell proliferation about 40% to 60% in 3D spheroid cell culture whereas in 2D, 80% of reduction in cell proliferation was found at the same dose (35). As many pharmacological agents are designed to target the cell surface receptors, the changes in expression level and pattern of receptors on cells grown in 2D culture might be the major underlying reason for increased drug sensitivity in 2D culture system.

Altogether, it has become obvious that the 2D culture raises biologically relevant issues in many applications including cell functional studies and drug testing. Researchers have tried to develop various 3D *in vitro* models to study normal and abnormal cellular functions in many pathologies in biomimetic manner, which can also contribute in significant reduction in use of the animal model.

1.2.2 Role of mechanical properties of ECM in human tissues

In biological system, stiffness has been used to collectively represent the mechanical properties of a biological substrate. By definition, rigidity or stiffness of a material is defined as the extent to which a material resists deformation in response to an applied force (36). In our body, different organ systems exhibit a wide range of organ stiffness ranging from very stiff (e.g. bone (20000 kPa)) to very soft (e.g. brain and lung (5 kPa)) tissues (36,37). There are

two types of mechanical forces observed at tissue level: 1) endogenous forces generated by cytoskeletal contractibility of cells, and 2) exogenous force exerted by the cells' locality.

Given these wide range of tissue stiffness specific to each organ, various cell types in each tissue/organ can fulfil their physiological functions within the defined normal range of stiffness. Historically, researchers have emphasized on the role of biochemical regulators such as cytokines, growth factors and enzymes in many cell biology functions. Evolutionally, as all cell types are equipped with mechanosensitive properties to respond to mechanical stimuli from their surroundings, any changes in mechanical properties of ECM could have an impact on cell behaviours such as proliferation, migration and differentiation (38). Given the importance of mechanical properties of ECM, its role has been evaluated in various research areas spanning from organ development, stem cell differentiation, tissue homeostasis and pathogenesis of many diseases (16).

Role of Mechanical properties of ECM in Embryogenesis: The mechanical properties of the ECM have been shown to play a critical role in shaping the embryo from the earliest stage to terminal differentiation step (39-41). Generally, changes in intracellular tension along the gradients of tissue stiffness determines embryonic stem cell fate by paving the direction in which cells have to move and grow in (42). For instance, in tissue patterning for lung development, the tips of respiratory buds have thinner basement membrane than other areas creating stiffness gradient to attract resident mesenchymal stem cells into this area for budding morphogenesis (43).

Role of Mechanical properties of ECM in Stem cell differentiation: According to the observations in Engler *et al* study, human mesenchymal stem cell (hMSC) fate can be directed with changes in substrate rigidity. Specific range of substrate elasticity, fine-tuned to mimic *in vivo* tissue stiffness, could induce hMSC differentiation towards particular tissue types. For instance, soft matrix with brain tissue stiffness are neurogenic whereas stiff matrix corresponding to bone and muscle stiffness are osteogenic and myogenic respectively (44-46). Moreover, findings from Shih *et al* suggest that the stiff matrix induce osteogenic differentiation of hMSC in a FAK (Focal Adhesion kinase)-Erk1/2 (Extracellular signal regulated kinase 1/2) activation dependent manner (47).

Role of Mechanical properties of ECM in Disease Progression: Many fibrosis associated pathologies like pulmonary fibrosis, liver cirrhosis and systemic sclerosis are associated with excessive matrix deposition and increased matrix rigidity (48-50). In a traditional view, abnormal ECM synthesis and deposition has been widely accepted as the outcome of the disease status in these pathologies. However, it is increasingly recognised that the increase in matrix stiffness plays an active role in promoting fibroblast activation in a positive feedback fashion. Constitutive activation of fibroblasts leads to aberrant collagen deposition followed by crosslinking of collagen, which stiffens the tissue and impairs tissue homeostasis over time. Moreover, tissue stiffening induces the imbalance between ECM degradation enzymes like MMPs (Matrix Metalloproteinase) and ECM maintaining enzyme like TIMPs (Tissue inhibitors of MMPs) (51). This imbalance will eventually turn the fibrosis into an irreversible state.

Interestingly the similar phenomenon of ECM stiffening effect has been observed in tumour progression. Stiffness of solid tumour like breast cancer is 10 times higher than that of normal tissue (52). Indeed, hardening of tissue is one of the hallmarks of tumours. Like myofibroblasts in fibrotic tissue, cancer associated fibroblasts (CAF) present in tumour tissue produce excessive amount of ECM protein called desmoplasia (53). The pronounced effect of the matrix stiffening in tumour environment is aberrant cell proliferation. Mammary cells on the stiff matrix are carcinogenic with upregulation of proliferation related genes and downregulation of apoptotic genes (52). Further, increased stiffness in tumour tissue are also associated with increased cancer cell invasiveness, increased lysyl oxidase activity for collagen cross linking and decreased MMP activity (54).

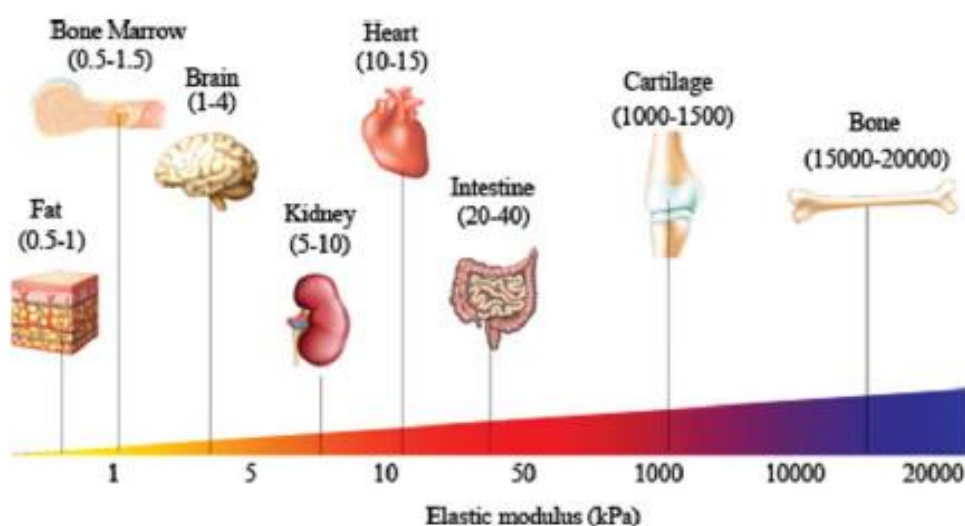


Figure 1.5 Overview of tissue specific stiffness across the various organs in human. Different stiffness levels of specific organs are described in elastic modulus. [Cox *et al* 2011,(37)]

1.3 Lung Disease

1.3.1 Lung inflammation and repair

In order to discuss the lung inflammation and its repair mechanism, it is essential to understand the normal lung architecture. The primary function of lungs is gas exchange. The inhaled air by active respiratory mechanism pass through the whole respiratory tract including nose, larynx, trachea and multiple generations of bronchi and bronchioles which ends in the functional units of lungs called alveoli. The alveolus is the only place where gas exchange takes place between the pulmonary capillaries and alveolar lumen. The alveolar membrane is formed by multiple cell types such as *type I alveolar cells* which are the main cell type in the lining of alveolar epithelium, *type II alveolar cells* which produce surfactant and serve as the progenitor cell for type I alveolar cell during repair and *alveolar macrophages* which function as the first line defence mechanism of the airway against infection and other toxic inhaled substances (55).

The lungs respond to infection and injury by non-specific acute lung inflammation. Inflammation is characterised by the recruitment of various types of immune cells at different time points, vasodilation with increased vascular permeability and many soluble factors generated from infiltrated immune cells including chemokines, cytokines and growth factors (56). The purpose of acute inflammation is to limit hazardous stimuli such as infections to prevent their dissemination into systemic circulation and promote the nonspecific and specific immunity and encourages the tissue repair process (57,58). However complete resolution of acute inflammation after cessation of injury is critically important to prevent its progression into chronic lung inflammation (59).

Abnormal immune response, persistent injurious stimuli or incomplete resolution of acute inflammation can lead to chronic lung inflammatory conditions (60,61). Acute lung inflammation and repair process will be discussed more in the following sections.

Following lung injury, neutrophils are the first innate immune cells recruited into alveolar space from the circulation in the early phase of injury in response to chemokines and cytokines produced from alveolar macrophages and epithelial cells. More than 2/3 of all circulating white blood cells can be entered lung injury site within few minutes following the chemokine gradient created in the inflammation site.(62,63). However, entering into the alveolar space occurs in the expense of alveoli integrity because they have to transmigrate through the alveolar membrane which is highly organised with four types of ECM protein called type IV collagen, laminin, heparin sulphate proteoglycans and nidogen (64). Neutrophils use the proteolytic degradation method to migrate out of the pulmonary capillaries and enter into alveolar space where they release many cytotoxic agents including free oxygen radicals, inflammatory mediators such as prostaglandin and leukotriene, many other proteases (elastase) and antimicrobial proteins like lactoferrin into alveoli to clear the foreign substances or to combat the infection (65). During this stage, these toxins are also harmful to the local alveolar epithelium and endothelium inducing them to undergo apoptosis and necrosis (62,66). Both direct and indirect effect of leucocytes migration are implied on the integrity of alveolar and pulmonary endothelium causing the increased permeability of pulmonary membrane leading to alveolar oedema (67). To minimise these effects, like in other wound healing process,

the injured epithelium is sealed by a temporal scar formed by collagen deposition from resident lung fibroblasts (57).

In addition to neutrophils, other immune cells such as monocytes and lymphocytes are also attracted to the inflammation site at different stages of inflammation. The recruited monocytes to the inflammation site mature into various functional subtypes of macrophage which can significantly increase the total number of local macrophages during inflammation (68). The phenotypes of macrophages change during the course of inflammation owing to their plasticity in response to micro environmental changes including the different types and concentration of soluble factors presented to them (69). They play a mainly pro-inflammatory role in early stage of inflammation but later they switch to an anti-inflammatory role and participate in repair process of lung injury. For example, pathogen associated molecular patterns (PAMPs) on viruses or bacteria can induce the proinflammatory phenotype in early phase of inflammatory cascade but apoptotic cells at late phase of inflammation render macrophages with an anti-inflammatory phenotype (70).

Following inflammation, alveoli become oedematous and filled with debris containing the pathogen (live/dead), apoptotic cells and granules released from the immune cells (67). During the repair phase, all debris are needed to be scavenged to restore the normal function of the lungs. Alveolar macrophages are the best scavengers for phagocytosis of tissue debris and dead cells (71). In terms of macrophage recognition for phagocytosis, phosphatidylserine (PS) is a good recognition marker which is normally present in inner side of cell membrane and only exposed when the cells are in early apoptosis phase(72). There is no clear-cut distinction between inflammation and repair phase.

Activated neutrophils and recruited macrophage also expose PS to induce the initiation of repair phase even when they are in activated stage before they undergoes the programmed cell death (73). Moreover, in addition to scavenger functions, macrophages can reprogram into the anti-inflammatory type upon recognition of PS on apoptotic cells and produce many anti-inflammatory mediators like interleukin (IL)-10, transforming growth factor (TGF)- β and other growth factors like insulin like growth factor and hepatocyte growth factor (HGF) to control the inflammation (70,74).

During repair phase, the damaged alveolar epithelium requires full reepithelialisation. Type II alveolar pneumocytes are regarded as an important progenitor cell which can migrate, proliferate and differentiate into type I, lining alveolar epithelium to reform the new alveolar membrane. Many signalling pathways like TGF β 1 are involved in the type II alveolar cells proliferation and differentiation into type I lining epithelium (75). Tissue fibroblasts also help in regeneration of type I epithelium by producing growth factors like keratinocyte growth factor (KGF) and HGF which have proven effect on type II cell differentiation (76). However, many questions about the heterogeneity of type II cells, the signalling pathways involved in type I cells differentiation from type II cells and characterisation of new progenitor cells in lungs are yet to be answered.

Together with reepithelialisation, the collagen scar formed during inflammation process must be processed and removed. Collagen fibres are mainly digested by a group of enzymes called matrix metalloproteinases (MMPs) mainly secreted from the immune cells like neutrophils and macrophages (77). Moreover, being a protease enzyme, MMP can activate many soluble factors including pro/anti-

inflammatory cytokines like IL1 β , macrophage inflammatory protein [MIP]-1 α and TGF β 1 by altering their activity and bioavailability (stimulating pro form to active form or cleavage from the extracellular matrix pool) (78). Neutropenic animal models showed severe fibrosis condition with impaired collagen lysis due to the lack of MMPs (79). These observations also prove the dual role of neutrophils and macrophage in lung inflammation and repair phase.

Once inflammation is triggered following lung injury, in some lung diseases like asthma, chronic obstructive pulmonary disease (COPD) and acute respiratory distress syndrome (ARDS) there is an uncontrolled chronic inflammatory response during the repair process. It is now recognised that in many chronic lung pathologies, some of the main signalling pathways that control the regulation of transcription factors like nuclear factor kappa B (NF- κ B) and activator protein 1 (AP1) show persistent activation in immune cells following acute inflammation leading to amplification of immune responses (80).

In addition to immune cells, recent data has indicated that the structural lung cells like fibroblasts, epithelium and smooth muscles can behave like immune cells by secreting various cytokines including growth factors and chemokines that lead to augmentation of inflammation. For instance, in asthmatic patients, the expression of eotaxin, monocyte chemoattractant protein (MCP)-3,4 and granulocyte monocyte colony stimulating factor (GM-CSF) are found to be upregulated compared to control subjects (81,82). These cytokines, produced either constitutively or following activation, from the structural cells of the lung may influence the initiation and amplification of ongoing lung inflammation towards the chronic inflammatory stage with fibrotic complication (83).

Therefore, intracellular mechanisms/signalling in response to inflammatory and injurious stimuli, leading to increase the synthesis of inflammatory mediators in those cells are of great interest to control the progressive inflammation (84-87).

1.3.2 Progression to fibrosis

Fibrosis characterises chronic diseases of many major organs such as lung, kidney and liver that results in end stage failure of these organs and consequently is a major cause of morbidity and mortality. Generally, fibrosis is characterised by (1) excessive accumulation of extracellular matrix (ECM) proteins, (2) hyperproliferation of fibroblasts and (3) their differentiation into myofibroblasts in clusters, termed fibrotic foci, that collectively lead to disruption of normal tissue architecture (88). Many acute and chronic pulmonary diseases such as idiopathic pulmonary fibrosis, chronic asthma, interstitial pulmonary pneumonia and chronic granulomatous diseases like sarcoidosis are also associated with lung fibrosis as an end stage complication (89).

Excessive collagen deposition or incomplete resolution of scar during lung inflammation may impair gas exchange and affect the respiratory function. During the repair phase following lung inflammation, the collagen deposition must be resolved completely and replaced by normal functional alveolar epithelium. Collagen deposited during inflammation must be digested by enzymes like MMPs when the inflammation cascade subsides. Imbalance in ECM homeostasis such as decreased or inadequate secretion of MMPs from fibroblasts or macrophages during lung injury or persistent activation of fibroblasts with aberrant collagen production and cross linking has shown to be

complicated as lung fibrosis with excessive ECM deposition following lung injury (64,90,91).

Impaired cellular cross-talk between the two main structural cells types, airway epithelium and fibroblasts was also proposed as a central mechanism in the pathogenesis of lung fibrosis evidenced by the appearance of co-localisation of epithelial apoptosis area and α -SMA positive myofibroblasts in the fibrotic lungs. Moreover, in *in vitro* co-culture experiments, it was shown that mechanical injury to airway epithelium can affect the co-cultured fibroblast behaviours such as increased α -SMA expression and collagen production (type I and III) (92). In addition to direct cell to cell contact, soluble mediators such as connective tissue growth factor (CTGF) and prostaglandin (PGE₂) produced from epithelium and fibroblasts have been found to be involved in both cell types' cross talk in either paracrine or autocrine manner (93-100). Other growth factors such as platelet derived growth factors (PDGF) and TGF- β released from anti-inflammatory monocytes/ macrophage (101) has been shown to have pronounced effect on the proliferation of type II alveolar epithelium and myofibroblast differentiation in lung fibrosis (76).

A number of recent studies suggest that the persistent fibrotic response in chronic fibrosis is considerably more complicated than previously thought. Multidirectional interactions between multiple factors such as impaired cellular cross-talk, stiff ECM and soluble factors like TGF- β have been shown to be involved in lung fibrogenesis.

Nevertheless, due to the complexity of disease, there is still lack of comprehensive *in vivo* and *in vitro* experimental models of lung that can recapitulate the most important manifestations of the diseases in human lung.

This has hampered the progress in comprehensive understanding of disease processes and also in developing anti-fibrotic agents.

1.4 Overview of current available lung fibrosis models

1.4.1 *In Vitro* Model

With the awareness of the shortcomings of the animal models, development of human based *in vitro* lung models as a platform for studying various lung pathologies has attracted substantial interest in recent years. Ideally engineered *in vitro* lung models should simulate different aspects of native tissue (e.g. combination of different cells types, appropriate ECM and scaffolds etc.) so that they can be used as platforms for investigating biological and pathological processes.

Most of the models used in the past for studying the lung inflammation and fibrosis pathology have primarily used the standard two dimensional cultural techniques. This approach clearly lacks consideration for the complexity of a 3D microenvironment in the lung where almost all resident structural and immune cells are exposed to a variety of either structural or soluble proteins like growth factors and cytokines in a unique spatial geometry. The stiffness of the planar tissue culture plate is one good example of the poor physiological relevance of two dimensional cultural systems.

As discussed before, depending on the type of organ, different tissues show a wide range of tissue stiffness from kPa to GPa scale and for normal lung tissue stiffness is around 5 kPa (102). However, the surface of tissue culture plate has the extreme stiffness about 2 to 4 GPa. Therefore, the results obtained from these surfaces cannot be representative of *in vivo* condition where the stiffness of the microenvironment is significantly different. Although ECM proteins (e.g.

collagen, fibronectin) coated surfaces have been used to understand the cell behaviours in response to ECM environment (103,104), this approach does not include the spatial and contextual drive from the 3D extracellular microenvironment. Compared to 2D planar matrix substrates, 3D-matrix interactions display enhanced cell biological activities in terms of integrin receptor expression and focal adhesion [69].

Therefore, there is a need for development of more biologically relevant 3D lung tissue models to be used as tools for better understanding of the basic interactions of key cells like epithelial cells, fibroblasts, endothelium and their surrounding extracellular matrix in steady-state and under pathological conditions such as lung fibrosis. Some 3D *in vitro* lung models have been developed to examine the physiologic functions like mucus secretion, cellular crosstalk (105-109), pathologic changes and even drug toxicity or drug responses (110,111). Decellularized human tissue and cell derived ECM scaffolds have also shown to be attractive culture systems for studying the effects of ECM on surrounding cell phenotype (88,112) but these systems still suffer from the disruptive effects of cell removal agents (e.g. detergents) used in decellularization process which can alter the ECM composition, and tissue availability is also a limiting factor (113).

With the help of recent advances made in tissue engineering, there are many methods available for creation of 3D microenvironment. Phase separation (114), electrospraying (115) or electrospinning methods (116) are commonly used methods for fabrication of fibrous scaffold. Depending on the type of cells (either epithelial or mesenchymal) and organ system (lung or bone), the choice of substrates for fabrication of 3D microenvironment becomes different. For

example, porous fibre sheets could be a favourable 3D alternative for culturing epithelial cells which normally grown on basement membrane. The porous fibre sheet can provide a closer morphological resemblance to the basement membrane of the tissue.

The other widely used method is cell encapsulation within biomimetic 3D hydrogels which can provide the microenvironment resembling the native extracellular matrix (ECM) (117). By definition, hydrogels are hydrophilic, physically or chemically cross-linked macromolecular networks formed by hydrophilic polymers swollen in water or biological fluids. Hydrogels with tuneable stiffness are ideal for growing the mesenchymal cells like fibroblasts, smooth muscles and vascular endothelium (105). By means of different polymerisation methods, different types of structures can be made from wide range of polymers for various purposes. Generally, hydrogels can be classified to synthetic (118), natural (119,120) or combined synthetic and natural gels (107,121). Synthetic microgels are attractive for many types of cell culture in tissue engineering because of their physical properties like mechanical strength and well defined shape and biological parameters such as resemblance to natural extracellular matrix (ECM) and the ability to entrap the cells within the gel. On the other hand, certain polymers like PEGDA (polyethylene glycol) diacrylate based gels exhibit poor cell attachment, explaining their cell unresponsive features and limited biodegradability (122). Different formulations of PEG that contain Arg-Gly-Asp (RGD) sequences however show enhanced cell binding and matrix metalloproteinase (MMP) sensitive degradation sequences and have higher biocompatibility (123). However, they have not been widely used because of complicated technology for processing.

In terms of natural polymers, collagen and gelatin are attractive biomaterials from the view that they are non-synthetic materials with high biocompatibility and low immunogenicity. However, because of their poor mechanical robustness, most of the natural hydrogels fail to create long lasting scaffolds even though cells can adhere to and proliferate within such hydrogels easily.

1.4.2 *In Vivo* Model

Most of the information about the lung fibrosis has been derived from post-mortem studies of lung tissue/slices from the patients with lung fibrosis or tissue samples from lung biopsies. In post-mortem studies, typically the disease status is at an advanced stage at the time when the tissue samples are processed, which makes it difficult to investigate the natural history of the disease progression. In order to overcome this limitation, scientists have used numerous *in vivo* models not only to study the development of lung fibrosis but also to test the efficacy of anti-fibrotic agents.

Various *in vivo* models (mainly mouse models) have been extensively used in the study of lung fibrosis for decades. Among those, a bleomycin induced lung model is the best characterised and commonly used model in lung fibrosis research because of its similarities to aspects of human disease and the availability of multiple routes for drug delivery. However, there are some common weaknesses in mouse models such as strain dependent outcomes, limited biological/clinical relevance to human and high cost. Some of limitations in biological relevance of animal models arise from differences in lung anatomy, ventilation mechanism, lung protein expression profiles, their immune system and duration of injury required for disease progression

compared to human (124). Therefore, there are still major concerns regarding the usefulness of *in vivo* models in predicting the pathophysiology and pathogenesis of lung fibrosis in human since currently no animal model faithfully recapitulates all features of pulmonary fibrosis (125-127).

1.5 Project overview

Many studies have investigated the pathogenesis of lung inflammation and fibrosis using conventional tissue culture plate and flask. It would be difficult to translate findings from those researches into *in vivo* condition because cells at tissue level *in vivo* reside within highly structured 3D architectural environment. Therefore, it is critical to validate existing results from *in vitro* 2D cell culture using more biomimetic and disease relevant *in vitro* models perhaps before undertaking *in vivo* studies. With the help of transwell culture technique, many *in vitro* single cell studies can be improved by performing co-cultures that enable investigating multicellular interactions (e.g. epithelial cells, fibroblasts and immune cells).

Although animal models could offer whole organism level understanding in many of these studies, their biological relevance to human disease remains questionable due to many fundamental intra species differences. Therefore, studies using human cells under tissue relevant culture conditions could complement, and perhaps in some cases replace, *in vivo* animal studies.

In order to investigate the effect of biomechanical property changes of ECM in fibrosis, only culture systems which display tissue relevant stiffness level can enable to understand the role of matrix stiffening in lung fibrosis. In considering biophysical cues such as dimensionality, investigation on lung injury and repair will need to be carried out under 3D cultures condition.

In this project, it was hypothesised that studying lung inflammation and fibrosis within a biomimetic extracellular matrix microenvironment will provide more physiologically relevant and reliable understanding of the cellular events involved in lung inflammation and initiation/progression of fibrotic response following lung injury.

In order to test this hypothesis, in the first instance, a number of biomimetic scaffolds were fabricated and characterised. This was followed by the use of these scaffolds to study a number of cellular and molecular events that underpin lung inflammatory and fibrotic responses.

To this end, I first developed and characterised a 3D culture system by electrospinning methods which has the similar spatial organisation of normal lung ECM. By using this optimised 3D lung model, we studied lung inflammatory response in *in vivo* like lung environment.

Secondly, in order to investigate the effect of stiffness/biomechanical property of ECM in lung fibrosis progression, we developed the bioengineered platform with tissue relevant stiffness by using ECM based hydrogel. With careful optimisation, we achieved the stiffness value of normal and fibrotic lungs in hydrogel system. By applying this platform, we studied the impact of ECM stiffness changes in the disease progression of lung fibrosis and investigated the mechanosensitive signalling pathways implicated in this process.

In this project, we can prove the importance of the spatial organisation and substrate stiffness of ECM in lung inflammatory response and lung fibrosis by using the bioengineered biomimetic *in vitro* lung models.

Chapter 2

A 3D Culture System for Investigating Inflammatory Responses in Human Lung

2 Chapter 2 – A 3D culture system for investigating inflammatory responses in human lung

2.1 Introduction

2.1.1 Role of lung fibroblasts in lung inflammatory responses

Inflammation is thought to be the central mechanism for many chronic lung disorders such as asthma, chronic obstructive pulmonary disease, and lung fibrosis although the specific features and anatomical sites affected in each disease are different. Immune cells recruited to the site of inflammation are regarded as the key effector cells in lung inflammation through the production of various proinflammatory cytokines (128). However, once inflammation is triggered, a chronic inflammatory response persists following lung injury. It is now accepted that in addition to immune cells either influxing into or already residing in lung tissue during inflammation, the structural lung cells such as airway epithelium, smooth muscles and lung fibroblasts, also play an important role in initiation and maintenance of chronic lung inflammation by secreting and responding to a plethora of inflammatory mediators such as cytokines, chemokines and growth factors (84-87). Through those mediators, they involve in the local regulation of inflammatory responses such as immune cell chemotaxis, transendothelial migration, cellular apoptosis and myofibroblast differentiation (129-131).

Fibroblasts are a major population of stromal cells characterized by a spindle shaped morphology and flat oval nuclei. Functionally they are main cell type responsible for lung integrity by synthesizing various ECM proteins and remodeling ECM in response to various physiological stimuli (132). However,

the cell proliferation rate, ECM production and other immunomodulatory functions of fibroblasts are different between tissues according to the functional requirement of different tissues (133).

An overview of the role of lung fibroblasts in inflammation is illustrated in Figure 2.1. As highlighted in Figure 2.1, fibroblasts produce many *chemotaxis factors* such as interleukin-8 (IL-8), monocyte chemoattractant protein (MCP-1,3,4), macrophage inflammatory protein-1-alpha (MIP-1- α), RANTES and eotaxin to induce endothelial activation and favor the circulating leucocytes entering the site of inflammation (83). Interestingly, fibroblasts can also express different vascular *adhesion molecules* like intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) to enhance monocyte- fibroblast adhesion and chemokine production in a reciprocal manner during inflammation (134,135). For instance, the secretion of MIP1 from macrophage requires the engagement with ICAM-1 on fibroblasts induced by TNF α (134). In addition to chemokines, growth factors such as granulocyte monocyte colony stimulating factor (GM-CSF), platelet derived growth factor (PDGF) and transforming growth factor-beta (TGF- β) produced from fibroblasts have shown to be implicated during the acute and chronic phase of lung inflammation in both autocrine and paracrine dependent manner (136-139). In addition, the paracrine effect of TGF β -1 induces stromal derived factor (SDF1) on fibroblasts and the interaction of SDF1 with CXCR4 interaction on T cells not only causes T cells recruitment towards the inflammation site but also induces retention of T cell in this site (140). In these ways, together with immune cells, lung fibroblasts facilitate the cytokine milieu that maintains tissue inflammation in active state.

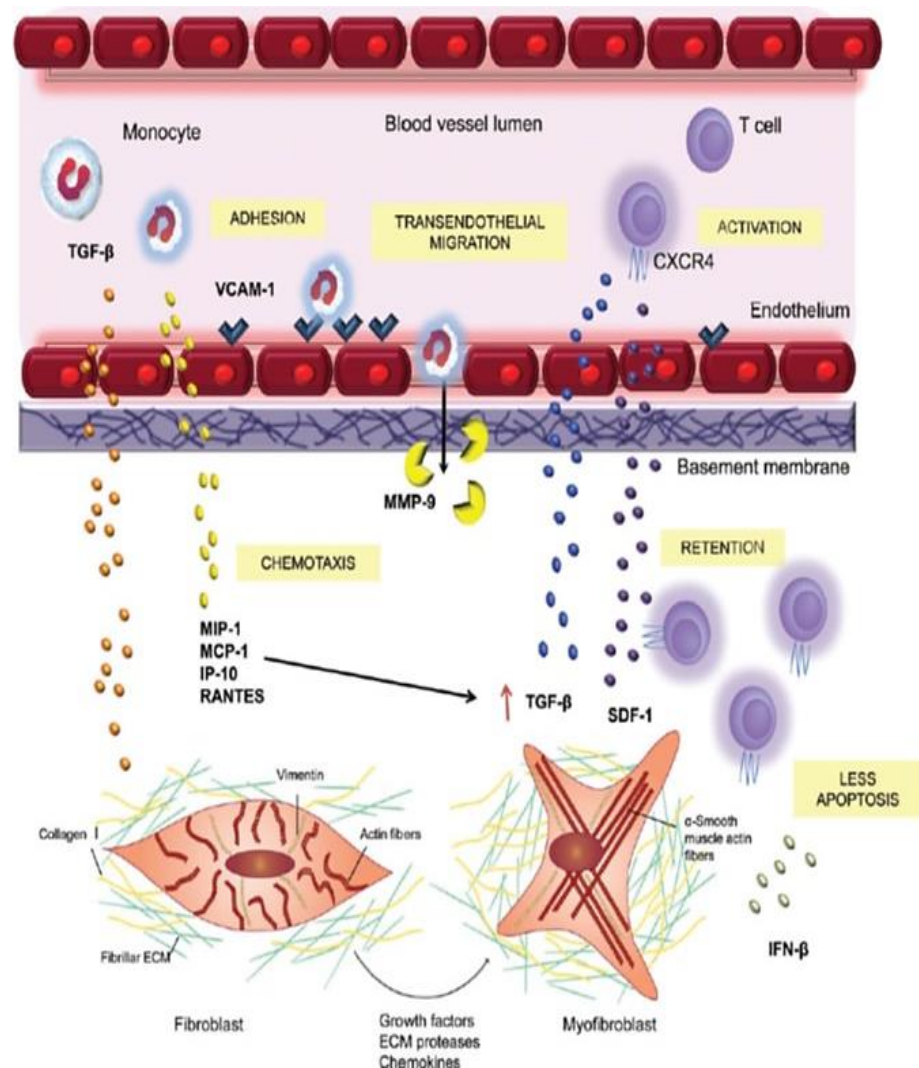


Figure 2.1 Overview of the central role of fibroblasts in inflammation. Activated fibroblasts induce the trafficking of circulating leucocytes towards the inflammatory site via the secretion of various chemokines and induction of adhesion molecules on the endothelium. Autocrine and paracrine effect of TGF β can induce myofibroblast differentiation and retention and less apoptosis of immune cells via SDF1-CXCR4 interaction. [Linthout *et al* 2014, (129)]

2.1.2 Role of NF- κ B transcription factor activation in lung inflammation

Not limiting to immune cells, pro-inflammatory cytokines in active inflammation have effects on fibroblasts such as increased proliferation, myofibroblast differentiation, and cytokine production from fibroblasts (141-

144). At the transcriptional level, the expression of a series of genes controlling inflammatory cytokines are under the major control of transcription factor NF- κ B (145). In addition to being a transcription factor controlling a number of inflammatory responses, NF- κ B activation is probably the earliest cellular event in response to exogenous stress and injury (146). Recently NF- κ B has been shown to be activated in the structural cells of lung such as airway epithelium and smooth muscles in association with increased transcription of some proinflammatory cytokines and growth factors (147-149).

In unstimulated cells, NF- κ B is bound to an inhibitor (I κ B) in the cytoplasm preventing it from entering the nuclei. With exposure to various external stimuli, I κ B is promptly phosphorylated, ubiquitinated and degraded by 26S proteasomes and release active NF- κ B into the nucleus (150) where it binds to a specific sequence in the promoter regions of target inflammatory genes (Figure 2.2) coding for inflammatory cytokines, chemokines, cell adhesion receptors and immune receptors (Table 2.1) (151). The well-known stimuli that can activate NF- κ B include various inflammatory cytokines like TNF α , IL1 β , activator protein C, viruses and oxidants.

Two cytokines, TNF- α and IL-1 β , produced by inflammatory cells especially monocyte/macrophages in the early inflammatory stage, are also shown to be capable of setting up a positive feedback loop in NF- κ B activation pathway which is important for perpetuation of a local inflammatory response (145). Moreover, these two cytokines are shown to be involved in the initial activation of fibroblasts for synthesis of various inflammatory mediators (86,137,139,152-154). Moreover, in the absence of RelB (I κ B-stabilizer) in *in*

vivo mouse model, uncontrolled persistent production of chemokines including MIP-1(1 α and 1 β), MIP-2, IP-10 and RANTES was observed following activation with inflammatory stimuli like TNF- α or lipopolysaccharide (155). More importantly TNF- α itself has been demonstrated to play a central role in the pathology of various pulmonary diseases such as asthma, chronic bronchitis, COPD and acute lung injury (156).

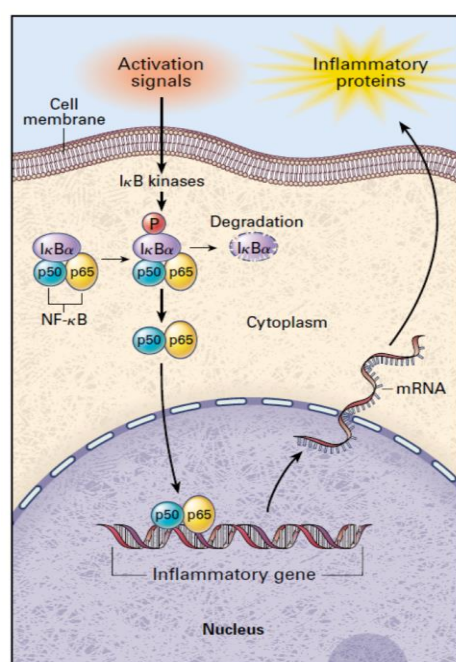


Figure 2.2 Schematic Diagram of NF- κ B activation. Activation of NF- κ B involves the phosphorylation and subsequent proteolytic degradation of the inhibitory protein I κ B by specific I κ B kinases. Then free NF- κ B moves into the nucleus and enhances the transcription of gene for inflammatory proteins. [Barnes *et al* 1997,145]

Therefore, the ability to study the molecular pathways controlling the inflammatory responses in fibroblasts, such as those involved in NF- κ B activation under *in vivo* like conditions will no doubt provide a better insight and understanding of lung inflammation, particularly in lung fibrosis where fibroblasts play a central role in pathogenesis. Thus, detection of NF- κ B activation can not only act as a sensitive probe for inflammatory responses in human lung but also provide a useful tool for monitoring the impact of drug intervention or progression of chronic lung inflammation.

Table 2.1 Inflammatory genes regulated by NF- κ B**Cytokines**

- Tumour Necrosis factor α (TNF α)
- Interleukin 1 β (IL1 β)
- Interleukin 2 (IL2)
- Interleukin 3 (IL3)
- Interleukin 6 (IL6)
- Interleukin 12 (IL12)

Growth Factors

- Granulocyte Monocyte colony stimulating factor (GM-CSF)
- Granulocyte colony stimulating factor (G-CSF)
- Monocyte colony stimulating factor (M-CSF)

Chemokines

- Interleukin 8 (IL8)
- Macrophage Inflammatory Protein 1 alpha (MIP 1 α)
- Macrophage Chemotactic Protein (MCP 1)
- RANTES

Inflammatory Mediators

- Inducible nitric oxide synthetase (iNOS)
- Inducible Cyclo-oxygenase 2
- 5 lipoxygenase
- Cytosolic Phospholipase A2
- C Reactive Protein
- 12- lipoxygenase

Adhesion molecules

- Intracellular adhesion molecules (ICAM1)
- Vascular adhesion molecules (VCAM1)
- E selectin

2.1.3 Two-dimensional vs three-dimensional culture systems

Generally, 2D cell culture refers to cells cultured on a flat surface such as conventional tissue culture plate in which cells are arranged in monolayer and interact with neighboring cells mainly through basal-lateral cell junctions. In contrast to 2D, cells in 3D cell culture are embedded within the ECM matrix in which cells can expose to either matrix or other neighboring cells in all

directions similar to *in vivo* conditions (18). Given the fact that 3D cultured cells are more functionally and morphologically adopted to *in vivo* microenvironment than their 2D counterparts, in recent years there has been substantial interest in developing 3D culture systems considering the spatial organization of the tissue architecture (18).

Most studies on lung inflammation including NF- κ B activation have been performed in conventional 2D culture systems. However, the limitations of 2D cell culture system are becoming more apparent (157,158). For example, *in vivo* fibroblasts are non-polar cells supported by ECM and exist in a 3D conformation within a particular geometry. Typically, when the cells are seeded on a rigid flat surface, they tend to become stretched in order to adapt to the commonly used flat rigid surface of tissue culture flasks/plates (159). This will not only affect cell morphology and polarity but also affect cell membrane tension, spatial organization of the cell surface receptors and intracellular tension (18). Due to changes in cell membrane tension, one of cell membrane functions called endocytosis/exocytosis is directly affected on 2D surface, which in turn influences the overall vesicular trafficking within the cytoplasm of the cells. Those processes are mechanistically related to many aspects of cell physiological functions such as phagocytosis, migration, reactive oxygen species production and secretion of soluble factors like cytokines including growth factors (160).

Human fibroblasts cultured on cell derived tissue matrix in 3D have greater cell attachment and higher proliferation than when cultured in 2D (161-165). Furthermore, cells cultured in 3D are thought to be able to withstand more stress when encountered cytotoxic agents owing to their natural biophysical

dimension (18,166). Thus, it is reasonable to suggest that studying the pro inflammatory responses, such as NF- κ B activation, in fibroblasts in 3D better reflects the functional property of these cells *in vivo* and can provide more accurate results for future applications including drug testing for controlling NF- κ B activation in lung fibroblast as new target for controlling lung inflammation.

As discussed in Chapter 1, there are many types of available 3D culture techniques such as electrospun scaffolds (106,167), 3D printed scaffolds (168) and spheroids culture (24). Among them, electrospinning is the one of the most effective technologies in fabrication of porous scaffolds with micro- and nano-scale fiber and pore sizes that present a close resemblance to native fibrillar ECM structure (169). The basic set up for electrospinning process is illustrated in Figure 2.3. An electrospinning platform consists of three basic components: a high voltage power, a spinneret, and a collector. Briefly, the applied voltage creates a cone shaped deformation of polymer drop coming out from spinneret called taylor cone. Within the defined range of electrical field, a liquid jet is formed once the electrostatic force on the deformed polymer drop can overcome the surface tension of polymer drop. The electrified jet is then sprayed toward the counter electrode, leading to the formation of a long and thin thread. As the liquid jet is continuously elongated and the solvent is evaporated, solid fibres with diameter as small as nano scale range are deposited on the collector plate (170,171). By using this technique, we have recently reported poly (ethylene terephthalate) (PET) electrospun scaffolds with nano- and micro-diameter fibre range. Interestingly, we and others have shown that such electrospun scaffolds with fibre diameters of approximately

240 ± 70 nm highly resemble the natural ECM of normal lung tissue (245 ± 83 nm) (105,169). The main advantage of this scaffold compared to other culture system is that it can support the long-term 3D culture of differentiated lung epithelial cells and fibroblasts up to 4 weeks (105,172).

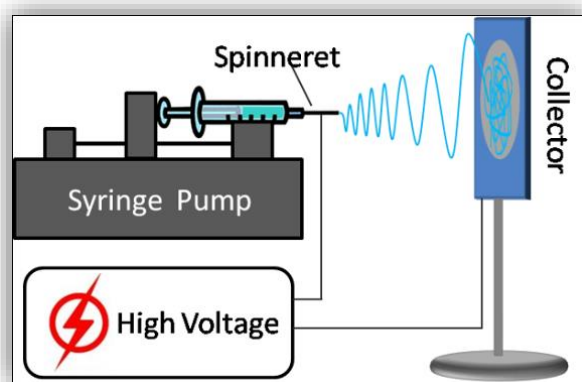


Figure 2.3 Schematic illustration of the basic setup of electrospinning.
[Haung *et al*, 2013]

In this chapter, we investigated the role of lung fibroblasts in lung inflammation by studying the activation of NF- κ B activation under biomimetic conditions. We also elucidated the importance of matrix dimensionality in the study of lung inflammation by comparing the NF- κ B activation between 2D and 3D culture systems.

2.2 Materials and Methods

2.2.1 Polyethylene Terephthalate (PET) scaffold fabrication by electrospinning

10% electrospun Polyethylene terephthalate (PET) was fabricated using published method described in Helen *et al* (2014) (105). Briefly, PET was dissolved in 1:1 trifluoroacetic acid (TFA): dichloromethane (DCM) (Fisher Chemicals, U.K.) to create a 10% (w/v) solution. The polymer solution was loaded into a syringe (10 mL) with an 18-gauge needle (BD Falcon, U.K.). The syringe was securely assembled on a syringe pump driver (Harvard Apparatus Ltd., U.K.). A grounded steel collector plate was positioned at 15 cm distance from the needle tip. Then PET polymer solution was pumped at a constant flow rate of 0.5 mL/hour at 14 kV for 4 hrs. Scaffold sheets were placed in a fume hood for 24 hrs to air dry. In order to have unique characteristics of scaffold such as thickness, diameter of fibres and pore sizes at each electrospinning, experimental conditions described in table 3.1 were specified for each electrospinning. The pore size of the scaffold was determined by using the SEM image analysis. According to Morris *et al* 2014, the pore size was defined by the longest distance within the defined pore on the same focal plane (172).

Table 3.1 Characteristics of PET scaffolds

Concentration of PET	Solvent (DCM:TFA)	Flow rate (ml/hr)	Needle Gauge	Voltage (kV)	Total Volume (ml)	Working Distance
10% (w/v)	1:1	0.5ml/hr	18G	14kV	2ml	15cm

2.2.2 Scanning electron microscopy for characterisation of scaffold

PET electrospun scaffolds were analysed by SEM (Scanning Electron Microscopy) (JEOL JMS-6060 LV microscope, U.K.) by mounting PET scaffold on carbon-coated electron microscope stubs and gold sputter coating for 4 minutes under an argon atmosphere (Blazers Union, SCD 030, BOC, U.K.) prior to analysis.

2.2.3 2D and 3D cell culture

The human fetal lung fibroblast cell line (MRC-5) was obtained from the American Type Culture Collection (ATCC) and routinely cultured in T75 flasks in Eagle's minimal essential medium (MEM) supplemented with 2 mM L-Glutamine, 10% FCS and penicillin-streptomycin (100 IU/mL / 100 µg/mL) at 37°C, 5% CO₂ in a humidified incubator until confluent. The culture medium was changed three times a week.

For 2D culture system, the cells were trypsinized with 1x trypsin-EDTA when they confluent, centrifuged at 350 *g* for 5 minutes at room temperature, seeded directly into a 24 well plate (Costar) in transfection experiment and HCL (1N) treated coverslip was used for cell seeding in immunostaining. 1.5 ×10⁵ cells in 500 µl per well was used for all experiments. Then the cells were incubated in a humidified incubator for 24 hrs to allow cell adherence before transfection. Passage numbers 10 - 20 were used.

For 3D cell culture, PET scaffold sheets were cut and sterilized under UV light (254nm), 15 minutes on each side and then incubated with 70% ethanol for 20 minutes in a non-tissue culture treated 12 well plates. Sterilized stainless steel rings (inner diameter - 1cm, outer diameter - 1.9 cm and height - 0.8 cm) were used to hold down the scaffold and limit cell attachment in the center of

scaffold. After placing steel rings on the top of the PET scaffold, it was then sterilized with an antibiotic/antimycotic solution (5%) (A5955, Sigma) overnight. Before cell seeding, PET scaffolds were washed with PBS x 2 times then presoaked in complete MEM culture medium for 1 hr. Cell suspension of 1.5×10^5 cells in 500 μ L of complete MEM medium was seeded on the PET scaffold inside the steel ring while placing the same amount of medium outside. After seeding, the plate was incubated for 24 hrs to allow cell adherence to the scaffold before transfection.

2.2.4 Immunostaining of NF- κ B /p65 subunit and TNF receptor I expression (2D and 3D culture) and confocal microscopy

Primary anti-NF- κ B/p65 (C-20) rabbit polyclonal IgG antibody from Santa Cruz Biotechnology (sc-109, CA, USA), TNF receptor I rabbit polyclonal IgG antibody from Abcam (19139) and Alexa Fluor[®] 488 goat anti-rabbit IgG secondary antibody (H+L) from Molecular Probes (Life Technologies, UK) were used in our experiments.

Briefly, the same culture condition for both 2D (coverslip) and 3D (PET scaffold) culture was used for immunostaining of NF- κ B and TNF- α receptor I expression. NF- κ B activation was determined in MRC5 lung fibroblasts by immunostaining for the intracellular position of p65 transcriptionally active subunit of NF- κ B as previously described (173). Experiments were stopped by 4% (w/v) paraformaldehyde for 20 min at room temperature after 1hr stimulation with TNF- α (2ng/ml) (Gibco, Life Technologies, UK). TNF- α receptor I expression was studied without permeabilization to detect the surface receptor expression. Cells were permeabilized with 0.2% (v/v) Triton for 20 min at room temperature and neutralized with 50 mM ammonium

chloride for 5 min. Cells were washed three times in PBS after each treatment. Unreactive binding sites were blocked with 5% (w/v) bovine serum albumin (Sigma) in PBS for 30 min. Cells were then incubated with primary anti-NF- κ B/p65, anti TNF receptor I antibody for 1 hr at room temperature (1:100 (v/v) in PBS). Cells were washed with PBS 3 times then incubated with Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody (H+L) (1:1000 v/v in PBS) for 1 hr at room temperature in the dark, washed 3 times with PBS, followed by incubation with 300 nM DAPI (Sigma-Aldrich) in the dark for 30 min. Images were captured using 40x objectives of Zeiss LSM710 confocal with a Zeiss Observer microscope (SLIM imaging, University of Nottingham).

2.2.5 Real time monitoring of NF- κ B activation in lung fibroblasts by Luciferase reporter system (2D and 3D culture)

Ready-To-Glow™ NF- κ B secreted luciferase reporter system (Cat No- 631743) was obtained from Clontech laboratories (Palo Alto, CA). Transfection was performed as described in protocol provided from lipofectamine 2000® transfection agent (Invitrogen, UK). Briefly, the ratio 1: 2.5 of pNF- κ B MetLuc2 reporter (μ g) and transfection agent (μ L) was used according to our optimization result (Appendix 1). Before addition, the culture medium was replaced with 400uL of Opti-MEM®. The cells were transfected for 5 hrs in incubators and then refreshed with complete MEM.

In 3D culture, the *in-situ* transfection method was used in our experiment and the same transfection procedure was followed as in 2D culture. One positive control with control vector was kept in both 2D and 3D experiments to determine transfection efficiency.

In order to test the dose dependent TNF α effect on NF- κ B activation in 2D and 3D systems, transfected MRC5 cells in both culture were stimulated with 3 concentrations of TNF- α (0.2, 2 & 20 ng/mL) for 24 hrs in duplicate wells after 24 hrs' transfection.

Culture medium was refreshed 1 hr before the experiment in order to reduce background reading. Then triplicates of 50 μ L supernatants from each well were collected and the assay was performed immediately according to the protocol provided (or samples were frozen at -20°C until analysis). A white 96 well plate was then transferred to a luminometer (FLUOstar Optima) and analyzed. Data were recorded as relative light units (RLU).

2.2.6 Alamar Blue Assay for cell viability on 2D and 3D culture

Following 24 hr incubation with different doses of TNF- α , viability of MRC5 cells cultured in 2D and 3D were determined by Alamar Blue assay (Invitrogen, life technologies, UK). Cells were incubated with Alamar blue dye (10% of the volume of fresh culture medium) for 4 hrs at 37°C with protection from the direct light. 100 μ L of triplicate supernatants were transferred to a 96-black well plate (Fisher Scientific) and read at λ_{ex} = 540nm / λ_{em} = 580nm (FLUOstar Omega) plate reader with fluorescence reading.

The Alamar Blue reduction by the cells expressed as fluorescence emission intensity units was measured on specific days of the culture period for different samples. The % reduction of Alamar Blue for each case was calculated using the following formula.

$$\text{percentage reduction of alamarBlue}^{\circ} = \frac{S^x - S^{\text{control}}}{S^{100\% \text{ reduced}} - S^{\text{control}}} \times 100$$

where S^X is the Alamar Blue fluorescence signal of the sample at day x , $S^{100\%reduced}$ is the signal of the 100% reduced form of Alamar Blue and $S^{control}$ is the signal from the control (the culture medium supplemented with 10 % Alamar Blue without cell). The 100% reduced form of Alamar Blue was produced by heating culture medium supplemented with 10 % Alamar Blue at 121°C for 15 minutes.

2.2.7 Statistical analysis

Statistical analysis was performed using Graph Prism 6, San Diego CA. Data are expressed as means $\pm$ SEM and analyzed by one way ANOVA with appropriate post hoc test for multiple comparisons. Significance level was set at $p < 0.05$.

2.3 Results

2.3.1 Development of 3D culture system using non-degradable PET electrospun scaffold

For developing the 3D cultures, we electrospun PET nanofibers (using the parameters summarized in Table 3.1) into fiber mats that were used for culturing fibroblasts in 3D. Scanning electron micrographs of PET scaffold confirms the random arrangement of nanofibers with non-beaded appearance (Figure (2.4 A and 1B)). Figure 2.4 C shows average fibre diameter (257.4 ± 16.28 nm) and pore size 1079 ± 80.74 nm (mean $\pm$ SEM) of 5 independent PET scaffolds. We have previously shown that these electrospun scaffolds have comparable geometry to human lung ECM and support fibroblasts growth and differentiation (105,172). Therefore, in line with our observations, fibroblasts grown on such scaffold are expected to maintain more *in vivo* like morphology, phenotype and functional properties compared to cells grown on 2D substrates.

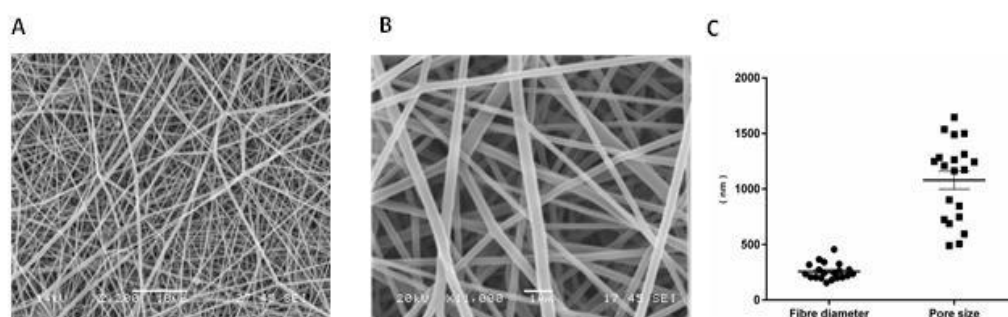


Figure 2.4 Acellular nanofiber PET scaffolds. Representative SEM images of 10% PET acellular electrospun scaffold dissolved in 1:1 (v/v) DCM: TFA are shown in different magnifications **(A)** (Bar = $10\mu\text{m}$) and **(B)** (Bar = $1\mu\text{m}$). Distributions of fibre diameters and pore sizes of electrospun scaffold are shown in **(C)** (4 random measurements from 5 individual scaffolds) (Mean $\pm$ SEM, $n=20$).

2.3.2 NF- κ B activation in response to TNF- α was detected in lung fibroblasts in 2D and 3D cultures by immunostaining

Using immunostaining method, we monitored nuclear localization of p65 subunit of NF- κ B in both 2D and 3D cultures as a surrogate for NF- κ B activation. Figures 2.5 and 2.6 show the NF- κ B/p65 immunostained images of MRC5 lung fibroblasts grown on 2D and 3D culture, respectively, after 1 hr stimulation with TNF- α (2ng/mL). Data clearly show that NF- κ B/p65 expression (green) remained cytoplasmic with almost no nuclear staining in unstimulated MRC5 cells (Figure 2.5 and 2.6, upper panel) whereas there was substantial nuclear NF- κ B/p65 staining (Figures 2.5 and 2.6, lower panel) following TNF- α stimulation. DAPI-Nuclei labeling (blue) was used to confirm the location of nucleus. This finding confirms NF- κ B activation in lung fibroblasts in response to proinflammatory stimuli TNF- α .

In addition to NF- κ B activation, the different morphology of fibroblasts was observed between 2D and 3D culture systems. Unlike cells grown in 2D which are aligned next to each other with limited cell-cell contact, fibroblasts grown in 3D have significantly higher cell-cell interaction forming networks (Figs. 2.5 and 2.6, right panel). There are also clear differences in the nuclear alignment between the two systems visualized after DAPI staining (Figs. 2.5 and 2.6, left panel) where cells in 2D cultures showed parallel nuclear alignment compared to a random nuclear arrangement in the 3D culture system. Given the similarities between the topography and dimensionality of the 3D fibrous scaffolds and the lung ECM (105,171,172,174), we believe the cellular orientation observed on 3D scaffolds is likely to be closer to their arrangement *in vivo*.

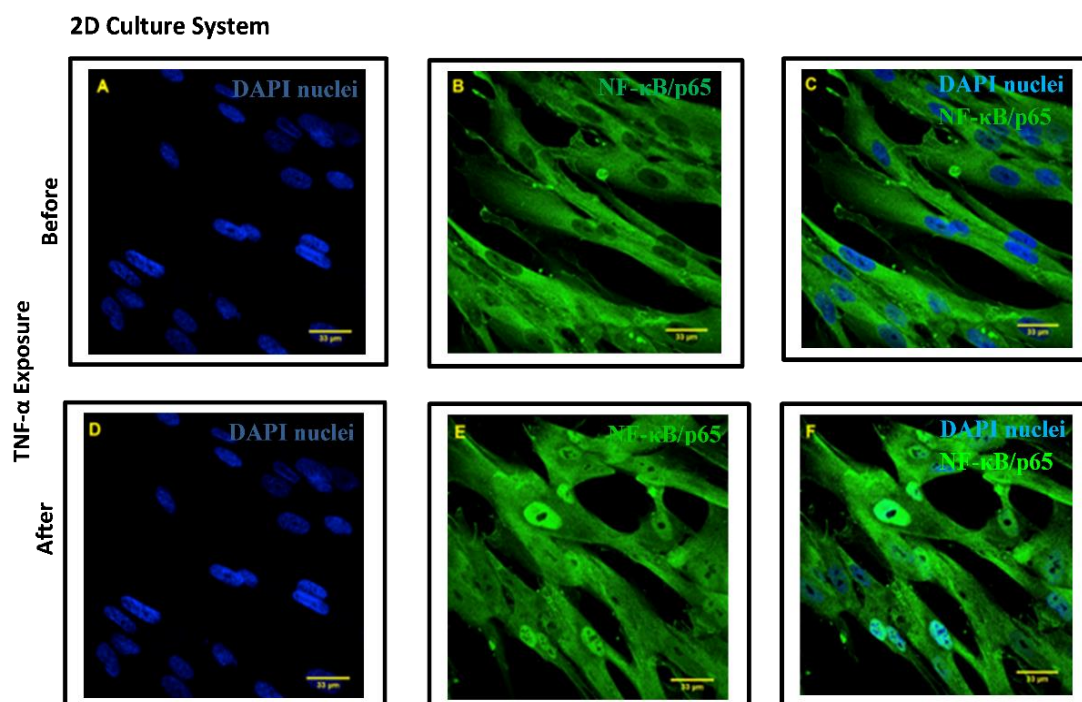


Figure 2.5 TNF- α induced NF- κ B Activation in MRC5 lung fibroblasts in 2D culture system. Translocation of NF- κ B/p65 was detected in MRC5 fibroblasts grown in 2D culture system after exposure to TNF- α (2ng/mL) for 1 hr by immunostaining (green color). Nuclei of lung fibroblasts were localized by DAPI (blue) (**A and D**). A-C represents cytoplasmic localization of NF- κ B/p65 without TNF- α -treatment whereas D-F represents nuclear localization of NF- κ B/p65 in MRC5 with TNF- α treatment for 1 hr. The nuclear localization of NF- κ B/p65 was overlaid by DAPI nuclear staining (**F**). (Bar =33 μ m, n=3).

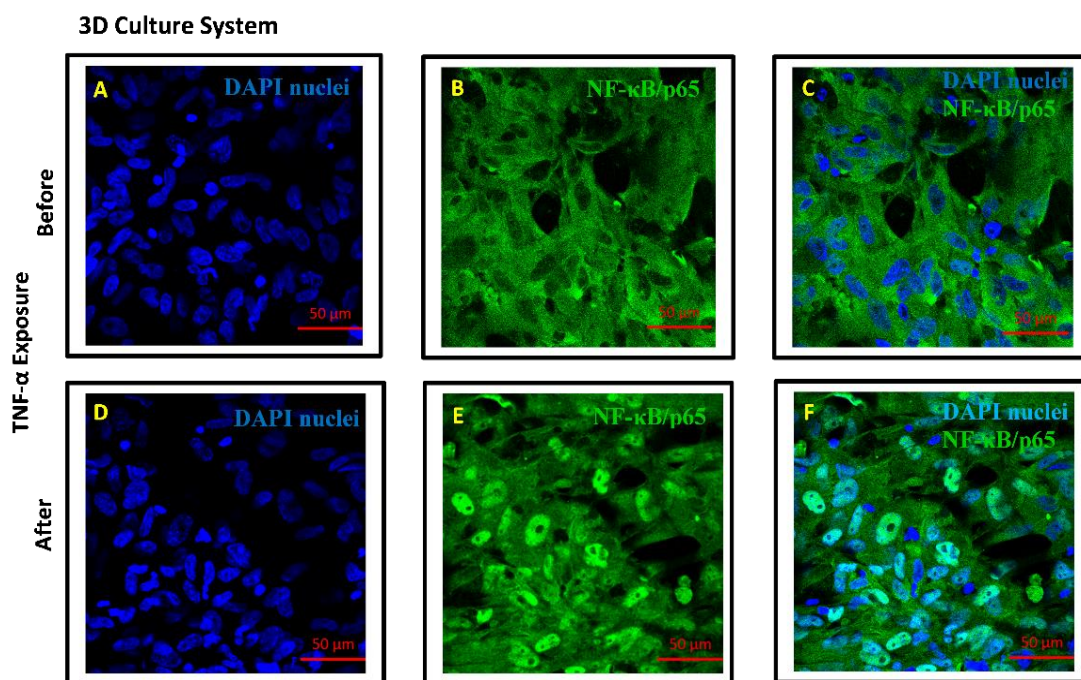


Figure 2.6 $\text{TNF-}\alpha$ induced NF- κ B Activation in MRC5 lung fibroblasts in 3D culture system. Translocation of NF- κ B/p65 was detected in MRC5 fibroblasts grown in 3D culture system after exposure to $\text{TNF-}\alpha$ (2ng/mL) for 1 hr by immunostaining (green color). Nuclei of lung fibroblasts were localized by DAPI (blue) (**A and D**). A-C represents cytoplasmic localization of NF- κ B/p65 without $\text{TNF-}\alpha$ -treatment whereas D-F represents nuclear localization of NF- κ B/p65 in MRC5 after $\text{TNF-}\alpha$ treatment for 1 hr. The nuclear localization of NF- κ B/p65 was overlaid by DAPI nuclear staining (**F**). (Bar =50 μm , n=3).

2.3.3 NF- κ B activation was detected in lung fibroblasts in 2D and 3D cultures by luciferase reporter assay

Having confirmed the NF- κ B activation in lung fibroblasts by immunostaining, to better quantify such activation we used a luciferase reporter assay. In this method, NF- κ B activation was monitored by the amount of luciferase protein secreted in the culture medium after TNF- α stimulation as the reporter construct expressing the luciferase gene is under the control of the NF- κ B promoter element. Firstly, both 2D and 3D cultures of fibroblasts were successfully transfected with a luciferase reporter vector after optimization of transfection efficiency with different ratio of transfection agent to control vector (Appendix 1). To ensure parity between 2D and 3D cultures, we also investigated transfection efficiency between the two culture systems. To this end, the same number of lung fibroblasts was transfected with control vector which was designed to express luciferase protein constitutively without NF- κ B promoter element. This was followed by culturing cells for 24 hr in 2D or 3D before measuring luciferase activity. The 24 hrs luciferase level after transfection was shown to be comparable between both systems (Figure 2.7 A).

To investigate the sensitivity of this assay for monitoring NF- κ B activation, fibroblasts cultured in 2D and 3D were stimulated with three concentrations of TNF- α with 10-fold increments (0.2, 2 and 20 ng/ml) for 24 hrs (Figure 2.7 B). As shown in Figure 2.7 B, all three concentrations of TNF- α significantly activated NF- κ B dependent luciferase production in MRC5 fibroblasts compared to unstimulated control in both 2D and 3D cultures. However, 2D and 3D cultures showed a very different activation pattern and sensitivity to

TNF- α stimulation. Despite the higher magnitude of NF- κ B activation in 2D cultures, cells became non-responsive to TNF- α stimulation in concentrations above 2ng/mL of TNF- α in contrast to 3D cultures in which cells remained responsive to TNF- α stimulation even at the highest concentration tested (i.e. 20ng/mL). Interestingly, fibroblasts grown in 3D had a dose dependent response to all TNF- α concentrations albeit the level of NF- κ B activation in 3D cultures was lower than 2D under the same experimental conditions (Figure 2.7 B).

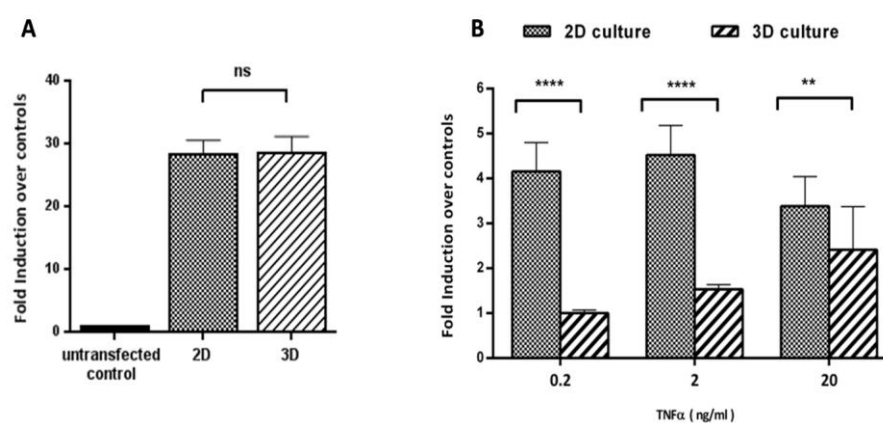


Figure 2.7 Detection of NF- κ B activation in 2D and 3D culture system by luciferase reporter assay. (A) Comparison of transfection efficiency between 2D and 3D culture systems. Constitutive luciferase secretion in both systems was measured at 24hrs after transfection with control vector under same experimental condition. Data expressed as fold induction over untransfected control value **(B)** Comparison of NF- κ B activation between 2D and 3D culture systems. Transfected MRC5 lung fibroblasts were stimulated with three different doses of TNF- α for 24 hrs in both systems after transfection. NF- κ B activation was measured as fold induction increment of luciferase reporter activity from respective untreated control. Data are mean $\pm$ SEM of combined results from three independent experiments and collected as triplicates samples from duplicate wells for each experiment **** $p < 0.0001$, ** $p < 0.01$, ns=not significant,(n=3) .

To assess any potential cell death after fibroblasts transfection and post TNF- α stimulation, we also determined the cell viability after 24-hrs exposure to different concentrations of TNF- α (Figure 2.8). This data showed no significant changes in cell viability between 3 concentrations of TNF- α in both 2D and 3D culture systems.

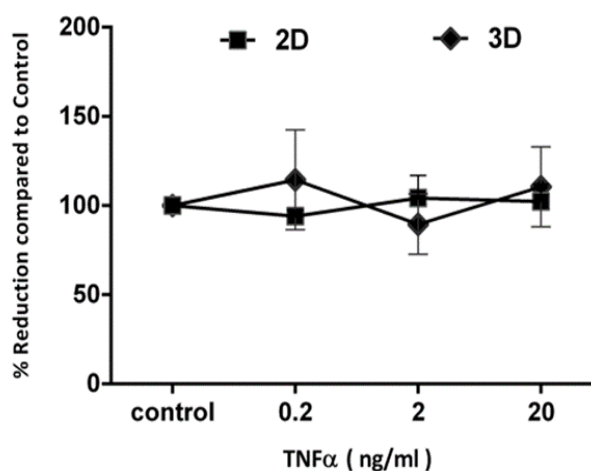


Figure 2.8 Assessment of cell viability between 2D and 3D culture system. Cell viability was assessed by Alamar Blue assay after 24 hrs stimulation with different doses of TNF- α . Untreated controls were defined as 100% and experimental data were expressed as percentage reduction compared to that of control. Data are mean $\pm$ SEM of combined results from three independent experiments and collected as triplicates samples from duplicate wells for each experiment ($n=3$).

2.3.4 Detection of TNF receptor I expression on lung fibroblasts (2D and 3D culture)

Given that NF- κ B activation in response to TNF- α is dependent on the surface expression of TNF- α receptor, it was reasonable to assume that the difference in the either pattern or level of TNF receptor expression between the two culture systems could drive difference in the NF- κ B activation level. Accordingly we examined the expression of TNF receptor type I (the main TNF receptor expressed on fibroblasts (175)) in cells cultured in 2D or 3D

systems. Interestingly, receptor expression pattern was found to be different showing localized expression in 2D culture (Figure 2.9 A and B) which was in contrast with homogenous expression pattern observed in 3D cultures (Figure 2.9 C and D).

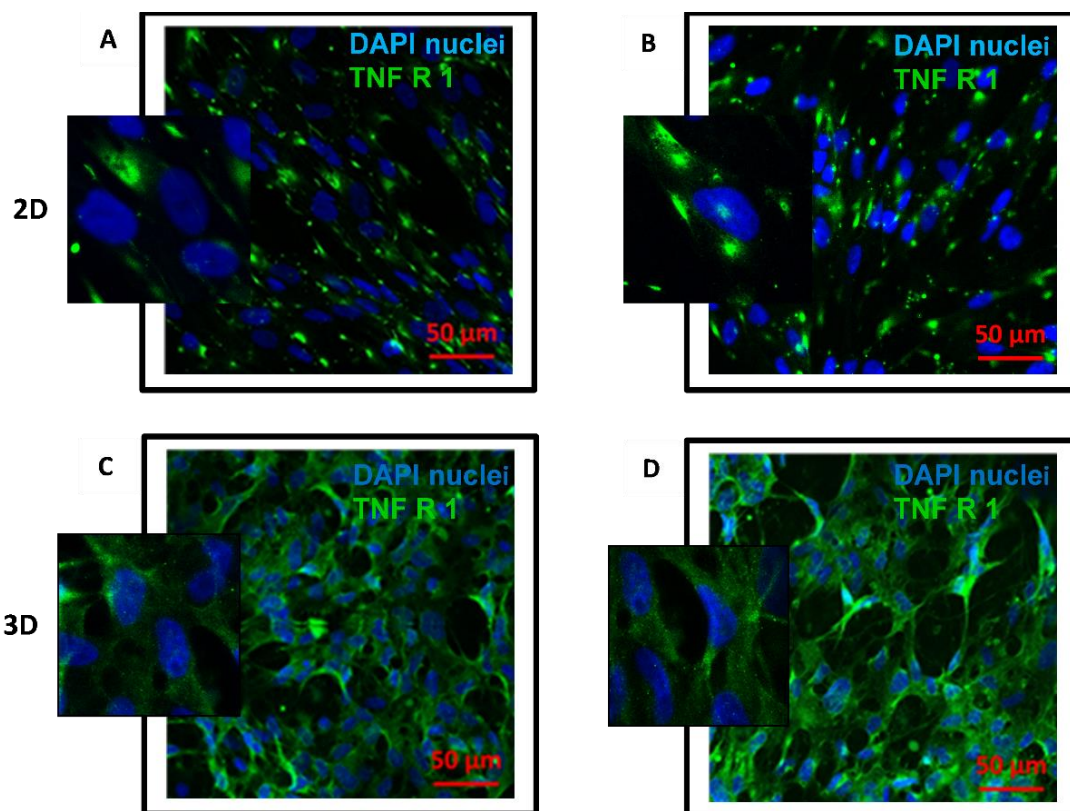


Figure 2.9 Immunostaining of TNF receptor I expression of lung fibroblasts in 2D and 3D culture systems. TNF- α receptor I on fibroblast surface was immunostained with Alexa Fluor[®] 488 secondary antibody (green) in both 2D (A-B) and 3D (C-D) culture systems and nuclei were labeled with DAPI (blue). (Bar =50 μ m, n=3)

2.4 Discussion

According to the observations in this chapter, both detection methods - immunostaining and luciferase assay are sensitive enough to detect NF- κ B activation in lung fibroblasts cultured in 2D or 3D. By applying these methods, we observed the NF- κ B activation in lung fibroblasts in both 2D and 3D culture. As NF- κ B has shown to be activated in many inflammatory lung diseases (176-178) and also studied in the *in vivo* model of inflammation in response to various stress stimuli (179-181), my findings confirm the active role of lung fibroblasts in inflammation evidenced by NF- κ B activation in lung fibroblasts in response to proinflammatory stimuli TNF- α . More importantly, there were significant differences in the activation pattern and dynamic range of NF- κ B activation responsiveness to TNF- α stimulation between 2D and 3D culture systems.

Such ‘bell-shaped’ pattern of NF- κ B activation in 2D cultures is in line with previous data showing a peak in NF- κ B activation in HEK293 cells in response to 5ng/ml TNF- α in 2D cultures followed by a drop at higher concentrations (182). We also claim the drop in NF- κ B activation at higher concentrations of TNF- α in 2D culture was not due to reduced cell viability in our experiment (Figure 2.7 C). This effect could be explained by the negative feedback effect of newly synthesized I κ B α at high level of NF- κ B activation. It has been shown that nuclear translocation of NF- κ B activates the I κ B α gene which has κ B binding sites. Thus, the buildup I κ B α can interfere with the binding of NF- κ B to DNA by binding and bringing it back to cytoplasm. It can also prevent further NF- κ B uptake by the nucleus (183,184).

These observations are consistent with data from mouse lung tissue showing dose dependent but low level NF- κ B activation in response to TNF- α compared

response from liver tissue where NF- κ B activation is at significantly higher levels but has a narrow dynamic range and drops with increasing concentrations of TNF- α (179). Overall, these data suggest fibroblasts cultured in 3D exhibit a more *in vivo* like pattern of NF- κ B activation in lung.

Another possible underlying reason of NF- κ B activation in 2D versus 3D might be associated with the observation of differences in TNF receptor expression pattern between two cultures (homogenous receptor expression pattern in 3D system vs the focal expression pattern in 2D system). As gap junction proteins can be expressed more in cell populations with higher cell to cell contact (185), we suggest that the formation of cellular networks in 3D culture system favors the formation of more gap junctions between the fibroblasts which allows the exchange of molecules including ions and second messengers via these junctions (185,186). Such differential TNF receptor expression pattern between 2D and 3D culture systems could at least in part explain the different dynamics of NF- κ B activation between two culture systems.

Stromal cells such as fibroblasts naturally grow on a 3D extracellular matrix bed that supports acquiring more physiologically relevant characteristics like receptor expression, proliferation and cytokine production (159). Fibroblasts grown on 3D porous scaffolds achieved more complex morphology closer to the architecture of normal lung tissue (105,174) and we propose that the observed NF- κ B activation pattern in 3D culture is more in line with such responses *in vivo* lung. This is supported by *in vivo* studies showing dose dependent NF- κ B activation in response to LPS challenge in lung inflammation of transgenic mouse model (179).

In summary, by using the 3D culture system, it clearly indicates the significance of the role of matrix dimensionality in pulmonary inflammatory response. It also again highlights the necessity of the biomimetic environment in the study of cellular functions especially in term of the magnitude of cellular response which can reflect the *in vivo* condition. Moreover, the 3D culture system and NF- κ B detection methods described in this study provide a simple but efficient tool for investigating proinflammatory responses non-invasively and in real time under physiologically relevant conditions. To further enhance the physiological relevance of this model, the 3D fibroblast layer and the NF- κ B detection system can be co-cultured with lung epithelial cells as well as relevant immune cells to create a multi cellular immunocompetent 3D model of human lung. Such a model will provide a powerful tool for studying cellular and paracrine cross-talk between lung fibroblasts, immune cells and other structural cells in the context of acute and chronic lung inflammation.

Chapter 3

Bioengineering an ECM based hydrogel platform to investigate the role of extracellular matrix stiffness in lung fibrosis

3 Chapter 3 – Bioengineering an ECM based hydrogel platform to investigate the role of extracellular matrix stiffness in lung fibrosis

3.1 Introduction

Collagen and elastin fibres form the main structural network of pulmonary parenchyma (187). In normal lung, the type I and type III fibres exist in 2:1 ratio and provide the tensile strength of lung ECM (88,188). By mixing fibres (collagen and elastic) with different properties, lung is designed to have high elasticity in alveolus that facilitates passive recoiling during expiration. The other types of ECM such as type IV collagen and laminin are mainly found in the basement membrane of the alveolus and airway (188,189).

3.1.1 Key features of lung fibrosis

In transition to the fibrotic state (e.g. in patients suffering from idiopathic pulmonary fibrosis (IPF)), the main types of collagen (types I and III) are elevated in the parenchyma. In early fibrosis, predominantly type III collagen is deposited in alveolar septum and interstitium whereas in the progressive established fibrosis state, type I collagen deposition is found in both active and scarring area of fibrotic lung contributing more than 80% of the total collagen in IPF lung (88). Moreover, the other types of ECM including fibronectin, hyaluronic acid, glycosaminoglycan and type V, VI, VII collagen are also elevated in fibrotic lung leading to the stiff lung parenchyma with impaired lung compliance at tissue level, ending in progressive dyspnoea (37,88).

Recent studies have indicated that altered biomechanical properties such as rigid matrix in diseased state have profound effects on important cell functions

like fibroblasts proliferation and contractility that are central to the pathogenesis of lung fibrosis (36,48,190). Matrix stiffness is six times higher in murine fibrosis model (bleomycin induced) than that of normal mice lung parenchyma (saline treated) (0.5kPa vs 3kPa) (191). Using native and decellularized human lungs, Booth and colleagues (2012) confirmed that the IPF lungs have significantly higher matrix stiffness, nearly 9 times higher than that of normal lung (2 kPa vs 17 kPa) (88). Initially, this high matrix stiffness was regarded as the outcome of the multiple fibrotic lesions in lung and not a major causative factor in pathogenesis. However, in contrast to this view, researchers have now focused on the extracellular matrix (ECM) as an active participant in the progression of fibrogenesis (Figure 3.1) (190,192).

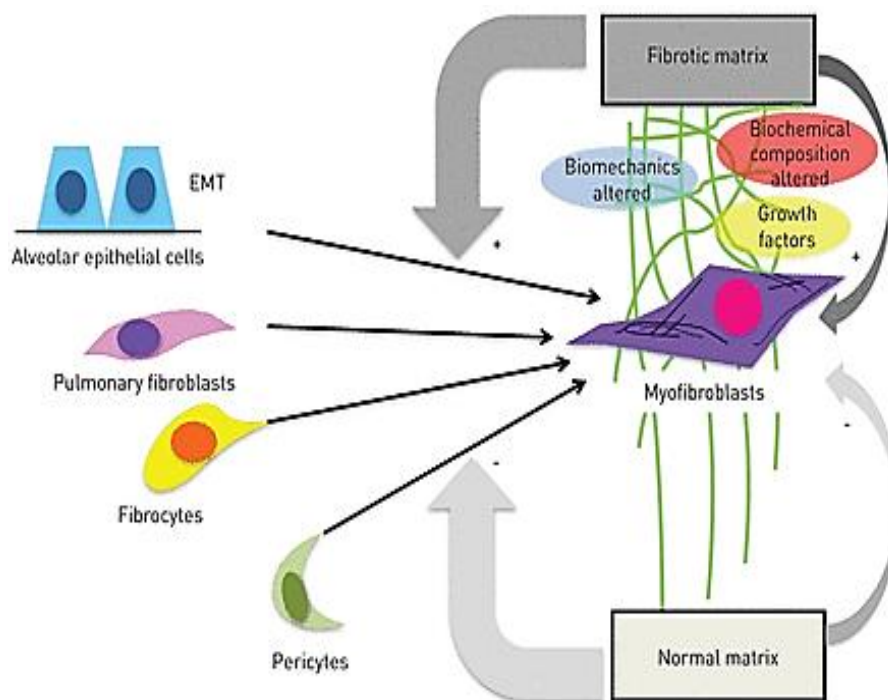


Figure 3.1 Overview of the active contribution of profibrotic extracellular matrix in the pathophysiology of pulmonary fibrosis. The profibrotic ECM with altered biomechanical and chemical properties has direct influence on the myofibroblast differentiation from various mechanisms [Shimbori *et al*, 2013, (190)]

Much attention has been given to the effect of matrix stiffness variation on key features of fibrotic state such as proliferation rate, myofibroblast differentiation (50,191,193) and epithelial-mesenchymal transition (EMT) (98,194).

Myofibroblasts are a transdifferentiated fibroblast phenotype, generally absent in normal lung parenchyma and found only in fibrotic lungs, mainly associated with active fibrotic sites (195). Morphologically, they are spindle shape with intracytoplasmic stress fibres and functionally, they share the contractile features of smooth muscle cells (196). The expression of α smooth muscle actin isoform (α -SMA), is the most widely used marker for myofibroblast transformation (197,198). Both *in vitro* and *in vivo* studies have confirmed that fibroblasts derived from fibrotic lung display a remarkable increase in the percentage of α -SMA positive myofibroblast populations (94,195).

Functionally, myofibroblasts are the major cells responsible for the synthesis of collagen which contribute to matrix composition within the lungs and connective tissue remodelling after lung injury (133). Under normal wound healing, myofibroblasts derived from resident fibroblasts and mesenchymal cells transiently present in injured areas for wound contraction but upon completion of the healing process, they undergo programmed apoptosis. In lung fibrosis, myofibroblasts become resistant to apoptosis and produce an excessive amount of collagen and other ECMs (96,199). In addition, fibroblasts isolated from fibrotic lung tissue are phenotypically different from non-fibrotic fibroblasts and they also exhibit altered responsiveness to growth factors. (200-203).

Given the importance of the matrix rigidity in lung fibrosis, Booth *et al* (2012) has shown that α SMA expression of fibroblasts grown on the stiff matrix of acellular IPF lung was higher than that of fibroblasts cultured on the acellular normal lungs independent of TGF- β (88). Moreover, inhibition of mechanosensitive signalling in experimental mouse fibrosis model induces the apoptosis of fibroblasts with the recovery from fibrosis features (146). Together, these findings suggest that myofibroblast transformation and fibroblast proliferation in lung fibrosis is strongly influenced by matrix rigidity state in lung fibrosis.

TGF- β 1 has been shown as one of the potent growth factors in the pathogenesis of lung fibrosis in induction of fibroblast proliferation, increased ECM production and myofibroblast differentiation (101,204,205). A recent study showed that down-regulation of one protein named TRIP-1 (Type II TGF- β receptor interacting protein-1) in primary lung fibroblasts induces three changes in fibroblasts behaviour such as increased α -SMA expression, collagen contraction ability and resistance to apoptosis. However, this effect cannot be abrogated by the blockage of Smad3 pathway which is the main signalling pathway implicated in TGF- β signalling (206). Additional intracellular signalling pathways such as Rho and Smad signalling have been implicated in myofibroblast differentiation in the pathogenesis of fibrosis in association with multiple extracellular stimuli (193,207). These findings again support the view that myofibroblast differentiation in lung fibrosis is associated with multiple signalling pathways and not limited to soluble factor like TGF β 1.

Regarding myofibroblast differentiation, there are the three possible origins of myofibroblasts (Figure 3.2) (96). The first most likely possibility is their differentiation from the resident lung fibroblasts population under certain inflammatory environments. The second suggestion is that a process known as the epithelial mesenchymal transition (EMT) in which alveolar airway epithelium (AEC) changes their morphology from cuboidal to spindle shape and lose their features such as E-cadherin and Zona occludens 1 expression and subsequently acquire mesenchymal phenotype such as α -SMA and fibroblastic specific protein-1 which are specific for myofibroblast and fibroblast respectively (208,209). The third alternative hypothesis has been recently proposed based on experimental animal models suggesting that bone marrow derived circulating fibrocytes can be recruited to lungs, where they can differentiate into myofibroblast and contribute to the fibrosis process (210).

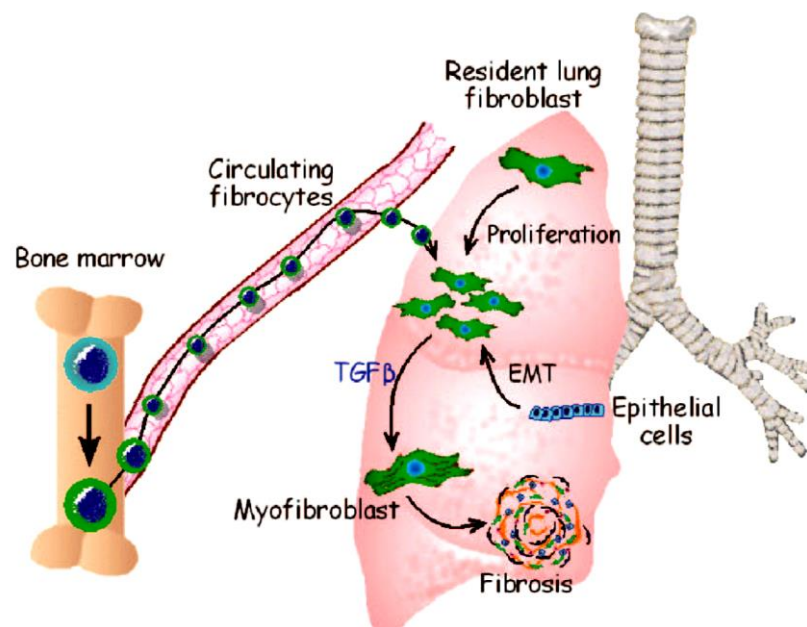


Figure 3.2 Potential Sources of Myofibroblasts in Lung [Strieter *et al* 2009, (61)]

Epithelial mesenchymal transition (EMT) is another hallmark of the fibrotic lungs associated with TGF- β 1 in both time and dose dependent manner. The presence of this phenomenon was proved *in vitro* in both primary and rat epithelial cell line in association with chronic exposure to TGF β 1 (98,208). In synergy with TGF- β 1, potent pro-inflammatory cytokines such as IL-1 β and TNF- α also enhance the TGF- β induced EMT features such as loss of epithelial markers like E-cadherin and cytokeratin, and increased expression of fibroblasts markers like α -SMA and vimentin (211). However, the association between the ECM stiffness and EMT changes in lung fibrosis are yet to be fully explored.

3.1.2 Biomimetic hydrogel scaffolds for investigating lung fibrosis

According to recent research focused on the role of matrix stiffening in lung fibrosis, it was obvious that biomechanical properties of ECM plays an important role in regulating fibroblast activation in response to inflammation and fibrosis. Being very rigid, a conventional tissue culture plate is 10^6 times higher than that of normal lung tissue (GPa vs. kPa range) (191). Studies aiming at the biology of fibrosis and inflammation using tissue culture plates cannot reflect the *in vivo* tissue microenvironment.

In this context, researchers have tested many *in vitro* culture systems with *in vivo* ECM features such as ECM rigidity and dimensionality which can allow the study of many pathologies under a biomimetic environment (212-215). Among many *in vitro* model systems, hydrogel cultures offer varying degrees of matrix biophysical and biochemical properties which allow culturing cells under 2D and 3D environments with spatial and temporal control (216-218). For those reasons, hydrogel cultures are now widely used as a versatile tool

for investigating fibroblasts behaviour under steady-state and pathological conditions (117,191,216,219,220).

With the advance of biomaterial engineering, a wide range of polymers are now used in hydrogel culture system for various applications in tissue engineering including stem cell engineering, drug testing and regenerative medicine (221-224). Basically, materials for hydrogel fabrication can be classified into two types, naturally derived or synthetic materials.

Naturally derived materials

Structurally, naturally derived polymers can provide more *in vivo*-like biochemical complexity of tissue. A good example of naturally derived materials is collagen, which is widely used in hydrogel culture systems for many applications. Owing to the reserved native binding site for integrins like the RGD (arginine-glycine-aspartic acid) sequence on collagen, cell attachment can be achieved within collagen hydrogel system (5,225,226). Collagen gel contraction assay is another useful application for testing fibroblast contractile function indirectly through hydrogel shrinking due to the exertion of contractile forces from cytoskeleton on the hydrogel (227,228).

Despite many advantages of using collagen as native ECM for cell culture, there are also some limitations with collagen hydrogel. The mechanical properties of collagen gels can be tuned within the limited range from the modulus ~ 0.0001 to 0.1 kPa (229). This range of modulus in collagen gels is too low to mimic the biomechanical effect of ECM in fibrotic state which is typically more than 15 kPa (88). Moreover, collagen based culture systems are not applicable in studies which require long term cell culture because they can

be easily remodelled by enzymes secreted from the fibroblasts such as MMPs (e.g. MMP-9 and MMP-2) (230). Being very soft nature and fluctuation of rigidity along the course of long term cell culture, the collagen hydrogel has assumed as an inappropriate model for studying fibrosis.

Hyaluronic acid (HA) is another widely used natural ECM protein in hydrogel synthesis (213,231-234). Although it has been modified with various functional groups like methacrylate and thiol groups to allow the formation of hydrogel with controllable modulus, unlike collagen gels, cell adhesions to HA is not guaranteed in many cell types. Some cell lines like dermal fibroblasts need additional surface coatings for attachment to HA hydrogel (235). In addition, care must be taken if we use HA hydrogel type in fibrotic studies because HA fragments (low molecular weight) have been shown to be associated with lung inflammation and it is assumed as one of the contributing factors in lung fibrosis progression (236). Therefore, given the potential contribution of HA fragments in fibrosis, it is not an ideal candidate for fabrication of hydrogel in such studies. As discussed in chapter 1, decellularized tissue are also attractive culture models for studying fibrosis because of conserved native ECM architecture with natural components (88). Some limitations such as tissue availability and labour intensive decellularization process still prevent the decellularized lung model from becoming widely used lung fibrosis model.

Synthetic Materials

Among many synthetic biomaterials, two materials namely polyacrylamide and poly (ethylene glycol) (PEG) are popular materials for hydrogel

fabrication and are widely in *in vitro* 2D cell culture (27,50,118,191,220,237). Such synthetic hydrogels can be formed from the commercially available monomer. By varying the concentration of chemical cross linker, synthetic hydrogels with a wide range of tuneable stiffness modulus can be fabricated. Being resistant to remodelling and enzymatic digestion by cells, they can be applied in long-term culture for fibrotic studies (191,193). However, they cannot inherently present natural biological cues for cell adhesion and require additional functionalisation of the inert surface by cell-adhesive ECM substrates such as collagen and fibronectin by means of bi-directional cross linkers like sulfoSANPAH (238). Because of low biocompatibility of synthetic biomaterials and high cytotoxicity of chemical cross-linkers, 3D cell encapsulation within the synthetic hydrogels is still challenging. To allow cell encapsulating within the PEG hydrogel, PEG must be conjugated with peptides like RGD and DGEA – protein sequences presented on the ECM for cell recognition and modified with methacrylate for the possibility of photo crosslinking (239). However, the synthesis of PEG hydrogel for 3D culture is more complex and cytotoxic due to free radicals and high UV exposure for crosslinking.

Gelatin (denatured form of collagen) is another well-known natural biomaterial used in the fabrication of hydrogels. During the denaturation process of collagen, covalent bonds which maintain collagen triple-helix structure are hydrolysed and release collagen α chain (40-90 kDa) called gelatin (5). Recently, a semisynthetic modified form of gelatin called gelatin methacrylate (GelMA) has been widely used in various types of cell culture for different applications (212,214,226,240,241). In GelMA synthesis, gelatin

is pre-modified by addition of methacrylic-anhydride groups for UV photo crosslinking. It has been shown that stiffness of GelMA hydrogel can be controlled across wide range of stiffness by varying degree of methacrylation, percentages of GelMA (wt/vol) and UV exposure time. Being a natural biomaterial like collagen, GelMA conserves the natural motifs of collagen like RGD sequence for cell adhesion and cell spreading without the need for additional functionalisation (214,226). Due to high biocompatibility for cell culture, encapsulation of cells within GelMA is also possible.

In contrast to polyacrylamide gels, the stiffness of GelMA hydrogels can be increased by increasing gelatin concentration, which mimics the fibrotic tissue in which tissue stiffening occurs due to increased deposition of collagen. Because of its photo-cross linkable nature, the stiffness modulus can increase to 30 kPa which allows simulating stiffness levels observed in lung fibrosis which usually displays stiffness more than 15 kPa and more stable culture system for long term (88,214). Overall, the properties of GelMA seem to be very relevant in fibrosis study and is superior to collagen.

Therefore, in this this chapter, to study the effects of biomechanical properties in fibrosis pathology, GelMA was selected as the material of choice for fabrication of fibrotic platform with tuneable stiffness levels relevant to normal lung and fibrotic lung. Using this platform, the impacts of biomechanical changes in ECM on cell behaviours including fibroblast proliferation, myofibroblasts differentiation and EMT.

3.2 Materials and methods

3.2.1 Development of micro-engineered hydrogel system with tuneable stiffness

Gelatin methacryloyl (GelMA) macromer as a white porous foam (kindly provided by Khademhosseini Lab, Harvard) was prepared as previously described (241). Basically there are three steps involved in the fabrication of GelMA hydrogel 1) 3-(Trimethoxysilyl) propyl methacrylate (TMSPMA) coating of glass slide (226), 2) Poly (2-hydroxyethyl methacrylate) (PolyHEMA) coating as previously described (226) and 3) fabrication of hydrogel with UV photo crosslinking.

TMSPMA treatment of glass slide (Appendix 2)

PolyHEMA coating of glass slide

TMSPMA treated glass slide was cut into 1 cm² glass chips. 4.5% (w/v) solution of poly-HEMA (Sigma-P3932, UK) was prepared in 100% ethanol by stirring at 50°C for 1 hr on heat plate. Then prepared glass chips were dipped in the warm poly-HEMA solution and then air dried in the fume hood for minimum 3 days (226). The coated glass chips were UV sterilised for 30 minutes before using in the experiment.

Photo crosslinking of GelMA on poly-HEMA coated slide

Briefly, different percentages of GelMA (5%, 10% and 15%) (wt/vol) were prepared by dissolving in 0.25% (wt/vol) of photoinitiator [Sigma-410896, UK] in PBS at 60°C. Briefly 150 µm height spacers were formed by 3 layers of scotch tape on the surface of petridish (Figure 3.3). 10 ul of different percentages of GelMA solution was placed in the middle of spacer and then

covered with a UV sterilised PolyHEMA coated glass slide. The whole construct was exposed to UV light for 9.5 seconds. 800 mW UV wavelengths was applied from 8cm distance from UV light source (Omicure). After UV photopolymerization, the polyHEMA coated slide was gently detached from the petridish surface and under the optimised conditions, the GelMA hydrogel was detached together with polyHEMA coated slide and then placed in 1 ml of the antibiotic/antimycotic solution (sigma) overnight for sterilisation before cell seeding. The mechanical properties of hydrogel were characterised according to established protocol (214).

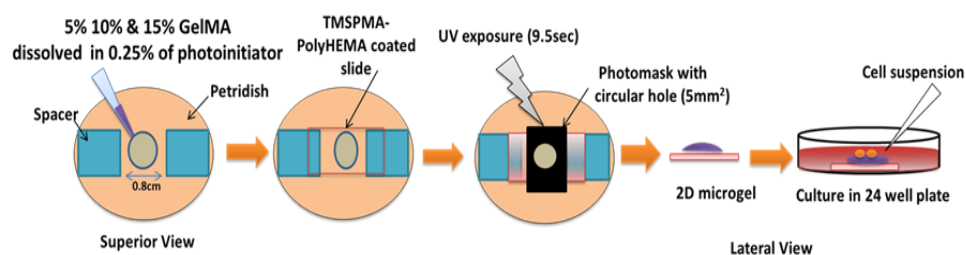


Figure 3.3 Schematic diagram for fabrication of GelMA hydrogel for cell culture.

3.2.2 Mechanical testing of hydrogel system

200ul of different percentages of GelMA were prepared using the same experimental parameters and left at 37 °C in DPBS for 24 hrs. Hydrogels were tested at a rate of 20% strain/min on an Instron 5542 mechanical tester (MIT, Boston). GelMA hydrogels made from prepolymer solutions of different concentrations were placed on flat holder for applying compressive force for measuring compressive modulus. Modulus were determined as the slope of the linear region corresponding with 0-5% strain. Three independent sets of experiments for each condition were performed in triplicate.

3.2.3 Cell Culture and Conditions

The human fetal lung fibroblast cell line (MRC-5) was cultured as described in Section 2.2.3. The airway epithelium cell line (A549) was obtained from the American Type Culture Collection (ATCC) and routinely cultured in T75 flasks in Dulbecco's minimal essential medium (DMEM) supplemented with 2 mM L-Glutamine, 10% FBS (Sigma) and penicillin-streptomycin (Sigma) (100 IU/mL/100 µg/mL) and HEPES buffer (10mM) at 37°C, 5% CO₂ in a humidified incubator until confluent. The culture medium was changed three times a week. When confluent, the cells were trypsinized with 1x trypsin-EDTA (Sigma), centrifuged at 350 *g* for 5 minutes at room temperature, seeded directly onto the prepared surface of GelMA with different percentages in a 24 well non-tissue culture treated plate (Falcon) and placed in a humidified incubator for 24 hrs to allow cell adherence on the gel and then transferred into new 24 well plate next day. Passage numbers 20 - 10 were used.

3.2.4 Alamar blue cell proliferation assay for detection of fibroblast proliferation in stiffness dependent manner

At different time points, proliferation of MRC5 fibroblasts cultured on the different percentages of hydrogels in 24 wells plate was determined by Alamar Blue cells proliferation assay as described in Section 2.2.6.

3.2.5 Immunostaining and confocal microscopy for α SMA expression in fibroblasts cultured on different percentages of GelMA hydrogel

Immunofluorescent staining and confocal microscopy were performed as described in our previous study (242). Briefly, the samples were fixed by using 4% paraformaldehyde for 20 minutes at RT and permeabilised by using 0.2%

100x Triton (Sigma) for 15 minutes at RT. For blocking nonspecific binding, samples were incubated 10% Goat serum for 30 minutes at RT and 5% bovine serum albumin for another 30 minutes at RT. Primary antibody - mouse α -SMA mAb, 1A4 (sigma) in 1:500 in PBS was incubated overnight at 4°C. Secondary antibody (1:500 goat anti mouse Alexa fluor 488 IgG_{2a}) was allowed to conjugate for an hour at RT. All immunofluorescent images under comparison were from the same experiment and also analysed together using same microscopy settings on the same day. Cell nuclei were stained with DAPI for 15 minutes.

Cells were then imaged with Zeiss LSM710 confocal with a Zeiss Observer microscope (SLIM imaging, University of Nottingham, UK) under 40x oil objective. Frame size of all images was 1024 x 1024 pixels, pinhole size of 1 AU and a line average of 8 with laser power of 2.

3.2.6 Western Blotting for α SMA protein expression in fibroblasts cultured on different percentages of GelMA

Before lysis, cells cultured on hydrogel were washed with cold PBS twice for 15 minutes on shaker at 4°C to remove protein absorbed in hydrogel and then lysed the cells using radio immune precipitation assay (RIPA) buffer with 1x protease and phosphatase inhibitor (Sigma) for 30 min on ice. Then protein lysates were centrifuged at 1300 rpm for 20 minutes at 4°C and collected the supernatant into new tubes. Then the protein concentration was measured by using detergent compatible (DC) protein assay (Bio-Rad) according to the manufacturer's instruction. The protein samples were stored at -80 °C until use.

Protein samples (8 µg) were prepared with 1x NuPAGE (Invitrogen) sample reducing agent which contains 50mM of dithiothreitol (DDT) and 1x NuPAGE LDS sample buffer (Invitrogen). Proteins were then denatured at 100°C for 5 minutes and separated by SDS-PAGE electrophoresis using mini PROTEAN electrophoresis system (Bio-Rad) for 30 minutes at 100V. The precast gel (4-20%) was used in electrophoresis. Following protein separation by electrophoresis, protein was transferred onto nitrocellulose membrane (GE healthcare) by wet transfer method by using 1x Tris Glycine buffer containing 20% methanol for 90 minutes at 90 V with a cooling pack inside the chamber.

Membranes were blocked with 5% BSA in TBS (tris buffer saline) with 0.1% Tween at RT for 1 hr and then incubated with primary antibodies overnight at 4°C. Anti-mouse αSMA (Sigma, A5228) (1:2500 dilution) (0.4 µg/ml) and anti-mouse-alpha tubulin (Abcam, 80779) (1:5000) dilution in 10% BSA in TBS with 0.1% Tween. Following primary antibody incubation, membrane was incubated with relevant HRP conjugated secondary antibody (1:25,000 for αSMA and 1:50,000 for alpha tubulin) for an hour at RT. The membrane was developed by chemiluminescent method by using Amersham ECL reagents from (GE healthcare). Protein blots were scanned and ImageJ was used to determine the mean density of protein signals. Alpha tubulin protein was used as a protein loading control.

3.2.7 RT-PCR for αSMA gene expression in fibroblasts cultured on different percentages of GelMA

Total RNA was harvested using pure Link™ RNA mini kit (Ambion) following Trizol extraction. First strand cDNA was generated using Superscript III (Invitrogen, Life Technologies) according to manufacturer's

instructions. qRT-PCR was performed on 20 ng total cDNA from independent samples, using reverse and forward primers specific for selected genes. Real time PCR was performed with Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent) by using Mx3000P QPCR system and data were also evaluated by comparative RT-PCR analysis of MxPro software (Mx3005P version). 18S rRNA was used as internal reference control to normalise the amount of the extracted RNA. Relative expression was normalised to 18sRNA reference gene. Primers were selected using Roche Universal Probes Library and purchased from Eurofins Scientific. Detail information of each primer was shown in Appendix 5. The nucleotide sequence of forward and reverse primers for α SMA and 18s rRNA are as follows;

α SMA forward 5'- GACCGAATGCAGAAGGAGAT-3'

α -SMA reverse 5'-CCACCGATCCAGACAGAGTA-3';

18S rRNA forward 5'-GTAACCCGTTGAACCCCATT-3'

18S rRNA reverse 5'-CCATCCAATCGGTAGTAGCG-3'

3.2.8 Statistical analysis

Statistical analysis was performed using Graph Prism 6. Data are expressed as means $\pm$ Standard deviation (SD) and analysed by one way ANOVA and two way ANOVA where appropriate with Bonferroni post hoc test for multiple comparisons. Significance level was set at $p < 0.05$.

3.3 Results

3.3.1 Mechanical Testing of hydrogel system

Mechanical properties of the extracellular matrix environment have shown to be important in lung fibrosis progression (192). GelMA is a well-established ECM based hydrogel system for different types of cell culture and its mechanical properties can be tuned easily (168,214,243). It is particularly relevant to studying lung fibrosis in which increased stiffness of ECM is mainly induced by collagen deposition. We first fabricated 5%, 10% and 15% GelMA hydrogels with 5mm² diameter and 100 μ m thickness. We then measured the mechanical properties of different percentage of GelMA hydrogels (Figure 3.4). As shown in Figure 3.4, different percentages of GelMA hydrogel showed varying degree of stiffness with 3.650 ± 0.1 kPa (5% GelMA), 15.15 ± 0.9 (10% GelMA) and 27.73 ± 0.8 kPa (15% GelMA). This range of stiffness in both compressive (Figure 3.4) and Young's modulus (Appendix 3) corresponds to the range of normal and fibrotic lung stiffness with 5% GelMA representing the normal lung stiffness while 10 and 15% being closer to fibrotic lung matrix (88).

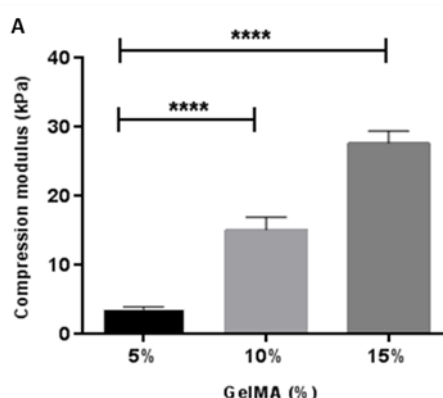


Figure 3.4 Mechanical properties of GelMA based hydrogel platform with different percentages of GelMA. The stiffness of hydrogel [5%, 10% and 15% (w/v)

GelMA] was measured in compressive modulus. Three independent sets of experiments for each condition were performed in triplicate. 10% and 15% (w/v) GelMA were significantly different (**** $p < 0.001$) compared to that of 5% (w/v) GelMA. (Mean $\pm$ SD)

3.3.2 Assessment of biocompatibility of GelMA for culturing lung fibroblasts

3.3.2.1 Assessment of cell adhesion on GelMA hydrogel

Adequate cell adhesion is essential for achieving the normal morphology and function of fibroblasts such as migration and proliferation. In order to test biocompatibility of the GelMA hydrogel as a cell culture system to study lung fibrosis, firstly we investigated fibroblast adhesion to GelMA hydrogel surface. MRC5 fibroblasts (3×10^4 /ml/well) were cultured on the 5mm^2 surface of GelMA with different stiffness for 7 days. Fibroblast adhesion and morphology were assessed by using cytoskeletal Phalloidin staining after 7 days of culture. As seen in Figure 3.5, lung fibroblasts adhere on the surface of 5%, 10% and 15% of GelMA with different stiffness levels. Despite the same number of fibroblasts adhered on the different percentages of GelMA on Day1, different morphology of fibroblasts was observed between different percentages of GelMA. Fibroblasts on 5% GelMA showed the multipolar spreading with network pattern whereas on 10% and 15% GelMA, different fibroblast polarisation pattern was observed. On higher percentages of GelMA, they aligned to each other with a bipolar pattern without any network appearance between the adjacent cells. In addition to different fibroblast polarisation pattern, another obvious finding between different stiffness levels was that the cell proliferation rate was significantly higher in higher percentage of GelMA (10 % and 15% GelMA) compared to

5% GelMA on day 7 of cell culture despite seeding the same cell density on the different percentages of GelMA.

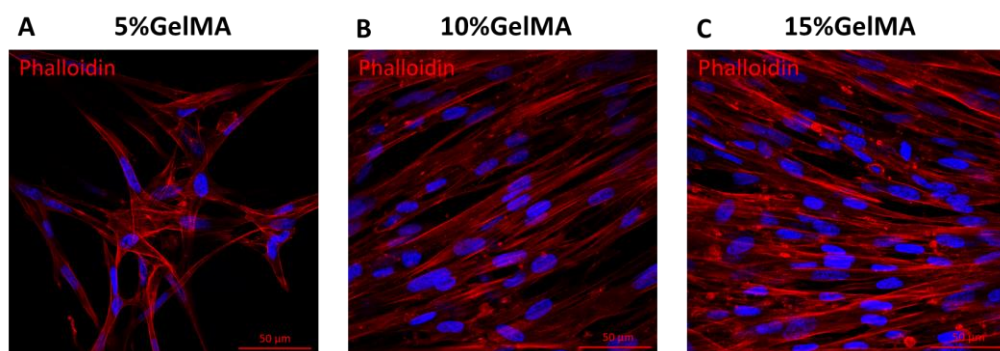


Figure 3.5 Assessment of fibroblast adhesion and morphology on the surface of GelMA hydrogel. Cytoskeleton images of lung fibroblasts stained with phalloidin were taken on day7 (A-C) after cultured on GelMA. Lung fibroblasts were cultured on different percentages of GelMA, in 5% GelMA (A), in 10% GelMA (B), in 15% GelMA (C). Fibroblasts were stained with Alexa Fluor 647 conjugated phalloidin (Red) and Nuclei were stained with DAPI (blue) (scale bar =50 μm, n=3)

3.3.2.2 Determination of ECM deposition from fibroblasts cultured on the hydrogel surface

To further assess GelMA compatibility for fibroblast culture, we also assessed the ECM deposition of fibroblasts, one of the main functions of fibroblasts *in vivo* and also important in wound healing and fibrosis. Since GelMA itself is a collagen derivative, we chose to investigate fibronectin deposition rather than collagen in order to avoid nonspecific detection. As shown in Figure 3.6, fibronectin deposition was observed across different percentages of GelMA over 7 Days of cell culture. We also included GelMA hydrogel without cells as a control to check nonspecific fibronectin staining on GelMA surface. According to Figure 3.6 lower panel, nonspecific fibronectin staining was not detected on GelMA control. However, absolute quantification of fibronectin staining was difficult to evaluate because of

different cell numbers after 7 days culture. Nevertheless, fibronectin deposition was clearly detected in all conditions of different stiffness levels.

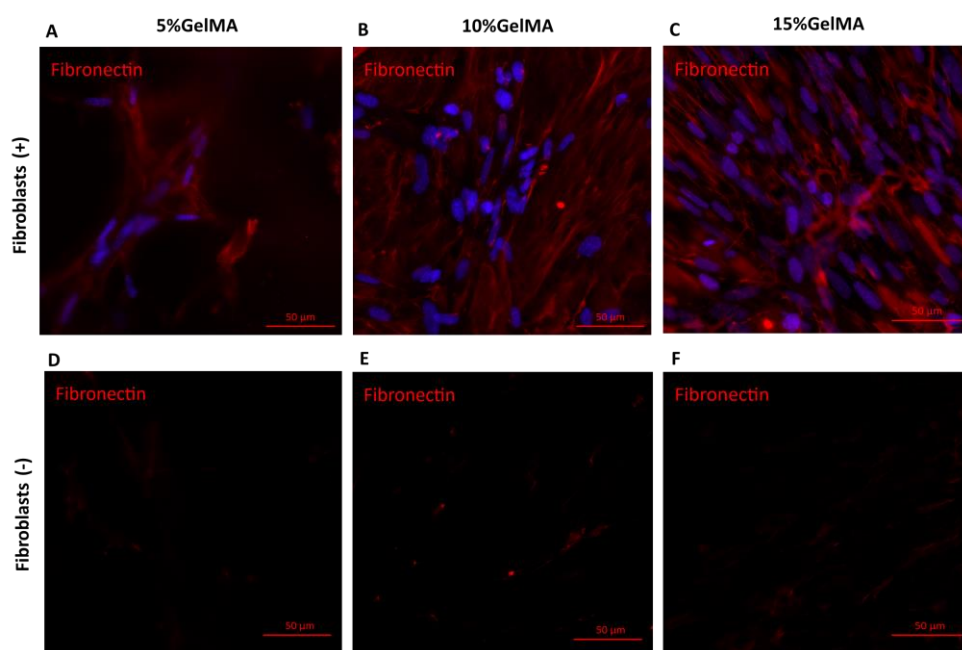


Figure 3.6 Assessment of Fibronectin deposition of MRC5 fibroblasts cultured on the different percentage of GelMA hydrogel (5%, 10% and 15%). The GelMA system with (A-5%, B-10%, C-15%) or without fibroblasts (D-5%, E-10%, F-15%) were stained with fibronectin -Alexa flour 647 (red colour) and nuclei were stained with DAPI (blue) (scale bar = 50 μ m & n=3).

3.3.3 Increased substrate stiffness promotes fibroblast proliferation

Using the GelMA hydrogels with different stiffness representing normal and fibrotic lung, we firstly investigated the fibroblasts' proliferative response to increasing stiffness of substrates. We studied the fibroblast proliferation over the duration of 5 days on GelMA hydrogel surfaces with different stiffness using the Alamar Blue assay. As shown in Figure 3.7- A, on a 5% GelMA surface, the fibroblast proliferation rate was significantly lower than that of the 10% and 15% GelMA surface on Day 3 and Day 5 even though all three surfaces have same initial cell seeding density (5×10^4 /ml/well). To better understand the potential synergy of matrix stiffness and serum in cell

proliferation, we further investigated the fibroblast proliferation on different stiffness levels with various serum concentrations (10%, 1%, 0.1%, 0%) in MRC5 cell culture media for 5 days. Interestingly, as shown in Figure 3.7-B, we achieved an increasing cell proliferation trend over 5 days on higher stiffness level (10% and 15% GelMA) only with 10% serum indicating that stiffness of substrate has major contribution in serum induced cell proliferation. On soft 5% matrix, the fibroblast proliferation decreased over time with all concentrations of serum. In other words, soluble factors in serum and stiffness of ECM or cell culture substrates contribute synergistically in fibroblast proliferative response.

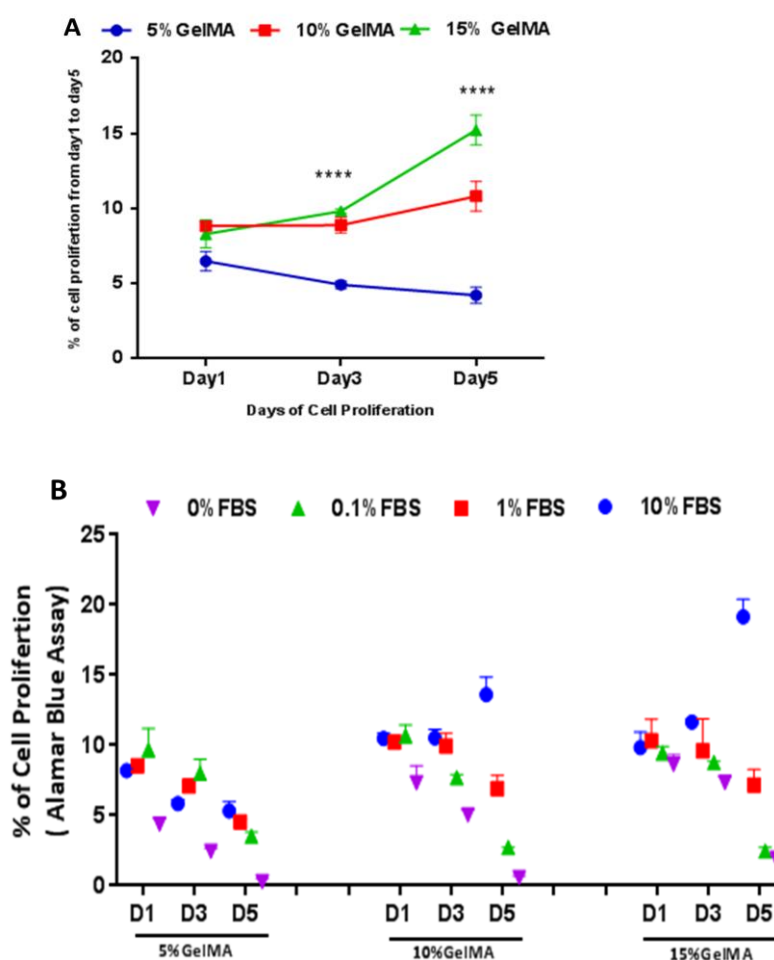


Figure 3.7 Increased substrate stiffness induces the fibroblast proliferation. (A) Percentages of fibroblast proliferation seeded on the surface of GelMA (5%, 10% and 15%) with different stiffness levels were compared between groups over 5 days of cultures (B) Stiffness induced cell proliferation was tested in different percentage of serum (0%, 0.1%, 1% and 10%) on different percentage of GelMA (5%, 10% and 15% GelMA) over 5 days. All cell proliferation rates in these experiments were assessed by the Alamar blue cell proliferation Assay at different time points, mean $\pm$ SD, (n=3). ****p<0.0001

3.3.4 Increased substrate stiffness promotes myofibroblast differentiation

As seen in Figure 3.8, there is a significant increase in α SMA mRNA and protein expression levels in lung fibroblasts with increasing substrate stiffness from normal lung (5% GelMA) to fibrotic lung (10% and 15% GelMA) values. In addition, confocal immunostaining also confirms the assembly of α SMA stress fibre in lung fibroblast cultured on higher stiffness hydrogels. Lung fibroblasts cultured on 10% GelMA hydrogels exhibit α SMA as short cortical stress fibres compared to the 15% GelMA where cells expressed long α SMA stress fibres across the whole cytoskeleton. Together these findings suggest that increased stiffness of substrate promotes the differentiation of human lung fibroblasts to myofibroblasts.

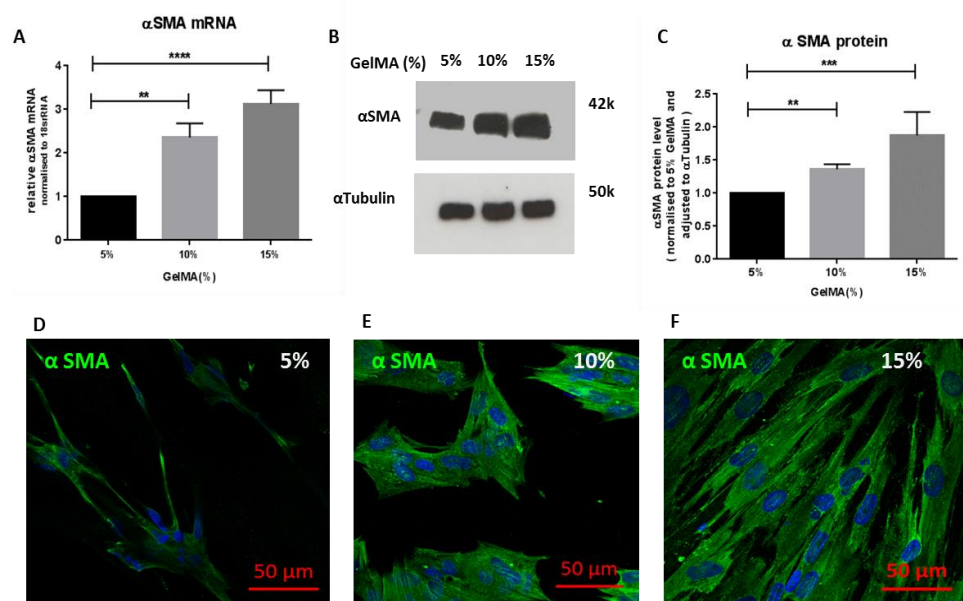


Figure 3.8 Increased substrate stiffness promotes αSMA gene and protein expression in lung fibroblast. (A) The level of αSMA mRNA level was examined in lung fibroblasts by the real time RCR after culturing on 5%, 10% and 15% of GelMA for 24 hrs. 18s rRNA was used as reference gene for loading control. The level on 5% GelMA was set at 1 for comparative analysis of RT-PCR. (B) The level of αSMA protein from lung fibroblasts cultured on 5%, 10% and 15% of GelMA for 3 days were determined by Western blot. α tubulin was used as protein loading control. Representative Western blot from 3 independent experiments are shown. Relative levels of αSMA protein expression were determined by densitometry of the blots from 3 independent experiments and normalised to α tubulin expression. (C,D,E) Confocal analysis of αSMA stress fibre formation by immunofluorescence staining at 3 days after gene silencing. Scale bar: 50μm, green= αSMA and blue= DAPI nuclei. Results are Mean ± SEM, n=3, **p<0.01, ***p<0.001, ****p<0.0001

3.3.5 Increased substrate stiffness promotes EMT in airway epithelium

In addition to myofibroblast differentiation, we also investigated the effect of increasing substrate stiffness on EMT, one of the key features of fibrosis in which epithelium loses their integrity and characteristics features and transform into fibroblast like cells. To study this, we used the A549 cell line which is widely used as a surrogate for type I alveolar epithelial cells. Firstly, we seeded 5×10^4 /ml of A549 cells on the GelMA microgel with relevant stiffness to normal lung and fibrotic lung. In this experiment, we detected increased expression of vimentin (fibroblast marker) and decreased expression of pan cytokeratin (epithelial marker) as markers for EMT transformation. As shown in the Figure 3.9, when we cultured A549 epithelium on GelMA hydrogel with different stiffness level for 5 days, the increased and differential filamentous expression pattern of vimentin was observed on 15% and 10% GelMA compared to that of 5% GelMA, which was more enhanced in the presence of TGF- β 1 (2ng/ml). However, we did not observe any significant changes in the pan cytokeratin expression with increasing stiffness of the substrate with or without TGF- β 1. Regarding epithelial cell morphology, on stiff matrix, most of the rounded epithelial cells became elongated adopting the fibroblast morphology, more obviously with or without TGF- β 1 supplement compared to soft matrix. On the soft matrix, we did not observe any morphology changes and vimentin expression with or without TGF- β 1 stimulation for 5 days. Together, our findings prove that substrate stiffness plays a major role in TGF- β 1 induced EMT process in fibrosis.

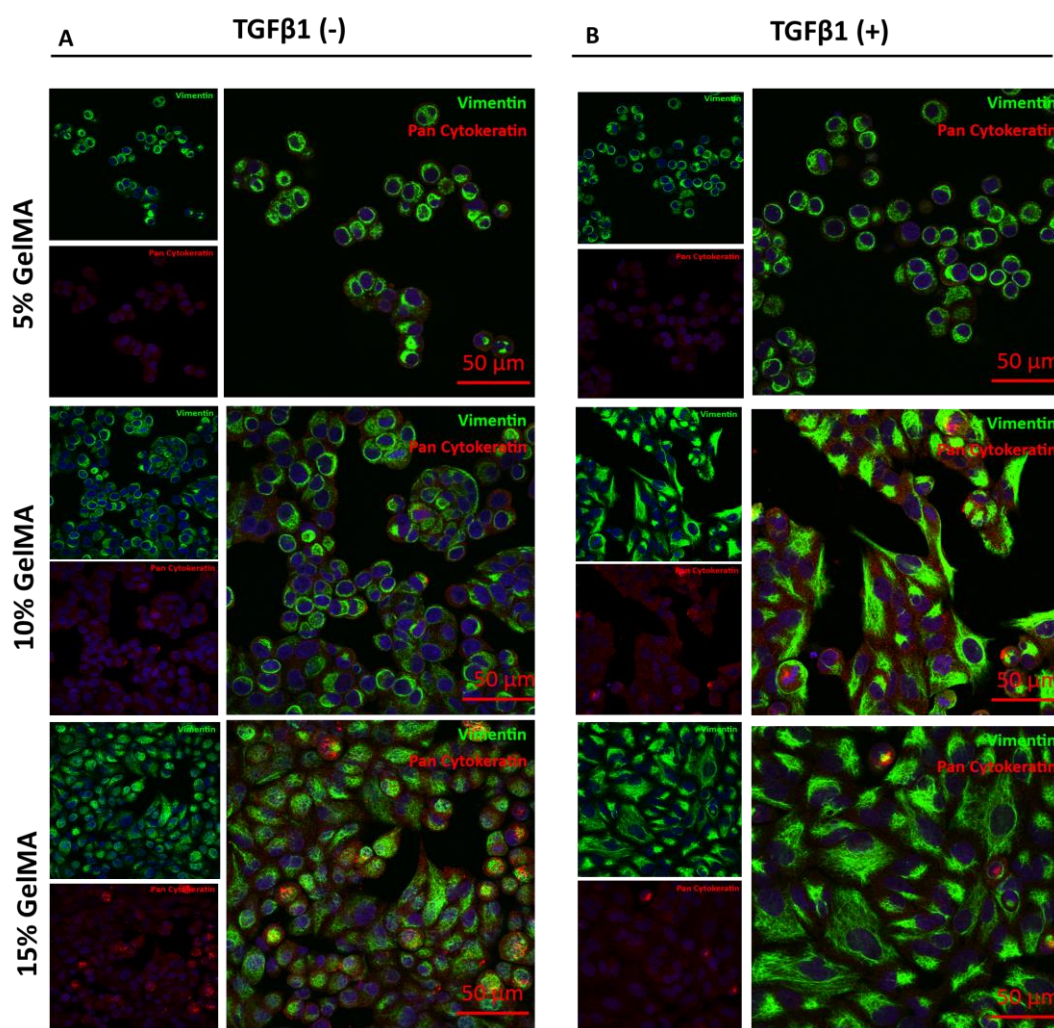


Figure 3.9 Increased substrate stiffness promotes TGFβ1 induced EMT process in A549 airway epithelium. A549 airway epithelium was seeded on the different percentages of GelMA for 5 days with (B) or without TGFβ1 (2ng/ml) (A). Confocal analysis of epithelium immuno-stained with vimentin- (Alexa Flour 488- green colour), pan cytokeratin –(Alexa Flour 647- red colour) and nuclei were counterstained with DAPI (blue) at 5 days after gene silencing. Scale bar: 50μm, n=3.

3.4 Discussion

In this chapter, we first optimised conditions for fabrication of GelMA hydrogels with different stiffness levels relevant to healthy and fibrotic lungs. According to our results, GelMA showed high biocompatibility with high cell viability, and could maintain the normal fibroblast functions including cell spreading and fibronectin deposition without the need for additional surface functionalisation. This further confirms that GelMA conserves the natural binding motifs such as RGD which are important for cell binding and spreading (117). Moreover, within the soft compliant matrix (approximately 2kPa), fibroblasts showed cell to cell interaction and assembled into network like structures whereas in the stiff microenvironment (>15kPa), bipolar shaped fibroblast are in parallel alignment. The impact of matrix stiffness on cell polarisation (morphology) has been reported in others cells such as epithelial cells (244) and endothelial cells (245). According to a number of studies reported recently, these different types of morphologies observed on soft and stiff matrix were found to be associated with changes in actin cytoskeletal organisation induced by changes in force generation within the cells when seeded on different rigidity of substrates (246,247).

Our data in this chapter confirm that stiffness of ECM have significant role in fibroblast activation such as increased proliferation and myofibroblasts transformation. Not limiting to fibroblasts, ECM stiffness driven cell proliferation effect has been consistently reported in the other systems of the cell types of body, including gliocytes (248), hepatocytes (49), and breast cells (52). Interestingly, stiffness induced fibroblasts proliferation process requires the high concentration of serum, which clearly indicates the

crosstalk/connection between cellular mechanosensing signalling and soluble factors signalling in the fibroblast proliferative response. This finding is also in line with the observation of Alam *et al* (2007) in which the adherent cells undergo apoptosis when cultured either in cell suspension or very soft gel even in the presence of very high concentration of growth factor (249). Therefore, it is reasonable to assume that cell proliferation we normally observe on conventional culture plate or flask is not only due to the presence of soluble factors in the supplemented serum in culture media but also driven by the extreme hardness of the tissue culture plate (~1GPa).

Although there are many signalling pathways related with cell proliferation, such as MAPK-ERK1/2, JNK pathways, P38, the signalling pathways that are specifically activated in stiffness induced proliferation in fibrosis remain unknown (250). Further studies are required to elucidate in this area for identifying new targets for the effective control of fibroblast proliferation in lung fibrosis. There are also few studies which explain possible underlying mechanisms of hyper-proliferative response on rigid matrix. One study has shown that ECM stiffness has an effect on the cyclin D1 expression, which might alter the speed of progression through the cell cycle G1 phase from G0 and G2/M phase during mitosis (19, 20). Additionally, impaired prostaglandin synthesis on rigid matrix was also shown to be related with the stiffness dependent cell proliferation. Increasing matrix stiffness was shown to suppresses the fibroblasts production of prostaglandin E₂ (PGE₂) which has antiproliferative effect together with reduction of expression of COX-2 (cyclooxygenase-2) which is required for PGE₂ synthesis (191).

Together with the observations presented from Haung *et al* (2012) using polyacrylamide hydrogel systems, our findings prove that the exposure to rigid matrix for longer duration (up to 5 days) can transform the fibroblasts to myofibroblast phenotype independent of TGF- β 1. They also identified Rho-ROCK (Rho associated coil coil kinase) signalling as an important intracellular mechanotransduction pathway in matrix rigidity sensing (146,191,193) in relating with the expression of α SMA. Such observations help better understanding of the intrinsic mechanisms involved in the regulation of matrix stiffness- induced myofibroblast differentiation.

In addition to the effects of ECM stiffness on fibroblast behaviour noted above, EMT phenomenon has been also linked with increased matrix stiffness in lung fibrosis. In contrast to stiffness induced fibroblast activation, both TGF- β 1 and stiffness of ECM have synergistic role in EMT process in lung fibrosis. Consistent with our results in human alveolar cell line -A549, stiffness induced EMT changes was also seen in a mouse epithelial cell line (194). All together, these results support the concept that changes in matrix stiffness play an active role in promoting fibroblast activation, EMT process and ongoing fibrosis (104,190).

To conclude, in this chapter we successfully developed a cell responsive ECM based hydrogel with tuneable stiffness that is relevant to normal and fibrotic lung by using GelMA. We confirm the significant role of matrix stiffening in the main pathologies of lung fibrosis such as fibroblast proliferation, myofibroblast differentiation and EMT. Further investigation to understand the mechanism underpinning stiffness driven cell proliferation and myofibroblast differentiation will be discussed in the following chapters.

Chapter 4

Investigating the role of ROCK signalling in stiffness induced myofibroblast differentiation in lung fibrosis

4 Chapter 4- Investigating the role of ROCK signalling in stiffness induced myofibroblast differentiation and cell proliferation

4.1 Introduction

4.1.1 ROCK Kinase - Structure and Function

ROCK (~160kDa) belongs to the family of serine/ threonine kinase proteins and is one of the major downstream effectors of small GTPase RhoA. Functionally RhoA- ROCK signalling controls cell adhesion and cell contraction via actin cytoskeleton reorganisation (251). Structurally ROCK has 2 domains- the carboxy (C) terminal RhoA binding domain and the amino (N) terminal kinase domain. There are two known isoforms of ROCK in humans, ROCK1 and ROCK2. These two isoforms have high amino acid sequence homology (65%) and identity (92%) in their kinase domains (252). Both isoforms are ubiquitously expressed in many tissues, but ROCK1 expression is more prominent in liver, spleen, lung, kidney and testis whereas ROCK2 expression is more abundant in heart and brain (253).

In its inactive state, ROCKs adopt the closed configuration with the two ends folded over each other. When activated, RhoA (GTP bound) binds to the RhoA binding domain at the C terminal end, followed by changing into the open configuration with exposing N terminal kinase domain (Figure 4.1). Through the N terminal kinase domain, ROCK mediates a series of downstream phosphorylation/dephosphorylation on multiple substrates (251).

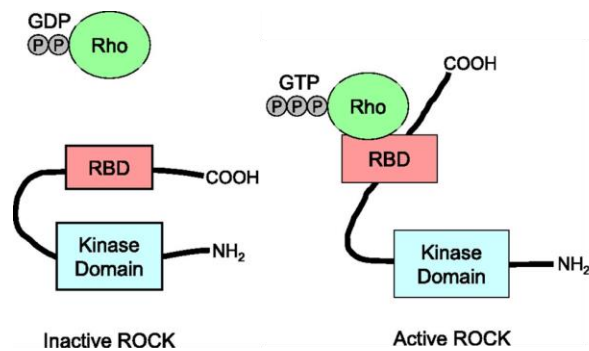


Figure 4.1. ROCK Structure in different activation status. In the closed inactive conformation, RhoA binding domains (RBDs) and the kinase domains of ROCK fold over each other (left). Activated GTP bound RhoA is able to bind the RBDs, shifting the ROCKs to an open active conformation (right) exposing and activating kinase domains. [Knipe et al 2014, (265)]

In particular, phosphorylation of the myosin binding subunit of myosin light chain phosphatase (MLCP) by ROCKs inhibits the dephosphorylation of myosin light chain (MLC). Persistent phosphorylated MLC mediates the polymerisation of the G actin (globular actin) into F actin (filamentous actin) and activates actomyosin contractile machinery of cell contraction with increased myosin II activity. Changes in actin polymerisation directs different physiological states of cells, such as cell adhesion, cell migration, cell contraction and cell apoptosis (252,254,255).

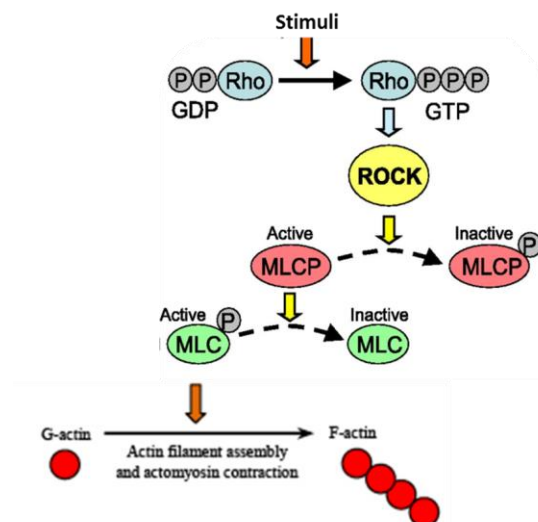
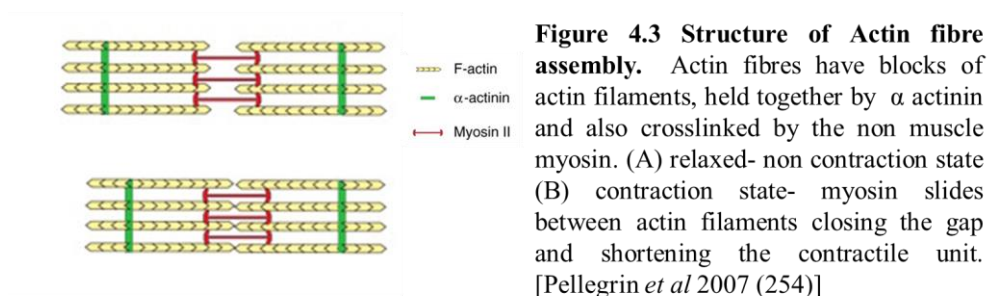


Figure 4.2 ROCK control of cytoskeletal organization. ROCKs are activated upon binding of GTP bound RhoA to kinase binding domain of ROCK. Activated ROCKs phosphorylate MLC phosphatase, inhibiting its ability to dephosphorylate MLC. Phosphorylated active MLC mediates the actin fibre assembly and cell contraction. [Knipe et al, 2014, (265)]

4.1.2 Role of ROCK signalling in regulation of myofibroblast differentiation in lung fibrosis

During cell contraction, stress fibres are the major contractile components of many cell types including fibroblasts, smooth muscles, and endothelial cells (254,256). They are usually composed of bundles of ~10 to 30 actin filaments, held together by the α actinin and cross linked by myosin II bundles which comprises two heavy and light chains (257). According to the structural arrangement of actomyosin bundles where bipolar bundles of myosin crosslink the bipolar arrays of actin bundles, the motor activity of myosin II bundles results in shortening of actomyosin bundles. The activation of myosin II that allows the interaction with actin bundles is controlled by the myosin light chain (MLC) phosphorylation (254,258).



In fibroblasts and smooth muscle cells, there are two kinase systems implicated in the process of MLC phosphorylation for assembling of stress fibre for cell contraction: 1) Calcium dependent myosin light chain (MLC) kinase (MLCK) and 2) Rho kinase mechanisms. In the calcium dependent mechanism, rapid increase in intracellular calcium increase the levels of MLC phosphorylation resulting in rapid and short-lived contraction type. However,

in the Rho kinase system, in response to specific stimuli, RhoA and its downstream ROCK kinase become activated and increase the MLC phosphorylation by means of two mechanisms: A) direct phosphorylation of MLC or B) by the inactivation of myosin phosphatase by phosphorylation. Through these two mechanisms, phosphorylated MLC can produce the prolonged and sustained muscle contraction type (Figure 4.2) (198).

In lung fibrosis, myofibroblasts are the most prominent cell type found in fibrotic foci. In the normal wound healing process, myofibroblasts contract the wound and undergo apoptosis after the healing process whereas in fibrosis, myofibroblasts become resistant to apoptosis and produce excessive amounts of extracellular matrix (ECM) proteins like collagen. Functionally, myofibroblasts share the contractile features of smooth muscle with the similar expression of smooth muscle actin isoform (α -SMA) which is normally absent in fibroblasts (61,96). However, α SMA expression seems to be regulated differently in smooth muscle and myofibroblast. The CArG response element in α SMA promoter is required for α SMA expression in smooth muscle but is not involved in α SMA expression in myofibroblast (259).

Although myofibroblasts exhibit contractile features like smooth muscle, a number of studies showed that the two cell types use different types of cell contraction mechanisms. In smooth muscle, the calcium dependent mechanism plays a more prominent role than Rho kinase system. This allows a rapid contraction response with sharp rise in intracellular calcium concentration. However, in myofibroblasts increased intracellular calcium concentration cannot induce contraction of these cells (260). Instead, in myofibroblasts, where sustained contraction is required for wound

contraction, Rho-ROCK kinase activation maintains the state of phosphorylated MLC for sustained contraction by inhibiting myosin phosphatase in a phosphorylated form (251,258,261).

Myofibroblasts which express high level of α SMA protein have been demonstrated to generate more force and exert a stronger traction in collagen contraction assays compared with fibroblasts which express only normal actin stress fibres (198,205,262). Interestingly, another study has shown that the expressed α SMA protein in myofibroblast became incorporated into the existing stress fibres and stress fibres containing α SMA generate more contractile force than normal stress fibres alone (196,263).

Recently Rho associated coiled-coil forming kinase (ROCK) has shown to be involved in stiffness driven myofibroblast differentiation in lung fibrosis (discussed in chapter 3) as an intrinsic mechanotransduction mechanism (146,264-266). Pharmacological ROCK inhibitors such as Y27632 or Fasudil (267,268) were shown to be efficient in preventing myofibroblast differentiation in both *in vitro* (193) and *in vivo* fibrosis models (146). In response to matrix stiffness, fibroblast- myofibroblast differentiation was mediated by activation of GTP bound RhoA which in turn activates the downstream ROCK kinase. Through the ROCK-MLC phosphorylation pathway, the G actin polymerises into F actin, initiating the assembly of the actomyosin stress fibre assembly. Under normal conditions, fibroblasts *in vivo* express very few or no stress fibres. They assemble stress fibres only when they encounter a mechanical stress (257). Therefore, it is not surprising that fibroblasts cultured on the extreme rigid support such as glass or plastic

substrates induce very thick stress fibres which is opposite to the cells cultured on soft matrix where very few thin stress fibres can be detected (254).

Myocardin related transcription factor A (MRTF-A) (also known as megakaryoblastic leukaemia factor) binds G actin as a transcriptional inactive form and sequesters in the cytoplasm. During ROCK mediated F actin fibre assembly, MRTF-A releases from G actin, translocates into the nucleus and coactivates the serum response factor element. Nuclear translocation of MRTF-A has shown to be involved in the activation and expression of fibrotic genes including α SMA and fibronectin (50,193,269) (Figure 4.4). This clearly exhibits ROCK's role in sensing matrix rigidity through MRTFA-SRF (serum response factor) dependent pathway in fibrosis.

In addition to MRTF-A-SRF pathway, similar ROCK mediated effect was recently reported in the regulation of the stiffness induced activation of cancer associated fibroblasts (CAF) in the tumour environment through the downstream transcriptional coactivators called YAP and TAZ. In CAF, it was shown that matrix stiffening enhances nuclear localisation of YAP and TAZ transcription factor through ROCK activation pathway (270). Therefore, it is expected that matrix stiffening during the lung fibrosis progression would have association with ROCK dependent YAP activation in lung fibroblasts (271,272).

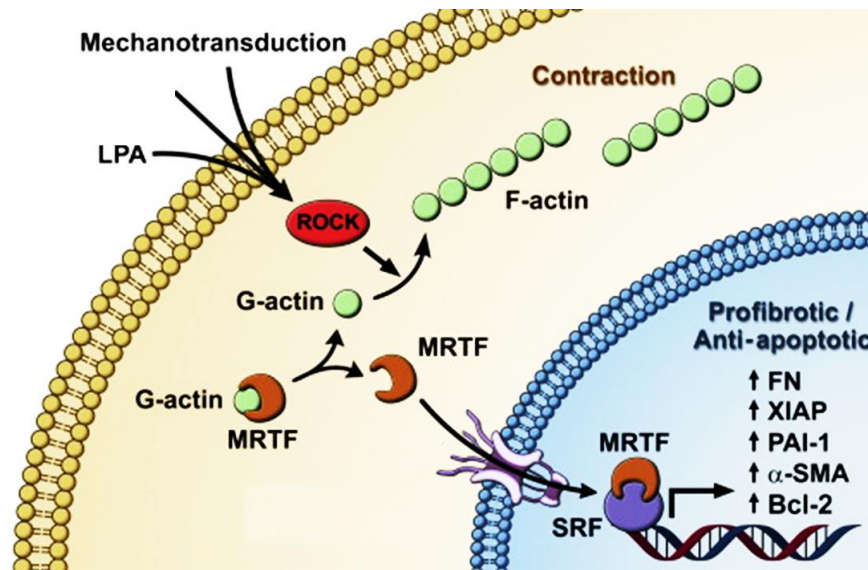


Figure 4.4 Rho- ROCK MRTFA signaling pathway in mechanotransduction.

The initial signaling of ROCK activation result in polymerization of F actin contractile fibres. Polymerization of F actin fibres release the MRTFA which normally binds with G actin in cytoplasm. The free MRTFA translocate into the nucleus initiating the transcription of the several profibrotic and antiapoptotic genes. [Crider et al 2010, (269)]

4.1.3 Two isoforms: ROCK 1 vs ROCK2

Given the high degree of homology within the kinase domain and the downstream pathway redundancy between two ROCK isoforms, it is not possible to determine the role of each isoform in stiffness-induced myofibroblast differentiation through using pharmacological inhibitors only (273). Although ROCK inhibitors have shown promising effect in controlling fibrosis, their off-target effect when used in high dose and non-selectivity against other kinases like protein kinase C related kinase (PRK) at the same concentration necessary for ROCK inhibition, limits their efficacy (268).

Advances in gene silencing using short interfering RNA (SiRNA) as well as progress made in generating knockout animal models, have the potential to

provide additional insight into selective ROCK isoform activity in different cellular functions. A growing body of evidence indicates the importance of ROCK isoforms in different pathologies (253). Several reports showed that ROCK isoforms have distinct contributions in many cancers. Increased ROCK2 has been reported in colon (274), bladder (275) and hepatocellular (276) cancers without affecting the ROCK 1 level. In contrast, ROCK1 but not ROCK2 was highly expressed in mammary (277) and prostate tumours (278). In vascular smooth muscle, ROCK 1 and 2 isoforms have different effect on the vascular smooth muscle morphology and the contractility of smooth muscles was mainly mediated by ROCK2 but not ROCK1(252,279).

Despite the discovery of different functions of ROCK isoforms in many tissues and pathological conditions, their respective roles in myofibroblast differentiation in lung fibrosis is yet to be elucidated. We believe that the detailed understanding of how each of the ROCK isoforms are involved in myofibroblast differentiation in lung fibrosis pathophysiology could pave the way for the development of more efficient therapeutic approaches to control lung fibrosis in an isoform specific manner (265,273).

It is certain that the surfaces of conventional tissue culture plates and glass slides are inappropriate to use in the study of cellular mechanobiology as their stiffness falls into the range of Gigapascals. To this end, to study mechanosensing signalling by fibroblasts, we developed microengineered ECM based scaffolds (discussed in chapter 2) with tuneable stiffness allowing to simulate stiffness of normal and fibrotic lung. Using this disease relevant platform, the role of each ROCK isoform in stiffness induced myofibroblast

differentiation in pulmonary fibrosis was investigated by SiRNA gene silencing methods in this chapter. In addition to myofibroblast differentiation, we also elucidated the role of ROCK regulation of stiffness induced fibroblast proliferation which is one of the major features in stiffness induced lung fibrosis pathology.

4.2 Materials and methods

4.2.1 Preparation of different percentages of GelMA hydrogels

We have developed the ECM based hydrogel platform with relevant stiffness corresponds to normal and fibrotic lung for testing stiffness induced myofibroblast differentiation and proliferation in lung fibrosis (Section 3.2.1). We applied the same hydrogel platform for testing ROCK isoform effect on stiffness induced myofibroblast differentiation.

4.2.2 Si RNA gene silencing of ROCK isoforms in lung fibroblasts on soft and stiff matrix

Human lung fibroblast cell line (MRC-5) obtained from ATCC, was routinely cultured as previously described (280) (referred to Section 2.2.4). Both SiRNA targeting human ROCK1 and ROCK2 (SiGENOME SMART pool) and non-targeting SiRNA control were purchased from Dharmacon (GE Healthcare) (Appendix 7). Cells were transfected by DharmaFECT1 transfection agent (GE healthcare) according to manufacturer's instruction. Lung fibroblasts (5×10^4) were seeded on the 5% and 15% GelMA hydrogel in non-tissue cultured treated 24 well plate overnight and changed into new plate before transfection. The efficiency and specificity of gene knock down was checked by RT-PCR and western blotting. Effective gene knock down was achieved at 50 nM of SiRNA of ROCK1 and ROCK2 in 1:625 dilution of transfection agent after 48-hrs transfection.

4.2.3 RT-PCR analysis for detection of expression of ROCK isoforms levels in lung fibroblasts after SiRNA silencing on soft and stiff matrices

ROCK1 and ROCK2 isoforms were knocked down on 5% GelMA and 15% GelMA individually and in combination by SiRNA gene silencing as described above. The level of ROCK1 and ROCK2 isoform mRNA levels were examined in lung fibroblasts by RT RCR at 2 days after transfection to assess specificity and efficiency of gene knockdown. 18s rRNA was used as reference control. The level of mRNA in sample transfected with non-targeted SiRNA was set at 1 for comparative analysis of RT-PCR.

Details methods used in RT PCR analysis for α SMA expression in this chapter was done as described in Section 3.2.7. Briefly Total RNA was harvested using pure LinkTM RNA mini kit (Ambion) following Trizol extraction. 100ng cDNA was synthesized using Superscript III first Strand Synthesis SuperMix (Invitrogen) according to manufacturer's protocol. Real time PCR was performed with Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent) and analysed with Agilent Mx3000P QPCR system.

Specific PCR primers contained the following sequences:

Human 18S rRNA forward	5'-GTAACCCGTTGAACCCCAT-3'
Human 18S rRNA reverse	5'-CCATCCAATCGGTAGTAGCG-3'
Human ROCK1 forward	5'-GATCCCAAATCGGAAGTGAA-3'
Human ROCK1 reverse	5'-CAAATCATATACCAAAGCATCCAA-3'
Human ROCK2 forward	5'-CCCATCAACGTGGAGAGC-3'
Human ROCK2 reverse	5'-AAGCAGGAAAATCTAAATCAAGGA-3'

18S rRNA was used as internal reference control to normalise the amount of the extracted RNA. RNA samples were analysed by using Mx3000P QPCR system and data were also evaluated by comparative RT-PCR analysis of MxPro software (Mx3005P version). Details information for each primer were described in Appendix 5.

4.2.4 Western Blot analysis for detection of ROCK1 and ROCK2 protein level in lung fibroblasts after gene silencing on soft and stiff matrices

The ROCK1 and ROCK2 protein levels were also confirmed by western blotting at 4 days after transfection. Details methods for Western blotting were described in Section 3.2.6. Anti ROCK1 (C8F7) and ROCK2 (D1B1) Rabbit monoclonal was purchased from Cell Signalling, UK. According to the manufacturer's (Cell Signalling) protocol, membranes were incubated with primary antibodies (ROCK1 and ROCK2) diluted in 1:1000 of 5% milk powder dissolved in TBS with 0.1% Tween for overnight at 4 °C. Then membranes were blocked subsequently with 5% milk dissolved in TBS with 0.1% Tween at RT for 1 hr followed by HRP conjugated secondary mouse anti rabbit antibody (Cell signalling) diluted in 1:500 dilution of 5% BSA dissolved in 0.1% Tween at RT for 1 hr. Blots were developed using ECL Prime chemiluminescence detection system (Amersham Biosciences). Protein blots were scanned and ImageJ was used to determine the mean density of protein signals. Alpha tubulin protein was used as a protein loading control.

4.2.5 Immunostaining, confocal microscopy and image Analysis for α SMA & F-actin fibre assembly and MRTF-A nuclear localisation in ROCK1/ROCK2 knockout fibroblasts on soft and stiff matrices

For this experiment, lung fibroblasts are cultured on for 4days after transfection for gene silencing of ROCK isoforms. Immunofluorescent staining and confocal microscopy were performed as described in Section 3.2.5.

All immunofluorescent images comparing 5% and 15% GelMA conditions are from the same experiment and analysed together using same microscopy settings on the same day. Primary antibody mouse α -SMA mAb, (1A4, Sigma) in 1:500 of PBS was incubated overnight at 4°C. Secondary antibody 1:500 of goat anti mouse Alexa fluor 488 IgG_{2a} in PBS was allowed to conjugate for an hr at RT. For phalloidin cytoskeletal staining, conjugated Alexa Fluor 647 Phalloidin (Invitrogen) was used (1:100 in PBS) for 1 hr incubation together with secondary antibody. Cell nuclei were stained with DAPI for 15 minutes.

Best signal option (sequential scanning) was chosen for multichannel laser scanning in confocal microscopy because only one detector and one laser are switched on at any one time, which effectively prevent overlapping between channels.

For detection of MRTF-A nuclear localisation, rabbit MRTF-A polyclonal (H140, Santa Cruz) in 1:50 dilution of PBS was incubated overnight at 4°C. Secondary antibody mouse anti rabbit Alexa Fluor 488 IgG (H+L) diluted in 1:200 of PBS was allowed to conjugate for an hour at RT after blocking with 10% goat serum (Sigma) for 30 minutes. Then the samples were counterstained with DAPI nuclei staining for 15 minutes to localise the

position of nucleus. For mean fluorescent intensity (MFI) measurement of MRTF-A, firstly overlaid images were separated into two channels (FITC-green and DAPI-blue) in ImageJ software (1.49v) and MFI measurement in nucleus and cytoplasm was performed only in green channel over an area of equal size whereas blue channel was used to localise the nuclear position. There was also no detectable background signal in control sample which has only secondary antibody and DAPI staining and background signal was also counterchecked by image J analysis. In order to identify nuclear translocation of MRTF-A, the MFI of MRTF-A staining in nucleus was compared to that of the cytosol of fibroblasts. The measurements were evaluated in random total 60 numbers of fibroblasts from images taken from three independent experiments for each condition and data was expressed mean $\pm$ SD. Significant increment of MFI in nucleus over cytoplasm was defined as MRTF-A nuclear localisation whereas no significant changes in MFI of MRTF-A in nucleus over cytoplasm was defined as no nuclear localisation of MRTF-A.

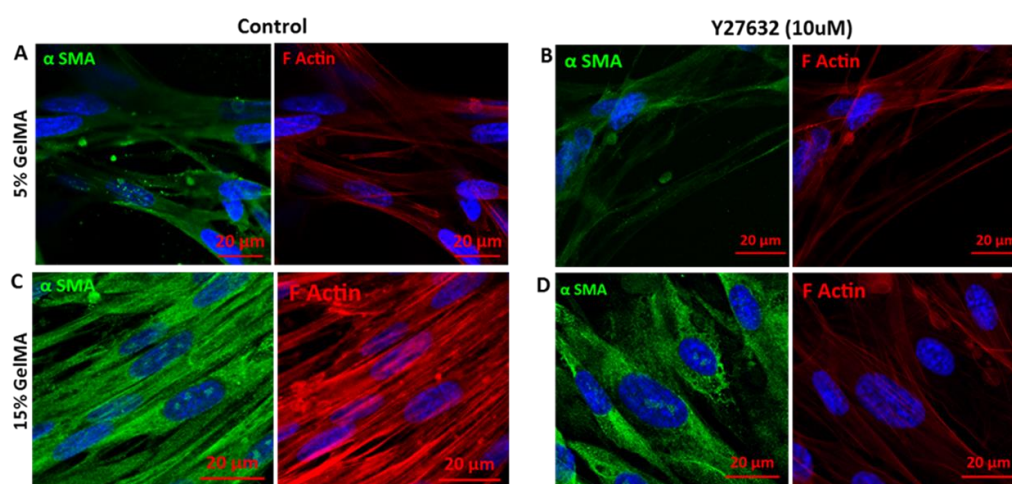
4.2.6 Statistical Analysis

Statistical analysis was performed using Graph Prism 6. Data are expressed as means $\pm$ Standard deviation (SD) and analysed by one way ANOVA and two way ANOVA where appropriate with Bonferroni post hoc test for multiple comparisons. Significance level was set at $p < 0.05$.

4.3 Results

4.3.1 Inhibition of ROCK signalling by Y27632 attenuates stiffness induced α SMA expression and fibre assembly in myofibroblast

Y27632 is one of the commonly used pharmacological ROCK inhibitors. To investigate the role of ROCK activation in stiffness induced myofibroblast transformation, lung fibroblasts cultured on 5% and 15% GelMA were treated with Y27632 (10 μ M) for 24 hr. On the stiff matrix (15% GelMA), Y27632 treatment caused a significant reduction in α SMA protein level (Figure 4.5 E) and a complete loss of α SMA fibre assembly compared to untreated controls (Figure 4.5 C and D). In addition, Y27632 treatment decreased the filamentous actin (F actin) assembly induced by stiff matrix. However, on the soft matrix (5% GelMA), we did not observe any significance changes in α SMA fibre assembly and α SMA protein level (Figure 4.5 E) and F actin assembly with or without Y27632 treatment (Figure 4.5 A and B).



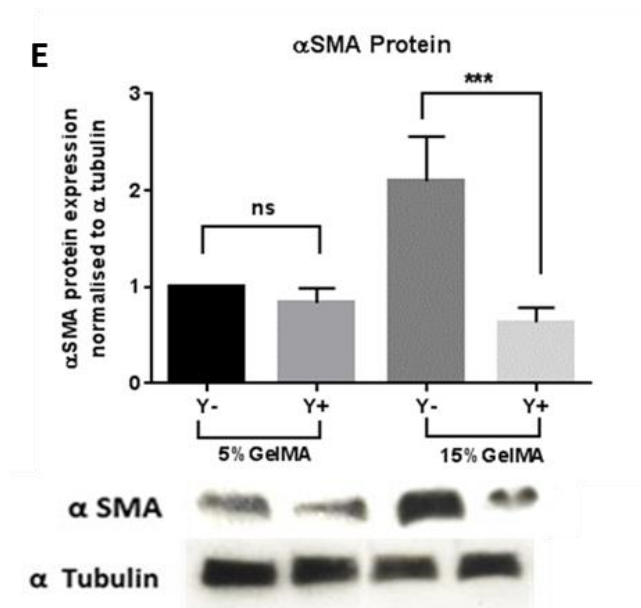


Figure 4.5 Y27632 inhibits stiffness induced myofibroblast differentiation. Lung fibroblasts are cultured on 5% (A, B) and 15% GelMA (C,D). The cells were treated with or without Y27632 (10 μ M) for 24hrs at 2 days after cells was cultured on different percentages of GelMA. The cells were stained for α SMA (Alexa Flour 488 - green) and F actin (Alexa Flour 647 red and examined by confocal microscopy. Nuclei were stained with DAPI. Scale bar =20 μ m. (E) The level of α SMA protein with or without treatment with Y27632 (10 μ M) on both 5% and 15% GelMA for 24hr were determined by Western blot. α tubulin was used as protein loading control. Relative levels of α SMA protein expression were determined by densitometry of the blots from 3 independent experiments and normalised to α tubulin protein. Results are Mean $\pm$ SEM, n=3, ***p<0.001, ns=not significant

4.3.2 Differential role of ROCK isoforms in α SMA expression and stress fibre assembly in fibroblasts grown on soft and stiff matrices

To further establish whether ROCK1 and ROCK2 have distinct function in regulation of stiffness induced α SMA expression, SiRNA gene silencing was used to knock down ROCK1 and ROCK2 isoforms specifically. As shown in Figure 4.6, ROCK1 and ROCK2 isoforms could be suppressed individually with high specificity without affecting the other isoform confirmed by RT-PCR and Western Blotting.

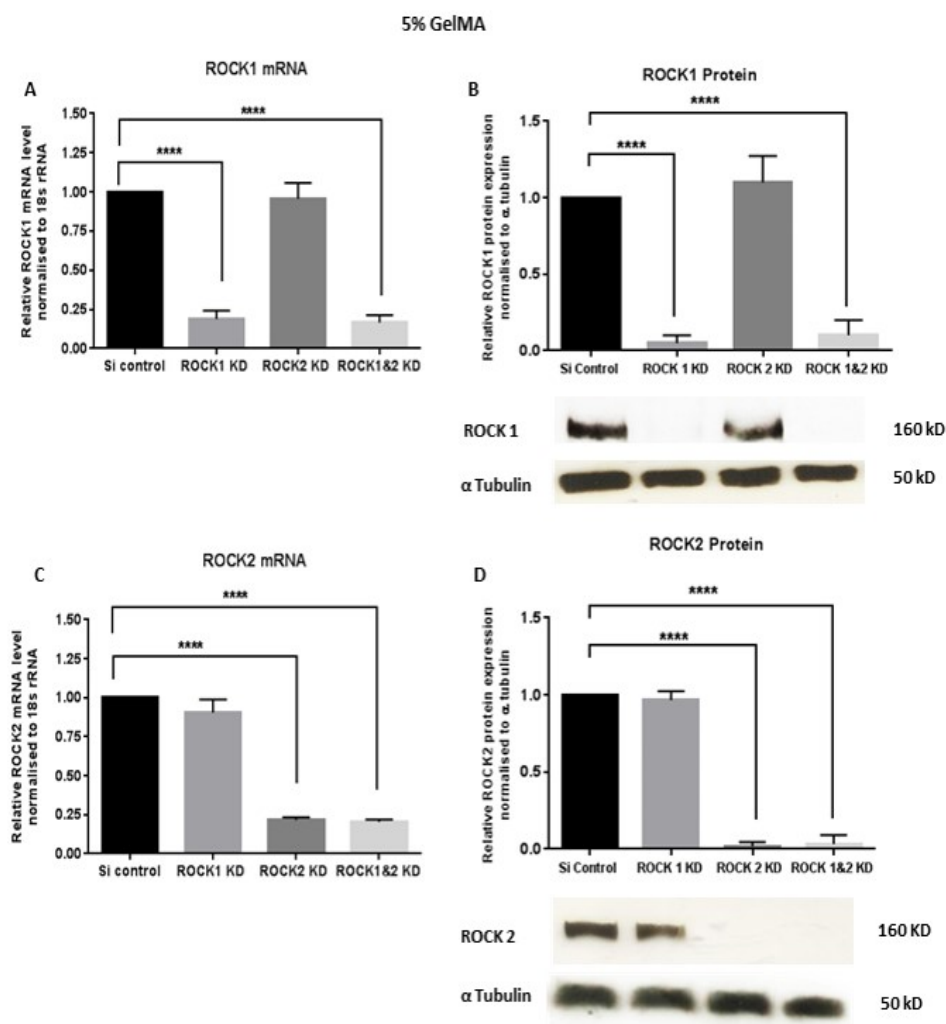


Figure 4.6 Specificity and efficiency of ROCK isoforms gene silencing in lung fibroblasts on 5% and 15% GelMA. ROCK1 and ROCK2 isoforms was knocked down on 5% GelMA (**A,B,C,D**) and 15% GelMA (**E,F,G,H**) individually and in combination by SiRNA gene silencing. (**A,C,E,G**) The level of ROCK1 and ROCK2 isoform mRNA level were examined in lung fibroblasts by RT RCR at 2 days after transfection to assess specificity and efficiency of gene knockdown. 18s rRNA was used as reference control. The level of mRNA in sample transfected with non-targeted SiRNA was set at 1 for comparative analysis of RT-PCR. (**B,D,F,H**) The ROCK1 and ROCK2 protein level was also checked by western blotting at 4 days after transfection. α Tubulin was used as protein loading control in WB analysis (n=3). Relative levels of α SMA protein expression of 4 days after gene silencing were determined by densitometry (n=3) normalised to α Tubulin. ****p<0.0001, Results are Mean $\pm$ SEM, n=3.

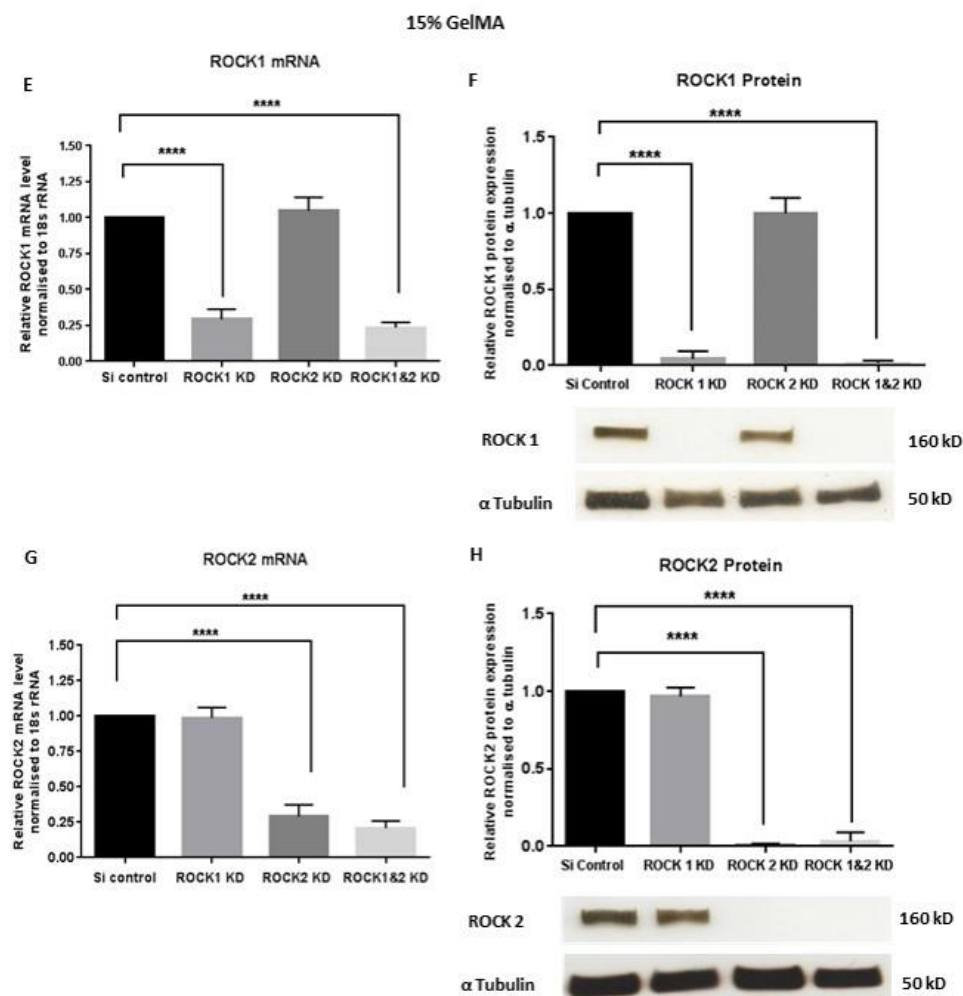


Figure 4.6 continued

We then analysed α SMA expression at gene and protein levels on lung fibroblasts cultured on 5% (soft) and 15% (stiff) matrices after ROCK1 and ROCK2 knockdown (Figure 4.7). RT-PCR analysis showed that silencing of ROCK isoforms individually or both isoforms simultaneously in fibroblasts cultured on soft matrix reduced α SMA mRNA expression by approximately 40% and 60% respectively without a significant change in α SMA protein level. However, paradoxically silencing individual ROCK isoforms, particularly ROCK 2, in fibroblasts cultured on stiff substrate led to a significant increase in α SMA mRNA expression whereas silencing both isoforms returned α SMA mRNA expression back to control levels. Western

blot analysis confirmed a similar pattern for protein expression in lung fibroblasts up to 4 days after transfection on stiff matrix. These data were in line with confocal microscopy showing the formation of thick α SMA stress fibre assembly in ROCK1 or ROCK2 deficient fibroblasts, on stiff matrix compared to ROCK1 or ROCK 2 deficient cells cultured on soft matrix where we did not observe the change in the α SMA fibre assembly compared to control ROCK expressed cells.

Importantly the knockdown of both ROCK isoforms simultaneously was associated with complete inhibition of α SMA stress fibre assembly in cells cultured on stiff matrix (Figure 4.7 F) indicating the primary function of ROCK isoforms in the α SMA fibre assembly rather than synthesis and breakdown of α SMA protein. In order to examine whether these findings are reproducible on another hydrogel system, we also repeated the same experiment on polyacrylamide hydrogel which is also widely used in stiffness studies (146,191,193,194,238). Consistent with data obtained from GelMA hydrogels, increased α SMA level with thick fibre assembly was observed on the stiff polyacrylamide hydrogels in the absence of one ROCK isoform (Appendix 4) proving that our findings are regardless of type of hydrogel system and mainly due to substrate stiffness.

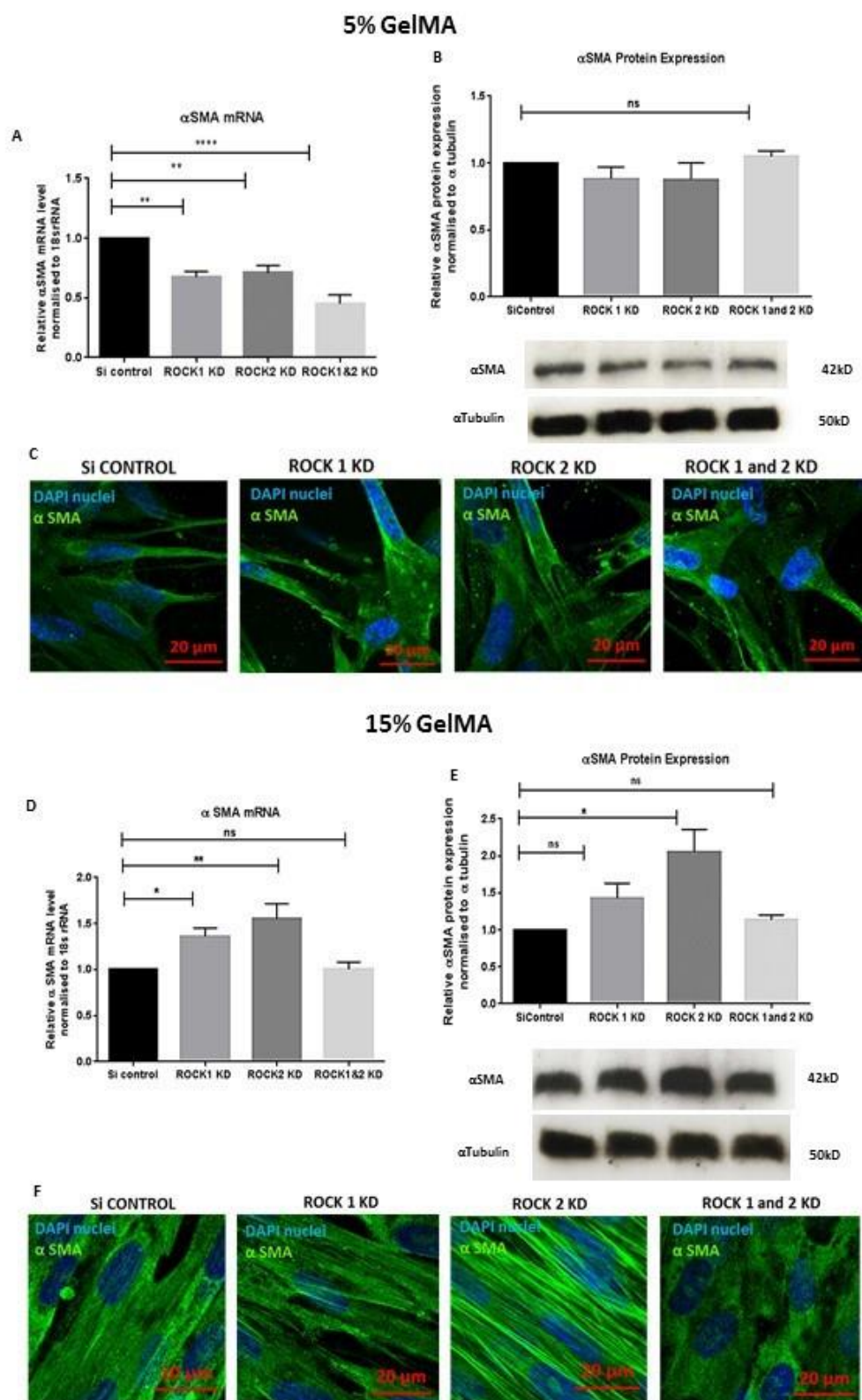


Figure 4.7 ROCK isoform regulation of α SMA (gene and protein expression) and stress fibre formation in lung fibroblasts cultured on 5% and 15% GelMA.

Lung fibroblasts are cultured on 5% (A, B, C) and 15% GelMA (D,E,F) for 2 Days for gene analysis and 4 days for protein level detection (B, E) after ROCK isoform gene silencing. The level of α SMA mRNA level was examined by the RT-PCR. rRNA 18s was used as reference control. The level of α SMA mRNA from Si Control was set at 1 for comparative analysis in RT-PCR. α Tubulin was used as protein loading control in WB analysis (n=3). Relative levels of α SMA protein expression at 4 days after gene silencing were determined by densitometry (n=3) normalised to α Tubulin. Results are Mean $\pm$ SEM, n=3, *p<0.05, **p<0.01, ****p<0.0001, ns=not significant. (C,F) Confocal analysis of α SMA stress fibre formation by immunofluorescence staining at 4 days after gene silencing. Scale bar: 20 μ m, green= α SMA and blue= DAPI nuclei staining.

4.3.3 Loss of α SMA fibre assembly in the absence of ROCK isoforms correlates with diminished F actin fibre on stiff matrix

F actin polymerisation from G actin monomer in cytoplasm is known to be upstream to the α SMA promoter activation in nucleus after activation by stiff matrix (193). Therefore, in addition to showing α SMA fibre assembly in ROCK isoforms depleted fibroblasts on soft and stiff matrices, we also tried to investigate any potential association between ROCK activation and F actin fibre formation under these conditions. As shown in Figure 4.8A, there was no change in the F actin fibres formation with ROCK isoform depletion on soft matrix. However, on stiff matrix, even though F actin staining did not show any significant increase in fibroblasts with individual silencing of ROCK isoform, it was significantly reduced when both isoforms are absent which also correlates with the loss of α SMA fibre assembly after knocking down of both isoforms (Figure 4.8B). These data clearly suggest a differential role for ROCK signalling in the control of F actin polymerisation in association with α SMA fibre assembly on soft and stiff matrices.

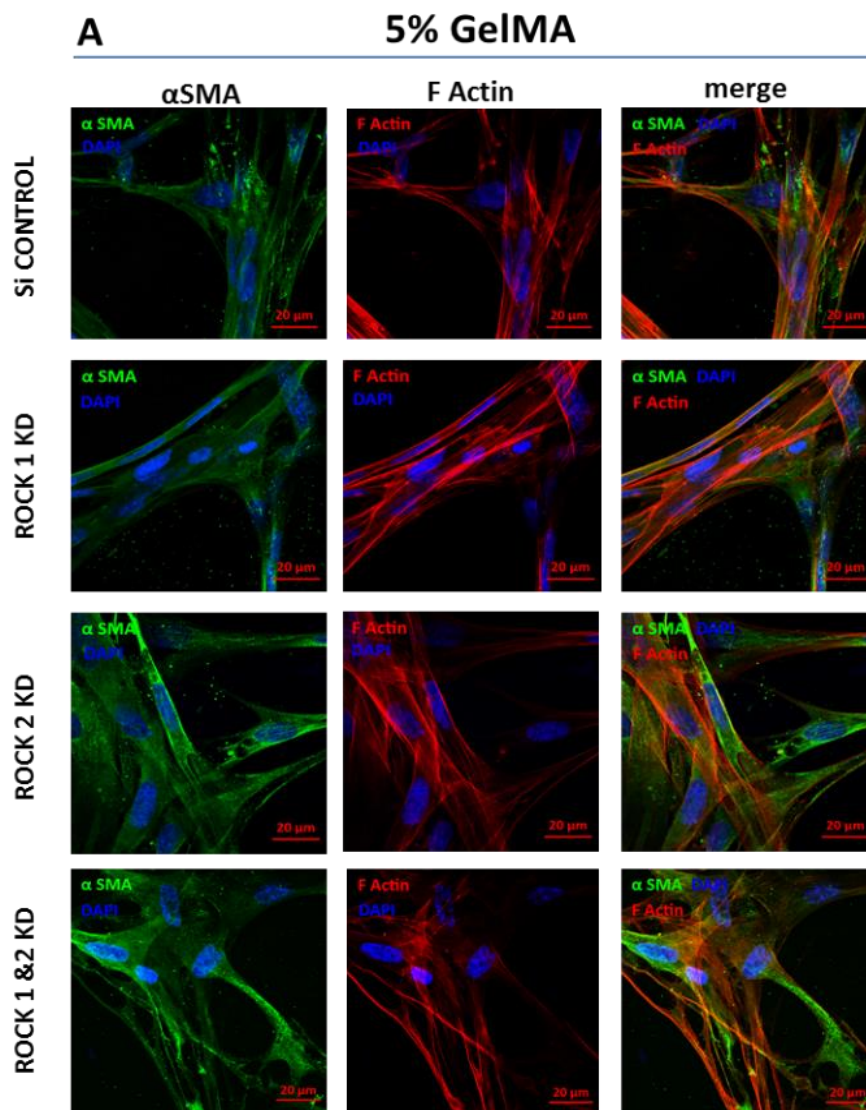


Figure 4.8. ROCK isoform regulation of F actin polymerisation in association with α SMA stress fibre formation in lung fibroblasts on soft and stiff matrices. Lung fibroblasts are cultured on 5% GelMA (**A**) 15% GelMA (**B**) for 4days after gene silencing of ROCK isoform. α SMA stress fibre (green) and F actin fibre (red) assembly was examined by confocal microscopy. Nuclei are stained with DAPI. Representative images from the three independent experiments. Scale bar =20 μ m, n=3

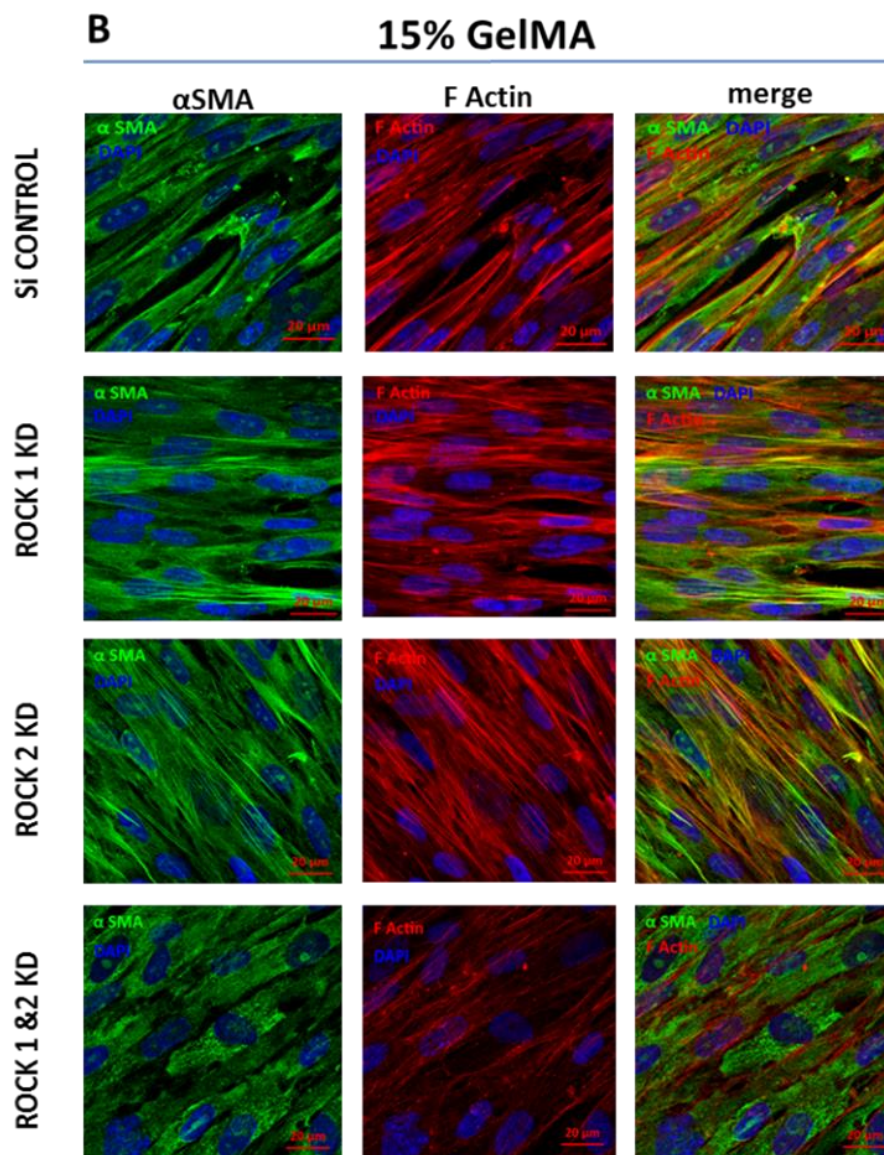


Figure 4.8 continued.

4.3.4 ROCK isoforms imbalance promotes nuclear translocation of MRTF-A in a stiffness dependent manner in favour of α SMA stress fibre assembly

Myocardin related transcription factor A (MRTF-A) has been shown to be involved in stiffness induced myofibroblast differentiation (193) by increasing F actin to G actin protein ratio. Generally, actin monomers are bound to MRTF-A in the cytoplasm. When the ROCK signalling is activated, G actin

monomers are released from MRTF-A molecules and polymerise to form F actin fibre assembly and MRTF-A is translocated into the nucleus where it promotes the fibrotic gene program. To determine the role of MRTFA activation in ROCK isoform dependent changes in stiffness induced myofibroblast differentiation, we examined the MRTF-A nuclear localisation in ROCK isoform depleted fibroblasts on 5% and 15% GelMA. Interestingly, immunofluorescent analysis demonstrated increased nuclear localisation of MRTF-A (30 to 40%) in lung fibroblasts with single ROCK isoform depletion on 15% GelMA compared to control (transfected with scrambled siRNA) and cells where both ROCK isoforms were knockdown (Figure 4.9-C,D). This was opposite to cells cultured on the 5% GelMA where the increased nuclear localisation of MRTF-A was not detected in all conditions (Figure 4.9-A,B). Taken together, these data support the important role of MRTF-A transcriptional factor activation in induction of α SMA gene expression and protein synthesis when one ROCK isoform was absent in lung fibroblasts cultured on a stiff matrix.

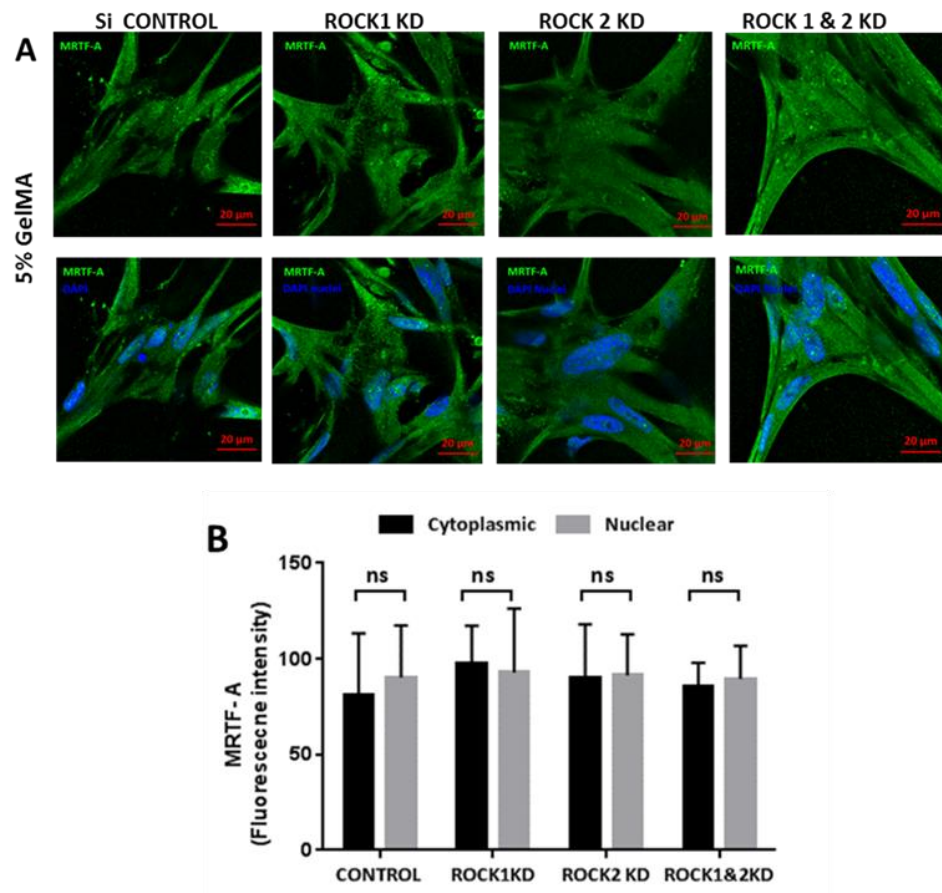


Figure 4.9 ROCK isoform regulation of matrix stiffness induced nuclear localisation of MRTF-A in lung fibroblasts. Lung fibroblasts are cultured on the 5% **(A, B)** and 15% GelMA **(C, D)**. The cells were fixed at 3 Days after gene silencing of ROCK isoform and stained for MRTF-A (Alexa Fluor 488 – green colour). Nuclear localisation of MRTF-A was examined by confocal imaging and overlaid with DAPI nuclei (blue colour) to confirm nuclear localisation of MRTF-A. **(B, D)** The mean Fluorescence intensity (MFI) of subcellular localisation of MRTFA was quantified by using ImageJ software. For each condition, MFI of nuclear and cytoplasmic MRTF-A in fibroblasts was measured from total 60 numbers of cells from three independent experiments. Scale bar =20µm, n=3, ****p<0.0001, ns=not significant.

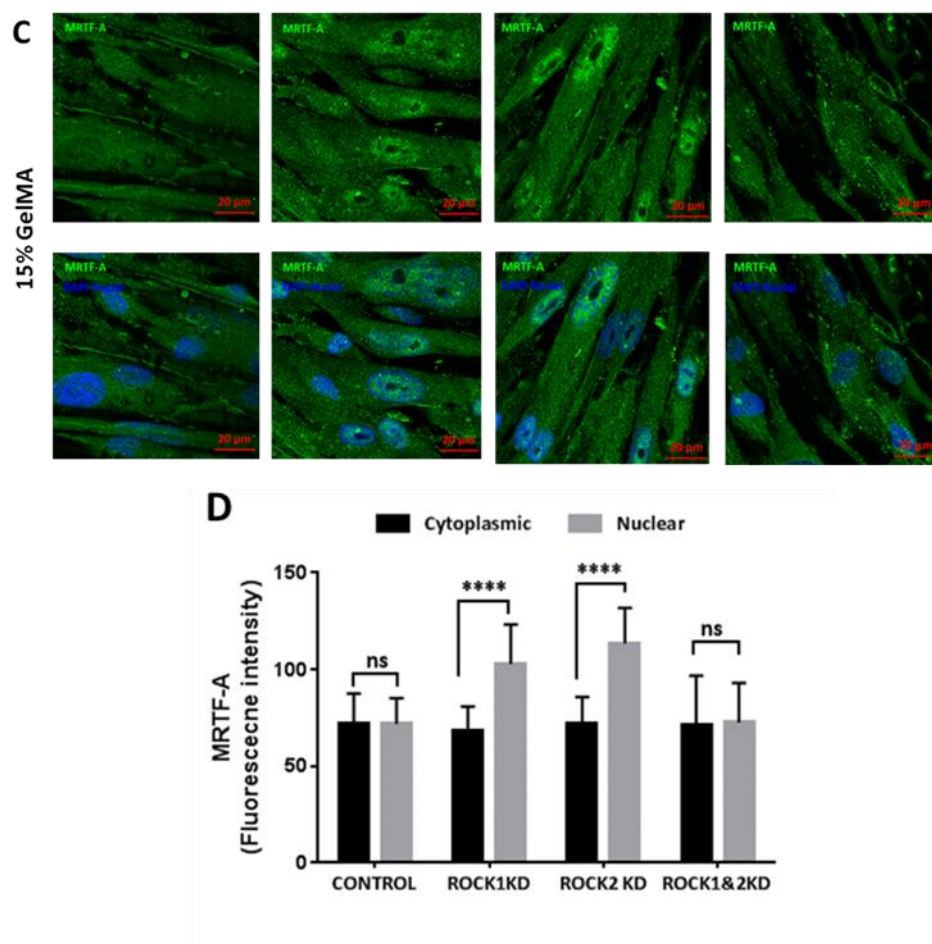


Figure 4.9 Continued

4.3.5 Inhibition of ROCK signalling does not attenuate stiffness induced fibroblast proliferation

As discussed in chapter 3, in addition to stiffness induced myofibroblast differentiation, increasing substrates stiffness also induces fibroblast proliferation. To this end, firstly we investigated the role of ROCK activation in stiffness induced fibroblast proliferation by using Y27632. In this experiment, lung fibroblasts cultured on 5% and 15% GelMA was continuously treated with 2 doses of Y27632 (10uM and 20uM) for 4 days. Cell proliferation was assessed by Alamar Blue cell proliferation assay at 4 days after treatment with Y27632 according to the protocol described in

Section 2.2.6. As shown in Figure 4.10 (A, B), inhibition of ROCK activation does not have any significant effect on fibroblast proliferation on both soft and stiff matrices at two doses of Y27632 compared to untreated control. The finding was also confirmed on soft and stiff matrices after ROCK isoforms were silenced individually or in combination in fibroblasts. For this experiment, ROCK isoforms were knock down in lung fibroblasts on soft and stiff matrices for 3 days before treatment with Y27632 for 4 days. Consistently with Y27632 treatment, absence of ROCK isoforms either one or combination does not have impact on stiffness induced cell proliferation (Figure 4.10- C, D). Taken together, it is reasonable to assume that ROCK signalling has profound effect on stiffness induced myofibroblast differentiation rather than controlling cell proliferation.

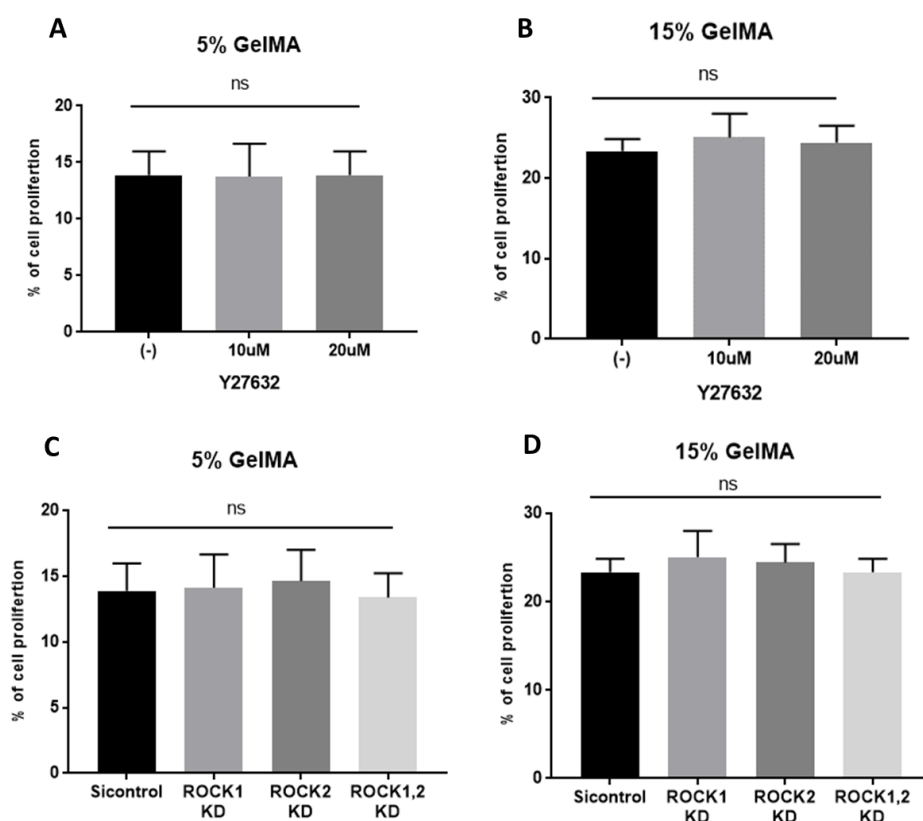


Figure 4.10 Inhibition of ROCK cannot prevent the stiffness induced fibroblast proliferation. Lung fibroblasts are cultured on 5% (**A, C**) and 15% GelMA (**B, D**) for 24 hrs before treated with Y27632 and gene silencing. For Y27632 treatment, the cells were treated with or without Y27632 (10 μ M and 20 μ M) for 4 days. Cell proliferation was assessed by Alamar blue cell proliferation assay at 4 days after treatment. For gene silencing, ROCK1 and ROCK2 isoform was knocked down on 5% GelMA (**C**) and 15% GelMA (**D**) individually and in combination by SiRNA gene silencing for 3 days. The cell proliferation was assessed by Alamar blue cell proliferation assay for 4 days after gene silencing. Mean $\pm$ SD, n=3, ns, not significant.

4.4 Discussion

Experiments using pharmacological inhibitors such as Y27632 and Fasudil have shown ROCK activation as a critical step in the stiffness induced myofibroblast transformation (191,193). In addition to showing high efficiency in *in vitro* experiments these pharmacological inhibitors have also shown good efficacy in preventing development of lung fibrosis as well as reversing the established fibrosis in the bleomycin induced lung fibrosis mouse models (146). However, given the non-selective nature of the ROCK inhibitors used in these studies, the relative contribution of ROCK 1 versus ROCK2 isoforms in the development of lung fibrosis is yet to be elucidated. Such information could not only enhance our understanding of lung fibrosis at the molecular level but could also pave the way for development of more effective intervention strategies for treatment of lung fibrosis.

The main aim of this study was to evaluate the importance of the balance between ROCK 1 and 2 isoforms and their relative contribution in stiffness induced myofibroblast transformation in lung fibroblasts. Using SiRNA gene silencing, we achieved highly specific knock down of ROCK1 and ROCK2 isoforms in spite of the high homology between them. This enabled us to investigate the effect of substrate stiffness on α SMA expression and assembly in ROCK1^{low}, ROCK2^{low} and ROCK1+2^{low} lung fibroblasts for the first time. The main conclusion from these experiments is that either of ROCK isoforms are sufficient for α SMA expression on soft matrix but both isoforms are required for α SMA stress fibre assembly and maintaining the α SMA protein level on stiff matrix suggesting the differential role of ROCK isoforms in myofibroblast differentiation on soft and stiff matrices. More importantly, one unexpected finding is that the absence of one type of ROCK isoform, as opposed to knocking down both isoforms simultaneously, the data show the myofibroblasts induced by stiff matrix transform into

a phenotype with increased level of α SMA with thick α SMA stress fibre assembly. This is opposite to the effect on a soft matrix where α SMA expression was dramatically reduced after knocking down only one ROCK isoform.

Additionally, the aggressive myofibroblast features are more prominent in the absence of ROCK 2 isoform suggesting a more dominant role for ROCK1 isoform in controlling α SMA gene expression and fibre assembly. Together these findings highlight the importance of ROCK isoform balance in maintaining fibroblasts normal phenotype and the fact that inhibiting individual ROCK isoforms (as opposed to simultaneous inhibition of both) could accelerate myofibroblast transformation on an already stiff matrix (e.g. fibrotic lung). Therefore, since on a stiff matrix, the total loss of α SMA assembly could be achieved only in the absence of both isoforms, it is reasonable to suggest that targeting both ROCK isoforms would be a safer pharmacological intervention for controlling myofibroblasts in fibrosis.

Interestingly our data show that knocking down both ROCK isoforms in cells cultured on stiff matrix do not reduce the α SMA protein expression back to the levels observed in cells cultured on soft matrix. This finding is inconsistent with our observations when using ROCK inhibitor Y27632 where α SMA protein level was reduced significantly within 24 hrs treatment with Y27632. The possible explanation is that Y27632 has additional effect in controlling α SMA synthesis and breakdown through other kinases rather than ROCK signalling. Furthermore we can argue that even though ROCK isoform gene silencing achieved high efficiency about 75% in our experiments, the stimulus from the stiff matrix was strong enough to activate the low level of ROCK kinase in myofibroblast to maintain the α SMA expression at high level. These data highlight the importance of a continuous drive from surrounding stiff matrix in influencing myofibroblast phenotype. Additionally, the loss of α SMA fibre assembly correlated with

diminished F actin polymerisation not only in the absence of both ROCK isoforms but also in Y27632 treated samples highlighting the critical role of ROCK activation in the contractile function of myofibroblast by controlling both α SMA fibre assembly and F actin fibre polymerisation on a stiff matrix.

MRTF-A activation has shown to be involved in stiffness-induced myofibroblast transformation as actin dynamics sensor (266). Our data showing an increase in nuclear translocation of MRTF-A in the absence of one ROCK isoform in myofibroblasts on stiff matrix suggest the possible link between increased α SMA expression with mature α SMA fibre assembly and changes in actin dynamics in these conditions. One explanation is that ROCK isoform imbalance on stiff matrix increased F - G actin ratio that favours the MRTF-A nuclear localisation and trans activates the α SMA gene expression. As the Rho-ROCK-MRTF-A signalling pathway has been implicated in controlling myofibroblast differentiation in lung fibrosis, our data further highlights the association between MRTF-A and ROCK isoform in stiffness induced myofibroblast differentiation. Further investigations are required to explain how MRTFA nuclear translocation affects increase in α SMA profile when one ROCK isoform is depleted in myofibroblast on stiff matrix.

According to our preliminary assessment on the role of ROCK activation in stiffness induced cell proliferation, ROCK isoforms do not seem to be involved in stiffness induced cell proliferation in contrast to myofibroblast differentiation. Therefore, it is reasonable to assume that stiffness induced cell proliferation might be controlled by signalling preceding ROCK activation or other specific mechanotransduction pathways rather than ROCK signalling. Recently, focal adhesion signalling at the cell-ECM interface has shown to be important in the early events of mechanosensing of the cells (281,282). It is interesting to elaborate whether the stiffness induced cell proliferation is controlled by the focal adhesion level preceding the ROCK activation.

In summary, our study provides a detailed understanding of the role of ROCK isoforms in the regulation of stiffness induced myofibroblast transformation and highlight the importance of ROCK isoform balance in lung fibrosis. This new insight could inform future attempts for developing therapeutic strategies that target ROCK activation for the treatment of lung fibrosis.

Chapter 5

Investigating the role of talin in Stiffness induced cell proliferation and myofibroblast differentiation

5 Chapter 5- Investigating the role of talin in Stiffness induced cell proliferation and myofibroblast differentiation

5.1 Introduction

Cell-matrix interactions are mediated via recognition of extracellular matrix proteins like collagen, fibronectin and laminin by various cell surface receptors and cell adhesion molecules (283). Integrins, a family of cell adhesion molecules, have been shown to be important cell surface receptors in mechanosensing of extracellular matrix (284-286). Many cellular functions such as cell proliferation, migration, adhesion and differentiation are associated with mechanical signals sensed by surface integrins. Activated integrins are dynamically coupled to the actomyosin system through many adaptor proteins such as talin, vinculin, filamin and paxillin (287). Amongst these proteins, recently talin is particularly interesting in matrix rigidity sensing because it can be unfolded following integrin activation upon exposure to stiff matrix (286,288,289). Generally, integrins are not able to connect directly the intracytoplasmic actin bundle owing to the lack of actin binding site (ABS) on their domains and talin provides indirect connection between integrins and F actin because it comprises both integrin and F actin binding sites on its two ends (Figure 5.1)(290).

5.1.1 Talin Structure: its domain and binding sites

Structurally, talin (270kD) has two domains, namely N terminal head domain (~50 kDa) and C terminal rod domain (>200 kDa) (291). Head domain can be further subdivided into three subdomains (F1, F2 and F3) and rod domains has 13 domains (R1-R13) (292). Functionally, as illustrated in Figure 5.1, head

domain binds to cytoplasmic tail of β subunit of integrin and involves in integrin activation whereas tail domain binds to F actin fibres which allows force transmission from integrin to cytoskeleton.

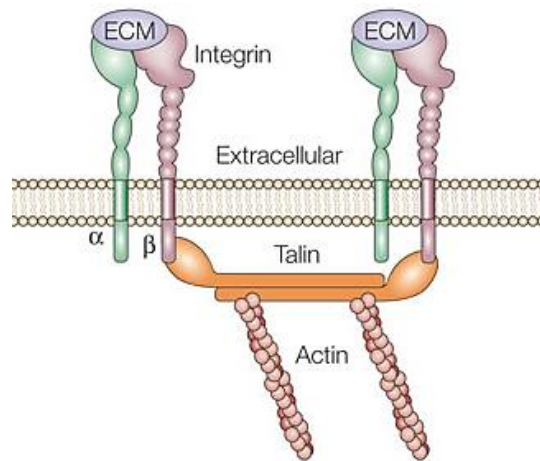


Figure 5.1 Structural basis of talin as cross bridge between integrin and actin network Head domain of talin dimer binds with β subunits of integrin whereas roddomain binds with F actin on the other end. [Gumbiner *et al* 2005, (293)]

Figure 5.2 illustrates the domain structure of talin molecules in relation with its binding partners such as integrin, actin and vinculin. The F3 subdomain of talin head has integrin binding site1 (IBS1) and F2 and F3 subdomains have actin binding site 1 (ABS1) (294). The rod domain contains vinculin binding sites (VBS 1-11), two additional actin binding sites (ABS2 and ABS3), a second integrin binding site (IBS2) and binding sites for other regulatory proteins like Kank (KN motif and ankyrin repeat domains) and RIAM (Rap1-GTP-interacting adapter molecule) (288,295,296).

In non-recruited physiological state, due to the molecular interaction between head domains (F2 and F3) and rod domains (R1-R2 and R9), talin molecules adopt the autoinhibitory ring shape and many binding sites on head and rod domains are unexposed (297,298). The regulatory proteins like KANK and

RIAM were shown to interfere the interaction between head and rod domains to release the autoinhibitory shape. Moreover, the functions of ABS2 on the head domain and IBS2 on the rod domain remain unclear and the role of talin in focal adhesion is likely to have more complexity than the simply acting as a connector between integrin and actin.

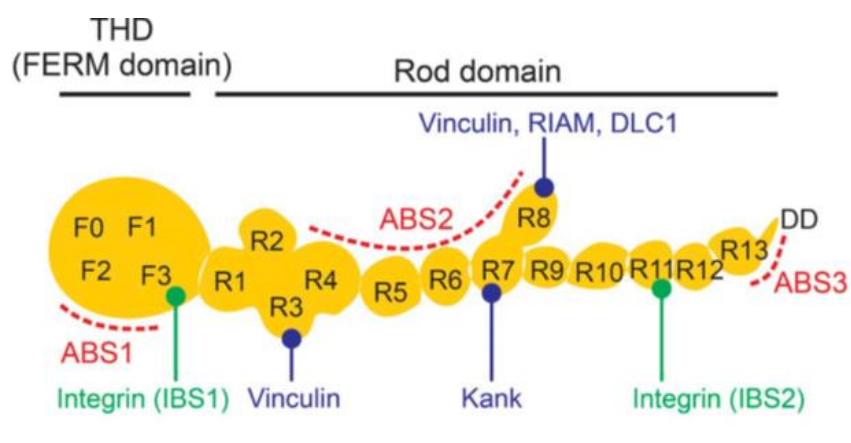


Figure 5.2 Structural basis of talin molecules. (A) Schematic diagram depicting the structure of talin. Talin head domain has four subdomains (F0, F1, F2, F3) containing integrin binding site 1 (IBS1) and actin binding site 1 (ABS1). Talin rod domain contains R1 to R13 helix bundles and a dimerization motif (DD) at the end of rod domain [Sun *et al* 2016, (281)].

5.1.2 Talin and its binding proteins in focal adhesion

Cells detect the biophysical properties of ECM mainly through focal adhesion (FA) during cell attachment or migration on the surface. Structurally, FA is a cluster of integrins that are formed at cell-ECM interface (281,285) and they are a frontier checkpoint in translation of biomechanical properties of ECM environment into an intracellular response through a chain of protein-protein interactions (287). FA usually begins with small areas (<200 μm) of matrix adhesion called the nascent adhesion or focal complex (299,300). Nascent adhesion can be seen in early stage of cell attachment and is usually transient

and force independent (287,301). They can transform into larger stable complexes known as classical FA and supermature FA in force dependent manner depending on the degree of matrix rigidity (300,302). Maturation of FA requires further clustering of integrins and recruitment of more adaptor proteins that leads to enhancement of the mechanical linkages between integrin and actin cytoskeleton (303).

Talin: interaction with Integrin

Integrins are transmembrane receptors composed of two units namely: α and β which are capable of bidirectional force transmission between the intracellular and extracellular compartments (304). The ectodomain of integrin, formed by both units, binds to extracellular ECM ligands such as collagen, fibronectin, and laminin whereas its cytoplasmic tail links to intracellular actin cytoskeleton via focal adhesion adaptors such as talin, filamin and vinculin (305). Depending on their structural compositions, activation mechanisms and their specificity to different ECM ligands, there are at least 24 different types of integrin $\alpha\beta$ heterodimers (18α and 8β) (287,306,307). Upon cell adhesion to ECM, integrins are transformed from low affinity to high affinity binding state for their specific ECM ligands through their conformational changes from bent to extended position in their ectodomains. Details are illustrated in Figure 5.3.

Once integrin binds to its specific ECM ligand, talin is recruited to the FA site. Specifically, the F3 domain on talin head interacts with membrane proximal NPxY motif on cytoplasmic tail of β integrin that induces integrin in active open conformation (308) and also increases the affinity of integrin to bind to its cognate ECM ligands- called “inside out” integrin activation (309,310).

These ECM ligand bindings in turn stabilise talin bound integrin in active conformation, leading to recruitment of more integrin binding adaptors to its tail for further maturation of FA - a process called “outside in” activation (304). Moreover, one of membrane phospholipids called phosphatidylinositol 4, 5 bisphosphate (PI (4, 5) P₂) was shown to enhance the integrin binding to multivalent ECM ligands called integrin clustering through talin activation. Moreover, some types of integrin themselves have shown to have the ability to serve as mechanosensors in sensing ECM stiffness through their level of expression (e.g. $\alpha 6 \beta$ integrin) (311).

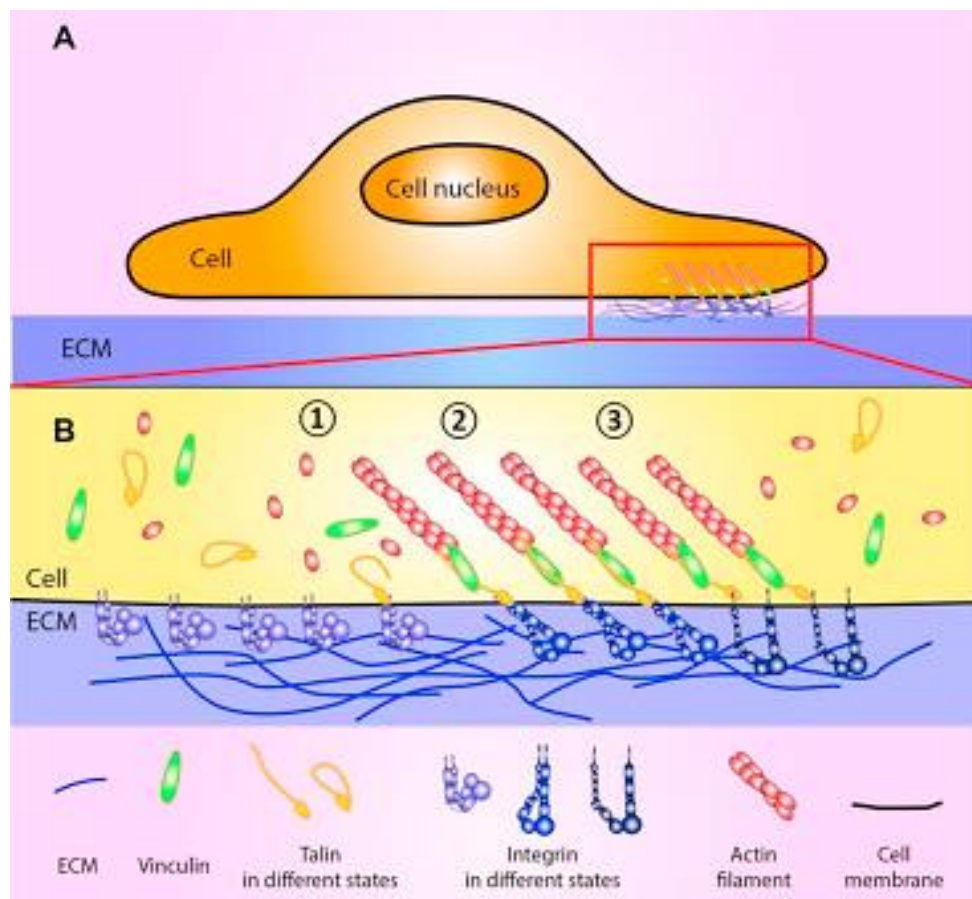


Figure 5.3 Schematic of overview of integrin mediated mechanotransduction [Sun *et al* 2016, (281)]. (A) Cell-ECM interface through focal adhesion. (B) Different components involved in focal adhesion maturation in force dependent manner. Without ECM ligands engagement, integrins are present in an inactive state with bent

configuration. This changes upon interaction with ECM following these steps: (1) Integrin binds to its specific ligands and starts recruiting focal adhesion adaptor molecules (talin, vinculin) (2) Talin binds to $\beta 3$ tail of integrin. F actin polymerises and binds to the opposite end of talin. Force transmits between ECM and actin cytoskeleton across the integrin-talin-actin linkage (3) Talin activates integrin into a high affinity binding state with extended open configuration.

Talin: interaction with Vinculin

Vinculin is another important focal adhesion adaptor molecule which is recruited to the focal adhesion complex in a force dependent manner following talin activation (312). Like talin, vinculin comprises of two domains: N terminal head domain and C terminal tail domain connected by a short linker (Figure 5.4). Vinculin head domain contains a talin binding site and the tail domain contains actin binding sites (313,314). Similarly, the vinculin binding sites are also masked inside helix bundles by the autoinhibitory interaction between head and tail domains of vinculin (314). Subsequent binding of the vinculin head to the vinculin binding site on talin is sufficient to disrupt the autoinhibitory configuration, creating an open conformation of vinculin (315,316). This open conformation also allows vinculin to bind actin bundles, further stabilising the integrin-talin-F actin linkages (289).

The finding that vinculins are not recruited to the focal adhesion site in talin deficient fibroblasts suggests the strong association between vinculin and talin. However, it is still unclear how vinculin is activated and recruited to the vinculin binding site on talin. The evidence that vinculin has localised in the focal adhesion site only when the force is applied also supports the hypothesis that the vinculin recruitment is force dependent (314). Talin unfolding by force generated from actomyosin exposes the cryptic vinculin binding sites in

tension dependent manner (317,318). When more force is applied on talin domains, talin unfolds throughout all domains, exposing more vinculin binding sites. The number of vinculin binding sites exposed depends on the magnitude of force applied on the talin. (282).

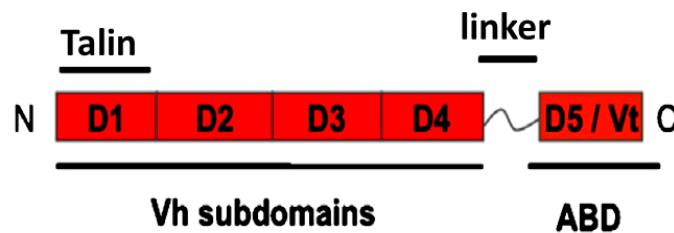


Figure 5.4 Schematic for domain structure of Vinculin with its binding sites. D1-4, vinculin head subdomains; D5/Vt, vinculin tail domains; ABD, Actin binding domains, [Ciobanasu *et al* 2013, (286)]

Talin: interaction with Actin Network

Some studies have shown that talin silencing also disrupts the actin cytoskeleton, cell spreading and focal adhesion formation (319). As discussed earlier, talin has 3 actin binding sites –ABS1 on head, and ABS2 and 3 on rod domains. Through these 3 binding sites on talin, activated integrin couples the actomyosin network leading to activation of downstream signalling pathways for cell differentiation, proliferation and migration. Among 3 binding sites (ABS1-3), ABS3 on talin's rod domain was shown to be attached by F actin after disruption of talin autoinhibitory conformation (320). The majority of cells failed to develop FA when the interaction between ABS3 and F actin was disrupted highlighting the importance of ABS3 in initial force transmission (321).

5.1.3 Talin1 vs Talin2

There are 2 talin isoforms, talin 1 and talin 2 which have nearly 80% homology and exhibit the same organisation pattern in their domains (322). Both genes are widely expressed but the level of expression of the two isoforms varies between tissues. Talin 1 is ubiquitously expressed but is more predominant in spleen, liver and lung (291). Talin 2 is highly expressed in brain and muscle and both isoforms appeared to be expressed at the same level in heart and kidney. Little is known about the structural and functional differences between talin 1 and talin 2 isoforms except a few studies reported in mouse cell lines. Recent evidence indicates that talin2 is functionally different from talin1 despite their high homology in protein sequence. Some studies have suggested that the two isoforms have different subcellular localisations within cells such as fibroblasts, muscle cells and macrophages (322). Interestingly, compared to talin 2, talin 1 is recruited to the focal adhesion significantly faster. Moreover, loss of talin 1 can be compensated by endogenous talin 2 in mouse fibroblasts and can be rescued by exogenous talin 2 in endothelium which normally expresses only talin 1 (282). However, talin 2 cannot replace talin 1 function in early embryogenesis resulting in non-viable talin 1 null mice (323). Interestingly, vinculin binding sites on R1-R3 domains of talin1 and talin2 recruit the vinculin in different ways. Unlike talin1, talin 2 recruits the vinculin independent of ABS3 and ABS2 (282). Furthermore, according to recent studies, talin2 has more affinity in binding to the tail of integrin β 1 than talin1 (324).

In the absence of any detailed studies using human cells or tissues, any functional differences between talin isoforms in human, particularly in the

context of mechanosensing for extracellular matrix stiffening and induction or resolution of fibrosis has remained elusive.

5.1.4 Role of talin in matrix rigidity sensing

Due to its complex structure with multiple folding domains and its ideal subcellular position (i.e. between integrin and actin) for mechanotransduction, talin is considered as novel mechano-responsive candidate molecule in matrix rigidity sensing. Force generated either from inside-out or outside-in signalling induces unfolding and structural rearrangement of talin domains to expose the multiple cryptic binding sites followed by recruitment of other cytoskeletal proteins which are involved in mechanotransduction (304).

Recent studies have shown that the length of talin in the resting state is about 60 nm which can extend as long as 350-400 nm under exposure to applied force (325). Moreover, it is interesting to note that talin unfolds at applied force around 5 pN (Pico newton) strength in *in vitro* tension models, which corresponds to the physiological force exerted in focal adhesion at the molecular level (326,327). In addition to talin head domain, rod domains are also sensitive to mechanical loads and contribute in mechanosensing of matrix.

Moreover, the role of talin in initial cell spreading and mechanosensing is also functionally linked with phosphorylation of FAK and vinculin binding to talin evidenced by gene silencing of talin correlated with absence of FAK phosphorylation and vinculin at FA site (319,328). On stiff substrates, cells experience the high traction force due to the resistance of matrix against cell contraction which makes talin domain undergo partial unfolding followed by the exposure of a cryptic vinculin binding site (282).

According to recent observations by Artola *et al* (2016), talin unfolding occurs only on matrix with stiffness level higher than threshold level (~ 5 kPa). Above threshold level, focal adhesion grows rapidly in a nonlinear fashion together with subsequent vinculin recruitment, FAK phosphorylation and YAP nuclear localisation. Their data also show that talin as a whole molecule is a requirement for force transmission in matrix rigidity sensing evidenced by the finding that only full length talin but not talin head or rod domain can rescue the force transmission (329).

In contrast to stiff matrix, soft matrix below the stiffness threshold level can deform easily by cell contraction and force transmission is kept at a low level. Instead of talin unfolding, slow mechanical loading causes integrin detachment from ECM ligands and no enhancement in adhesion growth including vinculin recruitment (Figure 5.5). As a consequence, robust actin fibres with large focal adhesions are formed on stiff matrix whereas less well developed focal adhesion and actin polymerisation is a feature of soft matrix (330).

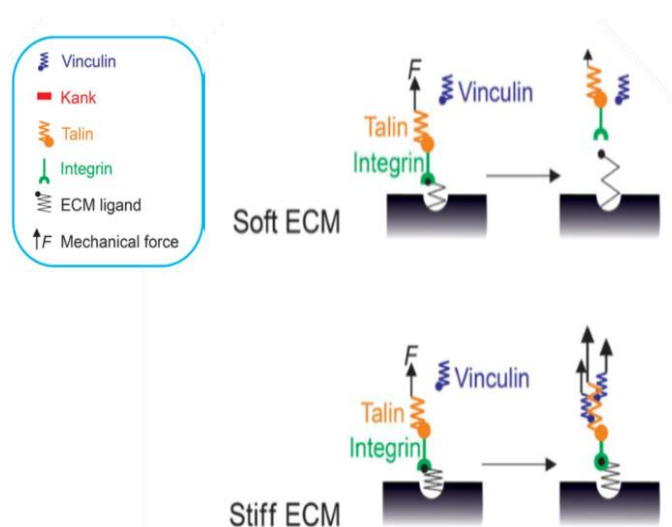


Figure 5.5 Schematic diagram showing differential regulation of talin dynamics on soft and stiff matrices [Sun *et al* 2016, (281)].

5.1.5 Talin: a potential target on fibrosis related disorders

As described above, previous studies provide strong evidence for the role of talin in probing matrix stiffness (192,194,331). Altered matrix stiffness with changes in ECM composition has been observed in many disease states in different organs such as lung fibrosis (88,191), liver cirrhosis (48) and cardiac hypertrophy (332). Not limiting to those, many studies have shown that the alteration in ECM mechanical properties alone can induce the malignant phenotype of cells (333). We (discussed in Chapter 3 of this thesis) and others have shown that stiffer ECM matrix can transform the fibroblasts into myofibroblasts independent of TGF β 1 in lung fibrosis (193,334).

Downstream to the activation of focal adhesion signalling through talin activation, many signalling pathways related to cell survival, cell adhesion and migration are activated. More importantly, drug resistance in fibrotic and malignant diseases are associated with ECM stiffening and controlling FA signalling can recover the drug sensitivity (250). Among many signalling pathways that controls the cell proliferation, it was shown that extracellular signal regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) signalling pathways are activated in stiffness induced cell proliferation in malignant tissues (250,335). In this context, experiments by Wang *et al* provide new insight about the importance of talin in regulation of cell proliferation. Talin deficient cells showed arrest in cell cycle progression which can be rescued only with C terminal talin tail re-expression but not the head domain (336). Although the role of ECM stiffness in both cancers and fibrosis is well

documented and talin has been shown to be important in matrix rigidity sensing, the specific role of talin in these diseases remains unknown. Therefore, we believe that understanding the detailed mechanism of talin-1 and talin-2 function and the cross-talk between them in stiffness induced fibrosis pathology could provide new insights into the molecular basis of aberrant fibroblast activation and pave the way for the rational design of novel treatment strategies to stop or ameliorate disease progression in fibrosis.

The aim for this chapter is to fully understand how different types of talin (talin-1 and talin-2) control the fibroblast activation including fibroblast proliferation and myofibroblast differentiation in response to stiffening of matrix in lung fibrosis. Instead of using an animal model, we tested the role of different forms of talin in human lung fibroblasts using bioengineered hydrogel with stiffness levels relevance to healthy or fibrotic lung.

5.2 Materials and methods

5.2.1 Si RNA gene silencing of talin1 and talin2 in lung fibroblasts and RT-PCR analysis for detection of efficiency of gene knockdown

The fabrication of soft (5%GelMA) and stiff (15% GelMA) hydrogels was performed as described in Section 3.2.1.

Human lung fibroblast cell line (MRC-5) obtained from ATCC, was routinely cultured as previously described in Section 3.2.3. SiRNAs targeting human talin1 (SiGENOME SMART pool- M012949-00-0005) or talin2 (SiGENOME SMART pool- M012909-01-0005) and non-targeting SiRNA control were purchased from Dharmacon (GE Healthcare, UK). The detail information for SiRNAs in the smart pools was provided in Appendix-7. Lung fibroblasts (4×10^5) were seeded in tissue culture treated 12 well plate overnight. Cells were transfected by DharmaFECT1 transfection agent (GE Healthcare) according to manufacturer's instruction. Effective gene knock down was achieved at 50 nM of both talin1 and talin2 SiRNA in 1:625 dilution of transfection agent after 2 days of transfection.

The efficiency of talin1 and talin2 gene knockdown was tested by RT PCR analysis at 2 days after transfection in tissue culture plate. Details for RT PCR analysis were discussed in Section 3.2.7. The level of mRNA in sample transfected with non-targeted SiRNA was set at 1 for comparative analysis of RT-PCR.

Specific PCR primers for this experiment contained the following sequences:

Human 18S rRNA forward	5'-GTAACCCGTTGAACCCCAT-3'
Human 18S rRNA reverse	5'-CCATCCAATCGGTAGTAGCG-3'
Human talin 1 forward	5'-TCTCCCAAATGCCAAGA-3'
Human talin 1 reverse	5'-TGGCTATTGGGGTCAGA-3'
Human talin 2 forward	5'-CTGAGGCTCTTTTCACAGCA-3'
Human talin 2 reverse	5'-CGGTGTTTGATATGGGAACC-3'

Human 18S rRNA was used as internal reference control to normalise the amount of the extracted RNA. RNA samples were analysed by using Mx3000P QPCR system and data were also evaluated by comparative RT-PCR analysis of MxPro software (Mx3005P version).

5.2.2 RT PCR analysis and Western Blotting for α SMA gene and protein expression in talin1 and talin2 knockout fibroblasts cultured on soft and stiff matrices

After 3 days of transfection, the talin1 and talin2 knockout cells were trypsinised and 5×10^4 /well/ml for each condition was seeded onto the 5% and 15% GelMA hydrogel in non-tissue cultured treated 24 well plate (Falcon) and transferred into a new plate on the following day. RNA extraction and protein extraction was done at 3 days and 4 days respectively after seeding on the soft and stiff hydrogel. Details Western blotting methods for α SMA protein were discussed in Section 3.2.6 and RT-PCR analysis for α SMA expression were described in Section 3.2.7.

5.2.3 Immunostaining, confocal microscopy and image analysis for α SMA and Ki 67 expression in talin1 and talin2 knockout fibroblasts cultured on soft and stiff matrices

Samples containing talin1 and talin2 knock down fibroblasts were fixed after cultured on soft and stiff matrices for 4 days. Immunofluorescent staining and confocal microscopy were performed as described in Section 3.2.5. All immunofluorescent images comparing 5% and 15% GelMA conditions were from the same experiment and analysed together using the same microscopy settings on the same day. The α SMA immunostaining was done as described in Section 3.2.5

For cell proliferation assessment, samples were incubated with rabbit Ki67 monoclonal (SP6, Abcam) in 1:100 dilution in PBS overnight at 4°C. Secondary antibody mouse anti rabbit Alexa Fluor 488 IgG (H+L)(Invitrogen) in 1:200 of PBS was used to conjugate for an hour at RT. Cell nuclei were stained with DAPI for 15 minutes.

Ki67 cell proliferation index was defined as the percentage of the number of Ki 67 positive cells over the total number of cells indicated by DAPI stained nuclei. The cells were counted in confocal image of 300 μ m². The average index for each condition was evaluated in random 18 images taken from three independent experiments and data were expressed as mean $\pm$ SD.

5.2.4 Statistical Analysis

Statistical analysis was performed using Graph Prism 6. Data are expressed as means $\pm$ Standard deviation (SD) and analysed by one way ANOVA and two-way ANOVA where appropriate with Bonferroni post hoc test for multiple comparisons. Significance level was set at $p < 0.05$.

5.3 Results

5.3.1 Detection of efficiency of talin gene silencing in human lung fibroblasts

To establish whether talin1 and talin2 have distinct function in regulation of stiffness induced fibroblast activation, SiRNA gene silencing was used to knock down talin1 and talin2 isoforms specifically. Firstly, talin genes were silenced in lung fibroblasts in tissue culture plate for 3 days. Gene silencing was done before seeding cells on hydrogels with different stiffness to avoid talin effect on early events of cell adhesion where talin is likely to play an important role in mechanosensing. The efficacy of the talin gene silencing was assessed using RT PCR at 3 days after silencing in tissue culture plate. As shown in Figure 5.6, SiRNA gene silencing can knockdown talin1 and talin2 gene expression level significantly in human lung fibroblasts with high specificity without affecting the level of other isoform at 3 days after transfection.

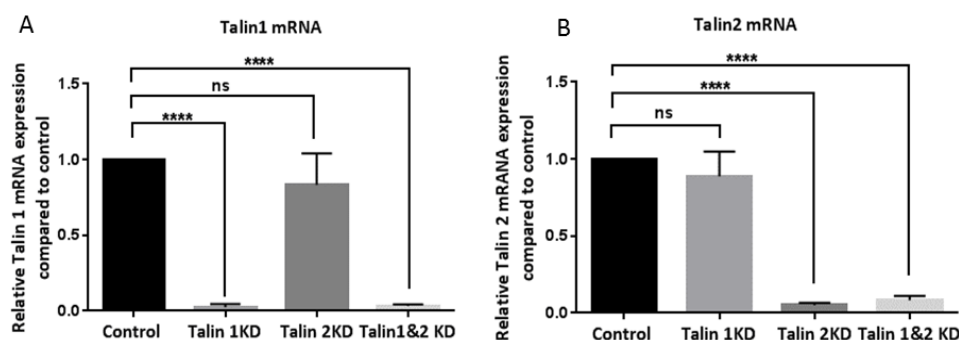


Figure 5.6 Specificity and efficiency of talin isoforms-talin1 (A) and talin2 (B) gene silencing in lung fibroblasts. Talin 1 and talin 2 isoform was knocked down on individually and in combination by siRNA gene silencing in tissue culture plate. The levels of talin 1 and talin 2 isoform mRNA were examined in lung fibroblasts by RT RCR at 3 days after transfection. 18s rRNA was used as reference control. The level of mRNA in sample transfected with non-targeted SiRNA was set at 1 for

comparative analysis of RT PCR. Results are Mean $\pm$ SD, n=3, ****p<0.0001, ns=not significant.

5.3.2 Investigating the role of talin1 and talin2 on initial fibroblast spreading on soft and stiff matrices

As talin molecules are important in initial integrin activation, we first determined the role of talin1 and talin2 on initial fibroblast spreading on matrix with different stiffness levels. For this experiment, talin1 and talin2 was knocked down for 3 days in human lung MRC5 fibroblasts in tissue culture plate and then talin1 and/or talin2 deficient cells were transferred onto soft and stiff matrix. We then studied cell attachment and spreading of talin1 and/or talin2 deficient fibroblasts on soft and stiff matrix by bright field microscopy (Olympus CRX 41 inverted microscope) with infinity analyse lumenera software at two different time points, 24 and 48 hrs for each condition. As seen in Figure 5.7, initial cell attachment was not affected on any of the matrices in the absence of either talin1 or talin2, as evidenced by the same number of cells present on both soft and stiff hydrogels. However, cell spreading was affected in the condition of talin1 knock down on both soft and stiff matrices, more obviously on soft matrix.

The talin1-deficient fibroblasts on soft matrix showed rounded morphology whereas the same fibroblasts on stiff matrix maintained cell spreading. The fibroblasts lacking both types of talin followed the similar morphology as talin1 knock down cells on both types of matrices.

In contrast, the cell spreading of talin2 knockout fibroblasts was comparable to the control treated with non-targeting SiRNA at 2 days after cell seeding on both types of matrices. Overall our observations showed that the differential

role of talin1 and talin2 in the regulation of fibroblast spreading between soft and stiff matrices.

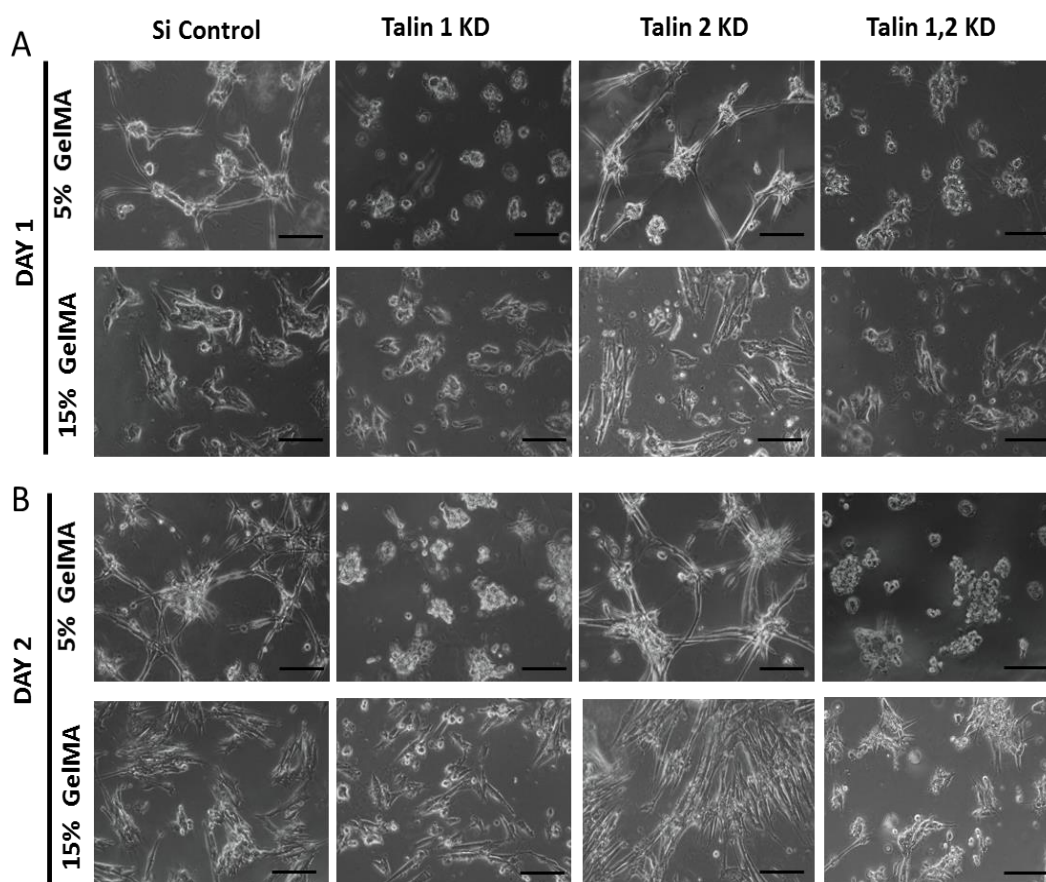


Figure 5.7 Fibroblast spreading was differentially regulated by talin1 and talin2 on soft and stiff matrices. Talin 1 and talin 2 knock out lung fibroblasts was seeded on soft and stiff matrices for 2 days. Bright field images (10x magnification) are taken with Olympus microscope (CRX41) using infinity analyse lumenera software at two time points, 24 hrs (A) and 48 hrs (B) after cell seeding on soft and stiff matrices. (n=3)

5.3.3 Talin1 but not talin2 controls the stiffness induced fibroblast proliferation

In chapter 3, we have shown that fibroblast proliferation was increased in a stiffness dependent manner. In order to determine the effect of talin1 and 2 on the stiffness induced fibroblast proliferation, we seeded the same number of talin1 and/or talin2 knockout fibroblasts on 5% (soft) matrix and 15% (stiff) matrix for the total duration of 4 Days and the cell proliferation was assessed

by Alamar Blue cell proliferation assay and by Ki67 staining. Alamar blue cell proliferation assay was performed as described in Section 2.2.6.

Ki67 is a cell proliferation marker that can be only detected in the nucleus of proliferated cells at the interphase of mitosis. As shown in the Figure 5.8, both cell proliferation percentage assessed by Alamar blue assay and Ki67 index showed that fibroblast proliferation was mainly controlled by talin1. The absence of talin1 prevented the cell proliferation on both soft and stiff matrices as opposed to the talin2.

Consistently with cell spreading discussed in previous section, lack of both talin1 and talin2 showed the similar level of reduction in cell proliferation as compared to talin 1 deficient cells. In contrast, lack of talin2 alone did not affect the fibroblast proliferation on both types of matrices. Therefore, altogether these data show that only talin1 is required for cell spreading and necessary for driving the fibroblast to be more proliferative on stiff matrix.

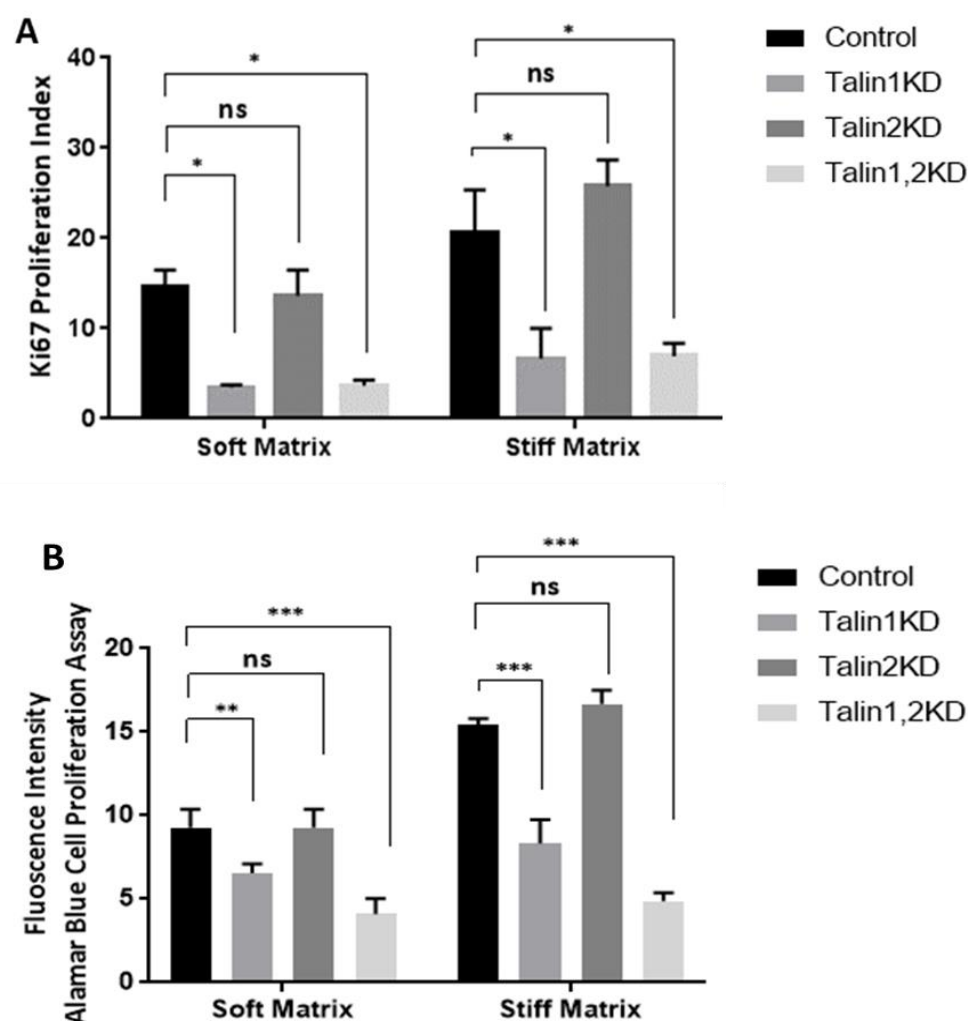


Figure 5.8 Increased cell proliferation induced by stiff matrix was diminished in the absence of talin1. The same number of talin1 KD, talin2 KD and talin1 and 2 KD fibroblasts was cultured on the soft and stiff matrices for 4 days. (A) Ki67 proliferation index (B) Alamar Blue cell proliferation Assay for quantitative comparison for cell proliferation in each condition after exposed to soft and stiff matrices for 4 days. Ki67 index was calculated from the percentage of Ki67 positive cells over total number of DAPI nuclei staining (C) Ki67 staining for detection of talin1 KD, talin2 KD and talin1 and 2 KD fibroblasts proliferation cultured on 5% of GelMA and 15% GelMA for 4 days on the soft and stiff matrice. Ki67-green DAPI-blue nuclei, scale bar equals to 100 μ m. The results are Mean $\pm$ SD, n=2, *p<0.05, **p<0.01, ***p<0.001, ns= not significant

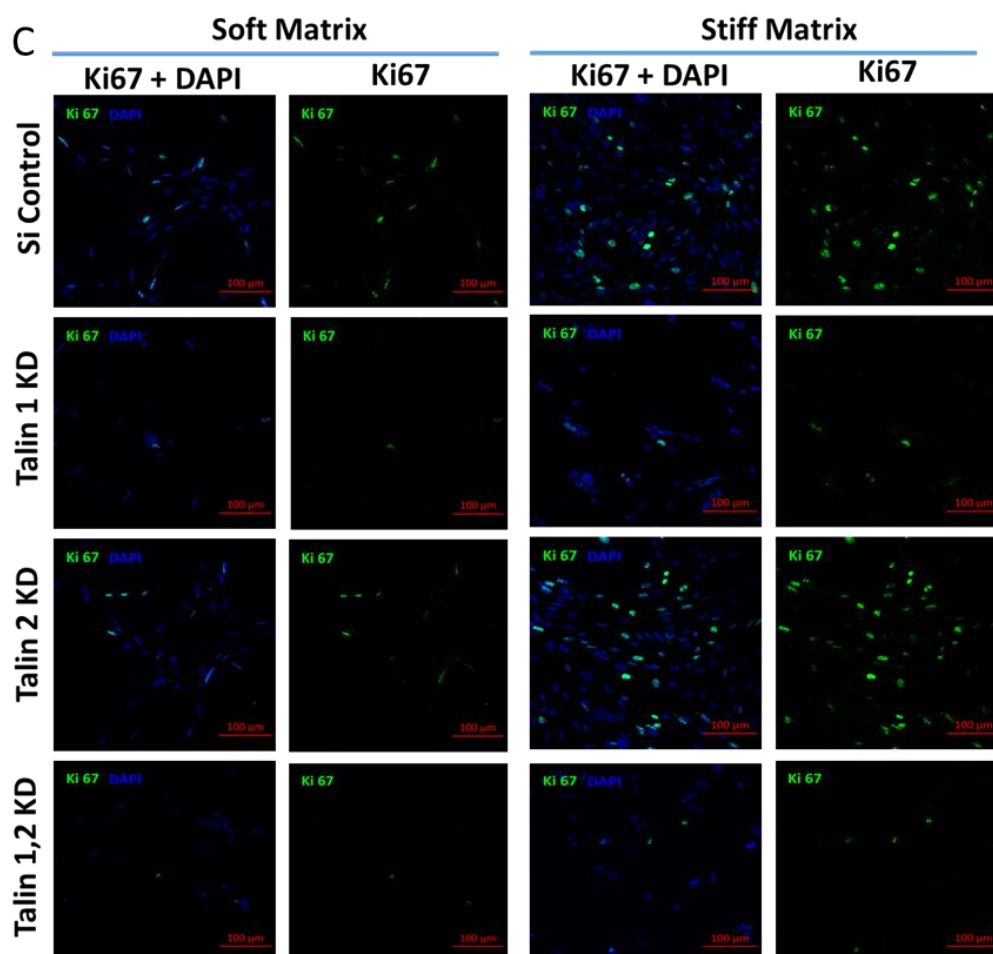


Figure 5.8 continued

5.3.4 Talin1 but not talin2 plays a dominant role in stiffness induced myofibroblast differentiation in human lung fibroblasts

To determine the effect of talin1 and 2 on stiffness induced myofibroblast differentiation, we investigated myofibroblast differentiation in talin1 and talin2 deficient lung fibroblasts cultured on soft and stiff matrices. As α SMA protein expression is a widely used marker for myofibroblast differentiation, we examined the α SMA mRNA and protein expression by RT-PCR and Western blotting in talin1 and/or 2 deficient fibroblasts. We firstly knocked down talin1 and 2 in lung fibroblasts in tissue culture plates and then exposed

to soft and stiff matrices for 3 days for RT-PCR analysis and 4 Days for Western blotting. It was not possible to perform the RT-PCR analysis and Western blotting for detection of α SMA level in talin1 knock out fibroblasts seeded on soft matrix where cell viability was significantly impaired after 3 days culture of soft matrix.

Interestingly on stiff matrix, the absence of talin isoforms either alone or in combination showed the differential regulation of α SMA expression. Lack of talin1 completely suppresses the α SMA expression whereas in the absence of talin2, the level of α SMA was paradoxically higher compared to control fibroblasts transfected with non-targeting SiRNA in both gene and protein expression profile (Figure 5.9). Consistently with these findings, absence of talin 2 induces more abundant and thick α SMA fibre assembly compared to control fibroblasts whereas talin 1 knock down showed the complete absence of fibre assembly on stiff matrix.

Altogether our data indicated that talin1 was mainly associated with stiffness induced increased α SMA expression and α SMA fibre assembly and talin2 has some antagonistic role over talin1 function in the control of myofibroblast differentiation.

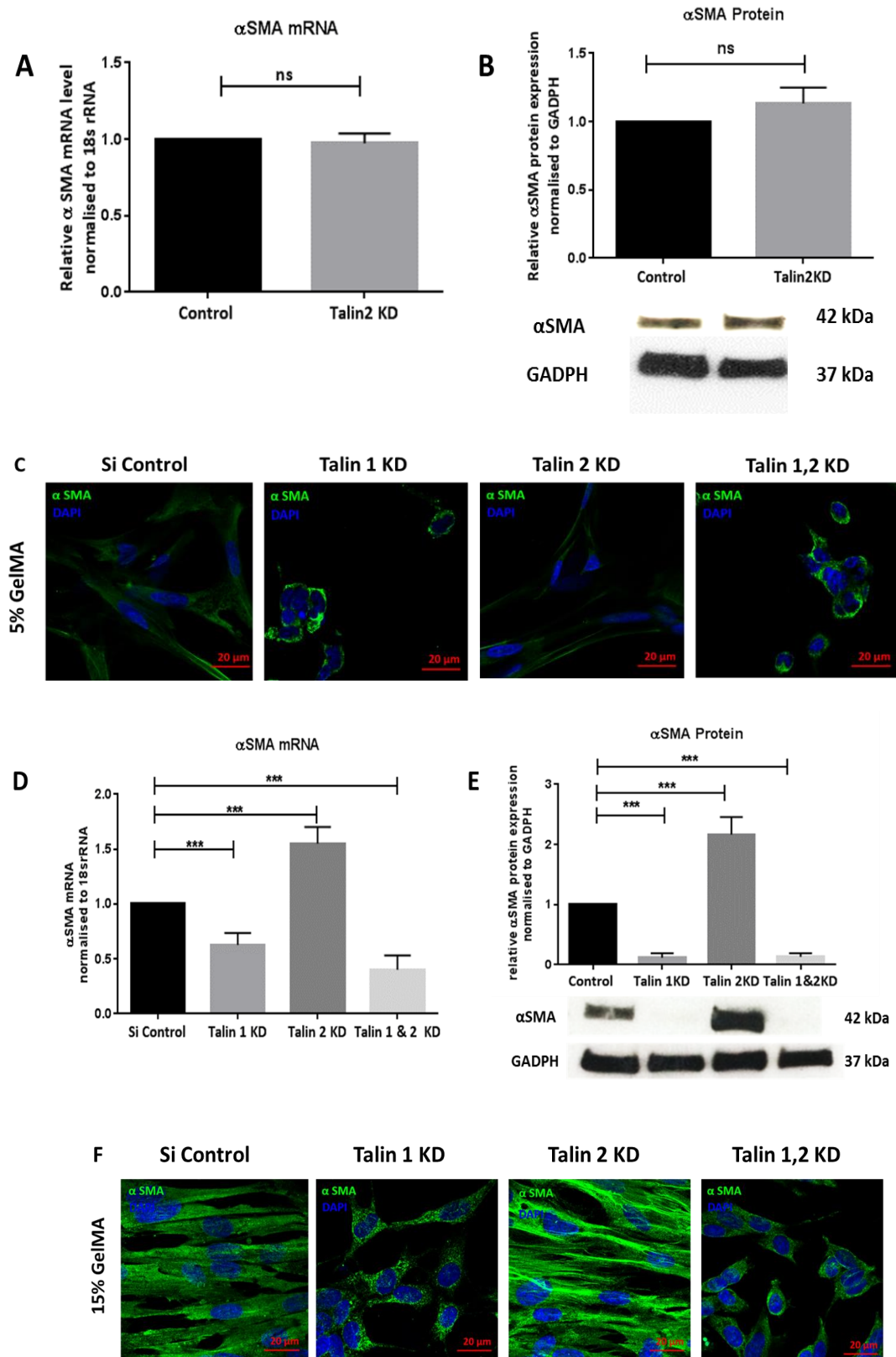


Figure 5.9 Talin isoform regulation of α SMA expression and stress fibre formation in human lung fibroblasts. Talin 1 KD, talin 2 KD and talin 1 & 2 KD lung fibroblasts were cultured on soft (5%) GelMA (A, B, C) or stiff (15%) GelMA (D, E, F) hydrogels. (A, D) The level of α SMA mRNA level was examined by the RT-PCR at day 3 after seeding on the soft and stiff matrices. 18s rRNA was used as reference control. The level of α SMA mRNA from Si Control was set at 1 for comparative analysis of RT PCR. (B, E) α SMA protein expression at day 4 after seeding on soft and stiff matrices was analysed by Western blot. GADPH was used as protein loading control. Relative levels of α SMA protein expression were determined by densitometry of the blots from 3 independent experiments and normalised to GADPH protein expression. (C,F) α SMA stress fibre formation in talin 1 KD, talin 2 KD and talin 1&2 KD lung fibroblasts was visualised by immunofluorescence staining and confocal microscopy at 4 days after cell seeding on the soft and stiff matrices. Scale bar: 20 μ m, Results are Mean $\pm$ SEM, n=2, ***p<0.001, ns=not significant

5.4 Discussion

Recently many researchers have studied the structure and function of talin including its role in integrin activation and in probing matrix rigidity where it functions as a mechanosensor. We and others (chapter 3 of this thesis) (88,193) have shown changes in ECM stiffness can induce fibroblast proliferation and myofibroblast differentiation hence contributing to the progression of lung fibrosis.

Although two structurally related talin isoforms are expressed in vertebrates, most researchers have mainly focused on talin1 which is more ubiquitously and highly expressed in mammalian cells compared to talin2. Therefore, although the role of talin1 in cell adhesion and migration is well recorded, the role of talin2 at cellular level has been understudied except few studies in mouse cell lines. The question that why certain types of tissue requires a second isoform of talin- talin2 remains unknown.

Using SiRNA gene silencing method, we studied the role of talin1 and 2 in cell adhesion, spreading, proliferation and myofibroblast differentiation in human fibroblasts. In order to elucidate the functions of talin1 and 2 in matrix rigidity sensing, we used micro-engineered tuneable ECM based hydrogels where matrix stiffness can be fine-tuned to simulate conditions in healthy and fibrotic lungs.

According to our data, in the absence of talin1 knock down cells, cell spreading seems to be more affected on soft matrix compared to stiff matrix as evidenced by the appearance of rounded fibroblasts on soft matrix after 4 days. In contrast, talin2 deficient fibroblasts showed cell spreading earlier on both soft and stiff

matrices compared to control fibroblasts proving that the dominant role of talin1 action over talin2 in cell spreading.

Interestingly, those findings are inconsistent with the findings in previous studies using different mouse cell lines in which talin1 and talin2 are equally important in cell spreading and matrix rigidity sensing (319). In mouse, cell spreading became affected and cells transformed into rounded morphology from bipolar appearance only in the absence of both isoforms. According to those findings, they concluded that talin1 and talin2 were redundant and could substitute each other in controlling cell spreading in the absence of one isoform. However, in our study using human fibroblasts, it was clearly shown that talin 2 cannot replace the role of talin 1 cells in cell spreading function indicating the different functions of talin isoforms in human fibroblasts.

Furthermore, we also studied the role of talin1 and talin2 isoforms in fibroblast proliferation on soft and stiff matrices. Our data showed a clear lack of cell spreading as well as decreased in cell proliferation in the absence of talin1 in human fibroblast on both soft and stiff matrices. These data clearly show that disruption in cell spreading after knocking down talin1 in fibroblasts can prevent the stiffness induced cell proliferation which is one of the major hallmarks of lung fibrosis. However, interestingly talin2 knock down cells can undergo cell proliferation at normal rate. Overall it is clear that talin1 plays a major role in controlling cell proliferation in human lung fibroblasts which seems to be associated with cell spreading. There are many main downstream cell signalling pathways for cell proliferation following FAK phosphorylation including P38, ERK and JNK signalling (250). Moreover, there are some evidences in mouse epithelial cell lines which showed that talin - C terminus

was associated with cell cycle progression and also suppress P21- inhibitor of cell cycle from G1 to S phase (336). However, we did not observe an increase in the level of P21 gene expression in human fibroblasts after knocking down talin 1 and/or 2 (data not shown). This inconsistency may be either due to species variation between human and mouse or variation between epithelium and fibroblast. Altogether the question of which cell proliferation signalling pathway(s) is mainly activated in response to matrix rigidity and following talin1 activation remains elusive.

Myofibroblast differentiation is an important feature of fibrotic lung lesions, which was shown to be associated with matrix stiffening in fibrosis. Given the importance of talin being a major adhesion adaptor molecule in force transmission from stiff matrix, we also studied the effect of talin isoforms on stiffness induced myofibroblast differentiation in the same hydrogel system. As discussed in Chapter 2, rigid matrix can induce the myofibroblast transformation by as evidenced by an increase in expression of α SMA as well as α SMA fibre assembly. Regarding myofibroblast differentiation, we have shown that talin1 but not talin2 mainly mediates myofibroblast differentiation on stiff matrix, evidenced by undetectable levels α SMA protein level with complete absence of α SMA fibre assembly in the absence of talin1 in human fibroblasts. These data confirm the clear association between talin1 and myofibroblast transformation on stiff matrix. Intriguingly, our data also showed that knocking down talin 2 in fibroblasts gave the paradoxical findings on stiff matrix (very thick α SMA fibres with high level of α SMA expression). This is the first time we report the differential function of talin isoforms relating with myofibroblast differentiation and it also explains the need for a second isoform

(i.e. talin2) in some tissues. Even though talin2 expression level in human fibroblast is much lower than that of talin1 (Appendix 8), it seems to have protective role in controlling myofibroblast differentiation. Further studies are required to investigate the underlying mechanism for how talin 2 opposes talin 1 function in controlling α SMA expression in fibroblasts.

In summary, it is interesting to report the antagonistic function of talin 1 and talin 2 in myofibroblast differentiation on stiff matrix and the role of talin1 in controlling fibroblast proliferation induced by stiff matrix in human fibroblasts. Our preliminary investigation of talin function in fibrosis provides the additional insight for understanding for the mechanosensing mechanisms for controlling the fibroblast proliferation and myofibroblast differentiation in fibrosis.

Chapter 6
General Discussion
&
Future Directions

6 Chapter 6- General Discussion and Future directions

ECM plays an active role in controlling cell behaviours such as proliferation, differentiation and migration by the virtue of the contextual cues including dimensionality, molecular composition and stiffness (37). My doctoral work has focused on the role of two biophysical cues of ECM namely matrix dimensionality and stiffness in the context of inflammatory responses in the human lung which are linked to many lung pathologies such as asthma, acute respiratory distress syndrome and lung fibrosis.

Firstly, we have shown that matrix dimensionality plays a critical role in lung inflammatory responses. The pattern and intensity of inflammatory responses in lung fibroblasts, measured by NF- κ B transcription factor activation, was found to be different between 2D and 3D culture systems. That difference between 2D and 3D systems was correlated with the difference in surface TNF α receptor expression pattern on lung fibroblasts between 2D and 3D culture systems. Surface TNF α receptors on fibroblasts were expressed focally when cultured on 2D surface whereas their expressions in 3D culture system were more widespread and homogeneous.

In addition to the concept of formation of the gap junctions between the interconnected fibroblasts in 3D culture system discussed in chapter 2, the other possible underlying reason for localised receptor expression pattern in 2D culture in contrast to 3D culture system might be associated with the formation of mature focal adhesion complex when cultured on rigid substrate. This explanation was also supported by the observation of colocalization of receptor with focal adhesion molecules within mature focal adhesions (337). Therefore, the relationship between focal adhesion complex formation and receptor expression

pattern in 2D and 3D culture systems is another interesting area to be investigated for future research.

Moreover, we could generate the NF- κ B reporter transfected cell line in 3D culture system by in situ transfection on scaffold, which allows us to monitor real time inflammatory response after stimulation with different stimuli. The NF- κ B reporter was also stable in lung fibroblasts for more than 2 weeks after transfection according to our work (data not shown). As most of the 3D systems are available for end point analysis (17,338), this is the first time we report the long-term 3D culture system for continuous monitoring of inflammatory response in real time.

We used the NF- κ B reporter as a surrogate transcription factor for studying of the role of lung fibroblasts in inflammation in our experiments. Not limiting to NF- κ B activation, the developed 3D culture system with real time monitoring is also applicable to studying other inflammatory associated conditions such as allergen stimulation, drug testing, and infections. Using this 3D culture system, it may also possible to set up co-cultures with other cells such as epithelium or immune cells like macrophages or dendritic cells within 3D environment. Such multi-cell co-cultures on 3D biomimetic scaffolds could provide useful tools for better understanding the cellular cross talk in disease pathology in an *in vivo* like environment.

In addition to matrix dimensionality, I have studied how changing in the stiffness of matrix affects lung fibrosis in the second part of my doctoral work. In order to study this, an ECM based microengineered hydrogel with tuneable stiffness relevant to normal and fibrotic lung was developed and characterised. By using such disease relevant platform, my data highlighted the significance of changes

in matrix stiffness in driving fibrosis progression such as fibroblast proliferation and myofibroblast differentiation. In addition, we have discovered that the stiffness of cell culture substrate was also as equally important as growth factors present in serum in the cell proliferative response.

Controlling fibroblast proliferation and myofibroblast differentiation are the major targets for drug intervention in lung fibrosis (332). Mechanosensitive pathways involved in sensing matrix rigidity changes are the focus of current research to identify new targets for controlling disease progression in fibrosis. ROCK kinase has been shown to play an important role in the intrinsic mechanotransduction of ECM rigidity sensing in lung fibrosis but isoform selectivity cannot be achieved with pharmacological inhibitors like Y27632. To this end, the SiRNA gene silencing technique allowed us to study the isoform specific role of ROCK regulation in myofibroblast differentiation to investigate the differential regulation of ROCK isoforms (ROCK1 and ROCK2) in stiffness induced lung fibrosis. Interestingly, we have found that ROCK isoforms 1 and 2 can differentially regulate the α SMA expression on soft and stiff matrices. Knocking down either ROCK 1 or 2 inhibits smooth muscle actin (α SMA) expression in lung fibroblasts on soft matrix, whereas on stiff matrix it further enhances fibrotic phenotype as evidenced by increased α SMA expression. This finding highlights the importance of appropriate biologically relevant matrix stiffness levels in the study of cellular response in fibrosis research. For example, if we study the effect of growth factors such as TGF- β 1, CTGF and PDGF in fibrosis using rigid tissue culture plate which has abnormally high stiffness value, it is difficult to exclude the fibrotic effect driven by normal tissue culture plate.

In addition to the regulation of F-actin polymerisation by ROCK signalling, α SMA fibre assembly was found to be completely controlled by ROCK signalling evidenced by the absence of α SMA fibres in the fibroblasts in the absence of both ROCK isoforms. Unexpectedly, absence of one ROCK isoform can paradoxically increase the α SMA expression and enhance the myofibroblast differentiation. Taken together, we can conclude that ROCK signalling regulates the contractile function of myofibroblasts by controlling both normal stress fibre and α SMA fibres assembly in isoform non-specific manner. In other words, both isoforms are synergistically required for the assembly of both types of fibres. The same level of total α SMA observed on stiff matrix in the absence of both ROCK isoforms compared to control could mean that additional signalling pathways besides ROCK activation are involved in the stiffness induced myofibroblast differentiation. Regardless, although pharmacological ROCK inhibitor like Y27632 does not have isoform selectivity effect, it can be considered as a potential drug intervention in controlling the contractile function of myofibroblasts.

Along with increased α SMA expression in the absence of one ROCK isoform, MRTF-A nuclear localisation was also enhanced on stiff matrix in contrast to soft matrix. This new association raises further questions about preventing MRTF-A nuclear localisation as another potential target to control α SMA expression induced by rigid matrix. As reduction of nuclear import of MRTF-A would also impair the TGF- β 1 induced α SMA expression and ECM deposition (339), in line with our observation, it would be interesting to test the hypothesis about MRTF-A nuclear localisation as final common pathway for α SMA expression induced by both humoral (TGF- β 1) and biomechanical stimuli (stiffness) in lung fibrosis

(Figure 4.3). Although some of our new observations broaden the overall understanding of the effect of ROCK signalling over ECM stiffness induced myofibroblast differentiation by exploring the α SMA expression at different level of expression, there is still much work remaining in terms of elucidating the molecular mechanisms underpinning the whole process.

Interestingly, our data show that both ROCK isoforms regulate myofibroblast differentiation but not proliferation (340). It is therefore reasonable to assume that stiffness induced fibroblast proliferation is controlled at the level preceding ROCK activation. As integrin mediated focal adhesion (FA) complex at cell-ECM interface plays a critical role in early force transmission and cell communication with their mechanical environment, one FA adaptor called talin was targeted as a next potential candidate molecule in controlling matrix rigidity induced pathology. Among many potential mechanosensing molecules in FA, talin has recently attracted great interest in mechanotransduction because of its particular position that allows linking the cell surface integrin receptor and actin cytoskeleton for cell contraction on one hand and its structure which shows great plasticity and ability to unfold in response to mechanical force, leading to exposure of cryptic binding site (e.g. vinculin) and further FA maturation (286,287,291), on the other hand. To this end, we investigated the role of both talin isoforms in stiffness induced fibroblast activation using SiRNA gene silencing in human lung fibroblasts.

Interestingly, our data clearly indicate that knocking down talin1 in human fibroblasts renders them nonresponsive to changes in substrate stiffness affecting both cell proliferation and myofibroblasts differentiation evidenced by diminished cell proliferation and α SMA expression in talin1 deficient fibroblasts.

It seems likely that absence of talin1 uncouples force transmission between stiff ECM and actin cytoskeleton reported by Artola *et al* (329) and prevents downstream FA signalling. Despite many existing data on stiffness induced cell proliferation and myofibroblast differentiation, our finding shows, for the first-time, that both fibroblast proliferation and myofibroblast differentiation on stiff matrix can be controlled by targeting talin.

Many different proliferation pathways including MAPK-ERK1/2, JNK pathways and P38 pathways are implicated following activation of FA signalling (250). However, in some examples of cancer therapy, some tyrosine kinase inhibitors like MAPK inhibitor, which can control cell proliferation effectively in most cancers, fail to control cell proliferation in some cancers where associated with stiff matrix such as hepatocellular carcinoma and breast cancer. This further suggests that the stiffness induced cell proliferation was driven by multiple or specific cell proliferation pathways (250). Understanding cell proliferation pathways following talin activation would be a logical continuation of my work to find novel targets for controlling stiffness induced cell proliferation.

In addition to biomechanical factors, contribution of growth factors such as TGF β 1, PDGF, CTGF are also well documented in lung fibrosis. There are also some evidences about the cross talk between the integrin activation and tyrosine kinase receptor signalling that are well known to be regulated by many growth factors including EDGF, TGF β 1, CTGF in cell proliferation (249,250,337). The observation that EGFR signalling can be activated on rigid matrix without ligand engagement in some types of cancers associated with rigid matrix further raises the possibility of cross-talk between these two signalling pathways. The observation of apoptosis of adherent cells when cultured in suspension even in

the presence of high concentrations of growth factors provides the additional evidence for the cross-talk between integrin activation and growth factors signalling (249). To date, little is known about the downstream mechanotransduction signalling to talin activation in controlling cell proliferation and its cross-talk with growth factors signalling which are likely to be more complex than we expected. We believe that exploring this area in the future work would improve the overall understanding of how stiffness of ECM regulates fibroblast activation and also provides new insight into the interplay between the effect of ECM stiffness and growth factor in controlling cell proliferation.

Furthermore, I also investigated the effect of talin1 and talin2 isoforms in stiffness induced fibroblast activation/myofibroblast differentiation. Interestingly, in contrast to talin1, low level of talin2 in fibroblasts has a protective role in controlling contractile phenotype of myofibroblast evidenced by high α SMA expression and fibre assembly in talin2 deficient cells on rigid matrix. Reciprocal antagonist relationship between talin1 and talin 2 in controlling stiffness induced fibroblasts activation despite the high homology in their sequence provides some rational for targeting talin2 as a protective molecule in lung fibrosis. Interestingly, these observations are in contrast with findings from mouse model where talin 1 and talin 2 are shown to be equally important and substitutable in cell spreading and matrix rigidity sensing. This opens door to the question about the physiological relevance of data from animal models in this area and shows the importance of using human based models for investigating the mechanisms of stiffness induced fibrosis, its maintenance and resolution.

Moreover, while we now have a glimpse into how talin affects the stiffness induced fibroblast activation in general, this idea needs to be explored in disease specific approach. Idiopathic pulmonary fibrosis, for instance, where the disease progression is mainly mediated by aberrant fibroblast activation might have strong relation with appropriate talin activation. Therefore, we believe that investigating the role of talin in IPF patients can provide new insights into the molecular basis of aberrant fibroblast activation and pave the way for the rational design of novel treatment strategies to stop or ameliorate disease progression in IPF. Not limiting to IPF and lung fibrosis, it will also be reasonable to explore this new finding in other stiffness associated disorders such as breast cancer and liver cirrhosis.

ECM stiffness has also been shown to be effect on many other cell types such as alveolar epithelium and macrophage in lung fibrosis. As discussed on chapter 2, one of the major features in fibrosis associated diseases, EMT transition, was also shown to be associated with stiffening of ECM. Further evaluation of the role of ROCK and talin signalling in the regulation of stiffness induced EMT transition, also highly recommended for the future fibrosis research.

In conclusion, this thesis elucidated the significance of matrix dimensionality and substrate stiffness in inflammatory and fibrotic responses in human lung fibroblasts. We have successfully established a 3D electrospun scaffold and an ECM based hydrogel platform with defined stiffness relevant to lung fibrosis to study the fibrosis related pathology and investigated the specific mechanisms and molecular pathways involved in lung inflammatory responses and fibrosis. Future advances and development of these systems into coculture model could further

clarify the molecular interactions of lung fibrosis and produce more accurate disease models.

Chapter 7

References

7 Chapter 7- References

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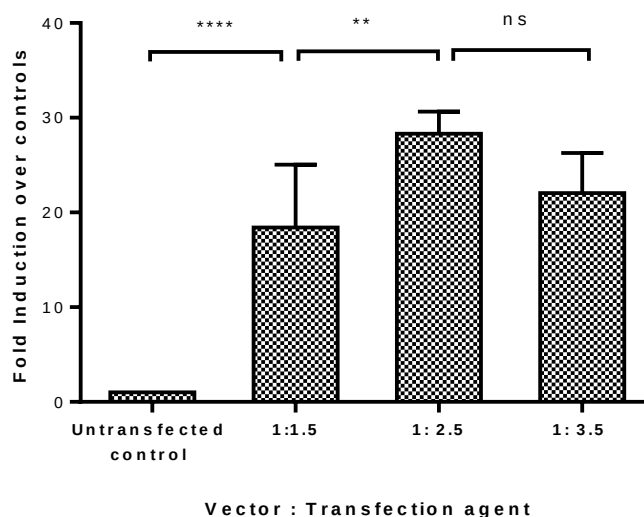
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8 Appendix

Appendix 1



Appendix 1. Optimization of the transfection efficiency in MRC5 lung fibroblasts by using different ratios of control vector to transfection reagent. The amount of vector and cell number was held constant at 0.8 μg and 1.5×10^5 cells per well respectively, and the ratios of vector to transfection agent were tested in three concentrations. Transfection efficiency was compared among three ratios of control vector: lipofectamine[®] 2000 transfection reagent. Data expressed as fold induction versus un-transfected control value. Data are mean $\pm$ SD of combined result from three independent experiments and data are collected as triplicates samples from duplicate wells for each experiment. **** $p < 0.0001$, ** $p < 0.01$, ns=not significant compared with untransfected control.

Appendix 2

TMSPMA coating of glass slides

Materials:

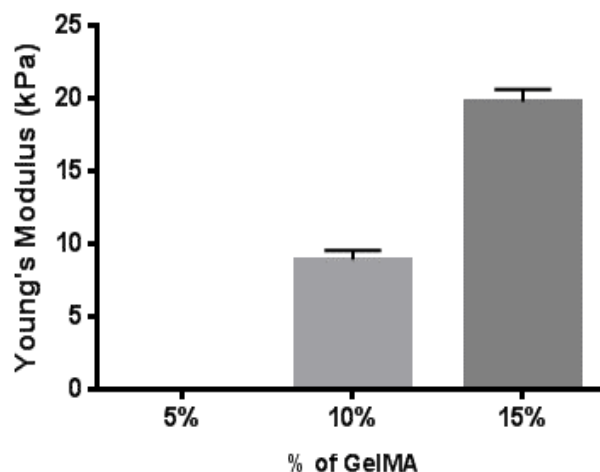
- Glass slides (Fisher Cat# 12-550-A8)
- Sodium hydroxide (NaOH) pellets - 10% (w/v) solution (Sigma Cat# S5881-1KG)
- 3-(Trimethoxysilyl)propyl methacrylate (aka TMSPMA) (Aldrich 440159-100ML)
- Reagent alcohol (Fisher Cat # A962P-4)
- Required PPE: Lab coat, safety glasses, gloves.

Protocol:

1. Put a glass beaker in a bucket of ice (*Caution*: this is a very exothermic reaction releasing a lot of heat), weigh 50 g of NaOH pellets, slowly add 450 mL of distilled water and have all pellets dissolved.
2. In the same beaker, place the slides in a staggered manner, make sure all of them contact with the NaOH solution.
3. Cover the beaker with a pyrex dish (*Caution*: do NOT use aluminum foil to cover as NaOH reacts with aluminum producing gaseous side products)
4. Let it sit overnight in the fume hood.
5. Discard the 10% NaOH solution into the proper waste bottle. Then with gloves, thoroughly rinse and rub each slide one by one under distilled water.
6. Dip each slide in 3 distinct 100% reagent alcohol baths and let it air dry (faster under the hood).
7. Wrap the glass slides with aluminum foil and bake for 1h @ 80°C.
8. TMSPMA treatment (under the fume hood):
 - a. Stack slides vertically and pour 3 mL of TMSPMA on top of the stack using a syringe.
 - b. After 30 min, flip the stack upside down to get even coating.
9. Cover the beaker with aluminum foil and bake the whole assembly in the beaker overnight @ 80°C.
10. Clean the slides in reagent alcohol again with 3 baths and air dry.
11. Wrap the glass slides with aluminum foil and bake again for 1-2 hrs @ 80°C.
12. Store the TMSPMA coated glass slides wrapped in aluminum foil at room temperature.

* This procedure needs to be performed prior to the coating of glass slides with poly(2-hydroxyethyl) methacrylate (PolyHEMA) or polyethylene glycol (PEG).

Appendix 3



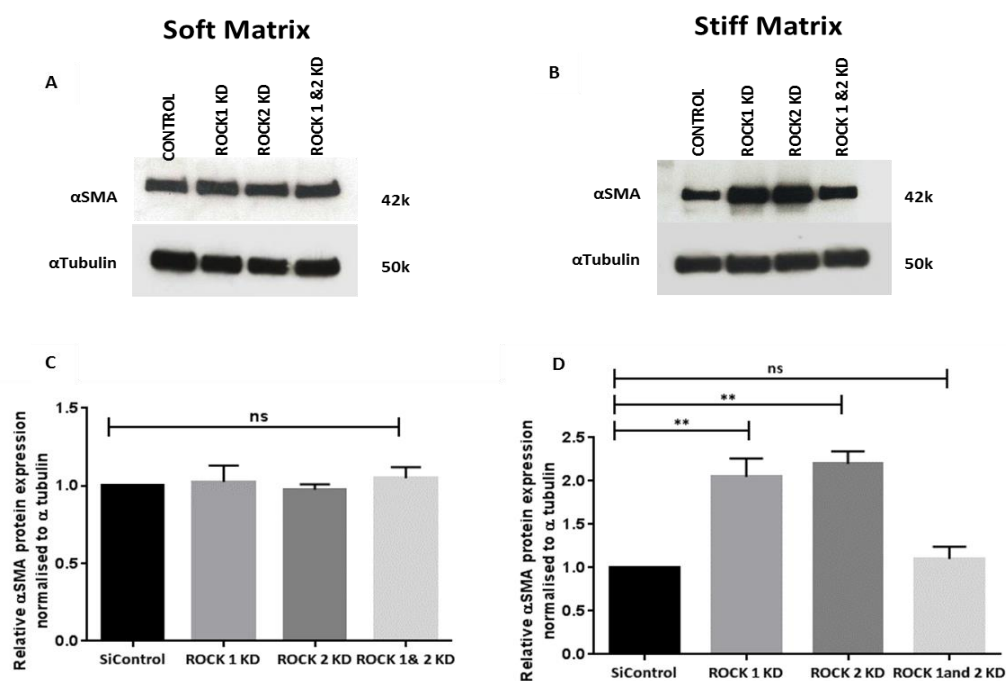
Appendix 3. Mechanical properties of GelMA based Hydrogel platform with different percentages of GelMA in Young's Modulus. The stiffness of hydrogel [5%, 10% and 15% (w/v) GelMA] was measured in Young's modulus. 10% and 15% (w/v) Mean $\pm$ SD, n=3. 200ul of different percentages of GelMA were prepared using the same experimental parameters and left at 37 °C in DPBS for 24 hrs. Hydrogels were tested at a rate of 20% strain/min on an Instron 5542 mechanical tester for Young's modulus. GelMA hydrogels made from prepolymer solutions of different concentrations were fixed between two clamp-type holders vertically for applying tensile force for measuring Young's Modulus. Both modulus was determined as the slope of the linear region corresponding with 0-5% strain. Three independent sets of experiments for each condition were performed in triplicate. Data from 3 independent experiments were pooled and statistically expressed as mean $\pm$ standard deviation (SD). (This measurement was assisted by Harvard MIT group)

Appendix 4

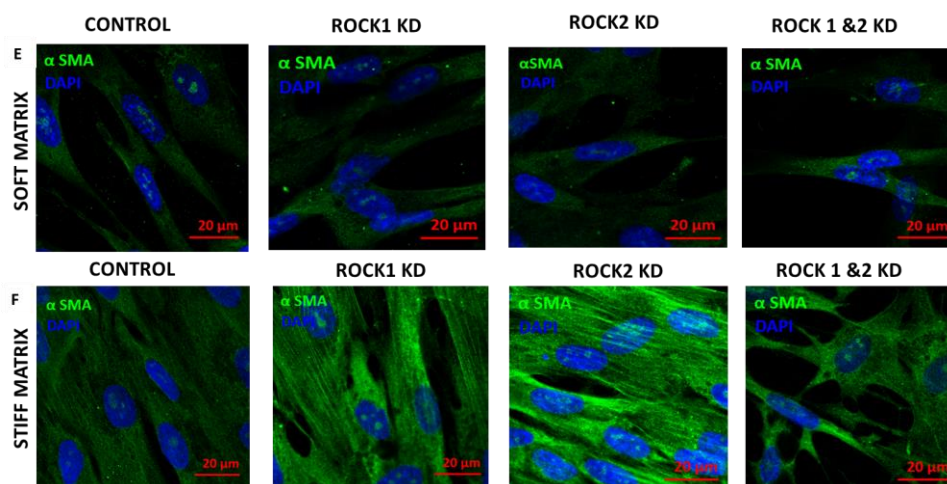
Fabrication and Mechanical Properties of Polyacrylamide Hydrogel System

	Soft Matrix	Stiff Matrix
Acrylamide (%)	4	8
Bisacrylamide (%)	0.3	0.264
Young Modulus (E)	3.24 ± 0.58	19.66 ± 1.19

Polyacrylamide Hydrogel System



Appendix 4 (continued)



Appendix 4. ROCK isoform regulation of α SMA (gene and protein expression) and stress fibre formation in lung fibroblasts cultured on Soft and Stiff polyacrylamide gels. Lung fibroblasts are cultured on soft and stiff polyacrylamide system for 4 days for protein level detection (A, B) after ROCK isoform gene silencing. α Tubulin was used as protein loading control in WB analysis (n=2). (C, D) Relative levels of α SMA protein expression of 4 days after gene silencing were determined by densitometry (n=2) normalised to α Tubulin. Results are Mean $\pm$ SD, **p<0.01, ns=not significant. (E, F) Confocal analysis of α SMA stress fibre formation by immunofluorescence staining at 4 days after gene silencing. Scale bar: 20 μ m, green= α SMA and blue= DAPI nuclei staining

Appendix 5

OLIGONUCLEOTIDE SYNTHESIS REPORT FOR PRIMERS

Oligo Name	Sequence (5'→3')	Yield [OD]	Yield [ug]	Yield [nmol]	Vol for 100 pmol/ul	Tm [°C]	MW [g/mol]	GC Content
1 αSMA Forward	5'- GACCGAATGCAGAAAGGAGAT-3' [20]	7.8	197	31.7	317	57.3	6224	50%
2 αSMA Reverse	5'-CCACCCGATCCAGACAGAGTA-3' [20]	7.1	191	31.5	315	59.4	6080	55%
3 18S Forward	5'-GTAACCCCGTTGAACCCCAT-3' [20]	6.7	192	31.8	318	57.3	6037	50%
4 18S Reverse	5'-CCATCCCAATCGGTAGTAGCG-3' [20]	7.2	202	33.1	331	59.4	6102	55%
5 ROCK1 Forward	5'-GATCCCAAAATCGGAAGTGAA-3' [20]	7.8	201	32.6	326	55.3	6159	45%
6 ROCK1 Reverse	5'-CAAATCATATACCAAAAGCATCCAA-3' [24]	9.6	244	33.6	336	55.9	7267	33%
7 ROCK2 Forward	5'- CCCATCAACGTGGAGAGC-3' [18]	6.2	171	31.2	312	58.2	5494	61%
8 ROCK2 Reverse	5'-AAGCAGGAAAAATCTAAATCAAGGA-3' [24]	10.1	243	32.7	327	55.9	7436	33%
9 Talin1 Forward	5'-TCTCCCAAAATGCCAAGA-3' [20]	4.7	125	20.7	207	55.3	6039	45%
10 Talin1 Reverse	5'-TGGCTATTGGGGTCAGA-3' [20]	3.7	103	16.5	165	59.4	6213	55%
11 Talin2 Forward	5'- CTGAGGCTCTTTTCACAGCA-3' [20]	5.4	157	25.9	259	57.3	6068	50%
12 Talin2 Reverse	5'-CGGTGTTTGATATGGGAACC-3' [20]	6.2	180	30	300	57.3	5997	50%

Appendix 6

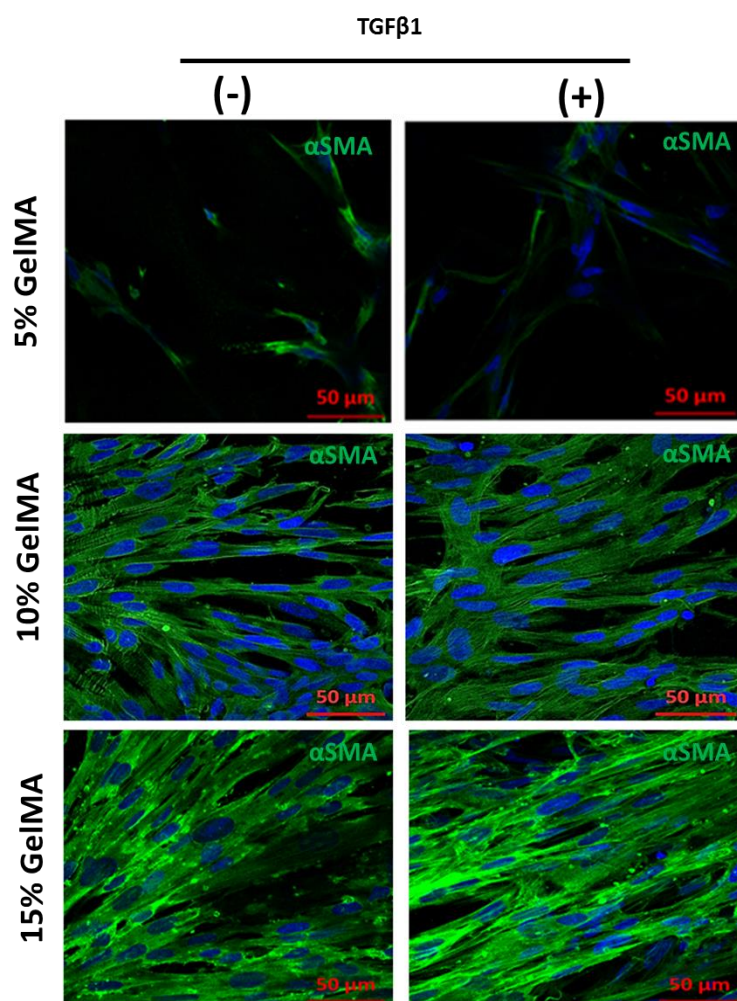


Figure 3.9 TGF- β 1 does not seem to have any additive effect on α -SMA expression in MRC5 fibroblasts on 5% and 15% GelMA hydrogel. MRC5 fibroblast were cultured on different percentages of GelMA (5% GelMA and 15% GelMA) with or without TGF- β 1 (2ng/ml) for 5days. Cells are fixed and stained with DAPI-nuclear blue staining & α -SMA - Alexa Flour488 Green staining, and analysed by confocal microscopy (n=2).

Appendix 7



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Product Transfer

Dharmacon Ref#: SO-2537606G

MSDS, Product Manuals, and Protocols are available online at dharmacon.gelifesciences.com/resource-library.

M-004610-02-0005, siGENOME Human ROCK2 (9475) siRNA - SMARTpool, 5 nmol

siGENOME SMARTpool siRNA D-004610-02, ROCK2

Target Sequence: GUAGAAACCUUCCAAUUC

Mol. Wt. Ext. Coeff.

13,373.0 (g/mol) 375,135 (L/mol-cm)

siGENOME SMARTpool siRNA D-004610-03, ROCK2

Target Sequence: GCAACUGGCUCGUUCAAUU

Mol. Wt. Ext. Coeff.

13,388.0 (g/mol) 367,036 (L/mol-cm)

siGENOME SMARTpool siRNA D-004610-05, ROCK2

Target Sequence: GCAAAUCUGUAAUACUCG

Mol. Wt. Ext. Coeff.

13,385.9 (g/mol) 380,564 (L/mol-cm)

siGENOME SMARTpool siRNA D-004610-18, ROCK2

Target Sequence: GCAGCAAUGGUAAGCGUAA

Mol. Wt. Ext. Coeff.

13,415.9 (g/mol) 367,481 (L/mol-cm)

M-003536-02-0005, siGENOME Human ROCK1 (6093) siRNA - SMARTpool, 5 nmol

siGENOME SMARTpool siRNA D-003536-01, ROCK1

Target Sequence: GGACACAGCUGUAAGAUUG

Mol. Wt. Ext. Coeff.

13,388.0 (g/mol) 369,350 (L/mol-cm)

siGENOME SMARTpool siRNA D-003536-02, ROCK1

Target Sequence: GAAGAAACAUUCCUAAUUC

Mol. Wt. Ext. Coeff.

13,358.0 (g/mol) 380,297 (L/mol-cm)

siGENOME SMARTpool siRNA D-003536-03, ROCK1

Target Sequence: GAGAUAGCAAGUCAAUUA

Mol. Wt. Ext. Coeff.

13,358.0 (g/mol) 379,051 (L/mol-cm)

siGENOME SMARTpool siRNA D-003536-05, ROCK1

Target Sequence: GCCAAUGACUACUAGGA

Mol. Wt. Ext. Coeff.

13,373.0 (g/mol) 375,313 (L/mol-cm)

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Product Transfer

Dharmacon Ref#: SO-2557692G

MSDS, Product Manuals, and Protocols are available online at dharmacon.gelifesciences.com/resource-library.**-012949-00-0005, siGENOME Human TLN1 (7094) siRNA - SMARTpool, 5 nmol****siGENOME SMARTpool siRNA D-012949-01, TLN1**

Target Sequence: GGAAUAACAGGGACCUUA

<u>Mol. Wt.</u>	<u>Ext. Coeff.</u>
13,373.0 (g/mol)	376,737 (L/mol·cm)

siGENOME SMARTpool siRNA D-012949-02, TLN1

Target Sequence: GAAGAGAUAGGUUCCCAUA

<u>Mol. Wt.</u>	<u>Ext. Coeff.</u>
13,373.0 (g/mol)	378,517 (L/mol·cm)

siGENOME SMARTpool siRNA D-012949-03, TLN1

Target Sequence: GAAGAUGGUUGCGGCAUU

<u>Mol. Wt.</u>	<u>Ext. Coeff.</u>
13,403.0 (g/mol)	363,298 (L/mol·cm)

siGENOME SMARTpool siRNA D-012949-04, TLN1

Target Sequence: GAGAUGAGGAGUCUACUAU

<u>Mol. Wt.</u>	<u>Ext. Coeff.</u>
13,373.0 (g/mol)	380,742 (L/mol·cm)

112909-01-0005, siGENOME Human TLN2 (83660) siRNA - SMARTpool, 5 nmol**siGENOME SMARTpool siRNA D-012909-02, TLN2**

Target Sequence: GGACGACCCUCCAUGUAC

<u>Mol. Wt.</u>	<u>Ext. Coeff.</u>
13,418.0 (g/mol)	363,921 (L/mol·cm)

siGENOME SMARTpool siRNA D-012909-03, TLN2

Target Sequence: GAUGUGCGAUCACCACUAU

<u>Mol. Wt.</u>	<u>Ext. Coeff.</u>
13,388.0 (g/mol)	373,088 (L/mol·cm)

siGENOME SMARTpool siRNA D-012909-04, TLN2

Target Sequence: GGCCACAAUUGAAUGCAUA

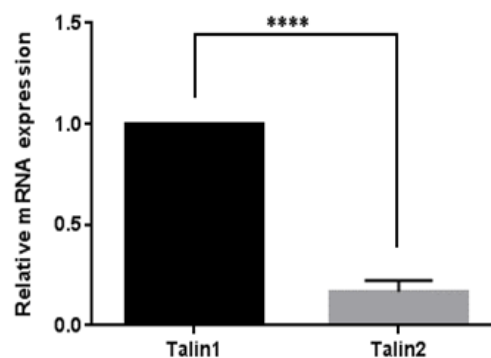
<u>Mol. Wt.</u>	<u>Ext. Coeff.</u>
13,373.0 (g/mol)	372,109 (L/mol·cm)

siGENOME SMARTpool siRNA D-012909-17, TLN2

Target Sequence: CCAGAAAGAGUGCGAUAAU

<u>Mol. Wt.</u>	<u>Ext. Coeff.</u>
13,400.9 (g/mol)	375,847 (L/mol·cm)

Appendix 8



Appendix 8 Talin 1 and talin 2 basal expression level in human lung fibroblasts.

The basal expression level of talin 1 and talin 2 was detected in human lung fibroblasts using RT PCR at 2 days after culturing cells in tissue culture plate. Basal talin1 and talin 2 isoform expression level was detected by RT RCR. 18s rRNA was used as reference gene control. Talin1 was set at 1 for comparative analysis of RT PCR. n=2, mean $\pm$ SD, ****p<0.0001