MATRIX-MEDIATED REGULATION OF TYPE I COLLAGEN SYNTHESIS AND DEGRADATION IN CULTURED FIBROBLASTS.

Thesis presented by

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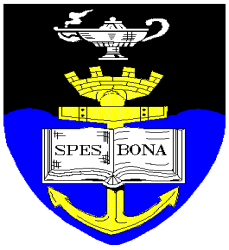

DEDICATION

To my wife Abigirl and my son, Kevin jnr, I thank you for your support, encouragement and love.

DECLARATION

I, Kevin Dzobo, hereby declare that the work on which this thesis is based is my original work (except where acknowledgments indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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For there is one God, Jehovah Almighty and Jesus Christ my Saviour.
Not to us, Oh Jehovah, but to your Name be the glory because of your steadfast love and faithfulness! Psalms 115:1

Abbreviations

A_{260}	Absorbance at 260 nm
α	Alpha
Ab	Antibody
ATCC	American Type Culture Collection
APS	Ammonium persulphate
β	Beta
BHT	Butylated hydrotoluene
bp	base pair
BSA	Bovine serum albumin
CBF	CCAAT Binding Factor
cDNA	complementary deoxyribonucleic acid
C_t	Threshold cycle
CoA	Coenzyme A
dCTP	deoxy-cytidine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dn	dominant negative
DNA	Deoxyribonucleic acid.
dNTP	deoxy-nucleotide tri-phosphate
ds	double stranded
DTT	Dithiothreitol
ε	Extinction coefficient
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal related kinase
ESI-MS	Electrospray ion mass spectrometry
FACITS	fibril-associated collagens
FAK	Focal Adhesion kinase
FBS	Fetal bovine serum
FCS	Fetal calf serum
aFGF	acidic-Fibroblast Growth Factor

bFGF	basic-Fibroblast Growth Factor
γ	Gamma
g	gram
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde -3-phosphate dehydrogenase
Gly	Glycine
GPI	glycosyl-phosphatidyl inositol
GSH	Glutathione
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IUPAC	International Union of Pure and Applied Chemistry
IOAC	International Organisation for Agriculture and Chemistry
IC_{50}	Concentration required for fifty percent inhibition.
kDa	Kilo Dalton
kV	kilovolts
λ	Lambda
L/h	Litres per hour
Luc	Luciferase
M	Molar
m^{-2}	per square meter
MAB	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
Me	Metal
MeCP	Methylated cytosine phosphate
MEK	Mitogen-activated protein kinase kinase
MIDAS	metal-ion-dependent adhesion site
µg	microgram
µL	micro-litre
µM	Micro molar
mg	Milligram
ml	Millilitre.
mm	Millimetre
MMP	Matrix Metalloprotease

M_r	Molecular weight
MRE	Matrix responsive element
MT-MMP	membrane-type matrix metalloprotease
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N_2	Atmospheric nitrogen
%	Percent
OD	Optical density
PBS	Phosphate buffered saline
CMV	cytomegalovirus
PDGF	Platelet-derived growth factor
p-ERK	phosphorylated Extracellular signal related kinase
PI-3 K	Phosphatidyl inositol 3 kinase
PMSF	Phenylmethylsulfonyl fluoride
RER	rough endoplasmic reticulum
RIPA	Radioimmunoprecipitation assay
rpm	revolutions per minute
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
s^{-1}	per second
ss	single stranded
SDS	Sodium dodecyl sulphate
SV40	Simian Virus 40
TIMP	Tissue Inhibitors of Metalloprotease
TGF-α	Transforming Growth Factor-α
uPA	urokinase plasminogen activator
UV-Vis	Ultra violet-visible
V	Volts
w/v	Weight to volume ratio.
Zn	Zinc

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7.2 Methods

ABSTRACT

Stromal cells and the extracellular matrix (ECM) components provide the microenvironment that is pivotal for cell growth, motility, attachment and differentiation. Fibroblasts are some of the cells responsible for the synthesis of most of the extracellular matrix proteins. Type I collagen is the most abundant extracellular matrix protein in the human body and is found in tissues requiring high tensile strength. In this study we investigated the effect of a pre-formed fibroblast-derived extracellular matrix on the expression of type I collagen and associated matrix metalloproteinases in fibroblasts. Both cellular and secreted type I collagen protein levels were decreased in fibroblasts grown on a pre-formed fibroblast-derived matrix compared to controls. This decrease was associated with a decrease in pro-α2(1) collagen mRNA while pro-α1(1) mRNA remained unchanged. These results suggested that the regulation of type I collagen occur at both transcriptional and post transcriptional levels. To determine transcriptional regulation of the α2(1) collagen promoter, deletion constructs were used in promoter assays. Deletion analysis identified the -375 to -107 region in the COL1A2 promoter as containing a potential matrix responsive element (MRE). Using siRNA technology, this study provided evidence that Sp1, possibly in combination with other transcription factors, binds to the COL1A2 promoter to repress the expression of the COL1A2 gene in the presence of the fibroblast-derived ECM. The stabilities of collagen α1(1) and α2(1) mRNAs were measured by blocking transcription with actinomycin D and measuring the rate of disappearance of the transcripts. Similar decay curves were obtained for both α1(1) and α2(1) mRNAs, matrix versus control, implying that the fibroblast-derived matrix does not affect the stabilities of the type I collagen mRNAs.

Kinase inhibition studies demonstrated that the fibroblast-derived matrix activates the MEK/ERK signaling pathway in fibroblasts. This was confirmed through the use of the dominant negative mutant MEK (dn MEK). The use of the dominant negative Ras, N17Ras, showed that the activation of the MEK/ERK signaling pathway is Ras-dependent. The fibroblast-derived matrix did not induce a change in protease expression patterns as shown by the mRNA levels, promoter activity and gelatinase activity. Cell-matrix interactions play fundamental roles in the development and function of various tissues. These interactions transmit an array of information that

influence gene expression patterns. The ECM has been proposed to act as a reservoir for a number of growth factors that can be released from the ECM to allow extracellular signaling to proceed without the need for new protein synthesis. No apparent effect could be attributed to the conditioned media or a suspended matrix when used in these experiments. Thus it appears no soluble factor, that would affect type I collagen synthesis, was released into the growth media, and that the fibroblasts required direct contact with the matrix for the response to occur. By using purified matrix components, type I collagen and fibronectin, it was shown that there is a specific negative feedback mechanism via type I collagen within the fibroblast-derived matrix.

We further explored the effects of the α2β1 and α3β1 integrin receptors on the expression of type I collagen in fibroblasts cultured on a fibroblast-derived matrix. Blocking α2β1 integrin function through the use of monoclonal antibodies reversed the effect of the matrix on type I collagen gene expression whilst blocking α3β1 integrin function did not reverse this effect. Furthermore, fibroblasts cultured on the fibroblast-derived matrix dramatically up-regulated the steady state levels of mRNA encoding the α2 and β1 integrin subunits, compared to fibroblasts cultured on plastic. The steady state levels of the mRNA encoding the α3 integrin subunit did not change, matrix versus plastic. The transcription of the α2 and β1 integrin subunit genes was influenced by the substratum. Our results demonstrate that the extracellular matrix controls the expression of α2 and β1 integrin subunits and that the regulation is exerted at least on the transcriptional level. This study provides novel insights into the mechanisms by which a fibroblast-derived matrix activates the MEK/ERK signaling pathway in fibroblasts and is the first such report to show the presence of a matrix-responsive element within the proximal α2(1) collagen promoter region. The MEK-ERK pathway and transcription factors binding to the MRE might be targeted in diseases where there is altered type I collagen synthesis such as fibrosis.

CHAPTER 1 INTRODUCTION

1.1The Extracellular matrix

The Extracellular Matrix (ECM) is the relatively stable structural material that lies under the epithelia and surrounds connective tissue cells (Hay, 1991). It is composed of a variety of macromolecules, with the four major classes of macromolecules being the collagens, proteoglycans, elastic fibres and glycoproteins. Most eukaryotic cells are in contact with an ECM whose composition and structure vary with developmental stage (Gout and Huot, 2008). The cells producing these ECM components continue to interact with their own ECM and the ECM produced by other cells. The most typical form of ECM is that found in connective tissue, comprising mainly type I collagen and the fibroblasts that produce the ECM. The connective tissue is surrounded by specialised ECM called the basal lamina, which underlies epithelial cells.

Many properties of the cell such as shape and movement are related to and are dependent on the organisation of the ECM. The interaction between the ECM scaffolds and cells represents the basis for organ structure and function (Stetler-Stevenson *et al*, 1993). In a healthy organism, the ECM forms a stable physical environment for cells and the ECM also undergoes a slow and regulated turnover leading to structural remodelling. The best recognised role of the ECM is the ability of its major constituent, the collagens, to polymerise and provide structural support to connective tissue, vasculature and cartilage and to generate the basement membrane. The ECM plays at least three important roles:

- Mechanical: tensile and compressive strength and elasticity.
- Protection: buffering against extracellular change and retention of water.
- Organisation: control of cell behaviour by binding of growth factors and interaction will cell-surface receptors.

Extracellular matrices have many specialised roles. The matrix of bone and tooth enamel for example are highly mineralised to withstand compression,

whilst the ECM that forms the cornea of the eye is transparent to light and highly elastic ECM is found in tendons. The ECM is also involved in regulating other biological functions. It serves as a reservoir for a variety of growth factors, such as TGF-β, TNF-α, VEGF and IL-3. Furthermore, ECM proteins can directly generate cellular responses. Type IV collagen has been shown to stimulate DNA synthesis in liver cells while fibronectin can act to inhibit tumour angiogenesis and metastasis (Ruoslahti, 1999). The ECM can also bind to and regulate the availability of matrix metalloproteinases (MMPs), which are involved in regulating matrix turnover and in modulating other biological functions.

The extracellular matrix is composed of a three-dimensional scaffold of collagen, fibronectin and other proteins, interlaced with proteoglycans. The properties of the extracellular matrix components provide a considerable amount of complexity to the potential interactions between cells and the ECM as well as between various matrix molecules (Yamada, 1991). The most important cells responsible for extracellular matrix synthesis are the fibroblasts and other mesenchymal cells. The balance between extracellular matrix synthesis and degradation plays a crucial role in extracellular homeostasis, and disruption of this balance causes alteration in ECM integrity. This can result in deleterious consequences including establishment of fibroproliferative disorders such as organ fibrosis. ECM synthesis is regulated through the action of growth factors and cytokines whilst degradation of the ECM is accomplished in part by matrix metalloproteinases.

Although many studies have been directed at understanding the interaction between the ECM and cells, most of these use artificial matrices of purified extracellular matrix proteins such as fibronectin and laminin (Fukuda *et al*, 2006; Elliot *et al*, 2005; Feng *et al*, 2005; Che *et al*, 2006). In fact, most studies defining mechanistic responses of cells and cancer cells to ECM signals have utilised isolated cultures of cloned and immortalised cells grown on a rigid surface comprising one isolated ECM component such as laminin or type I collagen (Bissell *et al*, 2002). These studies have vastly increased our knowledge on the molecular constituents and structures within focal adhesion

complexes, integrin receptors and their interaction with the ECM. However, a 2-D surface imposes an artificially rigid environment on the cell that contrasts with the pliability, molecular complexity and three-dimensionality (3-D) of the normal stromal environment *in vivo (*Beacham and Cukierman, 2005; Wozniak *et al*, 2004). Thus to study cells in a microenvironment that more reliably recapitulates a natural microenvironment rather than tissue culture plastic, investigators have cultured cells in 3-D support systems (Fischbach *et al*, 2007; Kim, 2005; Yamada and Cukierman, 2007). Recent studies have demonstrated the ability of the 3-D ECM to regulate gene expression in a variety of cell types and conditions. In monocytes, a different subset of genes is regulated depending on the 3-D ECM on which the cells are cultured (Adams and Watt, 1993). Cells also elicit different signaling pathways in response to matrix rigidity, indicating that both the biophysical as well as the biochemical states of the ECM are important in tissue homeostasis (Wozniak *et al*, 2004).

Many pathological processes such as fibrosis, invasion and metastasis are characterised by dynamic changes in ECM synthesis, deposition and turnover, thereby altering the mechanical properties of the ECM. Changes in rigidity of the stromal ECM could modify *in vivo* matrix adhesion properties and structure with subsequent changes in signaling mechanisms and cellular phenotypes (Wozniak *et al*, 2003, 2004). In addition, the matrix organisation is very important for efficient performance of cellular function. Fibroblasts grown in a relaxed matrix display a decreased level of cell proliferation in response to stimulation by PDGF when compared to those grown in monolayers. This was shown to be due to decreased levels of receptor autophosphorylation (Grinnel *et al*, 1999, 2005), suggesting that cell proliferation may be regulated by interaction between the cells and the ECM.

In vivo-like 3-D culture systems can achieve a close approximation to biochemical and biophysical properties of the *in vivo* microenvironment, without sacrificing opportunities for experimental manipulation (Beacham and Cukierman, 2005). During tumour invasion, the degradation of the basement membrane facilitates a more direct contact between neoplastic epithelial cells

and the adjacent mesenchymal compartments. In addition, during tumour growth, fibroblasts have been shown to be associated with the tumour mass and help in the production, and eventually alter the tumour ECM. This altered ECM is also used by epithelial cells and the fibroblasts for growth and as a pre-intravasation microenvironment. These and several other observations suggest that *in vivo*-like mesenchymal matrices represent a more accurate way to mimic *in vivo* microenvironments, providing an advantage over artificial culture systems in assessing the physiological growth properties of both stromal cells such as fibroblasts and cancer cells.

1.2 The Collagens

Collagens are the most abundant of the ECM proteins. Collagen fibrils and proteoglycans are ubiquitous components of the ECM and are present in all connective tissues. So far 27 collagen types have been characterised and the number may continue to increase (Sderhall et al, 2007; Veit et al, 2006; Pace *et al*, 2003). The collagens occur in unique tissue- and cell-specific patterns, arising during development in defined temporal and spatial patterns, and exhibit different functional properties (Linsenmayer, 1991). Collagens are involved in cell attachment, differentiation and in chemotactic processes. In many immunopathological processes, they act as antigens and also as the defective component in certain pathological conditions. Their primary function is to maintain the three-dimensional shape of tissues.

Collagens are homo- or heterotrimeric proteins built up from type-specific α chains to form stretches of triple-helical domains. The α chains are composed of a series of triplet Gly-X-Y repeats in which X and Y can be any amino acid, but frequently X is proline and Y is hydroxyproline (Linsenmayer, 1991; Prockop *et al*, 1979). Since every third amino acid in the α chain is a glycine, this allows narrow turns (Myllyharju and Kivirikko, 2001, 2004). The other common amino acids in collagens are proline and lysine. Non-collagenous domains include the fibronectin type III repeat and the A-domain of von Willebrand factor. In fibril-forming collagens a single triple-helical domain comprises more than 95% of the molecule while other collagens have multiple triple-helical domains (Prockop *et al*, 1979; Prockop and Kivirikko, 1995). A

triple-helical domain consists of three separate α-chains, twisted in the form of a left handed helix (Prockop and Kivirikko, 1995; Ramakrishman *et al*, 1998; Rossert *et al*, 2000) and stabilised by inter-chain hydrogen bonds. The triple-helical region is wound in such a way that the peptide bonds are hidden within the interior of the molecule. This makes the triple-helical region highly resistant to attack by general proteases such as pepsin. The only enzymes that are capable of cleaving the helical region are the collagenases (matrix metalloproteinases-MMPs).

Each of the 27 known collagen types has been assigned a number designated by roman numerals e.g. type I, II, III (Bornstein and Sage, 1980, 1989). Where all collagen chains are identical they are all designated α1. Where two chains occur they are designated α1 and α2 and so on .The ratio in which the chains occur is also included in the complete designation of a collagen molecule. Collagens can be subgrouped into several classes: fibrillar collagens, fibril-associated collagens with interruptions in their triple helix (FACITS), network-forming collagens, filamentous collagens, anchoring collagen fibrils, transmembrane-domain containing collagens, non-collagen collagens (Table 1.1) (Prockop and Kivirikko, 1995; Pace *et al*, 2003).

There are five types of collagens within the fibrillar collagen group: type I, II, III, V and XI. Type I, II and III are the most abundant collagens in this group while type V and XI are the minor fibrillar collagens. They provide a scaffolding network in the extracellular matrices of the tissues in which they are expressed, largely skin, bone, tendon, blood vessels, cornea, sclera and cartilage (Pace *et al,* 2003). The fibrillar collagens have rigid uninterrupted triple-helical domains of more than 300 Gly-X-Y repeats and the procollagens have globular amino (N)-and carboxyl (C)-terminal extension peptides that differ substantially in length and sequence characteristics (Ramirez *et al*, 1990; Prockop and Kivirikko, 1995). Fibrillar collagens are synthesised as procollagens, containing N- and C-terminal propeptides that are cleaved after secretion into the extracellular space. In addition to their scaffolding functions, fibrillar collagens also serve as attachment sites for other constituents of the ECM and cell surface receptors such as the integrins. Type I collagen is the

prototype fibrillar collagen and most of the information available has been obtained from studies on this collagen as it is the most abundant collagen isolated from many connective tissues (Ghosh, 2002).

Table 1.1 Members of the collagen family

Subfamily	Type	Chains	Isoforms
Fibril-forming collagens	I	α1(I), α 2(I)	$[α1(I)]_2$ α 2(I)
	II	α1(II)	$[α1(II)]_3$
	III	α1(III)	$[α1(III)]_3$
	V	α1(V), α2(V),α3(V)	$[α1(V)]_2$ α 2(V)
			[α1(V)α2(V)α3(V)]
	XI	α1(XI), α2(XI),α3(XI)	[α1(XI) α2(XI)α3(XI)]
			and α(V)/ α(XI) chimeras
Network-forming collagens	IV	α1(IV), α2(IV),α3(IV)	$[α1(IV)]_2$ α 2(IV)
		α4(IV), α5(IV),α6(IV)	[α3(IV) α4(IV)α5(IV)]
			α1/ α2(IV), α5/α6(IV)]?
	VIII	α1(VIII), α 2(VIII)	$[α1(VIII)]_2$ α 2(VIII)
	X	α1(X)	$[α1(X)]_3$
Micro-fibrillar collagens	VI	α1(VI), α2(VI),α3(VI)	[α1(VI)α2(VI)α3(VI)]
FACIT collagens	IX	α1(IX), α2(IX),α3(IX)	[α1(IX) α2(IX)α3(IX)]
	XII	α1(XII)	$[α1(XII)]_3$
	XIV	α1(XIV)	$[α1(XIV)]_3$
	XVI	α1(XVI)	$[α1(XVI)]_3$
Transmembrane collagens	XIII	α1(XIII)	$[α1(XIII)]_3$
	XVII	α1(XVII)	$[α1(XVII)]_3$
Bundle-forming collagens	VII	α1(VII)	$[α1(VII)]_3$

1.2.1 Type I Collagen

Type I collagen is expressed at high levels in tissues requiring high tensile strength but is known to induce specific signals upon cell attachment and that it is vital for several cellular functions including migration and proliferation. It is composed of a heterotrimer of two α1(1) and one α2(1) polypeptide chains. The chains are encoded by separate genes, with the α1(1) chain being encoded by a gene on chromosome 17 and α2(1) being encoded by a gene on chromosome 7. Type I collagen α chains are synthesised by many cell

types such as fibroblasts in connective tissue, osteoblasts in bone and odontoblasts in dentine matrices.

1.2.1.1 Type I collagen synthesis

The collagen mRNAs are translated in the rough endoplasmic reticulum (RER) where they are modified. Two α1(1) and one α2(1) chains form a triple helix with N-terminal and C-terminal extension peptides. Glycosylation occurs on the asparaginyl and hydroxylysyl residues by the addition of either glucose or galactose, whilst the proline and lysines residues are often hydroxylated and the collagen molecules are secreted into the extracellular space through secretory granules. The N- and C-terminal extension peptides are cleaved by procollagen N- and C-endopeptidase respectively (Fig 1.1) (Cummings *et al*, 2005). The N- and C-terminal extension peptides are essential in chain association, triple helical formation, intracellular transport and have been implicated in regulation of collagen synthesis (Widom, 2000; Uitto *et al*, 1979; Ghosh, 2000; Myllyharju and Kirivikko., 2001; Karsenty *et al*, 1998).

Fibers are formed by crosslinking several triple helices via inter-chain disulphide bonds. Concise regulation of collagen metabolism is extremely important as a disturbance may cause a large variety of pathological conditions. The overproduction of type I collagen has been implicated in many fibroproliferative disorders such as organ fibrosis, systemic sclerosis whilst decreased production of collagen or mutation results in conditions such as osteogenesis imperfecta and Ehlers-Danlos syndrome (Bornstein and Sage, 1989; Slemp and Kirschner, 2006; Mimura *et al*, 2006; Prockop and Kivirikko, 1984; Olsen, 1991). To date, many factors and signal transduction pathways have been found to be involved in the regulation of collagen gene expression in fibroblasts (Ghosh, 2002; Slemp and Kirschner, 2006; Mimura *et al*, 2006; Prockop and Kivirikko, 1984).

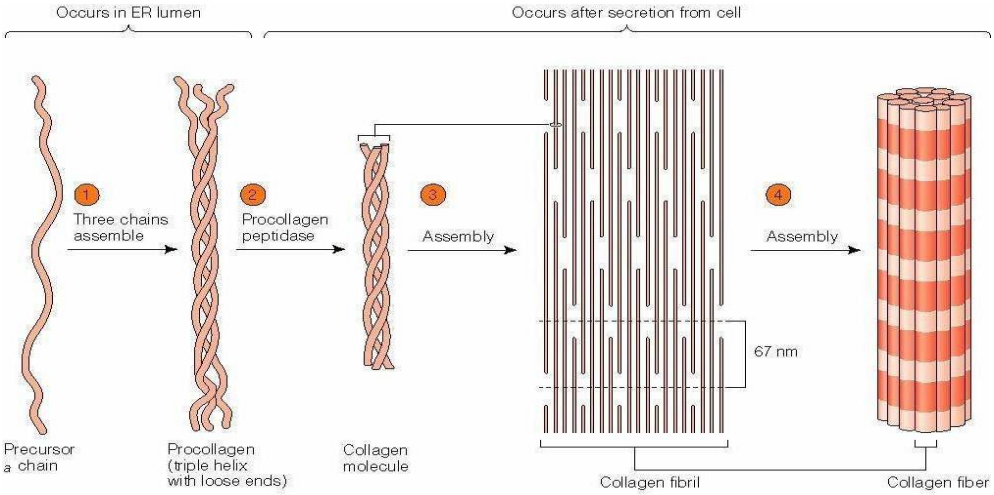


Figure 1.1 The synthesis of collagen in cells showing (1) the formation of the triple helix (2) the removal of N-terminal and C-terminal propeptides, (3) the incorporation of the mature collagen molecule into the fibres and (4) assembly of collagen fibres (Cummings et al, 2005).

1.2.1.2 Regulation of type I collagen synthesis

The type I collagen genes have been studied in detail with the human α2(1) collagen gene (COL1A2) proving to be an especially informative model to study the molecular mechanisms and cellular factors that control extracellular matrix assembly and degradation in both normal and pathophysiological conditions (Ghosh, 2002). Many nuclear factors have been implicated in regulating transcription of the COL1A2 genes in human and mouse models. There are subtle differences between mouse and human COL1A2 regulatory elements. It has also been proven that COL1A2 transcription is under the control of a complex regulatory network that includes multiple overlapping DNA elements and several other trans-acting factors.

1.2.1.2.1 Transcriptional regulation of type I collagen gene expression

Many studies have focussed on transcription as the primary level of regulation (Slack *et al*, 1993; Bou-Gharios *et a*l, 1996; Bornstein and Sage, 1989). Parker *et al* (1992) showed that α2(1) collagen synthesis is associated with differences in trans-acting factor binding to the proximal promoter of the gene. The regulatory regions within the two collagen promoters contain many repressor and enhancer elements (Ghosh, 2002; Ponticos *et al*, 2004). Several cis-acting regulatory elements and trans-acting protein factors are

involved in basal as well as cytokine-modulated type I collagen gene expression. The transcription of both COL1A1 and COL1A2 genes are regulated by elements in the promoter as well as in the first intron (Rossouw *et al*, 1987; Sherwood *et al*, 1990; Bornstein *et al*, 1996).

The COL1A1 and COL1A2 genes are co-ordinately regulated and the ratio of their steady-state mRNAs is tightly maintained at 2:1 (de Wet *et al*, 1987). Several transcription factors interact with upstream regulatory elements and control the basal, cell-type-specific gene expression (Ihn *et al*, 1996; Goldberg *et al* 1992). DNA methylation has been implicated in the downregulation of the collagen genes (Eckes *et al*, 1993; Ghosh, 2002). Gene inactivation by DNA methylation may be due to repression of binding of positive transcription factors or to increased binding of methyl-cytosine binding proteins (MeCPs) to methylated cytosine thus disrupting specific binding of transcription factor(s) in the regulatory region (Collins *et al*, 1997; Ghosh, 2002). The characteristic TATA box is present in both COL1A1 and COL1A2 promoters. A common CCAAT motif is also present in the two procollagen promoters to which the CCAAT binding factor (CBF) binds. CBF is a heterotrimeric protein comprising of three subunits, CBF-A, CBF-B and CBF-C (Maity *et al*, 1992). CBF has been shown to bind to the mouse COL1A1 and COL1A2 promoters to activate transcription (Maity *et al*, 1988).

Several other transcription factors have been identified and their binding sites located on the collagen promoter. Nuclear factor 1 is involved in replication as well as transcription control of several genes including COL1A2 (Rossi *et al*, 1988). Two negative factors, initiation factors 1 and 2 (IF1 and IF2), also interact with both COL1A1 and COL1A2 genes and their binding sites have been identified in the mouse promoters (Ravazzolo *et al*, 1991). Another transcription factor that has received much attention is Sp1, a zinc-finger transcription factor that binds to a GC-rich consensus sequence. Multiple binding sites for Sp1 have also been identified in the COL1A2 promoter. It appears Sp1 is involved in both downregulation and upregulation of COL1A2 promoter activity depending on the other transcription factors it associates with (Asano *et al*, 2009). Fli-1, a member of the ERG subfamily, has been

shown to be a repressor of COL1A2 promoter activity and functional interaction of Fli-1 with Sp1 is essential for this inhibitory action. A Fli-1 response element has been mapped between -353 and -186 bp of the COL1A2 promoter containing Ets and Sp1/Sp3 binding sites (Czuwara-Ladykowska *et al*, 2001). Evidence from many studies also implicates Sp1 and Sp3 along with other factors such as Fli1 and NF-1 in the control of type I collagen synthesis.

The AP-1 transcriptional complex contains Jun D, c-Jun and c-Fos that binds to their consensus sequence as homodimers or heterodimers. AP-1 has been shown to regulate type I collagen gene expression in fibroblasts (Katabani *et al*, 2005). Other proteins involved in collagen regulation include Smad 3 and Smad 4. Transient expression of Smad 3 and Smad 4 in human skin fibroblasts lead to stimulation of COL1A2 promoter activity. A Smad binding element was located at -263 to -258 bp and was shown to interact with Smads and reduce COL1A2 promoter activity (Chen *et al*, 1999, 2000).

1.2.1.2.2 Regulation of type I collagen gene expression by cytokines

Transcription of COL1A1 and COL1A2 is regulated by different cytokines with a number of transcription factors having been identified that have a regulatory function in the expression of the collagens (Uitto *et al*, 1979; Kahari *et al*, 1990, 1991; Trojanowska, 1998; Ihn *et al*, 1996). Cytokine responsive elements in the COL1A1 and COL1A2 genes have been identified and characterised (Rossert *et al*, 2000). Cytokines involved in regulation of type I collagen gene expression include TGF-β, interleukin-1, interferons and TNF-α, effecting mRNA levels, turnover and stability (Mauviel *et al*, 1991). TGF-β is known to stimulate transcription of COL1A2 promoter. Chen *et al* (1999) demonstrated that TGF-β stimulates human COL1A2 promoter activity through Smad signaling. Another cytokine, TNF-α, is known to counteract the stimulation of collagen gene expression by TGF-β through overlapping nuclear signaling pathways (Inagaki *et al*, 1995).

1.2.1.2.3 Regulation of type I collagen gene expression by oncogenes

Oncogenes, Ras, c-Myc, v-Fos and v-Src, play significant roles in the regulation of cell growth and division in both normal and neoplastic cells. Ras downregulates COL1A1 and COL1A2 mRNA synthesis via an AP-1 site located in the first intron (Slack *et al*, 1993). Oncogenic Ras regulates type I collagen at the transcriptional and post-transcriptional levels. Oncogenic Ras has been shown to downregulate the function of an intronic AP1 site in the COL1A1 gene (Slack *et al*, 1993) and to activate ERK MAP kinases, which phosphorylate the linker region of Smad 2 and 3 to inhibit their nuclear accumulation and thus repress TGF-β signaling (Ghosh, 2002).

1.2.1.2.4 Post-transcriptional regulation

Inhibition of procollagen mRNAs translation by cleaved peptides has been shown to regulate collagen synthesis. The N- and C-terminal peptides of type I collagen inhibit collagen production by affecting polypeptide chain elongation and/or termination (Aycock *et al*, 1986; Wiestner *et al*, 1979). This suggests that collagen production is either induced or inhibited depending on the availability of specific proteases needed for the generation of the peptides from the procollagens and the production of the procollagens in the first place. This implies that collagen feedback regulation may be controlled at any stage of collagen biosynthesis from synthesis, secretion and extracellular proteolysis.

1.2.2 The ECM and Diseases

The synthesis and degradation of the extracellular matrix and its components such as collagens are in constant flux. Excessive accumulation of ECM components leads to fibrosis. Deficiency or mutations in ECM protein production is associated with conditions such as cancer, atherosclerosis, osteogenesis imperfecta and Ehlers–Danlos syndrome (Slemp and Kirschner, 2006). A balance must therefore be achieved between synthesis and degradation of ECM components to ensure that excess accumulation does not occur. Cells such as fibroblasts synthesise most of the collagens in fibrillar ECM but also secrete ECM-degrading proteases, growth factors and

chemokines. These cells are therefore important in achieving the balance necessary under normal conditions.

Currently no acceptable therapeutic strategies exist for the treatment of prevalent fibroproliferative disorders. A great deal of research is being pursued to understand the molecular basis for the development and progression of fibroproliferative disorders and other pathological conditions such as cancer in order to identify effective therapeutic approaches. Most of these disorders are characterised by either a decrease or an increase in the synthesis and deposition of collagen. Therapies against myofibroblasts (carcinoma-associated fibroblasts) are being considered as a way to control cancer. Such therapies can be seen as a 'normalisation step' of the myofibroblasts. Many pathological processes including invasion and metastasis as described below are characterised by dynamic changes in ECM synthesis, deposition and turnover, thereby altering the mechanical properties of the ECM. Changes in rigidity of the stromal ECM could modify *in vivo* matrix adhesion properties with subsequent changes in signaling mechanisms and cellular phenotypes (Wozniak *et al*, 2003, 2004).

1.3 Tumour cell invasion and metastasis

During metastasis tumour cells detach from the primary tumour site to invade adjacent tissues and travel to distant sites. The processes of tissue invasion and metastasis involve both a change in tissue microenvironment and also activation of extracellular proteases. The series of interactions which take place in these two processes involve the release of normal cellular contacts within the primary tumour, followed by invasion through the underlying basement membrane, the destruction of and passage through the ECM and lastly the penetration into blood or lymphatic vessels for circulation to distant sites. At the moment there is a poor understanding of the genetic and biochemical determinants in the regulation of tissue invasion and metastasis.

1.3.1 Tumour cell Invasion

The interstitial matrix stroma contains many cells including fibroblasts and interstitial proteins comprising predominantly type I and type III collagens,

fibronectins, glycoproteins and proteoglycans. The matrix functions as a support scaffold mediating cell attachment and determines overall tissue architecture (Liotta and Kohn, 2001). The matrix influences many processes such as morphogenesis and mitogenesis.

One of the key characteristics of malignant cells is their ability to override the microenvironmental control by the host in order to invade tissue to metastasise to distant sites (Coussens and Werb, 1996, 2002). Invasion and metastasis are mechanistically closely related since both utilise similar strategies involving changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases (Keleg *et al*, 2003). During the process of tumour invasion, tumour cell(s) must traverse the ECM as they cross tissue boundaries and interact with ECM components such as type I collagen molecules and stromal cells such as fibroblasts.

A great deal of effort has been made in understanding the molecular and cellular processes involved in cell-cell and cell-matrix interactions during tumour invasion and metastasis (Zigrino *et al*, 2005). Intra-epithelial tumors have to cross an intact basement membrane that separates the epithelium from the stromal tissue. During the transition from carcinoma in situ to invasive carcinoma, tumour cells degrade the basement membrane in order to traverse the underlying stroma (Liotta and Kohn, 2001; Stetler-Stevenson *et al*, 1993; Gout and Huot, 2008). In general, tumor cell invasion through the basement membrane is thought to be a three-step process (Stetler-Stevenson *et al*, 1993; Gout and Huot, 2008). Firstly, neoplastic cells attach to the underlying basement membrane. Secondly, the malignant cells produce proteolytic enzymes to degrade the basement membrane and thirdly, the tumor cells pass through the basement membrane and penetrate the stromal tissue.

Invasion of the ECM is not unique to tumour cells, but is also performed by normal cells. Trophoblasts invade the endometrial stroma and blood vessels to establish contact with the maternal circulation during the development of hemocorial placenta (Stetler-Stevenson *et al*, 1993). During the process of

angiogenesis, endothelial cells invade basement membranes as well as the interstitial stroma. The difference between normal and tumour cells is that in normal cells the invasive phenotype is controlled whereas in tumour cells it is deregulated.

The loss of the basement membrane has been positively correlated with invasive carcinomas which may be due to a combination of decreased synthesis of ECM proteins and increased degradation of the same proteins. The interaction between tumour cells and the basement membrane has been described as the most critical stage during the initiation of the metastatic cascade (Stetler-Stevenson *et al*, 1993; Gout and Huot, 2008). Recent studies have contributed to our understanding of the role of the host stromal tissue in promoting tumour cell invasion and metastasis. Within a tumour-host microecology, stromal and tumour cells exchange signals that modify the local ECM, stimulate migration, proliferation and survival (Hanahan and Weinberg, 2000). The three steps occurring during tumour invasion of the ECM are briefly described below. These are (1) attachment or adhesion of tumour cells to the ECM (2) Degradation of the ECM (3) Tumour migration into the region of the matrix modified by proteolysis.

1.3.1.1 Attachment to the ECM

A number of specific cell-surface associated molecules or proteins and other diffusible factors have been implicated in the process of cell invasion and metastasis. These proteins modulate cell-matrix and cell-cell interactions and include integrins, cadherins, CD44, and the matrix metalloproteinases (MMPs). Adhesion and proteolysis regulate many processes associated with metastasis including how cancer cells interact with the host stroma or microenvironment, cancer cell migration, invasion, resistance to apoptosis and the ability to induce angiogenesis (Bacac and Stamenkovic, 2007). Adhesion molecules affect the metastatic potential of malignant tumours by serving as both positive and negative modulators and may direct tumours to specific tissues. Adhesion molecules on the cell surface play an important role in tumour cell migration and regulate the potential for epithelial cells to metastasise (Keleg *et al*, 2003).

A number of surface proteins involved in these interactions have been described and chief among them are the integrins. In addition to mediating specific binding of cells to components of the ECM, integrins transduce signals from the ECM into cells and regulate the expression of various genes including matrix degrading enzymes. Integrins recognise certain specific sequences in many adhesion proteins such as collagen, fibronectin and thrombospondin (Tiger *et al*, 2001; Aplin *et al*, 1990). When bound to the ECM, integrins aggregate on the cell membrane and associate in a molecular complex composed of adaptor, signaling and cytoskeletal proteins (focal adhesion complex). Actin fibres are converted into stress fibres that promote further integrin aggregation. This results in increased ECM binding as a result of this positive feedback loop. Integrins trigger both mechanical and chemical signals that regulate adhesive versus migratory interactions with the ECM.

Another important function of integrins in tumour metastasis is their role in cellular adaptation to changing tissue microenvironments as found in metastatic organs (Keleg *et al*, 2003). Highly malignant melanoma cells show an increased synthesis of the α2β1 integrin when cells are cultivated in a collagen matrix (Zigrino *et al*, 2005). Other cell receptors involved in mediating cell-matrix and cell-cell binding include cadherins and the immunoglobulin (Ig) superfamily. Normal epithelial cells communicate with the microenvironment through E-cadherin-mediated cell-cell interaction and the β1 integrin-mediated adhesion to the basement membrane. Cancer cells however have N-cadherin instead of E-cadherin and it plays an important role in invasion (Bacac and Stamenkovic, 2007). A positive correlation was found between the invasion into type I collagen of human cancer cell lines and the lack of E-cadherin (Mareel and Leroy, 2003).

1.3.1.2 Degradation of the ECM

ECM invasion is not a passive growth process but requires active biochemical mechanisms. Many proteases are involved in the proteolysis of the ECM proteins during processes such as cancer invasion and metastasis. These include MMPs, plasminogen activators, cathepsins and heparanases. Matrix metalloproteinases and their involvement in ECM degradation are described

in a later section (see section 1.5.1). Data from many researchers suggest that members of the MMP family also enhance tumour angiogenesis by triggering the angiogenic switch (Bergers *et al*, 2000). Released proteases degrade components of the ECM to reveal cryptic sites in extracellular matrices. Extracellular proteases are important in transforming cancer into an invasive and metastatic phenotype. Several studies have shown that proteases are upregulated, protease inhibitors are downregulated and inactive zymogen forms are converted into active enzymes in different/ several cancers (Bloomston *et al*, 2002; Bergers *et al*, 2000). The secretion of MMPs and their tissue inhibitors of matrix metalloproteinases (TIMPs) is balanced under normal conditions. During embryo morphogenesis, tissue remodelling, bacterial invasion and angiogenesis, the degradation of the ECM is done in a controlled manner (Liotta *et al*, 2001). During tumour invasion this process is deregulated.

1.3.1.3 Movement through the ECM

Once the ECM has been degraded and the basement membrane has been disrupted, cancer cells come into contact with structural and cellular components of the stroma, bringing them into contact with fibroblasts. Fibroblast behaviour is then modified to better serve the requirements of tumour cell growth, invasion, migration and survival (Bacac and Stamenkovic, 2007; Owens and Watt, 2001). Their phenotypes are altered to form myofibroblasts (cancer associated fibroblasts, CAFs). The aberrant phenotypic expression of fibroblasts surrounding tumours has been widely documented (Kalluri and Zeisberg, 2006; Olumi *et al*, 1999). CAFs are a source of proteolytic enzymes, growth factors, ECM proteins and participate in regulating the inflammatory response to tumour invasion. They have been shown to either increase or decrease ECM synthesis depending on the stage of the tumour (Kalluri and Zeisberg, 2006, Orimo *et al*, 2005).

As a tumour grows, the advancing front activates enzymes whilst the rear front remains attached to the ECM. Once the path for the tumour has been cleared, the tumour's rear front detaches from the ECM and it moves forward. The tumour cell attaches itself to the ECM ahead of its advancing front and

pulls itself forward. Tumour cells do not stop moving, even if the stimulus for movement has been removed. They migrate and penetrate tissue barriers until the host dies (Fidler, 2003; Chang and Werb, 2001). The three steps of ECM invasion as exemplified by the description above for tumour cells are linked and continuous and little information is available about the regulation of each step. The genes that control each step are still to be determined and it is hoped once this is done, this would contribute to a much better understanding of the process of invasion.

1.3.2 Metastasis

Metastasis is a complex, multistep and organ-selective process that is governed by many different classes of molecules (Yeatman and Nicolson, 1993). Loss of junctional contact between adjacent epithelial cells and cell-extracellular matrix associations are essential prerequisites for tumour cell detachment from the primary tumour (Tawil *et al*, 1996). These cancer cells are capable of establishing new tumours in locations remote from the site of the primary tumour and to subvert the tissue microenvironment in a way that is conducive to their continued proliferation and survival. The process of metastasis involves other processes such as angiogenesis, survival during circulation, intravasation and extravasation (Fidler, 2003; Gout and Huot, 2008). The host extracellular matrix or stroma is degraded by proteolytic enzymes and subsequently invaded by malignant tumour cells from the primary tumour. Tumour cells can penetrate the lymph node and blood vessels (intravasation) and enter circulation.

The stromal reaction to the presence of tumour cells is variable depending on the tumour cell properties and, in part, upon the local stromal composition. Assessment of the expression profile of stroma associated with invasive cancer revealed a gene profile reminiscent of that associated with wound healing. Genes found to be upregulated in the reactive stroma were found to have predictive value for both overall and metastasis-free survival of prostate and breast carcinoma patients (Bacac and Stamenkovic, 2007; Chang *et al*, 2005; Allinen *et al*, 2004). These observations suggest that the stromal response to carcinoma growth may hold the key to subsequent growth and

spread of the carcinoma. The modified stroma can be considered as a main tumorigenic agent 'as a whole' and is susceptible to therapeutic intervention sometimes called 'stroma-directed therapy' in cancer. However several key questions remain unresolved. Most importantly is the role played by stromal cells such as fibroblasts not only in the crosstalk with cancer cells but also the crosstalk between these stromal cells and the extracellular matrix.

1.4 Tumour Microenvironment

The tumour microenvironment was originally considered to only play a supporting role in metastasis and cancer progression but its active function in tumour development is now firmly established. Numerous studies have shown that tumour invasion and metastasis involve complex interactions between stromal and tumour compartments (Beacham and Cukierman, 2005; Wozniak *et al*, 2004). This profound role of the microenvironment in tumour development begins early in the neoplastic process, before cancer cells have crossed the basement membrane.

The tumour microenvironment includes the following:

- Cancer cells
- Non-cancer cells such as fibroblasts
- Secreted soluble factors
- Non-cellular material- ECM

The actual composition of each of these in the tumour microenvironment is highly variable. The conditions within the tumour microenvironment do not affect cancer cells only. The ECM, which forms the immediate environment of the invading malignant cells contains cells such as fibroblasts which biosynthesise the collagens among other proteins (Nakoman *et al*, 2005). Normal cells around a tumour exhibit altered characteristics compared to corresponding cells in normal tissue. Several studies indicate that fibroblasts within tumours harbour mutations that activate them into myofibroblasts or cancer associated fibroblasts (CAFs). Interestingly lung endothelial cells are transformed into CAFs by the presence of TGF-β (Zeisberg and Kalluri, 2004; Zeisberg *et al*, 2005, 2007; Gout and Huot, 2008)). Most mammalian cells are in contact with an ECM whose composition and structure vary with

development and diseases such as cancer (Gout and Huot, 2008). Inappropriate synthesis or degradation of any ECM molecules alters cell physiology and can cause disease (Kim, 2005; Wetzels *et al*, 1991; Nagle *et al*, 1995).

1.4.1 Cells synthesising the ECM

Fibroblasts are widely distributed and are the predominant cells producing the extracellular matrix proteins in the stroma (Kalluri and Zeisberg, 2006). In addition to being responsible for the production of the ECM, fibroblasts play a role in the regulation of differentiation and homeostasis of adjacent epithelia. Fibroblasts are key cellular component of tumours and are thought to facilitate angiogenesis and cancer progression. Pathologists have reported increased fibroblast proliferation and enhanced collagen deposition in the stroma surrounding many tumour types. This provided evidence that changes in the host stromal compartment occur concomitantly with epithelial transformation (Kunz-Schughart *et al*, 2002). Fibroblasts are the most abundant stromal cells in the desmoplastic response of tumours (Tuxhorn *et al*, 2002; Kunz-Schughart *et al*, 2002; 2003) and can be activated by various stimuli including growth factors such as TGF-β, EGF, PDGF, and FGF-2.

The most salient feature indicative of phenotypic switching to these activated fibroblasts (cancer-associated fibroblasts) is a myofibroblasts ultra structure (Beacham and Cukierman, 2005). These activated fibroblasts express α-smooth muscle actin, hence the term myofibroblasts (Fig 1.2) (Kalluri and Zeisberg, 2006). Markers used to distinguish activated fibroblasts from normal fibroblasts are vimentin and tenascin C. Activated fibroblasts also modulate the immune response through secretion of cytokines such as IL-1 (Rollins *et al*, 1989). Fibroblasts interact with their surrounding microenvironment through receptors such as integrins. Once fibroblasts acquire the activated phenotype, their proliferative activity increase and there is enhanced secretion of ECM proteins such as type I collagen and tenascin C. Activated fibroblasts also communicate with cells through secretion of growth factors and chemokines. Activated fibroblasts deposit high levels of collagen type I and III

to induce an altered ECM microenvironment, and also interact with the microvasculature by secreting MMPs and VEGF.

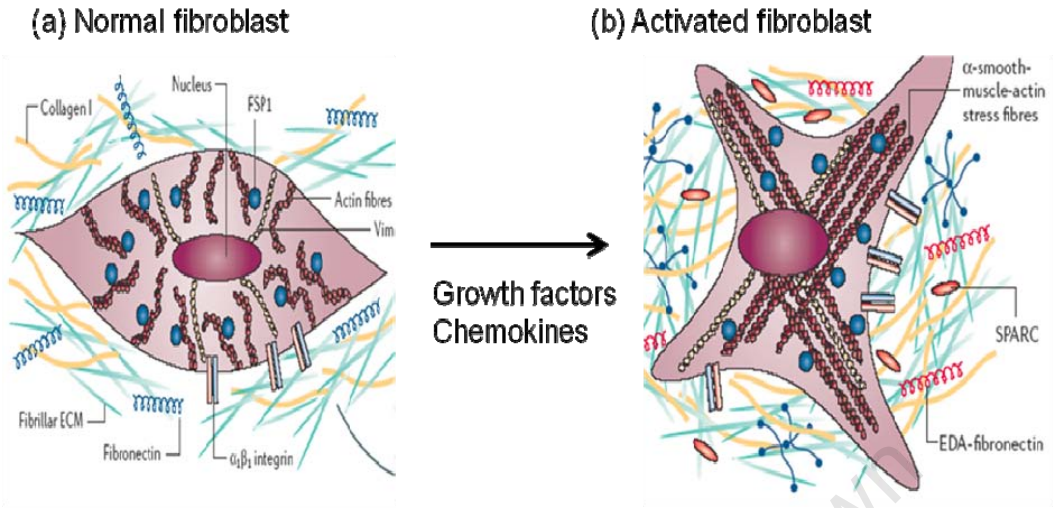


Figure 1.2 Activation of normal fibroblasts (a) by growth factors, proteases and chemokines results in an altered phenotype and the synthesis of large amounts of the ECM as shown in (b). (Kalluri and Zeisberg, 2006).

Activated fibroblasts are present in healing wounds and sclerosing tissues. Fibroblast activation in wound healing is reversed once the activating stimulus is removed. In tissue fibrosis however fibroblasts sustain their activated state often until organ death. Both cancer-associated fibroblasts and activated fibroblasts isolated from fibrotic tissue maintain their phenotype over several passages *in vitro*, until they enter senescence (Gout and Huot, 2008). Immortalised prostate epithelial cells grafted in mice together with CAFs, taken from the primary site, resulted in the development of intraepithelial neoplasia of the prostate (Olumi *et al*, 1999). This clearly indicates that CAFs have the potential to initiate tumour formation. CAFs are also thought to initiate the metastatic cascade through producing cytokines such as TGF-β that activate cancer cells and trigger their detachment from the primary tumour site. CAFs or activated fibroblasts secrete increased levels of ECM-degrading proteases such as MMPs particularly MMP 2, MMP 3 and MMP 9. This increases ECM turnover and alter ECM composition.

1.5 Enzymes responsible for the degradation of the ECM

The degradation of the ECM together with adhesion and detachment enable cells including tumour cells to move through the stroma. In the case of tumour cells, they secrete proteolytic enzymes and other factors which stimulate host

cells to secrete proteases to degrade matrix proteins. The degradation of the stroma is an irreversible process that can only be corrected through synthesis of new matrix proteins. Inhibitors of the MMPs constitute a viable strategy for interfering with the processes of ECM degradation.

1.5.1 Matrix metalloproteinases (MMPs)

MMPs comprise a family of at least 28 secreted or transmembrane enzymes collectively capable of processing and degrading various ECM proteins, of which about 22 MMPs have so far been found to be expressed in human tissues. MMPs share high protein sequence homology and have defined domain structures and thus, according to their structural properties, MMPs are classified either as secreted MMPs or membrane anchored MMPs, which are further divided into eight discrete subgroups. Secreted MMPs include minimal-domain MMPs, simple hemopexin domain-containing MMPs, gelatin-binding MMPs, furin-activated secreted MMPs and vitronectin-like insert MMPs, while membrane bound MMPs include type I transmembrane MMPs, glycosyl-phosphatidyl inositol (GPI)-linked MMPs and type II transmembrane MMPs (Egeblad and Werb, 2002; Fang *et al,* 2000.)

Crystal structures of MMPs, reveals the domain organization, polypeptide fold and specificity determinants (Bode *et al.* 1994). The crystal structures of the catalytic domains of most human MMPs in addition to porcine full length MMP-1 and human proMMP-2 have been resolved (Bode *et al*. 1994; Kiyama *et al*. 2006; Morgunova *et al*. 1999; Alexander, 2002). All MMPs are synthesized with a prodomain containing a leader sequence, which targets the protein for secretion (Sternlicht and Werb, 2001; Fang *et al*, 2000). The prodomain of MMPs contains a well conserved cysteine switch motif essential for maintaining a latent pro-MMP (Springman *et al*. 1990; Suzuki *et al*, 2004). The overall three dimensional structure of the hemopexin domain is a four-bladed propeller, with a calcium binding site nestled in the folds. Calcium seems to be required for some MMP/substrate interactions, and not others. Final activation of the MMPs often includes shedding of the hemopexin domain (Bode *et al*. 1999; Sternlicht and Werb, 2001.). Tissue inhibitors of MMPs (TIMPs) are secreted by fibroblasts, endothelial cells, and

chondrocytes (Liotta, 1992). These may guard against excessive breakdown of the ECM.

The interaction between the ECM components and cells plays a very important role in regulating the expression of matrix degrading proteases. Langhoz *et al* (2003) showed the important role of α2β1 integrin receptors in the regulation of MMP 1 expression in human skin fibroblasts. Zigrino *et al* (2005) showed that MT1 MMP expression is regulated by the activation of α2β1 integrins receptors. The α1β1 and α2β1 integrins are very important in the induction of MMP-1 secretion in human breast fibroblasts cultured on type I collagen (Moon *et al,* 2001; Nutt and Lunec, 1996). In addition to degrading ECM components, MMPs are involved in the processing of growth factors, cytokines, chemokines and adhesion molecules, hence they play an important role in maintaining tissue integrity. MMPs are involved in normal processes such as tissue remodelling, wound healing, trophoblast implantation and organ morphogenesis. Several studies have indicated an important role of the ECM in the induction of MMPs such as gelatinases (MMP 2 and MMP 9). MMPs also act as sheddases to cleave membrane-bound receptor molecules. Some of the most important MMPs are the fibrillar-degrading MMPs.

1.5.1.1 Fibrillar collagen-degrading MMPs

Fibrillar collagens are degraded by collagenases, MMP-1, MMP-8, MMP-13 and MMP-14 (MT1 MMP) at a specific site of the α chain resulting in generation of 3/4 N-terminal and 1/4 C-terminal fragments that are denaturated at body temperature. The fragments are further degraded by MMP-2, MMP-9 and MMP-13. Furthermore MMP-13 cleaves type I collagen in non-helical telopeptide while MMP-2 shows weak collagenolytic activity (Ala-aho and Kähäri, 2005). Obtaining information about the functional state of MMPs (pro-, active, or inhibited) is difficult. MMPs have an important role in the progression of many diseases and therefore profiling the levels of MMP activities rather than overall MMP abundance is necessary. This is because in pathological situations, tissue damage or other pathological effects are likely caused by active enzymes.

1.6 Focal Adhesion Complexes

Cell-matrix interactions are mediated mainly by integrins. Cytoskeletal and cytoplasmic proteins are recruited to the adhesion sites. Specialised structures called focal adhesion complexes are formed and these serve as a structural link between the ECM and the cytoskeleton. Other functions of focal adhesion complexes include serving as sites for signal transduction by recruiting a large number of signaling molecules, such as integrins. Proliferation and differentiation are some of the cellular processes controlled by adhesion-dependent signaling pathways (Wozniak *et al*, 2004; Schwartz and Yap, 2002; Parson, 2003). Beside integrins, other transmembrane proteins are also involved at the focal adhesion complex sites and these include integrin associated proteins (IAP), layilin, and growth factor receptors such as EGFR and VEGFR-2. There are many other proteins associated with the focal adhesion complexes and these include enzymes, adaptor proteins, cytoskeletal proteins, serine/threonine kinases and tyrosine kinases (Zanke *et al*, 1996; Ramirez and Rifkin, 2003). ECM-degrading proteins such as uPAR and MMPs are also included within the integrin-associated proteins group as some of the MMPs are found to be cell-associated through interactions with integrins. MMP1 is associated with collagen-binding I-domain of the α subunit and is required for α2β1 integrin-mediated keratinocyte migration on type I collagen (Sato *et al*, 2000; Xu *et al*, 2000, 2001).

1.6.1 Cell Adhesion Receptors

1.6.1.1 Integrins

Cell adhesion is very important in many pathological and physiological processes including wound healing, development, neoplasia and metastasis. Integrins are a family of heterodimeric adhesion receptors, that play a central role in transducing signals that provide support, regulate movement and affect gene expression (Aumailley and Gayraud, 1998; Hynes, 2002; Yamada, 2002; Juliano, 2004). They consist of non-covalently associated α and β subunits, with 18 different α subunits and 8 different β subunits having been detected in vertebrates. These combine to form 24 different integrin heterodimers (Fig 1.3) **(**Gullberg and Lundgren-Akerlund, 2002**).** Screening of the human genome identified 24 α and 9 β chains suggesting the number could be higher (Velling

et al, 1999; Humphries, 2000). About half of these heterodimers contain the β1 subunit (Al-Jamal and Harrison, 2008). The particular combination of α and β subunits determine ligand specificity, although a great redundancy is inherent in the system (Zutter and Santoro, 1990). Both subunits fold into an extracellular N-terminal globular head, which create the ligand-binding surface, a long stalk region connecting the ligand-binding head with the type I transmembrane stretch and the C-terminal cytoplasmic tail (Hynes, 2002; Humphries, 2000; Shimaoka and Springer, 2003).

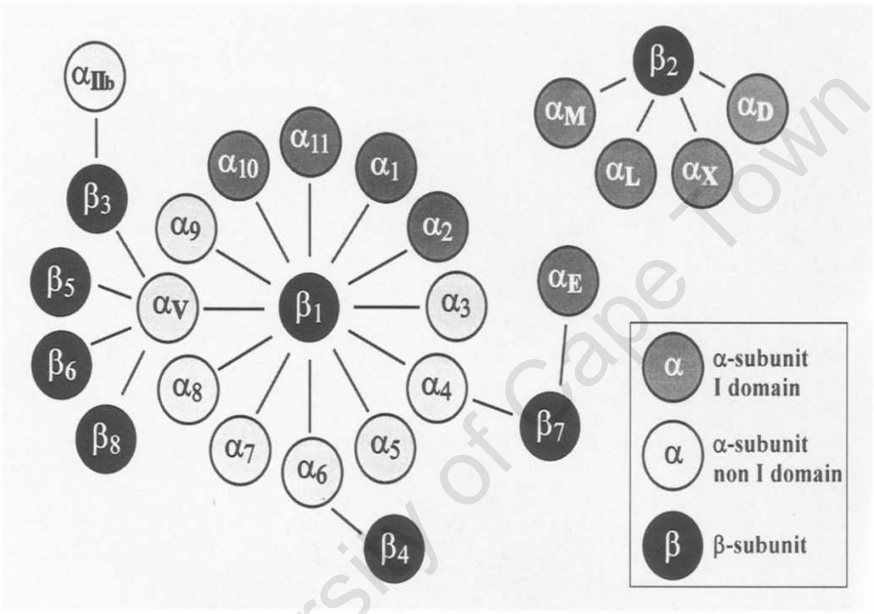


Figure 1.3 The integrin family. The currently known integrin chains of the vertebrate integrin family are shown. Integrin α subunits containing the I domain are labelled in dark gray. Integrins comprise non-covalently associated α and β subunits, with 18 different α subunits and 8 different β subunits having been detected in vertebrates. These combine to form 24 different integrin heterodimers (Gullberg and Lundgren-Akerlund, 2002**).**

The cytoplasmic tails are 10-50 residues long and do not contain any catalytic activity or consensus protein binding-motifs, except for the internalisation motif in β subunits. The only known exception is the β4-subunit with two fibronectin type II repeats in its uniquely long cytoplasmic domain (~1000 residues). Integrins bind to different macromolecules and transmit signals bidirectionaly, the so called 'inside out' and 'outside in' signaling. Under normal conditions, integrins exist in their inactive state bound to GDP. Their ligand binding affinity is regulated through conformational changes which can originate from the cytoplasmic domain or extracellular interactions (Adams

and Watt, 1990). Integrins bind ECM proteins and depending on the subunit composition will have different functions and ligand specificities. For example α5β1 binds fibronectin and α6β1 binds laminin.

X-ray crystal structural analysis of the α subunits revealed that the seven homologous repeats in the N-terminal part fold into a seven-bladed β propeller (Heino, 2000). The headpiece of the heterodimer is composed of this β propeller domain of the α subunit which closely interacts the β A domain (also known as the I-like domain) of the β subunit (Shimaoka and Springer, 2003). On the α subunit the most important regions involved in ligand-binding are clustered on the upper surface of the β propeller while binding motifs for regulatory divalent cations are exposed on the lower surface in the three of four last repeats. Half of the α subunits, such as the collagen binding α1, α2, α10 and α11, contain an I-domain (also known as the von Willebrand factor A domain) of around 200 residues inserted between the second and third propeller repeat (Shimaoka and Springer, 2003). The I-domain is exposed on the upper surface of the propeller and is involved in direct recognition and binding of the ligand (Humphries, 2000, Hynes and Zhao, 2000). The I domain contains a MIDAS site (metal-ion-dependent adhesion site). This characteristic MIDAS site binds negatively charged residues in the ligand (Plow *et al* 2000). The I-domain is able to fold correctly without being associated to the β subunit (Humphries, 2000).

The stalk region of α subunits lacking the I-domain are post translationally cleaved creating an N-terminal heavy chain and C-terminal light chain joined by a disulfide bond, except for α4 and α9. This acts as a lever, as the angle between the I-like domain and the hybrid domain controls the receptor conformation and the ligand binding affinity. The 'legs' of the integrins are composed of multiple globular domains known as the EGF-like repeats (Humphries, 2000; Heino, 2000). These EGF repeats are linked to a cytoplasmic tail domain via a single transmembrane domain. The β subunit has an N-terminal cystein-rich region, called a PSI-domain, homologous to other membrane proteins (Bork *et al*, 1999). This domain co-operates with a more C-terminal cystein-rich region in restraining the integrin in an inactive

state (Zutter and Santoro, 1990; Zang and Springer, 2001). The β subunits also contain an I-like domain with a MIDAS. When bound to an α subunit lacking the I domain, this I-like domain takes direct part in ligand binding and it is also involved in the actual association with the α subunit (Heino, 2000; Humphries, 2000). Some of the well-studied integrins include the collagen-binding integrins.

1.6.1.1.1 Collagen-binding integrins

The four integrins known to bind triple helical collagens are α1β1, α2β1, α10β1 and α11β1 (Knight *et al*, 1998; Xu *et al*, 2000). The α subunits of these integrins have the I domains and associate with the β1 subunit only. Ligand specificities of the four collagen binding integrins point to both unique and potentially overlapping physiological functions (Santoro *et al*, 1986; Heino 2000; Gullberg and Lundgren-Akerlund, 2002). α1β1 and α2β1 have been known for much longer and hence have been studied in detail while α10β1 and α11β1 have been recently identified. The α10β1 integrin was identified as a type II collagen-binding integrin in chondrocytes while α11β1 as a type I collagen-binding integrin on cultured muscle cells although studies later showed that α11β1 is not expressed on muscle cells *in vivo* (Camper *et al*, 1997, 2001; Velling *et al*, 1999; Tiger *et al*, 2001). These four integrins are expressed in different tissues, with α1β1 and α11β1 predominantly found in mesenchymal cells while both α1β1 and α2β1 are present in epithelial, stromal cells and platelets (Zutter and Santoro, 1990).

Ligand specificity differs among the four collagen-binding integrins. α1β1 mediates cell spreading on collagen type I, III, IV, V and XIII substrates. α2β1 mediates cell spreading on collagen types I-V but not on collagen type XIII. It appears that other factors are involved in the regulation of cell spreading. Recombinant I-domains from the four integrins have been used in binding assays and the results support the idea that these integrins differ in ligand specificity and perhaps function (Camper *et al*, 1997; Humphries, 2000). These four integrins also bind to other ECM proteins such as laminins.

There is no consistency in the expression pattern of collagen-binding integrins during metastasis. These integrins are up-regulated in some melanomas and

downregulated in others (Dahlman *et al*, 1998; Zutter *et al*, 1994, 1995) while two of these integrins, α1β1 and α2β1 have been shown to be negative regulators of collagen synthesis (Langholz *et al*, 1995, Kubota *et al*, 2003). The major collagenase, MMP 1, is upregulated by α2β1 and binds the I-domain of the α2 subunit, thereby suggesting that α2β1 downregulates collagen synthesis partly by causing its degradation (Langholz *et al*, 1995; Mimura *et al*, 2006). In fibroblasts seeded in a collagen lattice, α2β1 has been shown to induce MMP 13 in a p38 MAPK-dependent manner. α2β1 integrin has been implicated in numerous physiological and pathological conditions. During angiogenesis, α1β1 and α2β1 integrins interact with each other to regulate gene expression such as VEGF expression (Heino *et al*, 2008).

1.6.1.1.2 The α2β1 Integrin

Of the collagen-binding integrins, α2β1 integrin is the most studied. It was first identified on T-cells activated *in vitro* and was named VLA-2, but was later shown to be a collagen receptor on a number of cells including fibroblasts and fibrosarcoma cells (Santoro, 1986). It is a classical I domain-containing receptor (Zutter and Santoro, 1990; Takeuchi *et al*, 1997) and is widely expressed during embryonic development (Xu *et al*, 2000). Despite being widely expressed in human tissue no α chain is expressed in skeletal muscle. α2β1 integrin is the only collagen-binding integrin known to be expressed in platelets (Heino, 2000). Langholz *et al* (1995) showed the importance of α2β1 integrin in the regulation of MMP-1 expression in human skin fibroblasts. It was also demonstrated that membrane-type 1 (MT1) MMP expression is regulated by the activation of α2β1 integrin receptors (Koblinski *et al*, 2005; Xu *et al*, 2000; Montgomery *et al*, 1994).

The collagen receptor integrins are structurally distinct when compared to other extracellular matrix binding integrins. They have a specific 'inserted' or I-domain as a part of the α subunit (Heino, 2000) that is responsible for collagen recognition. α2β1-mediated stimulation of cell proliferation is dependent on cyclin E/cdk2 induction and α2β1-mediated cell migration involve the stress-regulated MAPK p38 (Hynes, 2002). The mechanism whereby integrin signaling results in stimulation or inhibition of cell

proliferation is unclear but might involve different transcription factors downstream of the signaling cascade. The use of monoclonal antibodies in inhibition studies has shown that both α1β1 and α2β1 have a role in VEGF-driven angiogenesis (Gullberg and Lundgren-Akerlund, 2002). α2β1 integrin is a promising target molecule for blocking inflammatory reactions in different disorders as it is expressed in leukocytes (Goldman *et al*, 1992). Other receptors involved in cell-cell and cell-matrix interactions include cadherins.

1.6.1.2 Cadherins

Cadherins are found in an amazingly wide range of organisms and their primary role is cell-cell adhesion via the extracellular transmembrane glycoproteins domains. Cadherins are calcium-dependent membrane proteins with an ectodomain consisting of five cadherin motifs (Prockop, 1998; Aplin *et al*, 1999). The two well known cadherins are E- and N-cadherin. Epithelial cells express E-cadherin whereas fibroblasts and neuronal cells express mainly N-cadherin (Kim *et al*, 1998, 2005). E-cadherin is a cell surface protein involved in homophilic cell-cell interactions, found at adherens junctions and associates with the actin cytoskeleton via catenins. The cell-cell adhesion is mediated by binding of the cytoplasmic tail of E-cadherin to cytoplasmic catenins, α-, β-, and γ-catenin (Takeichi, 1988, 1993; Jou *et al*, 1995). Just as integrins function to mediate cell-ECM interactions in anchorage-dependent survival, cadherins may also act in such a capacity, possessing a functional role in the regulation of intercellular adhesion-dependent survival. Changes in the structure or in the expression of the components of the E-cadherin/catenin complex, result in the suppression of cell-cell adhesion. E-cadherin expression decreases sequentially in progression from normal mucosa to adenoma and from adenoma to carcinoma (Gagliardi *et al*, 1995). N-cadherin expression induces a mesenchymal phenotype in breast carcinoma and in other epithelial cancer cells *in vitro* and it promotes invasion and metastasis *in vivo* (Hazan *et al*, 2000; Hazan *et al*, 2004). Recent studies have shown that exogenous introduction of N-cadherin was sufficient to confer invasive properties in bladder cancer cells by activating Akt (Rieger-Christ *et al*, 2004; Wallerand *et al*, 2008).

1.6.2 Integrin receptor-mediated signaling

Specialised cells are surrounded by different combinations of ECM proteins and express an array of tissue-specific integrin receptors. This diversity may represent one way to generate unique intracellular signals that give rise to tissue-specific phenotypes (Boudreau and Jones, 1999). The majority of signaling molecules implicated in ECM-integrin interactions appear to be rather ubiquitous mediators of signal transduction. Integrin-mediated signaling can be roughly divided into two categories. The first is 'direct signaling' in which ligation and clustering of integrins is the only extracellular stimulus. Adhesion to the ECM proteins can activate serine/threonine kinases, such as FAK and those in the mitogen-activated protein kinase (MAPK) cascade. Although many such direct signaling events have been described (Aplin *et al*, 1998), the mechanisms underlying these events have not been fully resolved. The second is 'collaborative signaling', in which integrin-mediated cell adhesion modulates signaling events that are initiated through other receptors, particularly receptor tyrosine kinases that are activated by polypeptide growth factors.

Several potential mechanisms for integrin-mediated signaling have emerged recently, with the focus placed either at the level of tyrosine kinase activation or within the downstream signaling cascade. In all cases, however, integrin-mediated adhesion seems to be required for efficient transduction of signals with origins at the cell surface and targets in the cytosol or nucleus might include FAK and the Ras/Raf/MEK/ERK pathway.

1.6.2.1 Focal Adhesion Kinase (FAK)

Signals from cell-matrix interactions are mainly transduced through tyrosine phosphorylation. Integrins are not known to exhibit any kinase activity and hence the need for active tyrosine kinases at the adhesion sites. Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that is localised at focal adhesion complexes and is activated in response to integrin clustering. FAK provides a link to several intracellular signaling cascades that lead to mitogenic responses, cell survival and cell migration (Ilic *et al*, 1998, 2008). Integrin-dependent autophosphorylation of FAK allows it to interact with

paxillin, tensin and Grb2/SOS, which in turn are able to activate downstream signaling mediators such as Src, Ras, and Raf that are implicated in growth control.

The use of FAK-null cells has shown that FAK is not vital for integrin activation but is important in cell migration (Ilic *et al*, 1998). The kinase domain of FAK contains several tyrosine residues that are phosphorylated and participates in autoregulation. Treatment of cells with tyrosine kinase inhibitors show that FAK and integrins associate independently of tyrosine phosphorylation (Miyamoto *et al*, 1995). Integrin-mediated FAK activation has been shown to require Rho-activity during the later phases of activation while the initial phosphorylation is independent of Rho-GTPases (Zhang *et al*, 2004). The FAK-phosphatidylinositol-3 kinase (PI3-K) interaction has been implicated in cell survival signaling via the Ser/Thr kinase Akt and in cell migration (Asano *et al*, 2004; Ilic *et al*, 1998).

1.6.2.2 Ras/Raf/MEK/ERK Signaling

The ras family, H-ras, K-ras and N-ras proteins have almost identical structures reflecting the similarity in their functions. Ras cycles between the GDP-bound inactive form and the GTP-bound active form (Chetoui *et al*, 2005; Leaner *et al*, 2005). Ras is activated by the guanine nucleotide exchange factor Sos1, which complexes with the adaptor protein Grb2. Grb2/Sos can bind directly to the Ras molecule associated with the inner leaflet of the plasma membrane. The binding of SOS to Ras causes a change in the Ras conformation and leads to the dissociation of GDP which allows Ras to bind GTP. In its activated form, Ras binds the Ser/Thr kinase Raf-1 that in turn initiates activation of the MEK/ERK MAPK pathway (Stewart and Guan, 2000).

The MAPK signal transduction pathway provides a common route leading to transcriptional regulation of genes that are crucial for cell growth and differentiation. Family members that are sequentially activated include MEK and ERK1/ERK2 (Stewart and Guan, 2000). MEK-dependent phosphorylation of ERK 1,2 results in their translocation to the nucleus, where they

phosphorylate and activate a number of transcription factors (Sun *et al*, 2003; Chen *et al*, 1996).

The mechanistic basis for the commonly observed integrin-mediated activation of MAPK, through Ras, is controversial at present. Several studies indicate the existence of a Ras-independent component in integrin-mediated MAPK activation (Chen *et al*, 1996) whilst many others show Ras involvement in integrin-mediated MAPK activation (Kubota *et al*, 2003; Ge *et al*, 2007). Three models have been proposed and two of these show Ras as a critical part in the activation of MAPK, with one model showing a Ras-independent MAPK activation. The first mechanism has FAK acting as a surrogate tyrosine kinase domain and the integrin engagement leads to FAK autophosphorylation, generating a binding site for the SH2 domain of Src. Src then phosphorylates FAK at several positions, where SH2-containing adaptor proteins such as Grb2 bind. Binding of Grb2 to FAK results in membrane localisation of SOS, a guanine nucleotide exchange factor, which in turn promotes GTP loading and activation of Ras. Activated Ras binds Raf and localises it to the membrane, where it is activated. Once activated, Raf phosphorylates and activates MEK which in turn phosphorylates ERK1/2. The second mechanism is FAK-independent activation of MAPK by integrins and involves the interaction of integrin subunits with the membrane protein caveolin. The Shc adaptor protein associates with caveolin, is tyrosine phosphorylated and recruited into integrin-associated complexes. Tyrosine-phosphorylated Shc is then bound by a Grb2-Sos complex and activation of MAPK occurs through the Ras-mediated pathway (Ge *et al*, 2007; Roux and Blenis, 2004). The third mechanism is Ras-independent and the localisation of Raf at the membrane is important for this activation but the membrane-associated components responsible are as yet to be identified.

These Ras-signaling cascades are only transiently activated because each normal Ras has low intrinsic GTPase activity that gradually inactivates its own signaling function by hydrolysing the bound GTP. Interestingly there is crosstalk between the Ras GTPase and PI3-K in that they interact and are able to activate each other (Stewart and Guan, 2000). The critical nuclear

targets of Ras/Raf/MEK/MAP kinase pathway include transcription factors such as Sp1 and Fos. The Fos protein forms a heterodimer with jun to yield the active AP-1 complex. The c-fos promoter has been well studied and the regulatory elements have been characterised (Treisman, 1994). Cancer cells are self-sufficient in terms of growth factors and their surface receptors are generally overexpressed. This can lead to the activation of Ras and subsequently the MEK/ERK pathway, facilitating the continued proliferation of cancer cells. Mutational activation of Ras has been reported in approximately 30 % of human tumours and might play a crucial role at the early steps of tumorigenesis and metastasis that triggers downstream signaling pathways that affect the expression of many genes (Ge *et al*., 2007; Uemura *et al*, 2003)..

Some of the protein tyrosine phosphatases involved in cell-ECM signaling events include PTEN, LAR and PTP. The tumour suppressor PTEN has been found to be a tyrosine protein phosphatase and a lipid phosphatase. When overexpressed PTEN inhibits stress fibre formation and thus inhibits cell spreading and migration with reduced activation of FAK and ERK (Gu *et al*, 1998). SHP-2 is another protein phosphatase shown to be involved in both integrin and growth factor signaling. SHP-2 is known to modulate the recruitment of Ras-GAP and the subsequent signaling via the Ras/Raf/ERK pathway (Ekman *et al*, 2002). The α2β1 integrin has been shown to mediate negative cell survival signaling by inducing dephosphorylation of Akt.

1.7 Definitions

The terms fibroblast-derived extracellular matrix (ECM) and matrix are used in the results section to mean the pre-formed matrix produced by fibroblasts in culture and then coated onto plastic culture dishes (Serebriiskii *et al*, 2008). The control used is the normal plastic culture dish. These definitions are important as some researchers use the term matrices to refer to artificial matrices of purified extracellular matrix proteins such as fibronectin and laminin (Fukuda *et al*, 2006; Elliot *et al*, 2005).

1.8 Objectives of the study

The recent development of fibroblast-derived matrices (Serebriiskii *et al*, 2008) have the potential to produce a new and physiological assay system in which to study interactions between cells and also between cells and the ECM. To date, the reciprocal regulatory effects of the ECM on cells such as fibroblasts have been elucidated only to a minor degree. This study investigated the regulation of type I collagen synthesis, a major component of the ECM, in the presence of a preformed fibroblast-derived ECM. Furthermore, the study explores the mechanisms of signaling involved in the type I collagen synthesis in the presence of the matrix and also the differences in type I collagen synthesis when fibroblasts are plated on the fibroblast-derived ECM and two matrix components, purified type I collagen and fibronectin.

1.9 Aims

1. To determine the effect of a fibroblast-derived ECM on type I collagen gene expression in fibroblasts
2. To determine the effect of the fibroblast-derived ECM on the gene expression and activities of type I collagen proteases
3. Functional mapping of the matrix responsive element (MRE) in the COL1A2 promoter
4. To identify possible transcriptional factors involved in the regulation of type I collagen gene expression by the fibroblast-derived ECM
5. To elucidate the signal transduction pathway involved in the matrix-mediated regulation of type I collagen genes
6. To determine the nature and origin of the stimulus from the fibroblast-derived ECM
7. To determine receptors involved in the interaction between the matrix and fibroblasts

CHAPTER 2

THE EFFECT OF A PRE-FORMED FIBROBLAST-DERIVED ECM ON TYPE I COLLAGEN GENE EXPRESSION

2.1 INTRODUCTION

Normal cells and tissues exist in a defined relationship with components of the ECM in their immediate microenvironment. Cell adhesion to the extracellular matrix (ECM) plays an important role in regulating cellular processes such as proliferation, migration, differentiation and survival (Giancotti and Ruoslahti, 1999; Hoshiba *et al*, 2006). In many pathological processes the interaction between cells and the surrounding extracellular matrix is altered. Gene expression patterns are changed and in doing so, cells synthesise an extracellular matrix that may differ from the normal ECM. During this process, cells normally degrade the extracellular matrix components such as types I and IV collagens, but elevated levels of these ECM components also has been reported in many pathological conditions such as fibroproliferative disorders.

Previous studies have shown that collagen gene regulation is a complex process whereby both the N-terminal and C-terminal extension peptide of type I collagen may function as negative feedback inhibitors of collagen biosynthesis in a variety of cells (Streuli and Bissell, 1990; Asano *et al*, 2004; Aycock *et al*, 1986; Bornstein and Sage, 1989). Collagen and fibronectin production are specifically induced in subconfluent cells by a pentapeptide (KTTKS) and residues 212-216 of the carboxy propeptide of the α1(1) procollagen protein (Aycock *et al*, 1986; Karsenty *et al*, 1995). This inductive effect is completely abolished as the cells become confluent. Many studies indicated that cellular behaviour and intracellular signal transduction are regulated by the type of ECM molecules (Giancotti and Ruoslahti, 1999; Koide *et al*, 1989; Nagaki *et al*, 1995). ECM composition can affect cell functions as well as matrix synthesis (Powell *et al*, 1997). In mammary glands for example, modification of the ECM at the stromal epithelial interface influence ductal

branching, end-bud development and epithelial proliferation in the prepubertal gland in mice (Silberstein *et al*, 1992; Sympson *et al*, 1994). In addition, the expression of COL1A1 is induced in attached fibroblasts compared to the same cells maintained in suspension (Dhawan *et al*, 1991) while in monocytes a different subset of genes is regulated, depending on the substratum on which the cells are cultured (Eierman *et al*, 1989).

ECM molecules such as fibronectin, vitronectin, laminins and collagens have been used before as substrates for studying gene regulation in cultured cells (Giancotti and Ruoslahti, 1999; Koide *et al*, 1989; Nagaki *et al*, 2002). However, none of these components, in their purified states, match the physical and biochemical characteristics of the natural ECM made by cells. Some of the most important ECM molecules include the collagens which are crucial in tissue structure and their structures also play a critical role in healing, in the pathology of diseases and as primary material for many tissue-engineering applications. The remodelling of collagen as a component of the extracellular matrix is integral to maintenance of tissue structure and function (Abraham *et al*, 2007; Moon *et al*, 2001, 2002; Mook *et al*, 2004). The process of wound healing and tissue remodelling depend on the degradation and the deposition of new extracellular matrix by fibroblasts (Diegelmann and Evans, 2004). Under certain pathological conditions such as invasion and metastasis, there is unregulated breakdown of collagen and therefore the assessment of collagen and its breakdown products is essential for monitoring and managing the patients (Abraham *et al*, 2006; Demers *et al*, 2000).

Several investigations have been done to identify the factors and the signal transduction pathways involved in collagen gene regulation in fibroblasts (Moon *et al*, 2001; Mook *et al*, 2003; Xiao *et al*, 2002). A better understanding of the role the ECM plays may offer opportunities to identify diagnostic markers and therapeutic targets for the many pathological conditions that are currently known. Therefore there is need to understand how the ECM controls fibroblast activity. A novel approach beyond the use of individual ECM components and collagen gels involves using natural, cell-derived 3D extracellular matrices, for example fibroblast-derived extracellular matrix

(ECM). These matrices are highly fibrillar and contain spaces originally occupied by the cells that generated the matrix. In this chapter, the effect of a fibroblast-derived ECM on the expression of type I collagen genes in normal and transformed fibroblasts was examined. This is the first time that such a natural fibroblast-derived ECM has been used to study the regulation mechanisms of type I collagen. The elucidation of the molecular basis of this process would be invaluable in the diagnosis, prognosis and therapeutic regimens not only in cancer but in other prevalent fibroproliferative disorders.

2.2 Results

2.2.1 Effect of fibroblast-derived ECM on cell proliferation

Fibroblast-derived ECM was prepared as described in section 7.2.3 and both normal (WI38 and FGo) and transformed (CT-1) cells were grown on the matrix or on plastic. To determine the effect of the fibroblast-derived ECM on cellular proliferation, the growth kinetics of WI38, CT-1 and FGo cells were determined using either direct cell counting or the MTT assay. Cell proliferation was followed over 72hrs and at each time-point cells were trypsinised and counted using a haemocytometer or the MTT assay was performed as described in section 7.2.4. In all cells, there was no noticeable differences between the proliferation curves of those plated on plastic and those on a matrix (Figure 2.1.A-C).

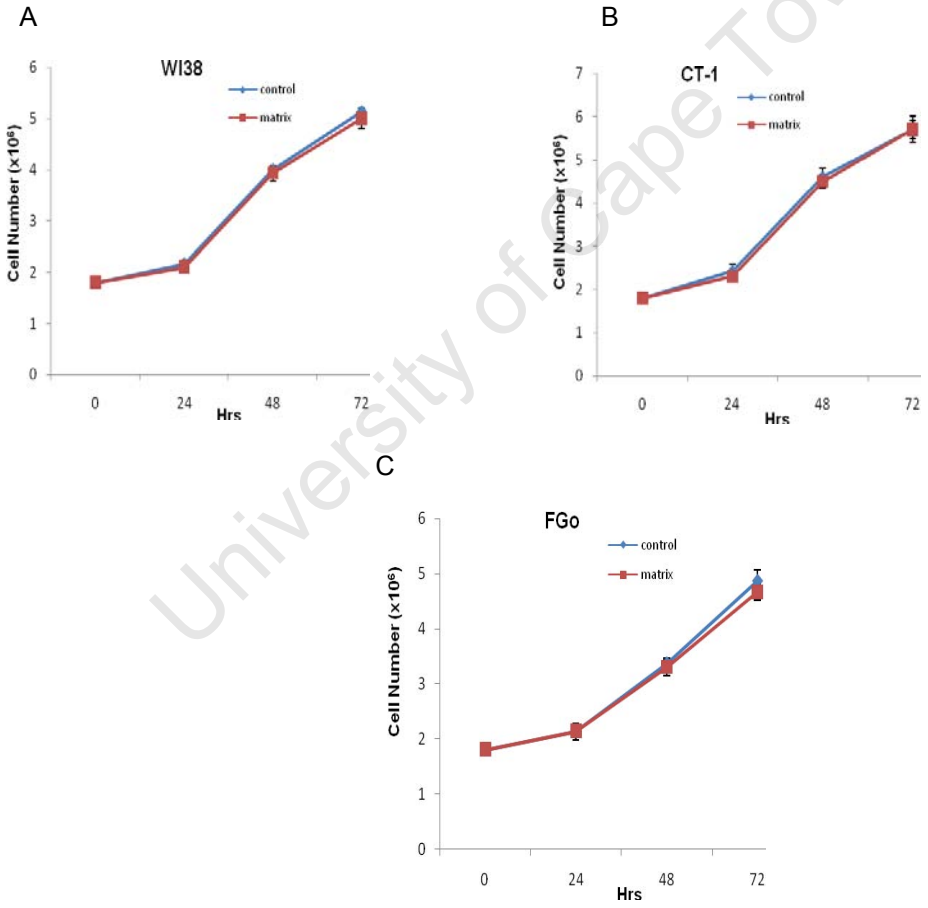


Figure 2.1 Influence of the fibroblast-derived ECM on cell proliferation. 3x 10^5 WI 38, CT-1 and FG_0 fibroblasts were plated on culture dishes with or without the fibroblast-derived ECM for 24 hrs and then grown in DMEM for the indicated times (24hrs, 48 hrs, and 72 hrs). Fibroblast-derived ECM was prepared as described in section 7.2.3. At the end of the experiment the fibroblasts were trypsinised and counted. A). Plot of WI38 cells. B). Plot of CT1cells. C). Plot of FGo cells. Control cells are shown as blue lines whilst those on matrix are shown as pink lines. There was no difference in growth rate between fibroblasts grown on a matrix and the control cells. Data are expressed as the mean ± S.D of three independent experiments.

Similar results were obtained using the MTT assay. These results suggest that the fibroblast-derived ECM does not alter the proliferation of the cells used in the study.

2.2.2 Effect of fibroblast-derived ECM on fibroblast morphology

To study the morphological differences between fibroblasts plated on plastic and those on matrix, fibroblasts were plated on plastic or on matrix for 48 hrs and then observed by phase-contrast microscopy. All pictures were taken under 100 x magnification. There were slight differences in the morphology of the fibroblasts plated on a matrix compared to those plated on plastic (Fig 2.2).

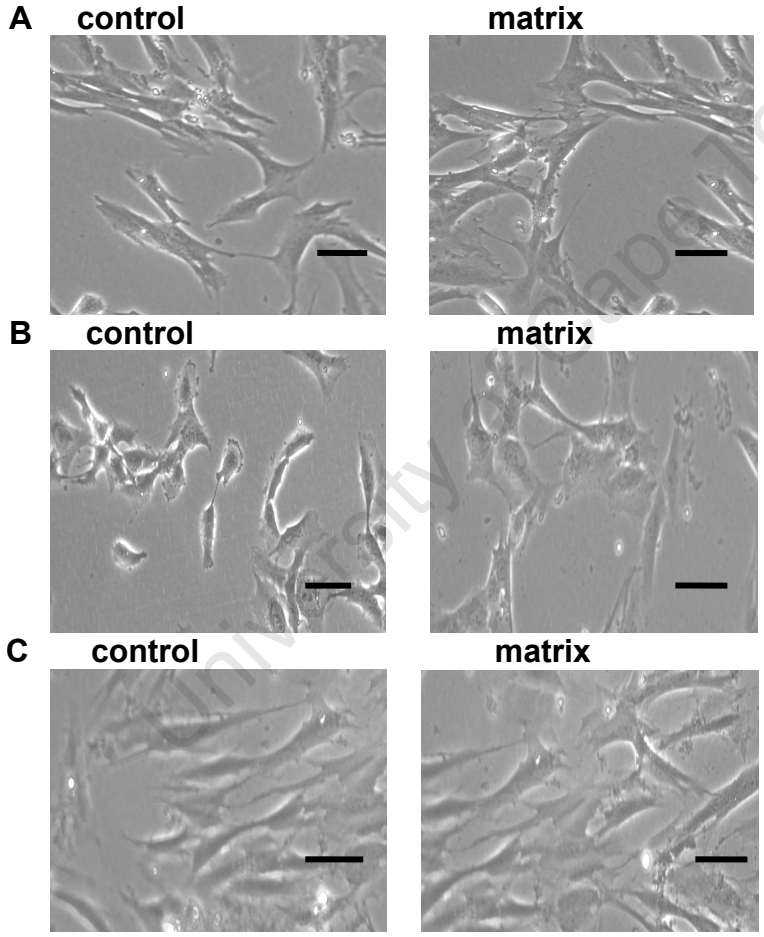


Figure 2.2 Effect of fibroblast-derived ECM on cell morphology.

WI38, CT-1 and FGo fibroblasts were seeded on dishes with or without fibroblast-derived ECM for 24 hrs and then grown in DMEM media for 48 hrs. Cells were observed under phase contrast at 100 x magnification: (A) WI38 on plastic or on fibroblast-derived ECM; (B) CT-1 on plastic or on fibroblast-derived ECM; (C) FGo fibroblasts on plastic or on matrix. Although fibroblasts on the matrix appear slightly elongated than those on plastic, there were no major changes in morphology. Scale bar is (▬) approximately 125 µm

Fibroblasts cultured on the fibroblast-derived ECM have a slightly elongated, spindle shaped morphology. The mechanism underlying this difference in morphology remains to be clarified.

2.2.3 Downregulation of type I collagen levels by fibroblast-derived ECM

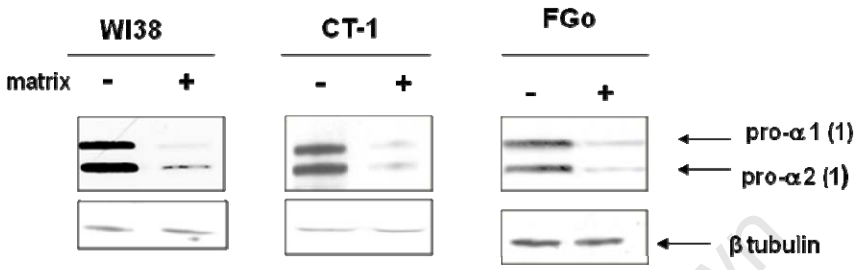
The effect of growing fibroblasts on a pre-formed fibroblast-derived ECM on type I collagen production in cellular lysates and the medium was investigated. Human fibroblasts were plated on plastic or on fibroblast-derived ECM for 24 hrs in complete medium, followed by growth in serum-free medium for 48 hrs in order to facilitate the identification of secreted collagens in the absence of serum proteins. Medium was digested with pepsin (section 7.3.1) and the remaining proteins were precipitated. Since type I collagen is resistant to pepsin digestion it remains intact while practically all other secreted proteins are digested. SDS polyacrylamide gel electrophoresis was performed to determine secreted type I collagen. For the analysis of intracellular collagens, cell lysates were fractionated by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with anti-type I collagen antibody as described in section 7.3.2. The antibody used in the study is made to type I collagen and interaction with the type I collagen chains does not necessarily show the α1 and α2 chains in the ratio 2.1. The results show that the levels of both intracellular and secreted type I collagen decreased significantly when fibroblasts were plated on a pre-existing fibroblast-derived ECM (Fig 2.3 A, C). A similar decrease was observed in WI38, CT-1 (a γ-radiated transformed cell line derived from WI-38 cells) and FGo skin fibroblasts. A time course analysis of the fibroblast-derived ECM-mediated inhibition of type I collagen gene expression in CT-1 cells showed that this was effective within 24 hrs of cellular contact with the pre-formed fibroblast-derived ECM (Fig 2.3 B).

2.2.4 Downregulation of collagen α2(1) mRNA levels by fibroblast-derived ECM

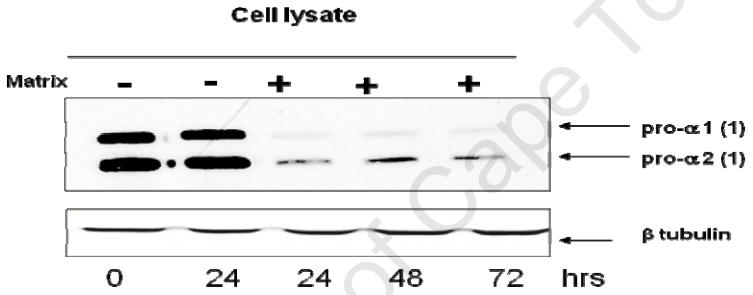
In order to understand the mechanism(s) by which the fibroblast-derived ECM mediates downregulation of collagen production, we examined the effect of matrix on the steady state levels of collagen mRNA. Fibroblasts were plated

on either plastic or matrix as previously described and total RNA was extracted for quantitation of α1(1) and α2(1) collagen mRNA by quantitative real time RT PCR using GAPDH as the normaliser. The matrix decreased the levels of α 2(1) collagen mRNA in all fibroblasts (Fig 2.4 A-C).

A Intracellular type I collagen



B Intracellular type I collagen



C Secreted type I collagen

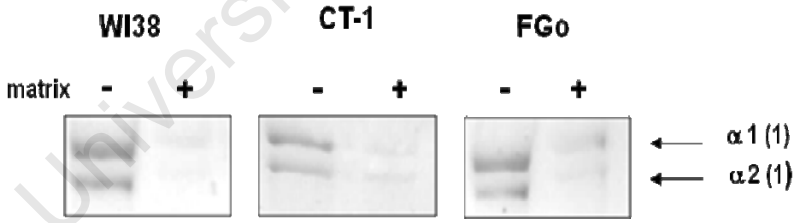


Figure 2.3 Effect of the fibroblast-derived ECM on the type I collagen synthesis.
(A) WI38, CT-1 and FGo fibroblasts were seeded on dishes with or without fibroblast-derived ECM for 24 hrs and then grown in DMEM for 48 hrs. Cell lysates (50 µg total protein) were separated by 7.5 % SDS PAGE and subjected to immunoblotting with goat anti-type I collagen antibody (1:1000). The secondary antibody used was donkey anti-goat IgG conjugated to horse radish peroxidase (1:1000). Arrows indicate the two α chains. β-tubulin was used as a loading control to confirm equal loading of samples. (B) Time-dependent effect of the fibroblast-derived ECM on the type 1 procollagen synthesis. CT-1 fibroblasts were treated as described above and incubated for the periods indicated (0, 24, 48, 72 hrs). Type 1 procollagen protein levels in the cell lysates were determined by immunoblotting as described in (A). (C) WI38, CT-1 and FGo fibroblasts were seeded on dishes with or without fibroblast-derived ECM for 24 hrs and then grown in serum-free DMEM for 48 hrs. The medium was harvested and secreted type I collagen was isolated by pepsin digestion (10 µg/ml) and TCA (10%) precipitation (section 7.3.1). Precipitated proteins were separated by 7.5 % SDS PAGE and the gels were stained with Coomassie BB. After destaining the collagen bands were identified based on the molecular weight markers. The media used in the quantification of secreted type I collagen was based on 1x 10^6 cells.

Interestingly, there was no change in α1(1) collagen mRNA levels when fibroblasts were plated on fibroblast-derived ECM. These results suggest that matrix-mediated regulation of α1(1) and α2(1) collagen gene expression occurs via different mechanisms. Since the reduction in mRNA levels for the three fibroblasts cell lines were similar, only CT-1 fibroblasts were used in a time-dependent study and other subsequent studies. The maximum reduction in α2(1) mRNA levels was already manifested after 24 hrs, following the same trend seen with the proteins (Fig 2.4 D). COL1A2 mRNA levels were

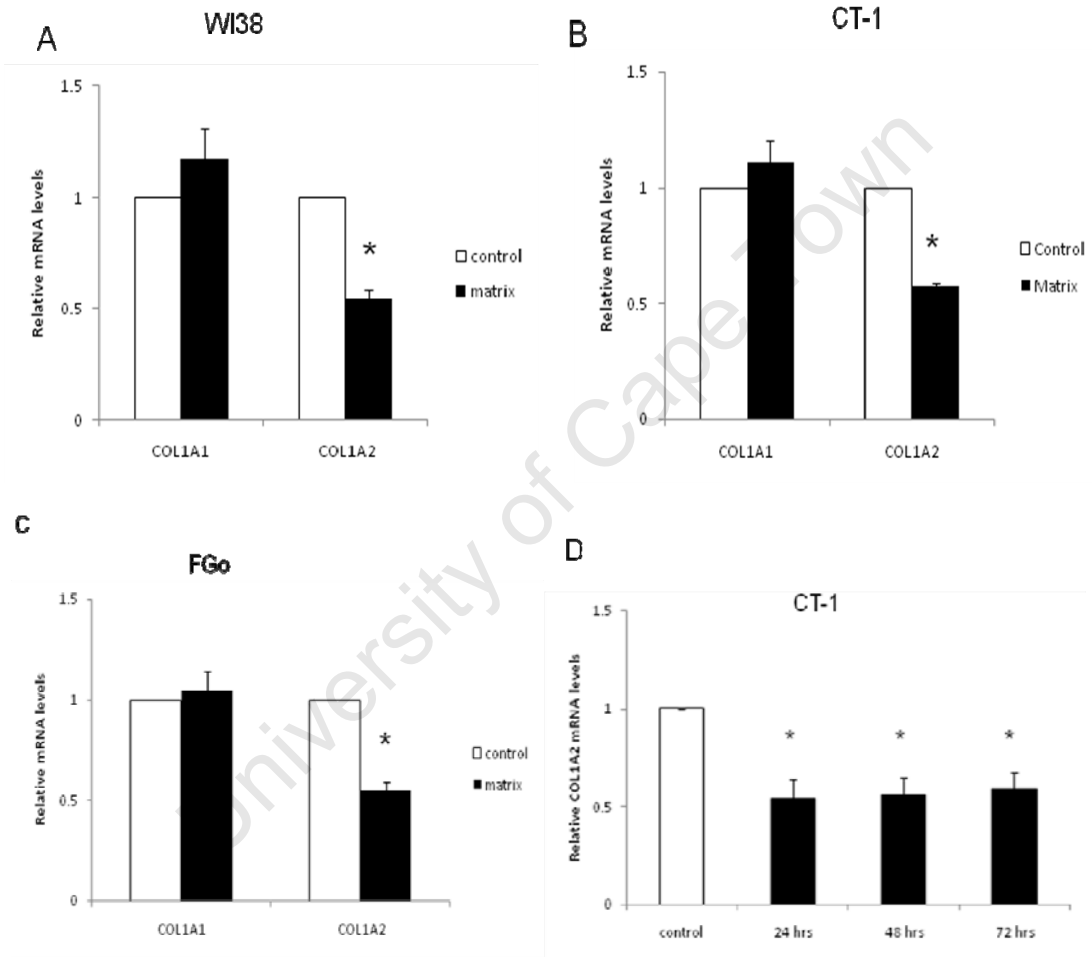


Figure 2.4 Effect of fibroblast-derived ECM on COL1A1 and COL1A2 mRNA levels.
WI38, CT-1 and FGo fibroblasts were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then cultured for 48 hrs. Cells were washed with PBS and RNA was extracted using Trizol reagent according to the manufacturer's instructions. Five micrograms of total RNA isolated from both control fibroblasts plated on plastic and fibroblasts plated on a matrix were used in cDNA synthesis and qRT-PCR was performed to evaluate COL1A1 and COL1A2 mRNA levels using GAPDH as a control as described in section 7.2.6. (A-C). The data is shown as mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic. (D) Time-dependent effect of the matrix on COL1A2 gene expression. CT-1 fibroblasts were treated as described above and incubated for the periods indicated for COLIA2 mRNA quantitation using qRT-PCR. The data is shown as the mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

significantly down in fibroblasts plated on ECM, suggesting transcriptional regulation.

2.2.5 Type I collagen mRNA stability

The effect of the fibroblast-derived ECM on the stability of α1(1) and α2(1) collagen mRNA was determined by plating fibroblasts on either plastic or ECM as previously described. After 24 hrs, actinomycin D was added to a final concentration of 1mg/ml (Mimura *et al*, 2006; Sohara *et al*, 2002). At designated time points after addition of actinomycin D (6, 12, 18 hrs), α1(1) and α2(1) collagen mRNA levels were determined by qRT PCR using GAPDH as the normaliser. The COL1A1 and COL1A2 mRNA levels for control and those plated on a matrix were expressed relative to the value at time 0 hrs (before addition of actinomycin) which was designated the value 1. The results demonstrate similar decay curves for both α1(1) and α2(1) collagen mRNA in fibroblasts plated on plastic and on a matrix (Figure 2.5, 2.6). The matrix therefore does not affect the stability of α1(1) and α2(1) collagen mRNA in fibroblasts.

2.2.6 Analysis of collagen degradation and matrix metalloproteinases

The COL1A1 mRNA levels did not change in the presence of the matrix while the α1(1) protein chain was significantly downregulated. This implies that the fibroblast-derived ECM not only inhibited the synthesis of type I collagen but could also be associated with its degradation. The effect of the fibroblast-derived ECM on the expression of major proteases of type I collagen was examined by quantitative real time RT PCR, immunoblot analysis and gelatin zymography. To evaluate the potential contribution of MMP1 in the matrix-mediated downregulation of type I collagen, real time RT PCR was used to determine the MMP 1 mRNA levels. The expression of MMP1 mRNA in the presence of the fibroblast-derived ECM did not show any significant difference as compared to that of controls, in any of the three cell lines (Fig 2.7). The promoter activity of MMP1 was also determined using the luciferase reporter gene in fibroblasts transfected with a construct containing the 4.3 kb MMP 1 promoter region fused to the luciferase (LUC) reporter gene (Znoyko *et al*, 2006). Similar to the mRNA levels, there were no significant differences in

promoter activity between fibroblasts plated on plastic and those grown on a matrix (Fig 2.8).

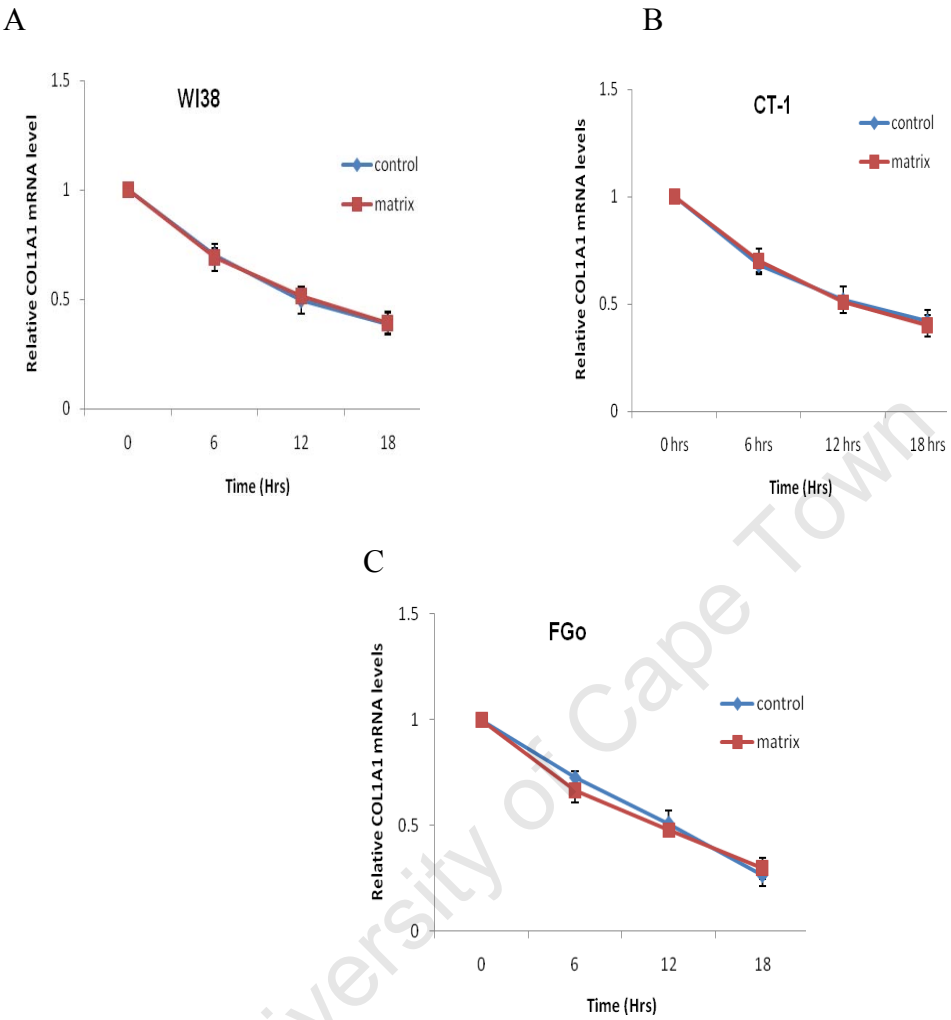


Figure 2.5 Effect of fibroblast-derived ECM on the stability of COL1A1 mRNA. WI38, CT-1 and FGo fibroblasts (A-C) were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then cultured for the periods indicated (6, 12, 18 hrs) after the addition of actinomycin D to a final concentration of 1mg/ml. Cells were washed with PBS and RNA was extracted using Trizol reagent as described in section 7.2.6.1. Five micrograms of total RNA was quantified by qRT-PCR using GAPDH as a control. The COL1A1 mRNA levels for control and those plated on a matrix were expressed relative to the value at time 0 hrs (before addition of actinomycin) which was designated the value 1. The data are shown as the mean ± S.D. of three independent experiments.

Collagen-degrading activity is considered to be one of the most important biological activities of MT1 MMP (Holmbeck *et al*, 1999). The effect of the fibroblast-derived ECM on the MT1 MMP protein levels was determined.

As shown in Fig 2.9, there were no significant differences in the protein levels of MT1 MMP in fibroblasts plated on the matrix compared to controls. The

bands corresponding to the pro-MT1 MMP and the active MT1 MMP were constitutively present in absence and presence of the fibroblast-derived ECM.

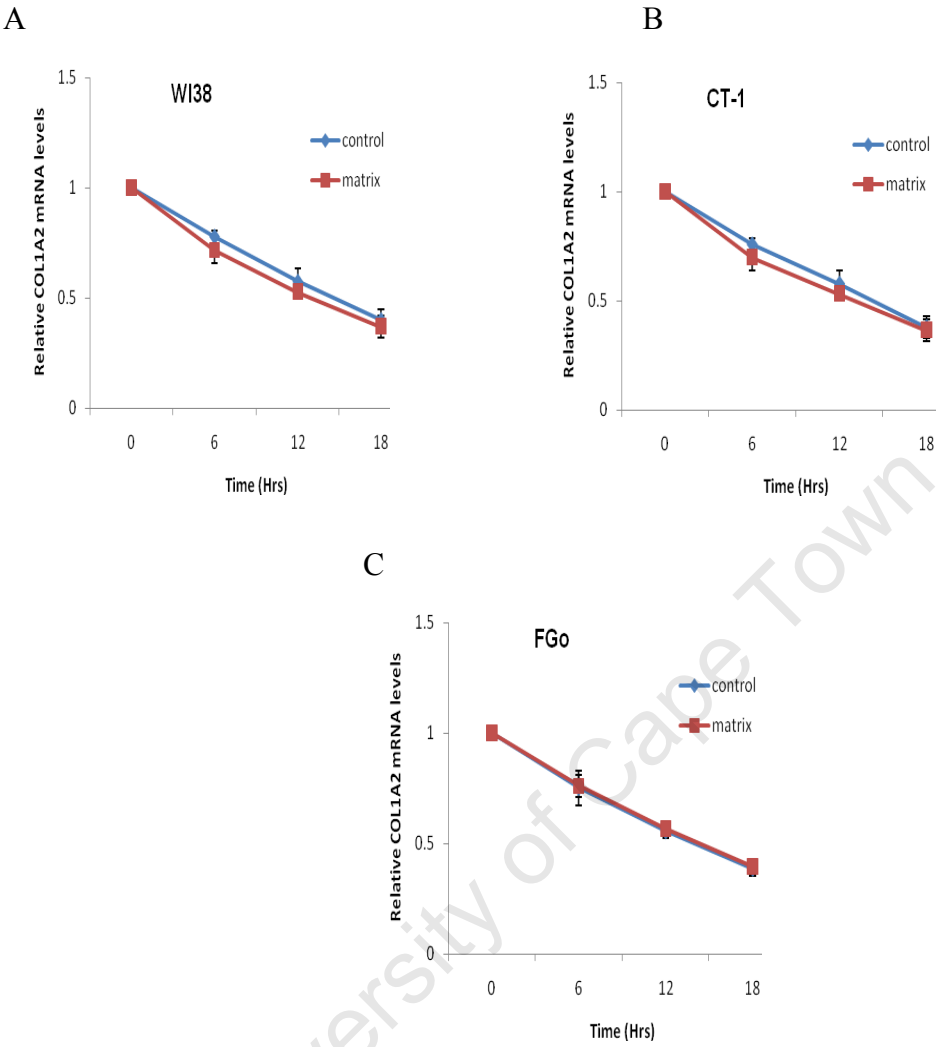


Figure 2.6 Effect of fibroblast-derived ECM on the stability of COL1A2 mRNA.
WI38, CT-1 and FGo fibroblasts (A-C) were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then cultured for the periods indicated (6, 12, 18 hrs) after the addition of actinomycin D to a final concentration of 1mg/ml. Cells were washed with PBS and RNA was extracted using Trizol reagent as described in section 7.2.6.1. Five micrograms of total RNA was quantified by qRT-PCR using GAPDH as a control. The COL1A2 mRNA levels for control and those plated on a matrix were expressed relative to the value at time 0 hrs (before addition of actinomycin) which was designated the value 1. The data are shown as the mean ± S.D. of three independent experiments.

The ¾ and ¼ fragments expected from type I collagen degradation by the collagenases were not observed on SDS-polyacrylamide gels implying that if any degradation is taking place then type I collagen was being degraded to smaller fragments by the combined action of collagenases and gelatinases. To evaluate the potential contribution of the gelatinases, MMP-2 and MMP-9, in the matrix-mediated downregulation of type I collagen, the MMP-2 and

MMP-9 mRNA levels were determined by real time RT PCR. The expression of MMP-2 and MMP-9 mRNAs in the presence of the fibroblast-derived ECM did not show any significant difference as compared to that of controls in any of the three cell lines (Fig 2.10).

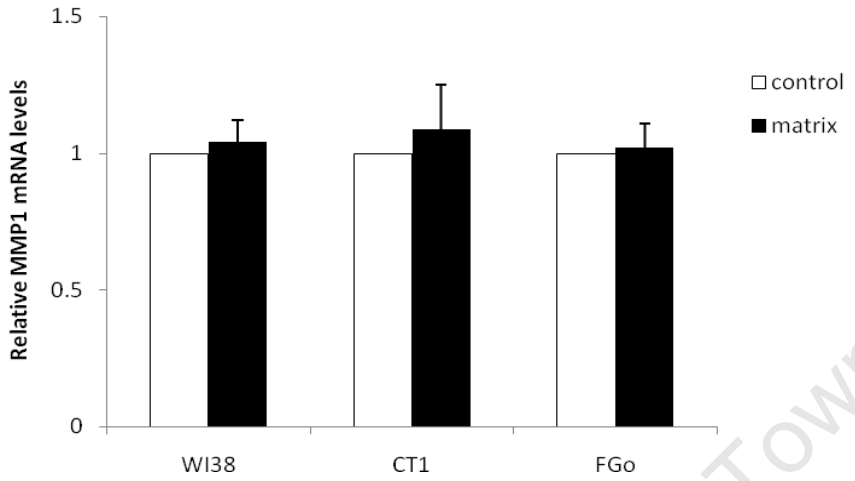


Figure 2.7 Effect of fibroblast-derived ECM on MMP-1 mRNA levels.
WI38, CT-1 and FGo fibroblasts were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then grown in DMEM media for 48 hrs. Cells were washed with PBS, RNA extracted using Trizol reagent and five micrograms of total RNA were used in cDNA synthesis for qRT-PCR to evaluate MMP-1 mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments.

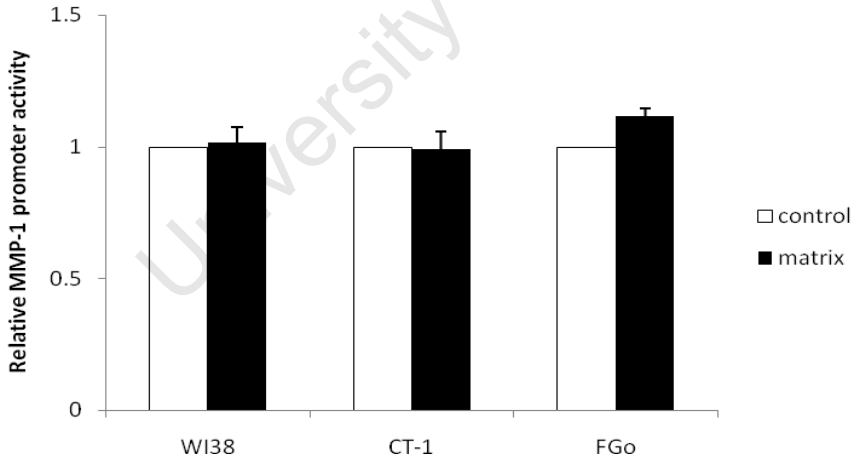


Figure 2.8 Effect of fibroblast-derived ECM on MMP-1 promoter activity.
WI38, CT-1 and FGo fibroblasts were transiently transfected with either empty vector or 4.3 kb MMP-1 promoter construct linked to the luciferase reporter gene. Transfectin reagent was used in all transfection assays as described in section 7.2.8. Transfected fibroblasts were harvested by trypsinisation and equal numbers of fibroblasts (3×10^5) were seeded in 6-well plates with or without fibroblast-derived ECM for 48 hrs. Cell extracts were obtained using passive lysis buffer and assayed for luciferase activity. Reporter activities are expressed as ratios of firefly luciferase to renilla luciferase. The fibroblast-derived ECM does not affect the promoter activity. The data is shown as mean ± S.D. of three independent experiments.

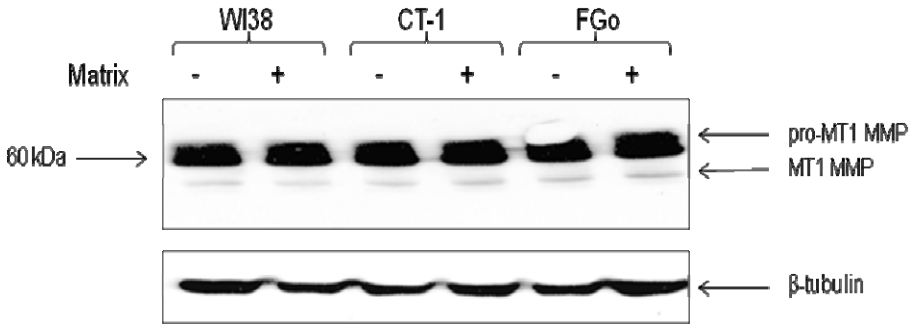


Figure 2.9 Effect of the fibroblast-derived ECM on MT1 MMP protein levels.
WI38, CT-1 and FGo fibroblasts were seeded on dishes with or without fibroblast-derived ECM for 24 hrs and after changing media, were cultured for 48 hrs. Cell lysates (50 µg total protein) were separated by 7.5 % SDS PAGE and subjected to immunoblotting with mouse anti-MT1 MMP antibody (1:1000). The secondary antibody used was goat anti-mouse IgG conjugated to horse radish peroxidase (1:1000). Arrows show the two bands corresponding to pro- and active MT1 MMP respectively (p-MT1 MMP and MT1 MMP). β-tubulin was used as a loading control to confirm equal loading of samples

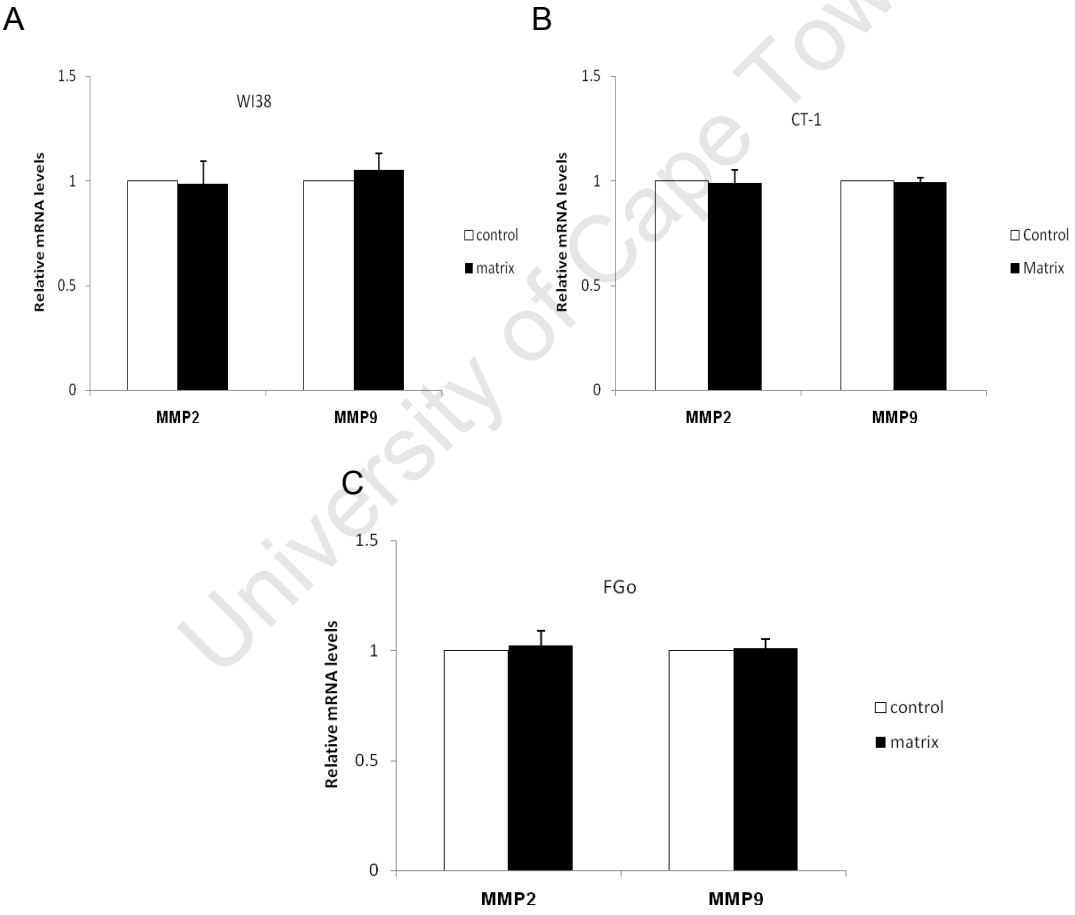


Figure 2.10 Effect of fibroblast-derived ECM on MMP-2 and MMP-9 mRNA levels.
WI38, CT-1 and FGo fibroblasts (A-C) were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then grown in DMEM media for 48 hrs. Cells were washed with PBS and RNA was extracted and five micrograms of total RNA were used in cDNA synthesis and qRT-PCR was performed to evaluate MMP-2 and MMP-9 mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments.

The levels of MMP mRNAs and overall MMPs protein abundance do not translate into active MMPs, therefore it is important to check the actual activities of the MMPs. The activities of the gelatinases, MMP-2 and MMP-9, were determined by gelatin zymography of the culture media. The fibroblast-derived ECM did not significantly alter the activities of either MMP-2 or MMP-9 after 24 hrs and 48 hrs of incubation (Fig 2.11). The minor differences in activities observed especially at 24 hrs might contribute to bring about the differences in type I collagen levels as observed in this study. There was a time-dependent activation of both latent pro-MMP-2 and pro-MMP-9 as the active enzymes are clearly visible. Active MMP-2 was visualised as a clear band at 67 kDa whilst active MMP-9 was also visible at around 88 kDa. The activity of MMP-9 was less prominent in all fibroblasts compared to the activity

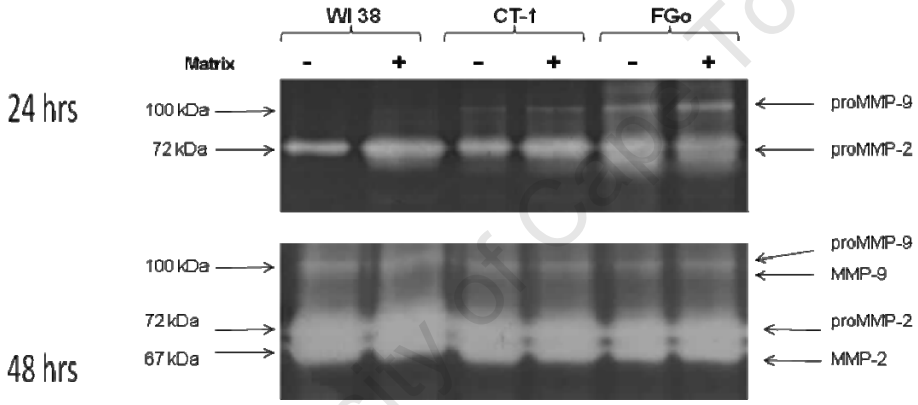


Figure 2.11 Effect of fibroblast-derived ECM on MMP-2 and MMP-9 activity.
WI38, CT-1 and FGo fibroblasts were seeded in dishes with or without ECM for 24 hrs and then incubated in DMEM for 24 hrs (upper panel) or 48 hrs (lower panel) at 37^0C. Gelatin zymography was performed on the media samples as described in section 7.2.7. Media samples (50 µg total proteins) were subjected to electrophoresis on 10 % SDS polyacrylamide gels containing 1 mg/ml gelatin. After electrophoresis gels were washed in 2.5 % Triton X-100 (to remove SDS), incubated in digestion buffer (50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.2 M NaCl and 0.02 % Brij-35) for 18 hrs at 37^0C then stained with Coomassie Brilliant Blue R250. After destaining, the gelatinolytic activity was observed as clear bands in the gels. Bands corresponding to pro- and low molecular weight, active MMP-2 and MMP-9 are clearly visible in the 48 hr samples (lower panel). Data are representative of three independent experiments.

of MMP-2. Not all proteases were investigated and therefore other proteases may be responsible for the difference between mRNA and protein levels. An alternative explanation could be decreased translation of the COL1A1 mRNA in the presence of the fibroblast-derived ECM and the possible involvement of microRNAs.

2.2.7 Functional mapping of the matrix-responsive element (MRE) in the human COL1A2 promoter

To determine whether the decrease in α2(1) mRNA levels in the presence of a matrix was due to an alteration in promoter activity, a series of 5' deletion constructs of the human COL1A2 promoter, spanning from -2389 bp to +54bp relative to the transcription start site, linked to the luciferase gene were used in mapping the promoter (Fig. 2.12). These COL1A2/luciferase reporter constructs were transiently transfected into WI38, CT-1 and FGo fibroblasts. Transfected fibroblasts were harvested by trypsinisation and equal numbers of fibroblasts (3×10^5) were seeded in 6-well plates with or without fibroblast-derived ECM. Six hours after transfection the media was changed and incubation was continued for 48 hrs. The luciferase activities were then measured. The fibroblast-derived ECM down-regulated the promoter activity in constructs COL -2389/LUC, COL -721/LUC and COL-375/LUC, matrix vs control. The downregulation was similar for the above constructs, but no significant change in the activity of COL -107/LUC construct was observed, matrix vs control. These findings suggest that the fibroblast-derived ECM effect was mediated via a regulatory element(s) located between residues -375 and -107 of the human COL1A2 promoter (Fig 2.12). Similar results were also obtained for WI38 and FGo fibroblasts.

This region of the COL1A2 promoter contains the binding sites for many transcription factors, chief among them being Sp1/Sp3, AP1 and the Smads that are implicated in the regulation of type I collagen synthesis (Kubota *et al*, 2003; Wang *et al*, 2005; Ghosh, 2002). Sp1 is implicated in both downregulation and upregulation of COL1A2 promoter activity depending on the other transcription factors it associates with. Sp1 has been found to be a repressor of COL1A2 promoter activity when it interacts with Fli-1 (Ramirez *et al*, 2006; Asano *et al*, 2009). The Fli-1 response element has been mapped between -353 and -186 bp of the COL1A2 promoter which contains Ets and Sp1/Sp3 binding sites (Czuwara-Ladykowska *et al*, 2001). Evidence from many studies implicates Sp1 and Sp3 along with other factors such as Fli-1 and NF-1 in the control of type I collagen synthesis in normal fibroblasts as well (Asano *et al*, 2009; Ghosh, 2002; Czuwara-Ladykowska *et al*, 2001).

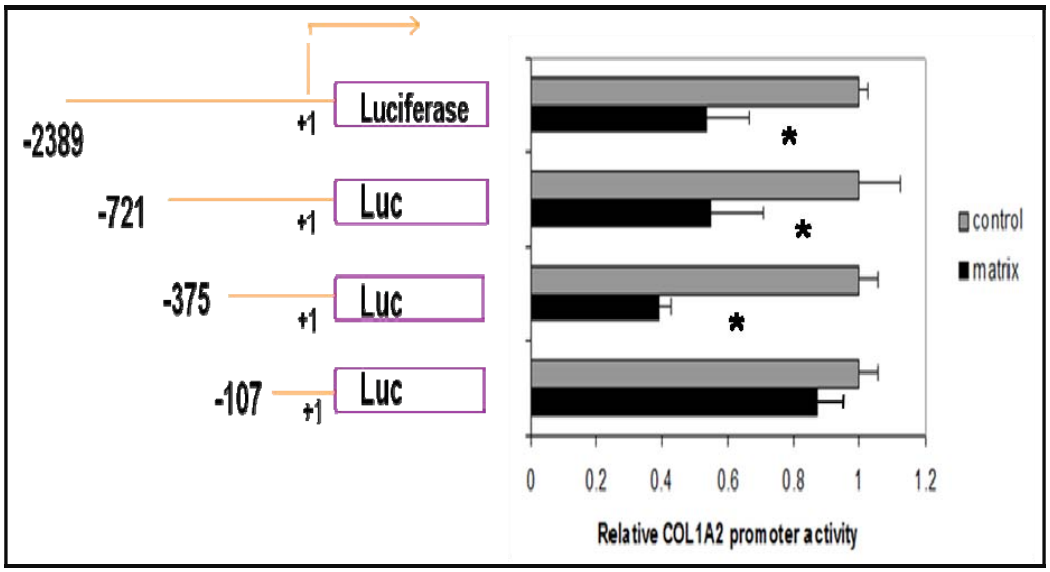


Figure 2.12 Promoter activity of the human proximal α2(1) procollagen promoters. CT-1 fibroblasts were transiently transfected with either empty vector or 5' deletion COL1A2 constructs linked to the luciferase reporter gene as shown. Transfectin reagent was used in all transfection assays as described in section 7.2.8. Transfected fibroblasts were harvested by trypsinisation and equal numbers of fibroblasts (3×10^5) were seeded in 6-well plates with or without fibroblast-derived ECM. Six hours after transfection the media was changed and incubation was continued for 48 hrs. Reporter activities were expressed as ratios of firefly luciferase to renilla luciferase. The fibroblast-derived ECM decreased α2(1) promoter activity significantly via a DNA responsive element located between nucleotides -375 and -107. Similar results were obtained when WI38 and FG0 fibroblasts were used. The data are shown as the mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic (control).

Further evidence that the matrix responsive element (MRE) could be located in the COL1A2 promoter, as suggested by both mRNA and promoter studies, comes from studies on SV-WI38 fibroblasts. SVWI38 cells synthesise type I collagen trimer comprising mainly α1(1) chains (Parker *et al*, 1989). When SV-WI38 fibroblasts were grown on a pre-formed fibroblast-derived ECM, COL1A1 mRNA and protein levels in cellular lysates did not change compared to controls. This is contrary to what was observed in WI38, CT-1 and FGo fibroblasts, where COL1A2 decreased significantly in the presence of the matrix.

2.2.8 Effect of Sp1 on type I collagen gene expression

Within the -375 to -107 region of the COL1A2 promoter are binding sites for many transcription factors including Sp1. To study the influence of Sp1 on matrix-mediated downregulation of type I collagen, a direct loss of function approach by silencing the endogenous Sp1 by RNAi was used (Lee *et al*,

2006; Tatenhorst *et al*, 2006). Since previous results for the three fibroblasts, WI38, CT-1 and FGo, were similar, only CT-1 were used in this and subsequent studies. Western blot analysis and real time PCR revealed that the siRNA duplex targeted to nucleotides of the human Sp1 gene led to marked reduction in the amount of endogenous Sp1 in CT-1 fibroblasts (Figure 2.13 A, B). The highest reduction of Sp1 was observed within 24 hours, so experiments addressing type I collagen gene expression using siRNA-transfected fibroblasts were performed using the 24 hour period. Since Sp1 siRNA successfully downregulated Sp1 mRNA and protein levels (in both cellular and nuclear proteins) in fibroblasts, it was used in experiments to examine whether this downregulation affected type I collagen gene expression.

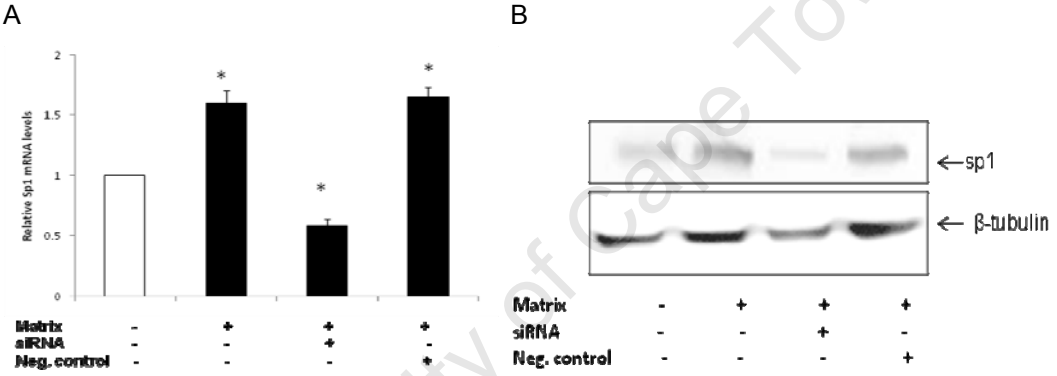


Figure 2.13 Effect of Sp1 small interfering RNA (siRNA) on Sp1 gene expression in fibroblasts. CT-1 fibroblasts were seeded on dishes with or without fibroblast-derived ECM for 24 hrs and then transfected with siRNA targeting endogenous human Sp1. An irrelevant siRNA molecule of similar size was used as a negative control in experiments where indicated. Incubation was continued for 24 hrs. (A) Cells were washed with PBS, RNA extracted using Trizol reagent and five micrograms of total RNA were used in cDNA synthesis. Quantitative RT-PCR was performed to evaluate Sp1 mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments (*$p \leq 0.05$). (B) Cell lysates (50 µg total proteins) were separated by 7.5 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with anti-Sp1 antibody. β-tubulin was used as a loading control to confirm equal loading of samples.

As shown in Figure 2.14 and Figure 2.15, transfection with siRNA duplex targeting Sp1, reversed the effect of the matrix on type I collagen protein and mRNA levels. The negative control siRNA when used in the presence of the matrix had no effect. Thus siRNA technology, provided evidence that Sp1, possibly in combination with other transcription factors, is involved in the matrix-mediated downregulation of the COL1A2 gene.

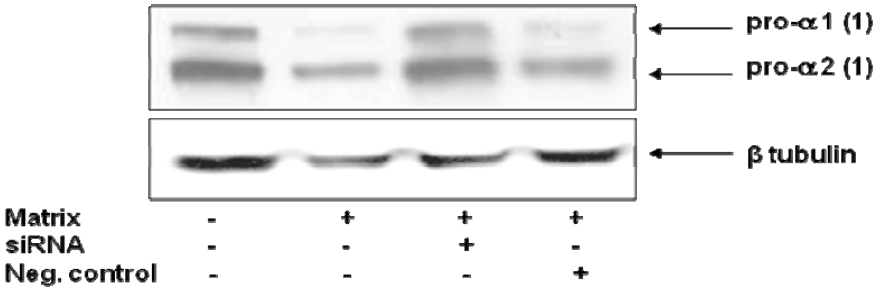


Figure 2.14 Effect of Sp1 small interfering RNA (siRNA) on type I collagen protein levels. CT-1 fibroblasts were seeded on dishes with or without fibroblast-derived ECM for 24 hrs and then transfected with siRNA targeting endogenous human Sp1. An irrelevant siRNA molecule of similar size was used as a negative control in experiments where indicated. Incubation was continued for 24 hrs. Cell lysates (50 µg total protein) were separated by 7.5 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with goat anti-type I collagen antibody (1:1000). The secondary antibody used was donkey anti-goat IgG conjugated to horse radish peroxidase (1:1000). Arrows indicate the two pro-α chains. β-tubulin was used as a loading control to confirm equal loading of samples.

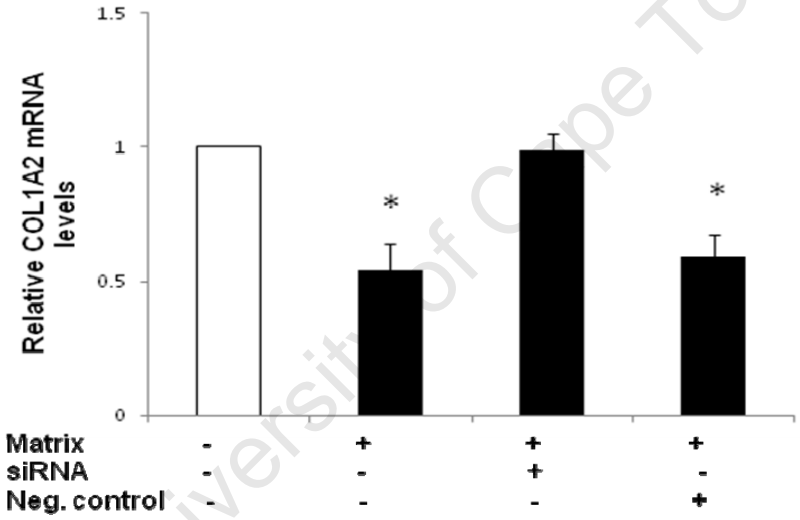


Figure 2.15 Effect of Sp1 small interfering RNA (siRNA) on COL1A2 mRNA levels in fibroblasts. CT-1 fibroblasts were seeded on dishes with or without fibroblast-derived ECM for 24 hrs and then transfected with siRNA targeting endogenous human Sp1. An irrelevant siRNA molecule of similar size was used as a negative control in experiments where indicated. Incubation was continued for 24 hrs. Cells were washed with PBS, RNA extracted using Trizol reagent and five micrograms of total RNA were used in cDNA synthesis. Quantitative RT-PCR was performed to evaluate COL1A2 mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

2.2.9 DISCUSSION

Traditionally the extracellular matrix is viewed as a relatively stable structural substrate for cells, but recent work has shown that it plays a prominent role in providing information to specific receptors on the cell surface and determine how they interpret biochemical data from their surrounding stroma (Stefanovic, 2005; Mauch and Kreig, 1990; Ramirez and Rifkin, 2003). The matrix contains specific receptor binding ligands and also conveys mechanical information to cells. It is now clear that cell-matrix interactions induce biological responses that are similar to those induced by growth factors, hormones and cytokines through specific receptors. In order to accurately determine how the properties of the matrix influence cellular behaviour, it is very important to be able to prepare experimental model systems that allow reliable reproduction of the *in vivo* matrix. Purified individual components of the ECM such as type I collagen, fibronectin and laminin have been used in previous studies and are all intended to mimic the ECM microenvironment, but the usefulness of these models is unknown. Under *in vivo* conditions, the ECM components are synthesised in a specific ratio to each other and mixing of these components *in vitro* will not truly represent the *in vivo* conditions under which cells synthesise and assemble the extracellular matrix. Accordingly, a natural fibroblast-derived ECM was used in this study in an attempt to closely simulate the *in vivo* environment.

This study showed downregulation of type I collagen gene expression when fibroblasts are plated on this fibroblast-derived ECM. The matrix-mediated downregulation of type I collagen synthesis resides mainly at the transcriptional level for the COL1A2 gene. There was no change in COL1A1 mRNA levels when the fibroblasts were plated on the matrix but there was significant downregulation of COL1A2 mRNA levels. There is compelling evidence that the steady-state levels of many proteins is regulated at multiple steps. When there is a large change in the amount of either mRNA or protein, it is likely that multiple steps in the metabolism of the mRNA and protein have been altered (Stefanovic, 2005; Lindquist *et al*, 2000). The steady-state level of the mRNA encoding the specific protein is determined not only by the rate of transcription, but also by the half-life of the mRNA in the cytoplasm.

Collagen mRNAs belong to the long-lived mRNAs and its stability depends on the culture conditions (Stefanovic, 2005). It has been reported that fibroblasts grown within Matrigel downregulate collagen α1(1) mRNA steady state levels by destabilising the mRNA (Eckes *et al*, 1993, 1999, 2000; Mauch and Kreig, 1990; Stefanovic *et al*, 1997). Therefore in this study, the stability of the α1(1) and α2(1) mRNAs were determined using Actinomycin D. There was no significant difference in the stability of both α1(1) and α2(1) collagen mRNA.. The next control step could be at the level of translation. Substantial evidence has accumulated in recent years showing that type I collagen synthesis is regulated at a post transcriptional level in various cell types (Eckes *et al*, 1993, 1999; Focht and Adams, 1984; Mauch and Kreig, 1990; Sato, 1995, 2000; Stefanovic, 1997, 1999, 2000, 2005). The possible degradation of type I collagen was therefore examined.

A correlation between the secretion of MMPs and TIMPs and collagen levels has been reported (Laliberte *et al*, 2001). We therefore analysed cell lysates and the media for MMPs, specifically MMP1, MT1 MMP, MMP 2 and MMP 9. Since MMP1 is one of the major type I collagenases its mRNA and promoter activity were determined. There were no significant differences in both MMP1 mRNA levels and promoter activities or MT1 MMP protein levels in the presence and absence of the fibroblast-derived ECM. There were no significant differences as well in MMP 2 and MMP 9 mRNAs levels and activities. It is likely that the fibroblast-derived ECM might affect other stages of collagen synthesis such as mRNA translation. It is also possible that a new area of regulation of type I collagen involving microRNAs is involved. Vast amount of information on the involvement of microRNAs in the regulation of other genes is available but no such information is available on the type I collagen gene. The substrate upon which cultured fibroblasts are maintained has been shown to be capable of phenotypic modification of cells (Vlodasky *et al*, 1980; Gospodarowcz *et al*, 1978). In our study there appear to be slight morphological differences between fibroblasts plated on plastic and ECM. Fibroblasts cultured within the fibroblast-derived ECM had a slightly elongated, spindle shaped morphology. The mechanism underlying this difference in

morphology remains to be clarified although it is possible that a loss of polarity may contribute to this effect (Beningo *et al*., 2004)

Deletion analysis showed that a 268-bp DNA element (-375/-107) in the human COL1A2 gene contains the matrix-responsive element (MRE) that mediates the matrix-downregulation of type I collagen synthesis. This region contains the binding sites for many transcription factors that affect type I collagen synthesis, with Sp1/Sp3, Ets1, the Smads and AP1 being well-known examples (Wang *et al*, 2005; Ghosh, 2002). These transcription factors interact with several co-factors and /or other promoter-bound complexes (Ihn *et al*, 1996; Sengupta *et al*, 2002). Wan *et al* (2001) reported that the expression of several transcription factors including Fli1, Sp1, c-jun and c-fos is required to activate or deactivate the transcription of many other genes. The COL1A2 proximal promoter is under the control of several clusters of cis-acting elements, which bind positively and negatively trans-acting factors (Ramirez *et al*, 2006). These proteins exert their different effects on transcription by binding to their respective sites and through interactions with other components of the promoter-bound protein complexes (Czuwara-Ladykowska *et al*, 2001). These combinatorial interactions amongst positive and negative nuclear factors determine the end point activity of the COL1A2 promoter under different cellular and experimental conditions. The present findings indicating Sp1-mediated downregulation of COL1A2 expression in fibroblasts extend the scope of functional Sp1 activity in fibroblast growth and differentiation. siRNA to Sp1 was used in this study and provided evidence that Sp1 has a negative regulatory role on type I collagen gene expression in fibroblasts in the presence of fibroblast-derived ECM. It has been shown before that Sp1 activates or represses different promoters depending on the other transcription factors it binds to or form complexes with (Santiago and Khachigian, 2004). Further investigation is needed to clarify the transcriptional machinery and to verify whether other transcription factors, such as Fli1, are also involved. These *in vitro* studies provide key insights into the role of Sp1 activity in COL1A2 transcription and could be studied further through whole animal analyses in transgenic mice bearing COL1A2 promoter mutations in the Sp1 elements.

Collagen remodelling by fibroblasts appear to have a critical effect on healing and in invasion and metastasis, thus gaining more information on how fibroblasts respond to its ECM is important. This study has shown that the gene expression of ECM proteins and type I collagen in particular can all be regulated by the environment with which cells interact. We propose that ECM-dependent regulation of proteins is part of a general mechanism whereby cells react to different substrata and establish necessary cell-matrix interactions essential for survival and differentiation. An improved understanding of the signaling pathways involved in cellular remodelling of collagen would be beneficial in understanding collagen diseases and many pathological conditions such as cancer cell invasion and metastasis. In conclusion, the present study has demonstrated that the proximal region of the COL1A2 gene promoter mediates the matrix–mediated downregulation of the human COL1A2 gene in fibroblasts. The present study offer the possibility of manipulating transcription factors as potential chemotherapeutic tools for modulating type I collagen in conditions such as fibrosis and in wound healing.

CHAPTER 3

SIGNALING PATHWAYS IN THE MATRIX-MEDIATED DOWNREGULATION OF TYPE I COLLAGEN SYNTHESIS

3.1 INTRODUCTION

The response of cells to extracellular stimuli is mediated by a number of intracellular kinases and phosphatases enzymes. The mitogen-activated protein kinases (MAPKs) for example are members of discrete signaling cascades that are focal pathways for diverse extracellular stimuli and regulate fundamental cellular processes. Most of these signaling cascades are activated through attachment of cells to the ECM via receptors such as the integrins. Integrin-mediated attachment to the ECM can activate several signaling pathways including focal adhesion kinase (FAK), PI3-kinase, MAPKs p38, JNK and the MEK-ERK pathways (Figure 3.1) (Giancotti and Ruoslahti, 1999; Ge *et al*, 2007; Chetoui *et al*, 2005; Ihn *et al*, 1996; Roux and Blenis, 2004; Pearson *et al*, 2001).

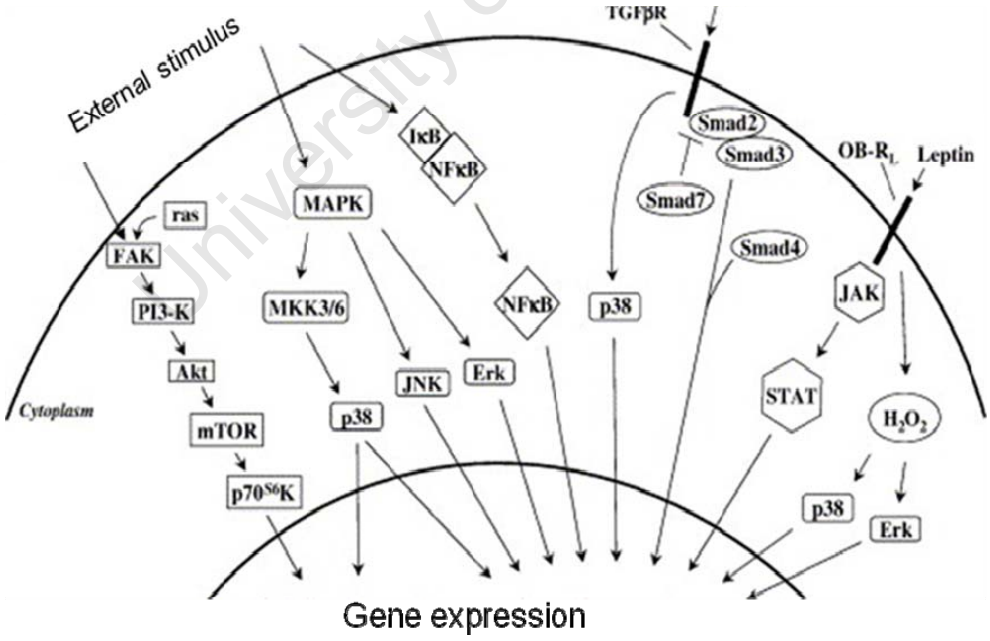


Figure 3.1 The various cellular signaling pathways found in fibroblasts. Most of these signaling cascades are activated by external stimuli via receptors such as the integrins. Integrin-mediated attachment to the ECM can activate several signaling pathways including focal adhesion kinase (FAK), PI3-kinase, MAPKs p38, JNK and the MEK-ERK pathways (Tsukada *et al*, 2006).

A number of observations strongly suggest that the activation of FAK by integrins plays a central role in initiating many of the signals that regulate growth of cells (Boudreau and Jones, 1999). Activation of the PI3-kinase pathway is mainly involved in cell survival whilst activation of the MAPKs by integrins provides a pathway to cell survival and to transcription of genes involved in cell growth and differentiation (Chetoui *et al*, 2005). p38 MAPK is generally activated in response to growth factors, cell stress and injury (Howe *et al*, 1998, 2002) whilst JNK is a well known positive regulator of cellular proliferation in fibroblasts. JNK activity is regulated by two upstream signaling kinases MKK4 and MKK7. The mechanistic basis for the integrin-mediated activation of the MAPKs is somewhat controversial and has not yet been fully elucidated. Both Ras-dependent (Lee and Juliano, 2004) and Ras-independent (Chen *et al*, 1996; Howe *et al*, 1998) mechanisms have been described. Another unresolved issue regarding integrin-mediated signaling is whether integrins themselves actually initiate the signaling or whether they simply modulate or enhance signals generated by soluble mediators such as growth factors (Boudreau and Jones, 1999, Chen *et al*, 1996).

The MEK-ERK signaling pathway is one of the principal cytoplasmic signal transduction systems governing eukaryotic cellular proliferation, differentiation and survival (Bobick and Kulyk, 2006; Chang *et al*, 2003; Johnson *et al*, 1988). The pathway consists of three serially phosphorylating protein kinases, Raf, MEK and ERK. This basic triple kinase cascade functions downstream of a diverse array of membrane receptors such as receptor tyrosine kinases (Schlessinger, 1994; Schlessinger and Bar-Sagi, 1995), G-protein coupled seven transmembrane receptors (Naor *et al*, 1997, 2008), integrins (Giancotti and Ruoslahti, 1999) and ion channels (Rane, 1999). Depending on the extracellular activating stimuli, the MEK-ERK pathway is triggered by a set of adaptor proteins. These adaptor proteins link the receptor to GDP/GTP exchange factors which subsequently activate small GTP-binding proteins such as Ras, Rap1 and Rac.

Ras is a membrane-bound GDP/GTP-binding protein that serves as a ‘molecular switch’ converting signals from the cell membrane to the nucleus.

The Ras family includes several distinct members such as H-Ras, K-Ras, M-Ras and N-Ras. The recruitment and involvement of Ras in the activation of the MEK-ERK pathway has been supported by many studies through the use of N17Ras, a dominant negative mutant used to block activation of MAPK (Stewart and Guam, 2000; Clark *et al*, 2000). Activation of Ras is followed by the sequential activation of Raf-1, MEK 1,2 and ERK 1,2 in the MEK-ERK pathway (Pinzani *et al*, 1998). Other studies indicate the existence of a Ras-independent component of integrin-mediated MAPK activation (Katsumi *et al*, 2004; Chen *et al*, 1996). Some of the observed differences may be due to the existence of several overlapping signaling pathways, with one pathway or another predominating in a particular cell type or experimental situation. Furthermore, activation of Raf-1 has been shown to regulate integrin-mediated ERK activation through MEK 1 (Barberis *et al*, 2000). Most of these studies have been done with plastic tissue culture dishes or using ECM components such as fibronectin and laminin.

The GTP binding proteins activate the Raf family of serine threonine kinases upstream of the triple kinase phospho-relay unit of the cascade (Bobick and Kulyk, 2006; Lewis *et al*, 1998).The phosphorylated ERK 1,2 subsequently translocate into the nucleus (Ge *et al*, 2007). Once in the nucleus, the kinases phosphorylate and activate several transcription factors including Elk-1and the fos proteins, resulting in various cellular responses including differentiation and regulation of specific metabolic pathways. In this study, a fibroblast-derived ECM was used in tissue culture to dissect the signaling pathway associated with the matrix-mediated downregulation of type I collagen gene expression using specific pathway inhibitors. Finally, to confirm the effects of the MEK-ERK signaling pathway and its associated upstream proteins on type I collagen gene expression, cultures of fibroblasts were transfected with the dominant negative MEK1 and N17Ras expression plasmids.

3.2 RESULTS

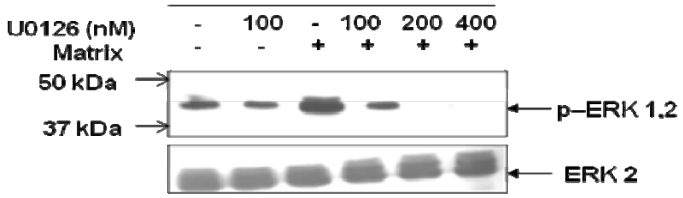
3.2.1 Analysis of the MEK-ERK signaling pathway in type I collagen gene regulation

The MAPK pathway is one of the major routes for signal transduction via integrins and it has been reported to be activated by plating cells on artificial matrices such as type I collagen (Wozniak *et al*, 2003, 2004). To determine the signalling pathway involved in the matrix-mediated downregulation of type I collagen gene expression, type I collagen levels were determined in cell lysates and the media, 24 hrs after adding the specific kinase inhibitors to fibroblasts seeded in dishes with or without the matrix. A dose dependent study was done to determine the concentration of U0126 and PD98059 required for MEK inhibition in the cell types used in the study. The levels of phosphorylated ERK 1,2 increased in cells grown on the fibroblast-derived ECM while type I collagen levels decreased significantly (Figure 3.2 A-B and Figure 3.3 A-B). In the presence of the MEK inhibitors, fibroblasts plated on matrix produced the same amount of type I collagen and mRNA as the control cells plated on plastic (Fig. 3.2 B-D; Fig. 3.3 B-D). U0126 and PD98059 were both able to reverse the matrix-mediated downregulation of type 1 collagen synthesis thus implicating the MEK-ERK signalling pathway in the matrix-mediated downregulation of type I collagen synthesis. This inhibition was not due to a reduction in cell growth or viability as the cell proliferation data showed that there is no significant difference between control fibroblasts and fibroblasts plated on a matrix (data not shown). The effects of U0126 and PD98059 on type I collagen synthesis and ERK 1,2 phosphorylation were identical and since PD98059 blocks only Raf-dependent phosphorylation/ activation of MEK1, it can safely be assumed that Raf-1 is involved as the upstream activator of MEK.

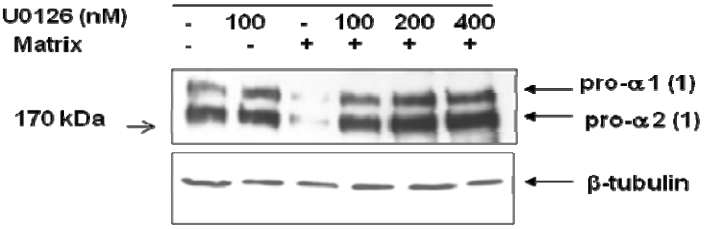
3.2.2 Analysis of the p38 MAPK, JNK and the PI3-kinase pathways

The JNK group of protein kinases are activated in response to a number of cellular stresses, including high osmolarity and oxidation (Ip and Davis, 1998; Kato *et al*, 2008). The p38 group kinases have been found to be involved in inflammation, cell growth, cell differentiation, the cell cycle, and cell death. It is clear, then, that the JNK protein kinases and p38 pathways share many

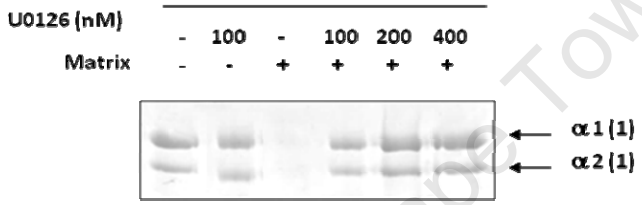
A. Phosphorylated ERK1,2



B. Intracellular type I collagen



C. Media type I collagen



D. COL1A2 mRNA

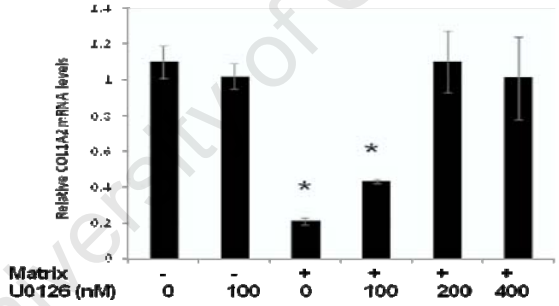
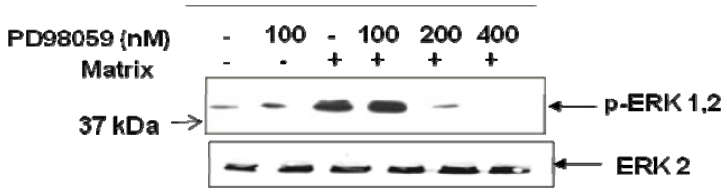
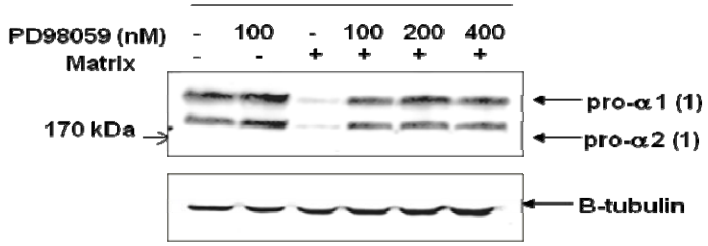


Fig 3.2 Effect of U0126 on matrix-mediated downregulation of type I collagen synthesis. The effect of the specific MEK 1,2 inhibitor, U0126, on type 1 procollagen gene expression was investigated as described in '*Materials and Methods*'. (A) CT-1 fibroblasts were seeded on dishes with or without fibroblast-derived ECM and then treated with the indicated concentrations of U0126 or 0.1% DMSO (as the solvent control) for 24 hrs. Cell lysates (50 µg total protein) were separated by 10 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with anti-*p*-ERK 1,2 antibody (1:500). The level of beta tubulin in the same sample was used as control. (B) CT-1 fibroblasts were treated as described above and cell lysates (50 µg total protein) were separated by 7.5 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting to evaluate type 1 procollagen protein levels as described in section 7.3.2. (C) Dose-dependent effect of U0126 on secreted type I collagen levels in the media. CT-1 fibroblasts were treated as described above, the medium was harvested and secreted type I collagen was determined as described in section 7.3.1. The media used in the quantification of type I collagen was based on equal cell numbers (2 x 10^6). (D) Dose-dependent effect of U0126 on COL1A2 mRNA levels. Fibroblasts were treated as described above, RNA was extracted using Trizol reagent and five micrograms were used for cDNA synthesis. Quantitative real time RT PCR was performed to quantitate COL1A2 mRNA levels. The levels of GAPDH in the same sample were used as the normaliser. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

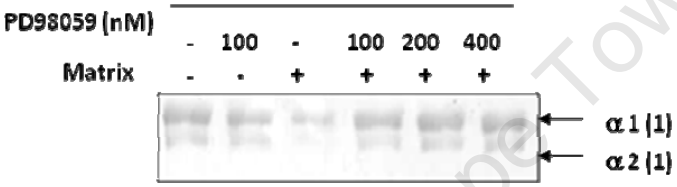
A. Phosphorylated ERK1,2



B. Intracellular type I collagen



C. Media type I collagen



D. COL1A2 mRNA

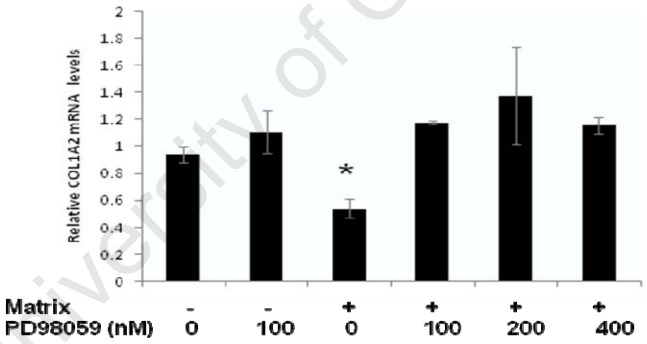


Fig 3.3 Effect of PD98059 on matrix-mediated downregulation of type I collagen synthesis. The effect of the specific MEK 1,2 inhibitor PD98059 on type 1 procollagen gene expression was investigated as described in '*Materials and Methods*'. (A) Dose-dependent effect of PD98059 on ERK 1,2 phosphorylation. CT-1 fibroblasts were seeded on dishes with or without fibroblast-derived ECM for 24 hrs and then treated with the indicated concentrations of PD98059 or 0.1% DMSO (as a solvent control) for 24 hrs. Cell lysates (50 µg total protein) were separated by 10 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with anti-*p*-ERK 1,2 antibody. The level of β tubulin in the same sample was used as control. (B) Fibroblasts were treated as described above and immunoblotting was performed to evaluate type 1 procollagen protein levels in the cell lysates (section 7.3.2). The levels of β tubulin in the same sample were used as controls. (C) Dose-dependent effect of PD98059 on secreted type I collagen levels in the media. Fibroblasts were treated as described above and the medium was harvested. Secreted type I collagen was determined as described in section 7.3.1. The media used in the quantification of type I collagen was based on equal cell numbers (2 x 10^6). (D) Dose-dependent effect of PD98059 on COL1A2 mRNA levels. Fibroblasts were treated as described above, RNA was extracted five micrograms were used in qRT PCR to quantitate COL1A2 mRNA levels. The levels of GAPDH in the same sample were used as the normaliser. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

similarities with the other MAP kinase cascades. The possible involvement of the p38 MAPK, JNK and PI3-kinase pathways in the matrix-mediated downregulation of type I collagen gene expression was investigated using their respective inhibitors, SB203580, SP600125 and wortmanin. As shown below in Figure 3.4 A-F, none of these inhibitors reversed the effect of the

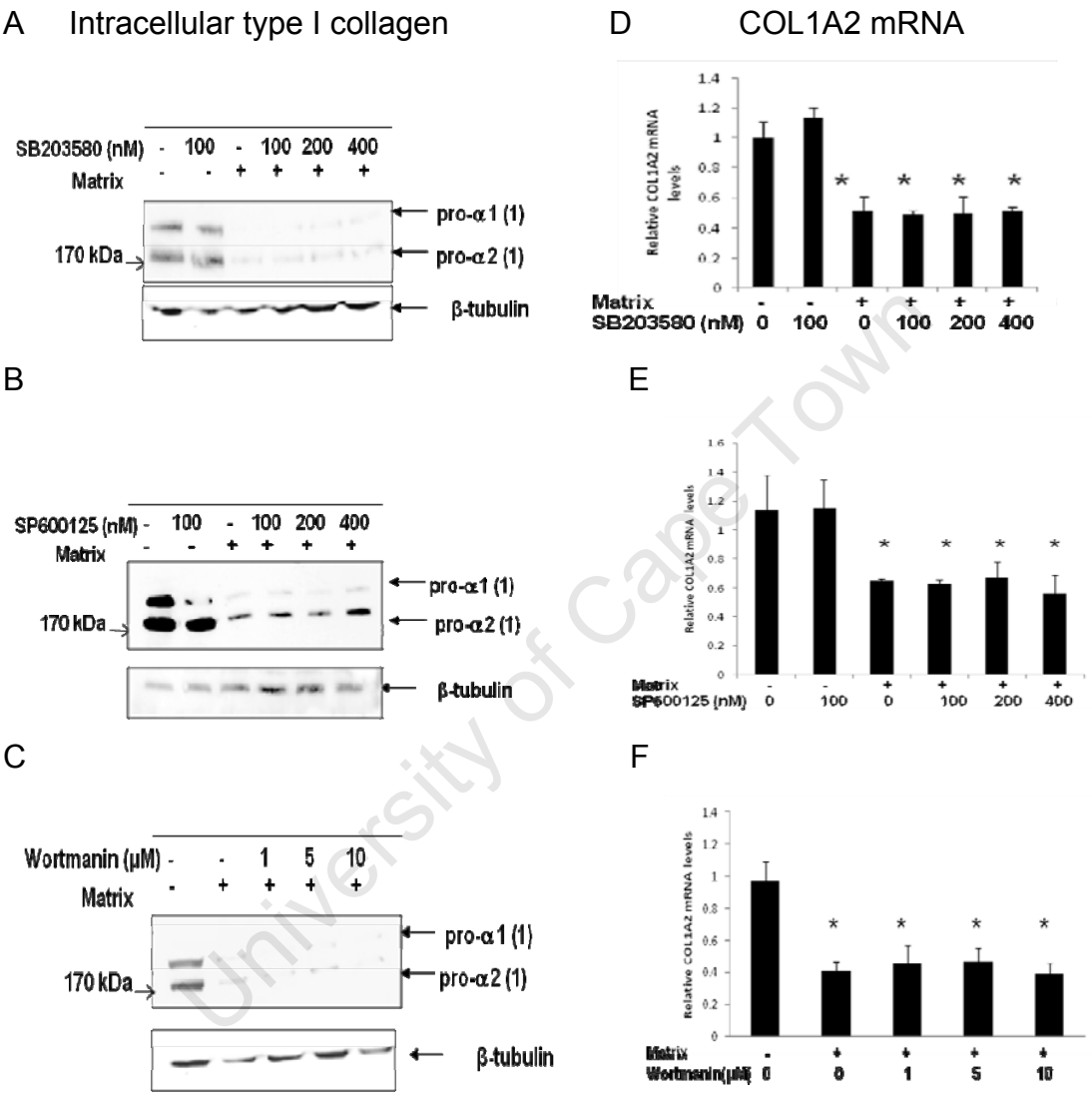


Fig 3.4 Analysis of the p38 MAPK, JNK and PI3-kinase pathways on matrix-mediated downregulation of type I collagen gene expression.
The effect of p38 MAPK inhibitor, SB203580, JNK inhibitor, SP600125 and PI3-K inhibitor, wortmanin, on type 1 procollagen gene expression was investigated as described in '*Material and Methods*'. CT-1 fibroblasts were seeded on dishes with or without fibroblast-derived ECM and then treated with the indicated concentrations of SB203580 (A), SP600125 (B) and wortmanin (C) or 0.1% DMSO (as a solvent control) for 24 hrs. Cell lysates (50 μg total protein) were separated by 10 % SDS polyacrylamide gel electrophoresis and immunoblotting was performed to evaluate type 1 procollagen protein levels using β tubulin as a control. Dose-dependent effect of SB203580, SP600125 and wortmanin on type I collagen COL1A2 mRNA levels. CT-1 fibroblasts were treated with SB203580 (D), SP600125 (E) and wortmanin (F) as described above, RNA was extracted using Trizol reagent and five micrograms were used for cDNA synthesis. Quantitative real time RT PCR was performed to quantitate COL1A2 mRNA levels, using GAPDH as the normaliser. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

ECM on type I collagen protein and mRNA levels as observed with the MEK-ERK inhibitors.

3.2.3 Confirmation of the involvement of the MEK-ERK signaling pathway in matrix-mediated downregulation of type I collagen synthesis

To substantiate the results obtained with PD98059 and U0126, fibroblasts were transiently transfected with a dominant negative (dn) MEK 1 construct. The dominant negative MEK1 contains serine to alanine substitutions at Ser^{218} and Ser^{222} . Because these serine phosphorylation sites are required for activation of MEK1, alanine substitution prevents activation. Preliminary experiments showed that 2µg dn MEK was sufficient to reduce the phosphorylation of ERK 1,2 significantly (Figure 3.5 A). Transient transfection studies confirmed that the MEK-ERK signaling pathway is involved in the matrix-mediated downregulation of collagen gene expression. Analysis of type I collagen and COL1A2 mRNA show that transfection with dn MEK reversed the matrix-mediated downregulation of collagen synthesis (Fig 3.5 B-C).

3.2.4 Analysis of COL1A2 promoter activity

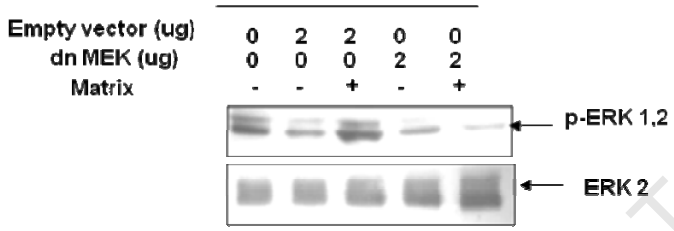
To investigate the role of the MEK-ERK signaling pathway in the transcriptional regulation of type I collagen gene expression, the COL1A2 promoter activity was determined in fibroblasts treated with U0126 and PD98059. Dose dependent studies established that 400 nM of each inhibitor was enough to reduce ERK 1,2 phosphorylation significantly hence this concentration was used to study the effect of the fibroblast-derived ECM on COL1A2 promoter activity. The MEK-ERK inhibitors, U0126 and PD98059, and the dn MEK reversed the matrix-mediated inhibition of COL1A2 promoter activity (Fig 3.6 A-C), thus confirming the MEK-ERK pathway as the signal transducer.

3.2.5 The activation of the MEK-ERK signaling pathway is Ras-dependent

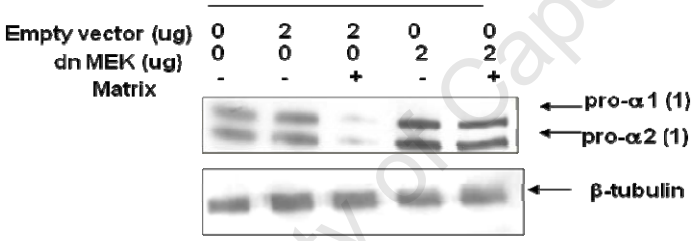
To investigate whether the matrix-mediated downregulation of COL1A2 through the MEK-ERK signaling pathway is dependent on Ras activation, a dominant negative mutant Ras, N17Ras, was used in transient transfection

assays. N17Ras is a GDP-bound negative mutant, used to analyse Ras dependent activation in many systems. The N17Ras binds GDP with preferential affinity over GTP, thus allowing N17Ras to inhibit endogenous Ras activation. Transfection of cells with N17Ras inhibited ERK 1,2 phosphorylation (Fig 3.7 A) and reversed the matrix-mediated inhibition of type I collagen and mRNA synthesis and promoter activity (Fig 3.7 A-C). Thus the matrix-mediated downregulation of type I collagen production through the MEK-ERK signaling pathway is Ras dependent.

A



B



C

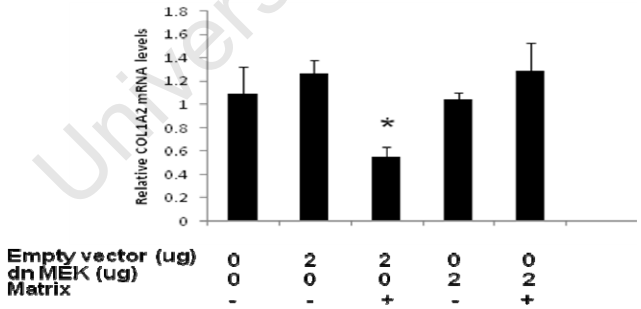
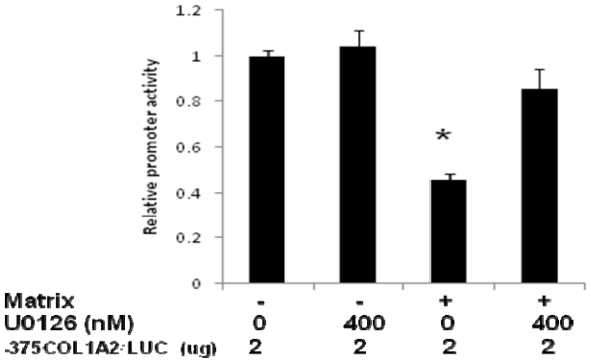
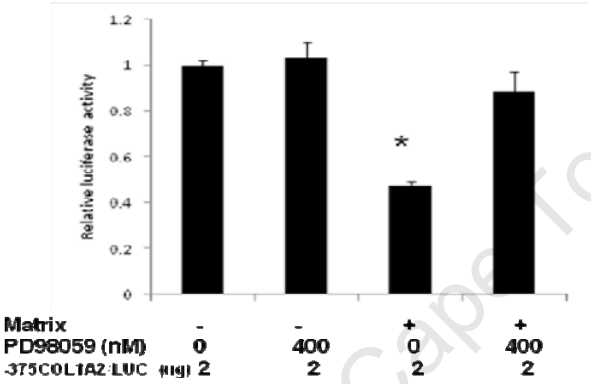


Fig 3.5 Effect of a dominant negative MEK 1 mutant on matrix-mediated downregulation of type I collagen synthesis. (A) CT-1 fibroblasts, transfected with 2 µg of the dn MEK or the empty vector, were trypsinised and seeded on dishes with or without fibroblast-derived ECM for 24 hrs. and immunoblotting was performed as described in section 7.3.2 to quantitate phosphorylated ERK 1,2 levels in cell lysates. The levels of β tubulin in the same sample were used as controls. (B) CT-1 fibroblasts were treated as described above and immunoblotting was performed to evaluate type 1 procollagen protein levels in cell lysates. The levels of β tubulin in the same sample were used as controls. (C) Effect of dn MEK1 on COL1A2 mRNA levels. CT-1 fibroblasts were treated as described in section 7.2.6, RNA was extracted using Trizol reagent and five micrograms were used for cDNA synthesis. Quantitative real time RT PCR was performed to quantitate COL1A2 mRNA levels using GAPDH as the normaliser. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

A. U0126



B. PD98059



C. dn MEK

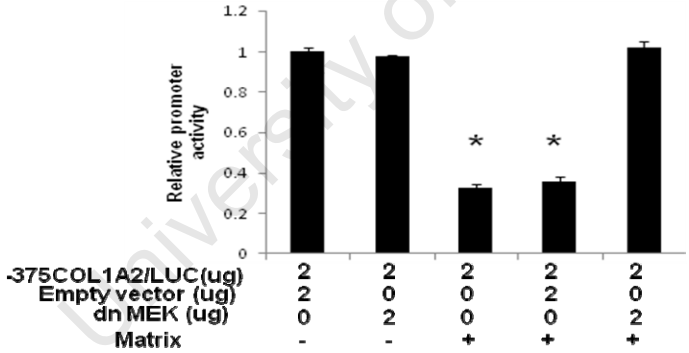
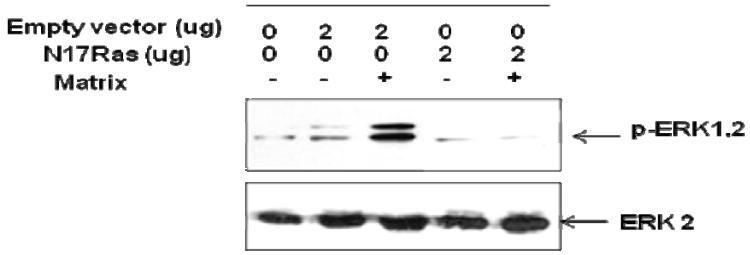
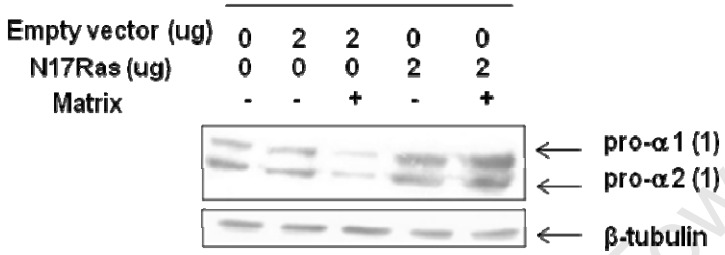


Fig 3.6 Effect of U0126, PD98059 and dn MEK on -375 COL1A2 promoter activity.
(A) Effect of U0126 on type I collagen promoter activity. Fibroblasts (3 x 10^5) were transfected with 2 µg of the -375 COL1A2/LUC construct, seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then treated with 400 nM of U0126 or 0.1 % DMSO (as the solvent control). Cell extracts were obtained using passive lysis buffer and were assayed for luciferase activity. (B) Effect of PD98059 on type I collagen promoter activity. Fibroblasts (3 x 10^5) were transfected with 2 µg of the -375 COL1A2/LUC construct, seeded in dishes with or without fibroblast-derived ECM for 24 hrs, and then treated with 400 nM of PD98059 or 0.1 % DMSO (as a solvent control). Cell extracts were assayed for luciferase activity. (C) Effect of dn MEK 1 on type I collagen promoter activity. Fibroblasts (3 x 10^5) were co-transfected with 2 µg of the -375 COL1A2/LUC construct and 2 µg of the dn MEK and seeded in dishes with or without fibroblast-derived ECM. Control fibroblasts were transfected with the empty vector. Cell extracts were obtained using passive lysis buffer and assayed for luciferase activity. Data are presented as the mean ± standard error (* $p < 0.05$), matrix vs control.

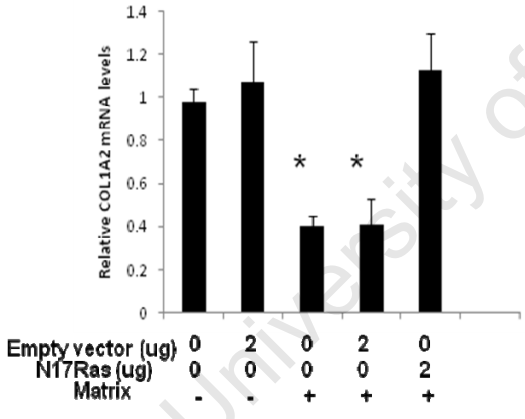
A. Phosphorylated ERK 1,2



B. Cellular type I collagen synthesis



C. COL1A2 mRNA levels



D. COL1A2 promoter activity

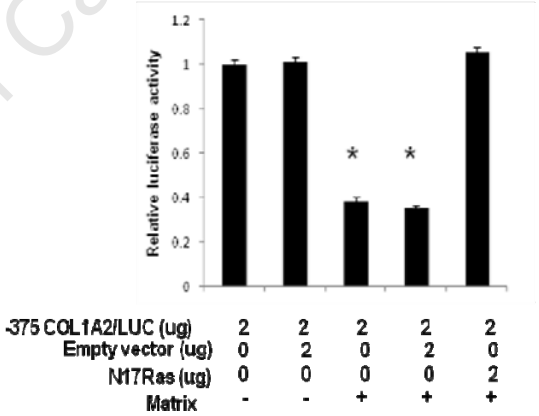


Fig 3.7 Investigation of Ras in the activation of MEK-ERK signaling pathway.
The effect of dominant negative mutant Ras (N17Ras) on ERK 1,2 phosphorylation and type 1 procollagen gene expression was investigated as described in '*Materials and Methods*'. (A) CT-1 fibroblasts were transfected with 2 µg of N17Ras or the empty vector. Transfected fibroblasts were trypsinised and seeded on dishes with or without fibroblast-derived ECM for 24 hrs. Cell lysates (50 µg total protein) were separated by 10 % SDS polyacrylamide gel electrophoresis and immunoblotting was performed to evaluate ERK 1,2 phosphorylation using β tubulin as a control. (B) Effect of N17Ras on type I collagen synthesis. Cell lysates (50 µg total protein) were treated as described above and subjected to immunoblotting with anti-type I collagen antibody. (C) Effect of N17Ras on COL1A2 mRNA levels. CT-1 fibroblasts were treated as described in section 7.2.6, RNA was extracted using Trizol reagent and five micrograms were used for cDNA synthesis. Quantitative real time RT PCR was performed to quantitate COL1A2 mRNA levels using GAPDH as the normaliser. (D) Effect of N17Ras on type I collagen promoter activity. CT-1 fibroblasts (3 x 10^5) were co-transfected with 2 µg of the-375 COL1A2/LUC construct and 2 µg of N17Ras and seeded in dishes with or without fibroblast-derived ECM for 24 hrs. Cell lysates were obtained using passive lysis buffer and assayed for luciferase activity. Data are presented as the mean ± standard error (* $p < 0.05$), matrix vs control.

3.2.6 DISCUSSION

The elucidation of the regulation of type I collagen gene expression is critical in understanding of pathophysiology of fibroproliferative disorders, wound healing, tissue remodelling and metastasis. The accumulation of ECM in general and type I collagen in particular is the chief pathologic feature of many fibrotic disorders (Border and Noble, 1994; Yamane *et al*, 2002, 2007). There is clear evidence that the activation of many signaling pathways including PI3K and MAPKs, may be involved in the regulation of type I collagen gene expression (Stefanovic, 2005; Mimura *et al*, 2006). Transmission of extracellular signals from the cell surface into the nucleus may involve activation of the mitogen-activated protein kinase (MAPK) signaling cascades via the ERK, JNK and/or the p38 pathways (Ge *et al*, 2007). Mammalian cells contain several extracellular signal-regulated kinases including ERK 1 and ERK 2. These kinases regulate cell processes stimulated by various extracellular agents and have been implicated in control of nuclear transcriptional activity (Treisman, 1994). The results shown in chapter 2 showed that the response of fibroblasts cultured on a fibroblast-derived ECM results in downregulation of type I collagen gene expression. The signaling events associated with this interaction are still unknown, understandably so, since the use of the fibroblast-derived ECM is not well studied.

The MEK-ERK signaling pathway is activated in response to a variety of external stimuli, including growth factors, cytokines and environmental stress (Mimura *et al*, 2006; Yamane *et al*, 2007). Using several different approaches, this study showed that MEK-ERK signaling is required for matrix-mediated downregulation of type I collagen gene expression. The involvement of Raf was proved by the results obtained with the MEK inhibitor PD98059 since it blocks only Raf-dependent phosphorylation/ activation of MEK1 and is not an effective inhibitor of MEK2 activation (Dudley *et al*, 1995). However U0126 is a more potent inhibitor of both MEK1 and MEK2, regardless of activation state, and it is for these reasons that studies were done with both inhibitors. In addition, PD98059 do not inhibit already activated MEK1 or MEK2, so the use of the two inhibitors would allow us to correctly conclude if the MEK-ERK pathway and Raf are involved in the matrix-mediated downregulation of type I

collagen gene expression. Treatment with these two inhibitors was associated with a similar inhibition of the matrix-mediated downregulation of type I collagen gene expression. To confirm that matrix-mediated downregulation of type I collagen gene expression occurs through the MEK-ERK signaling pathway, the dominant negative mutant MEK1 construct was used and it reversed the matrix-mediated downregulation of type I collagen gene expression. The p38 MAPK, PI3K and JNK pathways are not involved in the matrix-mediated downregulation of type I collagen gene expression. Inhibitors of these signaling pathways are well characterised and available commercially. These results suggest that the MEK-ERK pathway is critical in the regulation of type I collagen by the matrix in fibroblasts.

The MEK-ERK signaling pathway has been reported to play a role in modulating the transcription of many genes. The MEK-ERK signaling pathway is involved in EGF-mediated downregulation of type I collagen gene expression in fibroblasts (Mimura *et al*, 2006) possibly via transcription factors, such as c-fos, c-jun, Elk-1, Sp1/Sp3 and Ets-1 (Stewart and Guan, 1999; Leaner *et al*, 2005; Chetoui *et al*, 2005). This study confirmed that the MEK-ERK signaling pathway affects cellular gene expression via the Sp1 transcription factor. The expression of Ras negatively regulates c-fos and Egr-1 gene induction in many cell types including lymphocytes (Chen *et al,* 1996) and the activation of ERK is essential for numerous Ras-induced cellular responses including transcription activation of immediate early genes such as c-fos (Treisman, 1994; Hill and Treisman, 1995). Based on these reports, one can speculate the possible involvement of transcription factors such as c-fos in the matrix-mediated downregulation of type I collagen gene expression. Moreover, this study indicate that the MEK-ERK signaling pathway is involved in the modulation of type I collagen and supports the hypothesis that the inhibition or activation of the MEK-ERK signaling pathway may have therapeutic value.

Growing evidence indicates that external stimuli can activate the MEK-ERK signaling pathway through both Ras-dependent and independent pathways (Katsumi *et al*, 2004; Chen *et al*, 1996; Howe *et al*, 1998; Lee and Juliano,

2004). Experiments using Ras mutants have been instrumental in elucidating biological and biochemical functions of Ras. N17Ras is used extensively as a dominant negative mutant to probe Ras function because it interferes with Ras activation *in vivo* by forming non-productive complexes with nucleotide exchange factors (Stewart and Guam, 2000). In fact, overexpression of N17Ras is usually the sole indicator for determining whether a particular signaling event involves Ras activation. Ras binds Raf directly in a GTP-dependent manner which appears to be crucial for the activation of Raf (Stewart and Guan, 1999). Through the use of N17Ras, this study provided evidence that matrix-mediated downregulation of type I collagen was Ras-dependent.

The present study indicates that the matrix-mediated signaling occurs through the Ras/Raf/MEK-ERK signaling pathway. Additional experiments are warranted to examine the upstream events implicated in cell receptor-mediated Ras activation in fibroblasts. Possible receptors include growth factor receptors and integrins. Other reports suggest that protein phosphatase 2A (PP2A) is required for integrin-mediated activation of the MEK-ERK signaling pathway (Coppolino *et al*, 1995; Suzuki *et al*, 2004; Chetoui *et al*, 2005). However, it was proved that this was true only in Ras-independent activation of MEK-ERK signaling pathway. Since this study proved that Ras is involved, it follows that there may not be a role for PP2A in the integrin-mediated activation of the MEK-ERK signaling pathway. Crosstalk between the MEK-ERK pathway and other signaling pathways in the matrix-mediated downregulation of type I collagen need to be examined. In summary, this study showed that the fibroblast-derived ECM-mediated downregulation of type I collagen is via activation of the MEK-ERK signaling pathway and requires the activity of Ras. The Ras/Raf/MEK-ERK signaling pathway in turn might affect type I collagen gene expression via the Sp1 transcription factor, possibly in combination with other factors. These findings provide new insights into the mechanisms of signaling in fibroblasts and as such may have an impact on the regulation of fibroblast proliferation and differentiation, and fibroproliferative diseases.

CHAPTER 4

REGULATION OF TYPE I COLLAGEN SYNTHESIS VIA THE α2β1 INTEGRIN RECEPTOR

4.1 INTRODUCTION

Stromal microenvironments include the extracellular matrix, stromal cells such as fibroblasts as well as growth factors. These components have important regulatory roles in the growth and differentiation of cells. The ECM provides cell to cell and cell to matrix communications in addition to structural support. The mechanisms of interaction between cells and matrix, the cellular receptors that recognise the ECM molecules and the physiological response of the cells to attachment to the matrix have not been fully elucidated. The ECM proteins can induce diverse intracellular signals by providing both mechanical and chemical stimuli to cells. These signals will mitigate and be affected by other sources of cell signaling (Langenbach *et al*, 2006). ECM cues are very important in signaling pathways, therefore careful control of the matrix is important when studying downstream signaling events or cellular responses to soluble factors or external stimuli. Many studies have established that intracellular signal transduction depends on the type of extracellular components surrounding cells (Koide *et al*, 1989; Nagaki *et al*, 1995; Hoshiba *et al*, 2006; Giancotti and Ruoslahti, 1999)).

In order to have more a physiological *in vitro* system to analyse the characteristics of fibroblasts, it is becoming common to grow cells within support systems that attempt to simulate a natural microenvironment rather than on plastic (Fischbach *et al*, 2007; Kim, 2005; Yamada and Cukierman, 2007). Thin films of ECM proteins are used to study cell responses and behaviour. Thin film technologies provide the means of producing an ECM that can be quantitatively characterised and systematically modified to vary the characteristics of the ECM presented to cells. The different ECM surfaces employed in this study, vary in sites available for receptor engagement (fibroblast-derived ECM vs purified type I collagen vs purified fibronectin). In

this study the response of fibroblasts to a fibroblast-derived ECM was compared to purified matrix components (artificial matrices), comprising type I collagen and fibronectin. Purified type I collagen and fibronectin coated on tissue culture dishes as thin films have been widely studied (Giancotti and Ruoslahti, 1999; Gu *et al*, 2008; Koide *et al*, 1989; Nagaki *et al*, 2002). The ECM has been proposed to act as a reservoir for an increasing number of growth factors (Vlodavsky *et al*, 1980; Burgess, 2009) and these can be released from the ECM to allow extracellular signaling to proceed without the need for de novo protein synthesis.

Extracellular matrix components provide signals that control cellular development and whose control depends on integrin-mediated signaling pathways (Streuli *et al*, 1991). Fibroblasts express a rich array of integrin receptors on their surfaces (Heino, 2000; Hynes, 2002) and these are known to bind to collagen and fibronectin (Dickeson *et al*, 1997, 1998, 1999; Heino, 2000). The α5β1 complex mediates adhesion to fibronectin (Li *et al*, 2003) and the α1β1 and α2β1 complexes mediate adhesion to collagen (Heino, 2000; Dickeson *et al*, 1999). Integrins are the primary receptors for cellular adhesion to the ECM molecules and as a result act as crucial transducers of bidirectional cell signaling, regulating cell survival, differentiation, proliferation, migration and tissue remodelling (Giancotti and Ruoslahti, 1999).

In chapter 2 we have shown that the interaction between fibroblasts and the ECM resulted in the downregulation of type I collagen gene expression. Since type I collagen is the major component of the stromal matrix, we focused on the functional contributions of the α2β1 integrin, the primary collagen receptor. The functional contribution of the α2β1 integrin was then compared to that of α3β1 integrin. α3β1 integrin is expressed at high levels on fibroblasts and other cells where it is a major receptor for laminin-5. Knockout studies in mice have revealed important roles for α3β1 in maintaining tissue integrity (DiPersio *et al*, 1997; Kreidberg *et al*, 1996). Some reports however point to the fact that α3β1 is a promiscuous receptor that can bind to many other ligands besides laminin-5. We therefore examined the possibility that α3β1

could also be involved in the downregulation of type I collagen synthesis by the fibroblast-derived ECM.

Through the use of a suspended matrix and purified matrix components consisting only of type I collagen and fibronectin, this study shows that there is a need for direct contact between the fibroblast-derived ECM and fibroblasts in order for type I collagen synthesis to be downregulated. Furthermore, since type I collagen synthesis is downregulated only in the presence of the fibroblast-derived ECM (containing collagen) and purified type I collagen, and not in the presence of fibronectin, it indicates a specific negative feedback mechanism. Contrasting results have been published on the possible involvement of integrins in the activation of the MEK-ERK pathway (Chen *et al*, 1996; Lee and Juliano, 2004). Monoclonal antibodies were utilised to block the functions of the α2β1 and α3β1 integrins to study their effects on type I collagen gene expression. In this study we also explore the possible regulation of integrin gene expression by the fibroblast-derived ECM and their possible involvement in the downregulation of type I collagen synthesis. Changes in the pattern of expression of integrins alter the way that the microenvironment signals are perceived which may trigger the expression of different sets of genes. We determined the mRNA levels of the subunits of both α2β1 and α3β1 integrins and compared their expression to those of fibroblasts cultured on plastic.

4.2 RESULTS

4.2.1 Secreted factors are not involved in fibroblast-derived ECM-mediated downregulation of type I collagen synthesis

Many roles have been suggested for the ECM including the sequestration of biologically active growth factors. The nature and the origin of the stimulus responsible for the downregulation of type I collagen was investigated in several ways. The possibility that the results observed so far is as a result of the presence of secreted protein factors such as growth factors and cytokines, either released into the medium or trapped within the fibroblast-derived ECM was investigated. The effect of growth medium, exposed to fibroblasts, and a suspended fibroblast-derived ECM on type I collagen synthesis was studied in the presence or absence of the fibroblast-derived ECM.

Growth medium, exposed to WI38 fibroblasts, resulted in slightly higher type I collagen protein levels and had no effect on COL1A2 mRNA levels (Fig 4.1 and Fig 4.2). COL1A1 was omitted as the mRNA levels do not change in the presence of the fibroblast-derived ECM. Culturing fibroblasts with the growth media exposed to WI38 cells for 48 hrs, in combination with the fibroblast-derived ECM, resulted in type I collagen mRNA and protein levels comparable to that produced by the fibroblast-derived ECM alone. This suggests that factors secreted by WI38 fibroblasts into the media play no part in the downregulation of type I collagen gene expression. The possibility that the fibroblast-derived ECM itself contained sequestered protein factors such as growth factors, which had accumulated during the production of the matrix, was also studied. A pre-formed fibroblast-derived ECM was suspended in DMEM medium and then introduced to fibroblasts growing on plastic and on matrix. The suspended matrix floated in the media. After an incubation period of 24 hrs the levels of type I collagen mRNA and protein levels were determined as described in section 7.2.6 and section 7.3.2 respectively. No effect could be attributed to the presence of the suspended matrix (Fig 4.3 and Fig 4.4). Thus it appears that fibroblasts require direct contact with the matrix for the downregulation of type I collagen gene expression to occur. The use of suspected factors as positive controls is suggested in future studies.

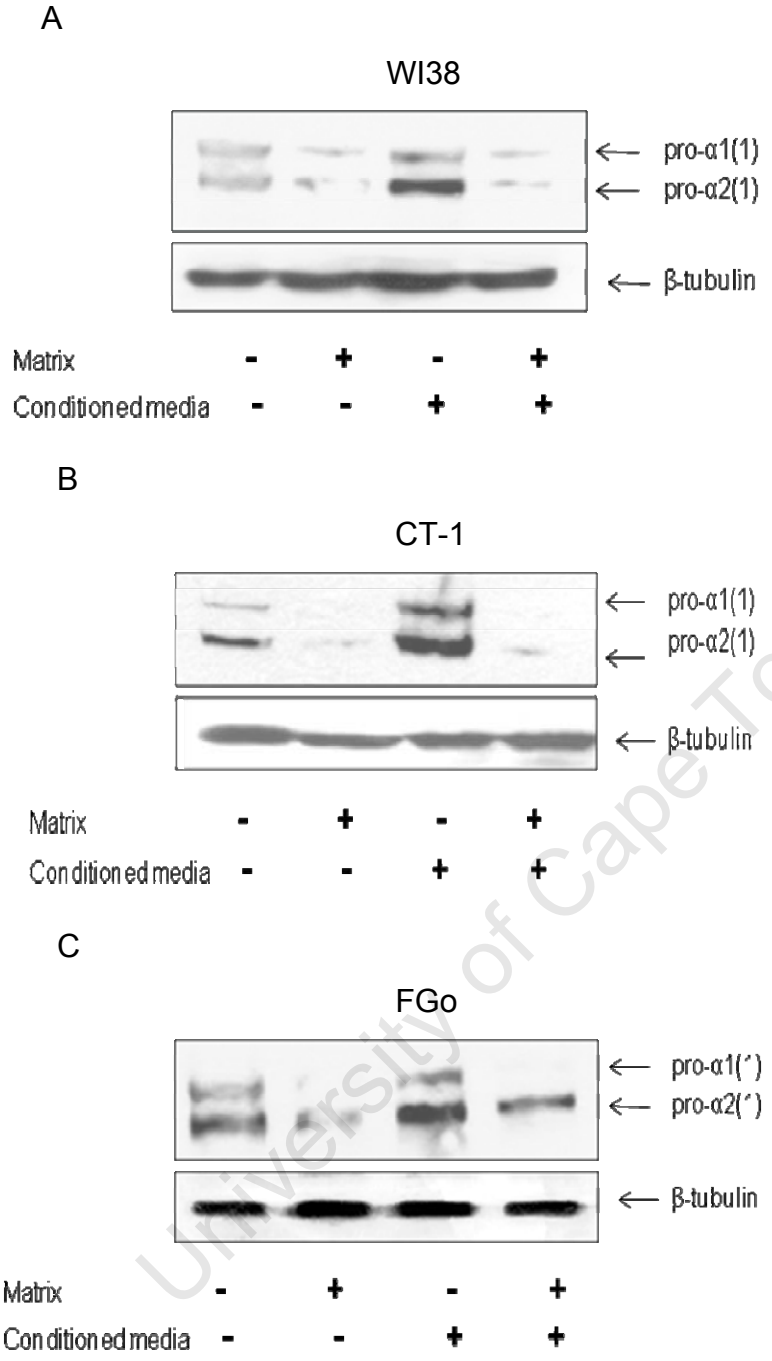


Figure 4.1 Effect of growth media, exposed to WI38, on matrix-mediated downregulation of type I collagen protein levels.
WI38, CT-1 and FGo fibroblasts (A-C) were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then cultured in media , exposed to WI38 fibroblasts, mixed with DMEM media, for 48 hrs. Cell lysates (50 ug total protein) were separated by 7.5 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with goat anti-type I collagen antibody (1:1000). The secondary antibody used was donkey anti-goat IgG conjugated to horse radish peroxidase (1:1000). Arrows indicate the two α chains. β-tubulin was used as a loading control to confirm equal loading of samples.

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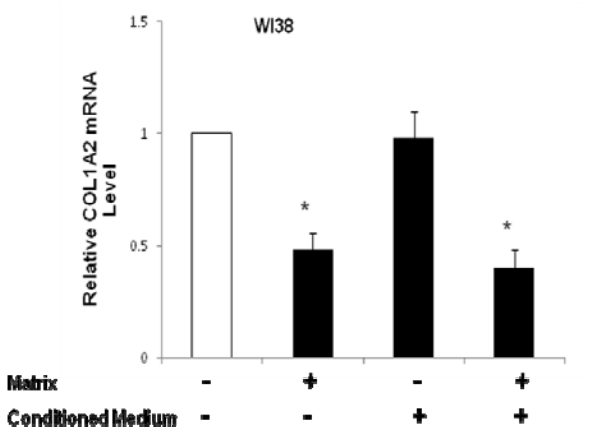

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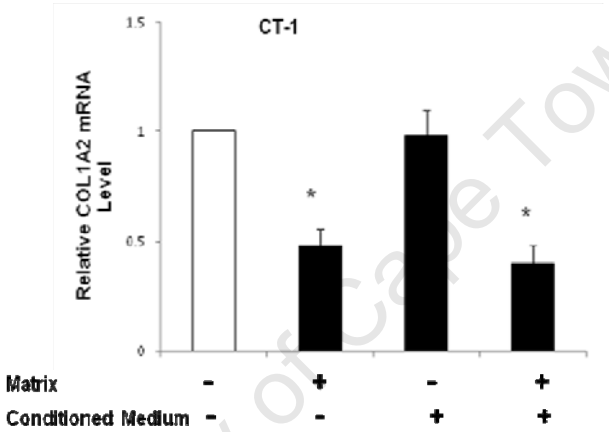

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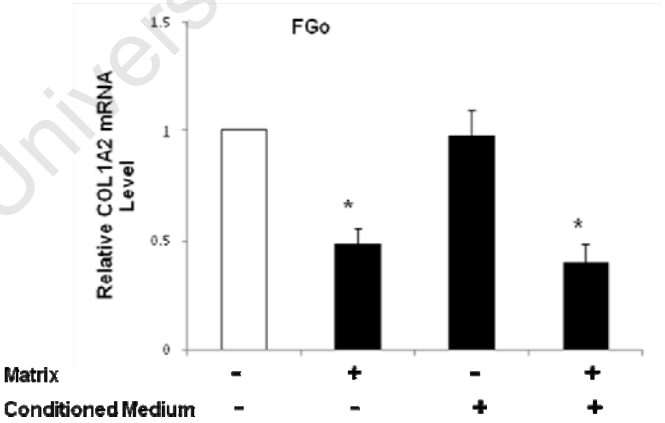

Figure 4.2 Effect of growth media, exposed to WI38 fibroblasts, on matrix-mediated downregulation of COL1A2 mRNA levels.
WI38, CT-1 and FGo fibroblasts (A-C) were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then cultured in media exposed to WI38 fibroblast, mixed with DMEM media, for 48 hrs. Cells were washed with PBS, RNA was extracted using Trizol reagent and five micrograms of total RNA were used in cDNA synthesis. Quantitative RT-PCR was performed to evaluate COL1A2 mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

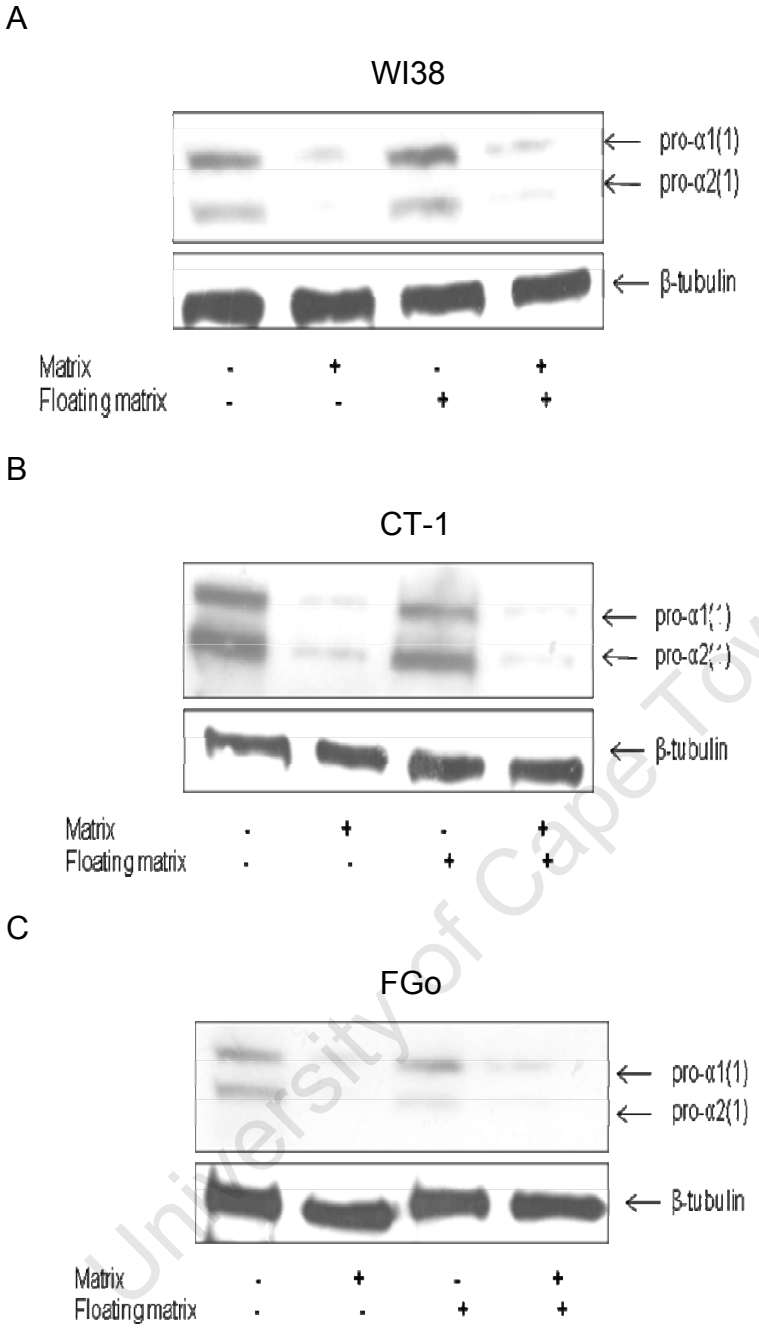

Figure 4.3 Effect of suspended matrix on matrix-mediated downregulation of type I collagen protein levels.
WI38, CT-1 and FGo fibroblasts (A-C) were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then the suspended matrix, in DMEM media, was introduced. Incubation was continued for 48 hrs. Cell lysates (50 ug total protein) were separated by 7.5 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with goat anti-type I collagen antibody (1:1000). The secondary antibody used was donkey anti-goat IgG conjugated to horse radish peroxidase (1:1000). Arrows indicate the two α chains. β-tubulin was used as a loading control to confirm equal loading of samples.

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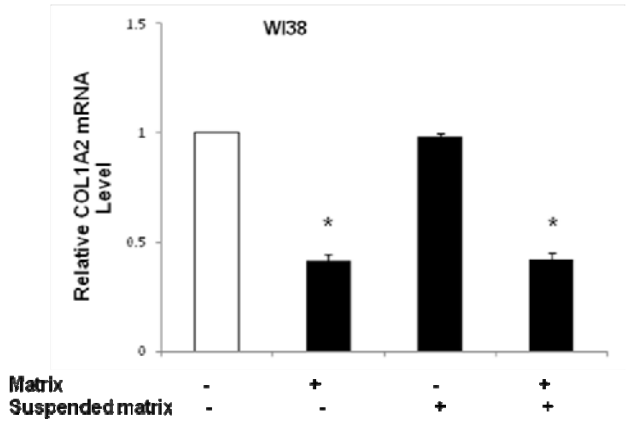

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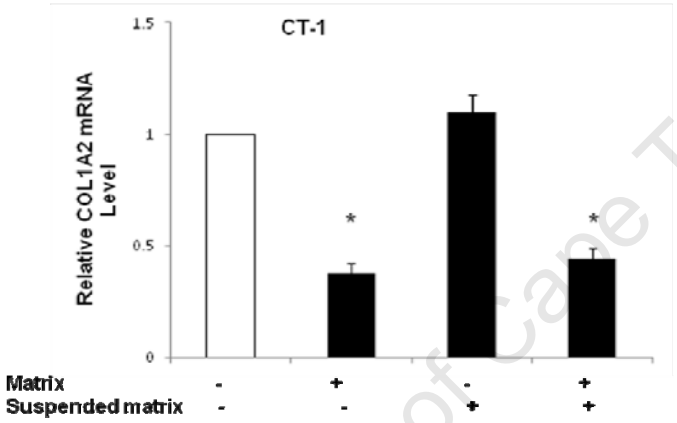

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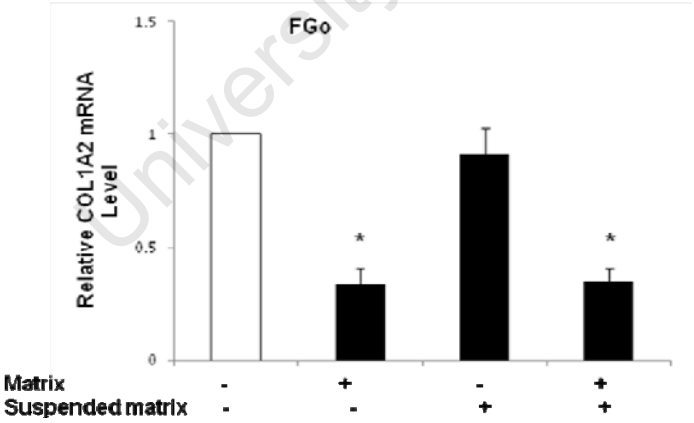

Figure 4.4 Effect of suspended matrix on matrix-mediated downregulation of COL1A2 mRNA levels.

WI38, CT-1 and FGo fibroblasts (A-C) were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then suspended matrix was introduced and incubation continued for 48 hrs. Cells were washed with PBS, RNA was extracted using Trizol reagent and five micrograms of total RNA were used in cDNA synthesis. Quantitative RT-PCR was performed to evaluate COL1A2 mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

4.2.2 Effect of matrix components on type I collagen synthesis

Results from section 4.2.1 appear to suggest that secreted factors are not involved in the matrix-mediated downregulation of type I collagen, therefore the nature of the stimulus from the fibroblast-derived ECM was explored using an additional approach. To identify the fibroblast-derived ECM components responsible for the downregulation of type I collagen gene expression, fibroblasts were seeded on artificial matrices such as purified type I collagen and fibronectin, the predominant components of naturally occurring interstitial extracellular matrix. Coated plastic dishes were prepared by allowing type I collagen or fibronectin solution to gel. The preparation of thin films of type I collagen or fibronectin has been characterised before (Langenbach *et al*, 2006; Elliot *et al*, 2003).

The use of purified matrix components (artificial matrices) of type I collagen or fibronectin, showed that the matrix-mediated effect was of structural origin. When fibroblasts were seeded on dishes coated with purified type I collagen, the synthesis of type I collagen was downregulated to about the same level as when seeded on the fibroblast-derived ECM. When seeded on dishes coated with fibronectin, however, type I collagen synthesis was not downregulated (Fig 4.5 and Fig 4.6). The decrease in the gene expression of type I collagen is not due to a reduction in cell growth as the cell proliferation results show that there is no significance difference between controls and fibroblasts plated on the matrix, purified type I collagen or fibronectin (Fig 4.7). Neither was there any significant difference in the rate of attachment to the plastic, fibroblast-derived matrix, purified type I collagen and fibronectin (data not shown).

4.2.3 Effect of blocking integrin function on type I collagen synthesis

The extracellular matrix provides signals that control cellular development and whose control depends mainly on receptor-mediated signaling pathways (Giancotti *et al*, 1999; Hanagata *et al*, 2006). The biological responses triggered by cell-matrix interactions have been compared to those transduced by hormones, cytokines and growth factors (Delcommenne and Streuli, 1995). Results obtained so far appear to exclude the involvement of secreted factors

therefore the focus was on receptors such as integrins that bind to components of the ECM. The integrin family of receptors are known to participate in a variety of transduction pathways (Hynes, 2002; Katsumi *et al*, 2004; Phillips and Bonassar, 2005).

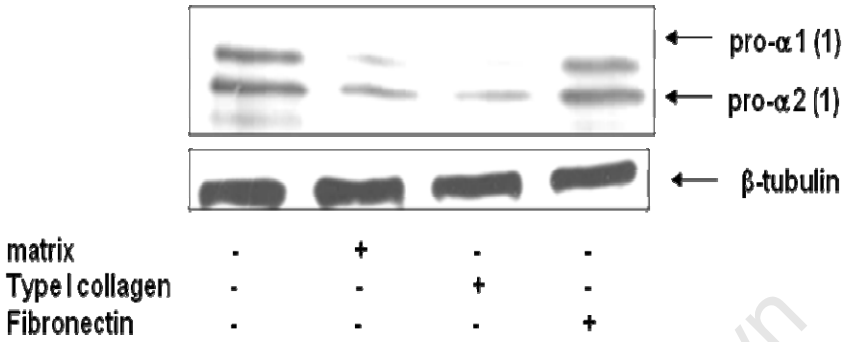

Figure 4.5 Effect of matrix components on type I collagen protein levels.
CT-1 fibroblasts were seeded in dishes with or without the fibroblast-derived ECM or its purified components, type I collagen and fibronectin, for 24 hrs and then incubated for 48 hrs. Cells were washed with PBS and proteins were harvested in RIPA buffer. Cell lysates (50 ug total protein) were separated by 7.5 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with goat anti-type I collagen antibody (1:1000). The secondary antibody used was donkey anti-goat IgG conjugated to horse radish peroxidase (1:1000). Arrows indicate the two α chains. β-tubulin was used as a loading control to confirm equal loading of samples.

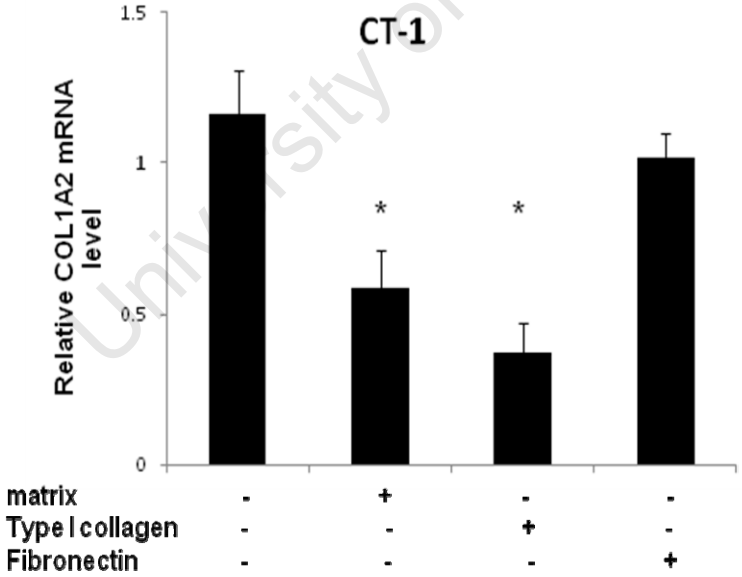

Figure 4.6 Effect of matrix components on COL1A2 mRNA levels.
CT-1 fibroblasts were seeded in dishes with or without the fibroblast-derived ECM or its purified components, type I collagen and fibronectin, for 24 hrs and then incubated for 48 hrs in DMEM. Cells were washed with PBS, RNA was extracted using Trizol reagent and five micrograms of total RNA were used in cDNA synthesis. Quantitative RT-PCR was performed to evaluate COL1A2 mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

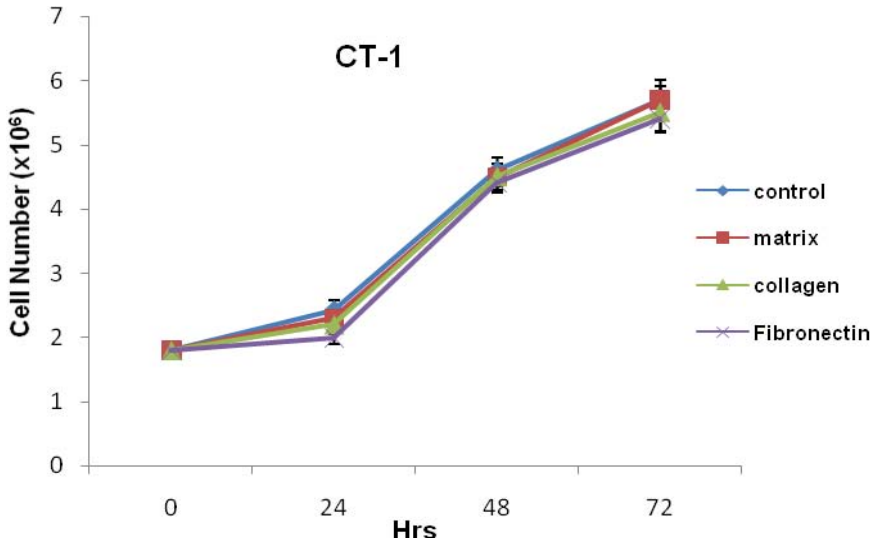

Figure 4.7 Influence of fibroblast-derived ECM and its components, purified type I collagen and fibronectin, on cell proliferation. 3x 10^5 CT-1 fibroblasts were plated on culture dishes with or without the indicated matrices for 24 hrs and then grown in DMEM media for the indicated times (24hrs, 48 hrs, and 72 hrs). Fibroblast-derived ECM were prepared as described in section 7.2.3. At the end of the experiment the fibroblasts were trypsinised and counted. There was no significant difference in growth rate between fibroblasts grown on a matrix and the control cells. Data are expressed as the mean ± S.D of three independent experiments.

The contributions of α2β1 and α3β1 integrins in the downregulation of type I collagen were evaluated using monoclonal antibodies to block the functions of the α2β1 and α3β1 integrins. Blocking α2β1 integrin function through the use of monoclonal antibody, MAB1998, reversed the inhibition of type I collagen protein (Fig 4.8) and COL1A2 mRNA levels (Fig 4.9) in cells cultured on ECM and this was through inhibiting ERK phosphorylation (Fig 4.10). Similar results were obtained when P1E6, another monoclonal antibody to human α2β1 integrin, was used (Fig 4.11-Fig 13). The results show that the α2β1 integrin is directly involved in the downregulation of type I collagen synthesis while blocking the α3β1 integrin function through the use of monoclonal antibody P1B5 had no effect type I collagen protein levels (Fig 4.14), COL1A2 mRNA levels (Fig 4.15) and ERK phosphorylation (Fig 4.16). These results suggest that matrix-mediated downregulation of type I collagen occurs via the interaction of collagen in the matrix with the α2β1 integrin receptor rather than α3β1 integrin receptor and that this in turn results in the activation of the Ras/MEK/ERK signaling pathway.

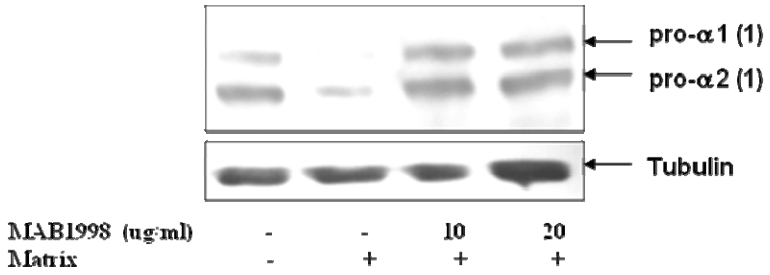

Figure 4.8 Effect of blocking α2β1 integrin with MA1998 on type I collagen protein levels. CT-1 fibroblasts were preincubated with the indicated concentration of MAB1998 antibody for 30 mins at 37^{0}C under standard cell culture conditions prior to addition to dishes with or without the fibroblast-derived ECM and then incubated for 48 hrs in DMEM media. Cells were washed with PBS and proteins were harvested in RIPA buffer. Cell lysates (50 µg total protein) were separated by 7.5 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with goat anti-type I collagen antibody (1:1000). The secondary antibody used was donkey anti-goat IgG conjugated to horse radish peroxidase (1:1000). Arrows indicate the two α chains. β-tubulin was used as a loading control to confirm equal loading of samples.

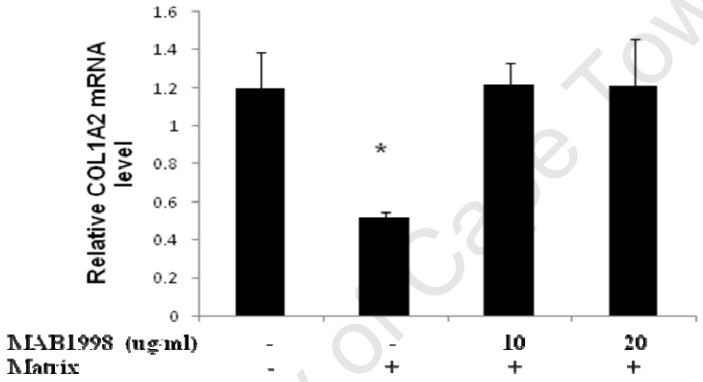

Figure 4.9 Effect of blocking α2β1 integrin with MAB1998 on COL1A2 mRNA levels. CT-1 fibroblasts were preincubated with the indicated concentration of MAB1998 antibody for 30 mins at 37^{0}C under standard cell culture conditions prior to addition to dishes with or without the fibroblast-derived ECM and then incubated for 48 hrs in DMEM media. Cells were washed with PBS, RNA was extracted using Trizol reagent and five micrograms of total RNA were used in cDNA synthesis. Quantitative RT-PCR was performed to evaluate COL1A2 mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

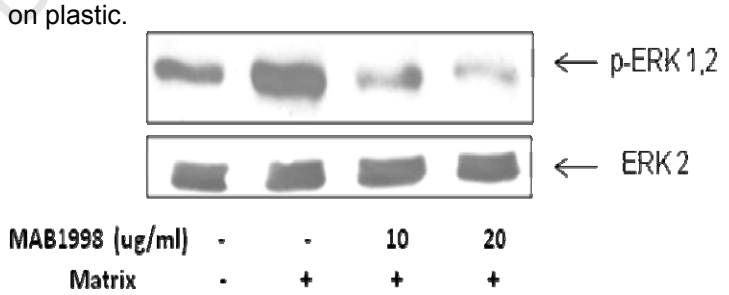

Figure 4.10 Effect of blocking α2β1 integrin with MAB1998 on phosphorylated ERK 1,2 levels. CT-1 fibroblasts were preincubated with the indicated concentration of MAB1998 antibody for 30 mins at 37^{0}C under standard cell culture conditions prior to addition to dishes with or without the fibroblast-derived ECM and then incubated for 48 hrs in DMEM media. Cells were washed with PBS and proteins were harvested in RIPA buffer. Cell lysates (50 µg total protein) were separated by 7.5 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with anti-p-ERK 1,2 antibody (1:1000). β-tubulin was used as a loading control to confirm equal loading of samples.

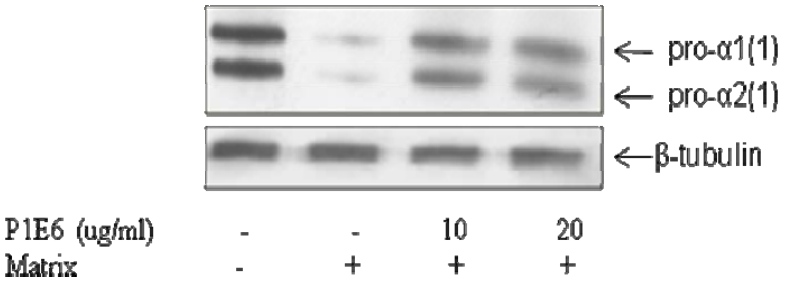

Figure 4.11 Effect of blocking α2β1 integrin with P1E6 on type I collagen protein levels. CT-1 fibroblasts were preincubated with the indicated concentration of P1E6 antibody for 30 mins at 37^{0}C under standard cell culture conditions prior to addition to dishes with or without the fibroblast-derived ECM and then incubated for 48 hrs in DMEM media. Cells were washed with PBS and proteins were harvested in RIPA buffer. Cell lysates (50 µg total protein) were separated by 7.5 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with goat anti-type I collagen antibody (1:1000). The secondary antibody used was donkey anti-goat IgG conjugated to horse radish peroxidase (1:1000). Arrows indicate the two α chains. β-tubulin was used as a loading control to confirm equal loading of samples.

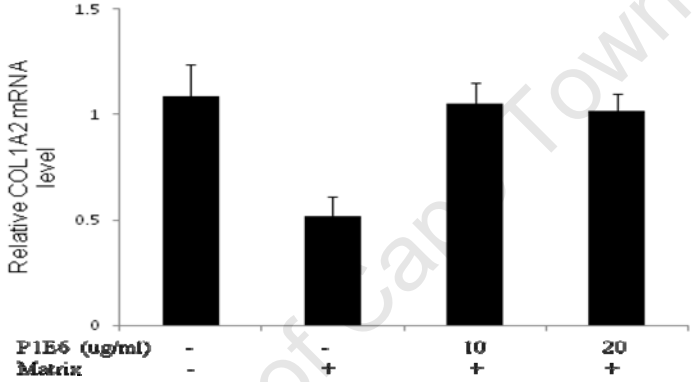

Figure 4.12 Effect of blocking α2β1 integrin with P1E6 on COL1A2 mRNA levels. CT-1 fibroblasts were preincubated with the indicated concentration of P1E6 antibody for 30 mins at 37^{0}C under standard cell culture conditions prior to addition to dishes with or without the fibroblast-derived ECM and then incubated for 48 hrs in DMEM media. Cells were washed with PBS, RNA was extracted using Trizol reagent and five micrograms of total RNA were used in cDNA synthesis. Quantitative RT-PCR was performed to evaluate COL1A2 mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

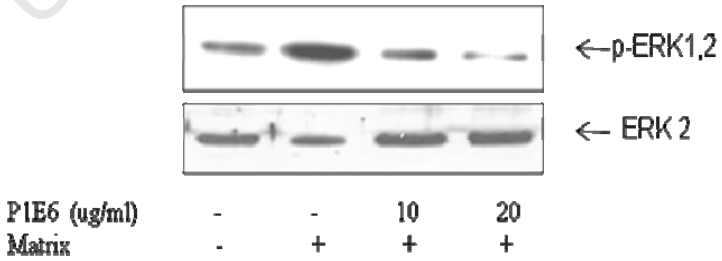

Figure 4.13 Effect of blocking α2β1 integrin with P1E6 on phosphorylated ERK 1,2 levels. CT-1 fibroblasts were preincubated with the indicated concentration of P1E6 antibody for 30 mins at 37^{0}C under standard cell culture conditions prior to addition to dishes with or without the fibroblast-derived ECM and then incubated for 48 hrs in DMEM media. Cells were washed with PBS and proteins were harvested in RIPA buffer. Cell lysates (50 µg total protein) were separated by 7.5 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with anti-p-ERK 1,2 antibody (1:1000). β-tubulin was used as a loading control to confirm equal loading of samples.

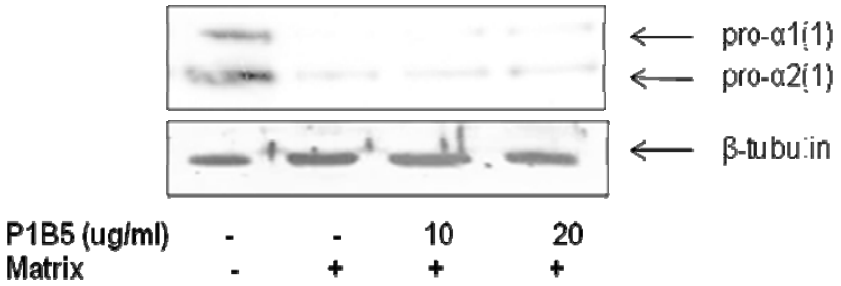

Figure 4.14 Effect of blocking α3β1 integrin with P1B5 on type I collagen protein levels. CT-1 fibroblasts were preincubated with the indicated concentration of P1B5 antibody for 30 mins at 37^{0}C under standard cell culture conditions prior to addition to dishes with or without the fibroblast-derived ECM and then incubated for 48 hrs in DMEM. Cells were washed with PBS and proteins were harvested in RIPA buffer. Cell lysates (50 µg total protein) were separated by 7.5 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with goat anti-type I collagen antibody (1:1000). The secondary antibody used was donkey anti-goat IgG conjugated to horse radish peroxidase (1:1000). Arrows indicate the two α chains. β-tubulin was used as a loading control to confirm equal loading of samples.

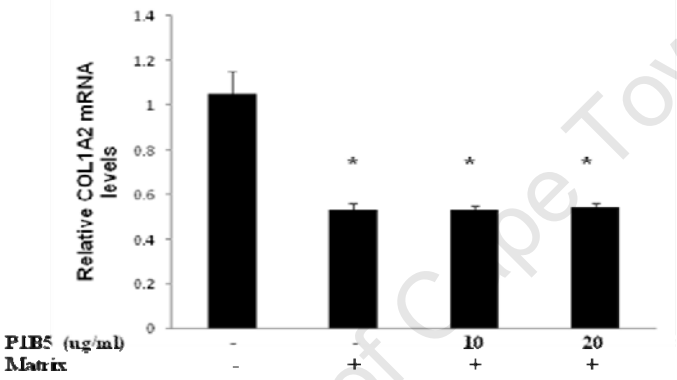

Figure 4.15 Effect of blocking α3β1 integrin with P1B5 on COL1A2 mRNA levels. CT-1 fibroblasts were preincubated with the indicated concentration of P1B5 antibody for 30 mins at 37^{0}C under standard cell culture conditions prior to addition to dishes with or without the fibroblast-derived ECM and then incubated for 48 hrs in DMEM. Cells were washed with PBS, RNA was extracted using Trizol reagent and five micrograms of total RNA were used in cDNA synthesis. Quantitative RT-PCR was performed to evaluate COL1A2 mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

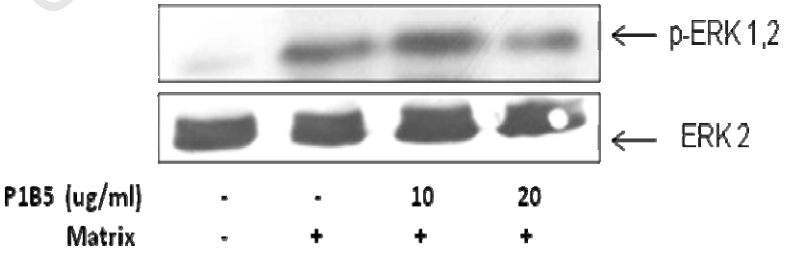

Figure 4.16 Effect of blocking α3β1 integrin with P1B5 on phosphorylated ERK 1,2 levels. CT-1 fibroblasts were preincubated with the indicated concentration of P1B5 antibody for 30 mins at 37^{0}C under standard cell culture conditions prior to addition to dishes with or without the fibroblast-derived ECM and then incubated for 48 hrs in DMEM. Cells were washed with PBS and proteins were harvested in RIPA buffer. Cell lysates (50 µg total protein) were separated by 7.5 % SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with anti-p-ERK 1,2 antibody (1:1000). β-tubulin was used as a loading control to confirm equal loading of samples.

4.2.4 Effect of fibroblast-derived ECM on integrins mRNA levels

The effect of the fibroblast-derived ECM on integrin mRNA levels was determined by real time PCR analysis. The expression of integrin β1, α2 and α3 mRNAs were determined after plating fibroblasts on plastic or on ECM. Fibroblasts cultured on the fibroblast-derived ECM up-regulated the steady state levels of mRNAs encoding α2 and β1 integrin subunits, in comparison to fibroblasts cultured on plastic (Fig 4.17). These results demonstrate that the extracellular matrix controls the gene expression of α2 and β1 integrin subunits and that the regulation is exerted at least at the transcriptional level. There was no change in the mRNA levels of the α3 integrin subunit (Fig 4.17).

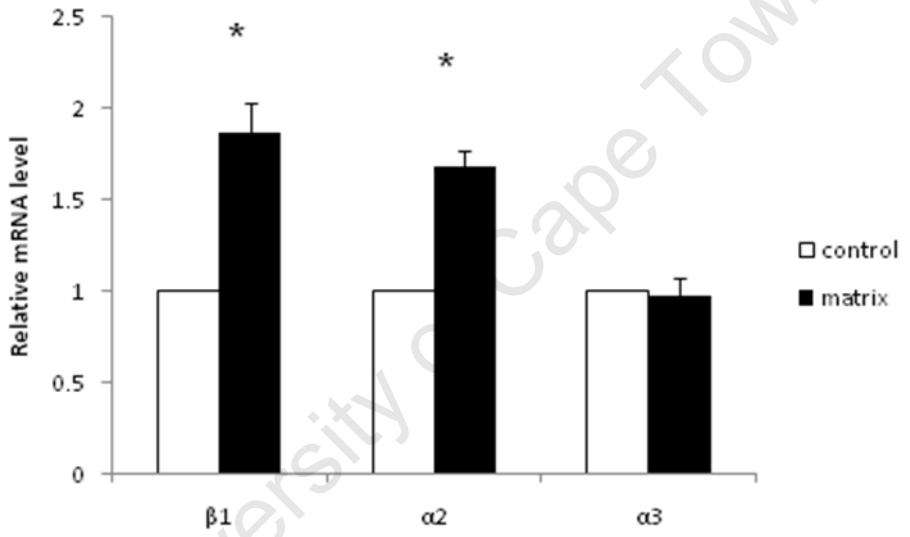

Fig 4.17 Effect of fibroblast-derived ECM on β1, α2 and α3 integrin mRNA levels.
CT-1 fibroblasts were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then grown in DMEM media for 48 hrs. Cells were washed with PBS, RNA was extracted using Trizol reagent and five micrograms of total RNA were used for cDNA synthesis. Quantitative real time RT PCR was performed to quantitate β1, α2 and **α3** mRNA levels. The levels of GAPDH in the same sample were used as the normaliser. The data are shown as the mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

4.2.5 Effect of fibroblast-derived ECM on E-cadherin mRNA levels

Cadherins mediate mainly cell-cell adhesion but are also involved in attachment to extracellular ligands and have been shown to change during processes such as tumorigenesis. The effect of the fibroblast-derived ECM on E-cadherin mRNA levels as determined by real time PCR analysis, showed that there was no significant difference between fibroblasts plated on ECM

compared to those plated on plastic (Figure 4.18). The transcription of the E-cadherin was therefore not influenced by the substratum.

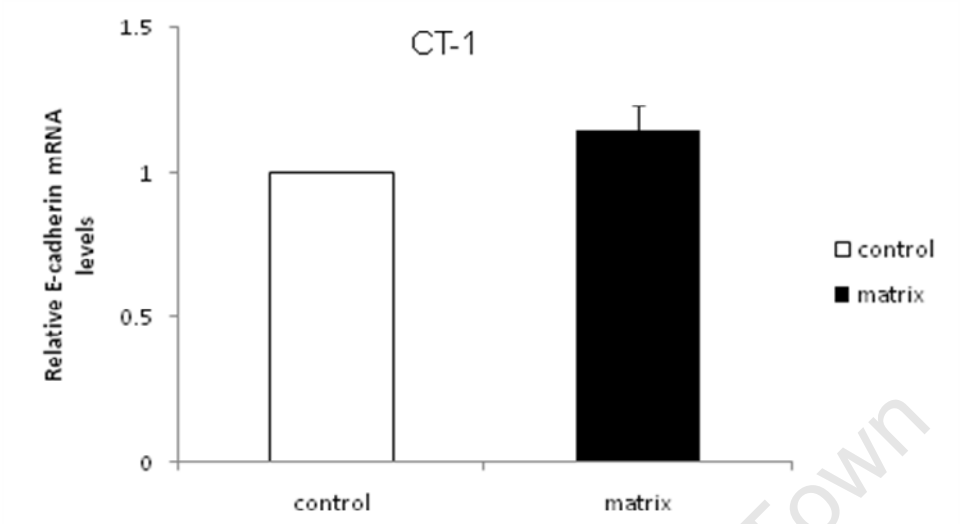

Fig 4.18 Effect of fibroblast-derived ECM on E-cadherin mRNA levels.
CT-1 fibroblasts were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then grown in DMEM for 48 hrs. Cells were washed with PBS, RNA was extracted using Trizol reagent and five micrograms of total RNA were used for cDNA synthesis. Quantitative real time RT PCR was performed to quantitate E-cadherin mRNA levels. The levels of GAPDH in the same sample were used as the normaliser. The data are shown as the mean ± S.D. of three independent experiments.

4.2.6 Effect of fibroblast-derived ECM on fibronectin, laminin α2 and COL5A3 gene expression

Our previous studies on the regulation of type I collagen synthesis by the fibroblast-derived ECM showed that different genes, in this case COL1A1 and COL1A2, can be affected by the ECM in different ways. We therefore examined the effect of the fibroblast-derived ECM on the expression of several other ECM genes. The expression of fibronectin, laminin-α2 and type IV collagen gene (COL5A3) was determined in the three fibroblasts cell lines WI38, CT1 and FGo in response to the presence of the fibroblast-derived matrix. There was a significant downregulation of fibronectin protein (Fig 4.19) and mRNA levels (Fig 4.20) whilst the fibronectin protein level in FGo appears to be unchanged. There was no change in the expression of laminin-α2 gene (Fig 4.21 and Fig 4.22) whilst the COL5A3 mRNA was upregulated in the presence of the ECM (Fig 4.23).

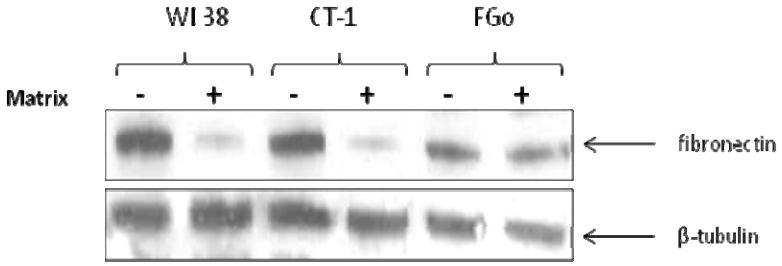

Figure 4.19 Effect of the fibroblast-derived ECM on fibronectin protein levels.
WI38, CT-1 and FGo fibroblasts were seeded on dishes with or without fibroblast-derived ECM for 24 hrs and after changing media, were cultured for 48 hrs. Cell lysates (50 µg total protein) were separated by 7.5 % SDS PAGE and subjected to immunoblotting with mouse anti-fibronectin antibody (1:1000). The secondary antibody used was goat anti-mouse IgG conjugated to horse radish peroxidase (1:1000). β-tubulin was used as a loading control to confirm equal loading of samples

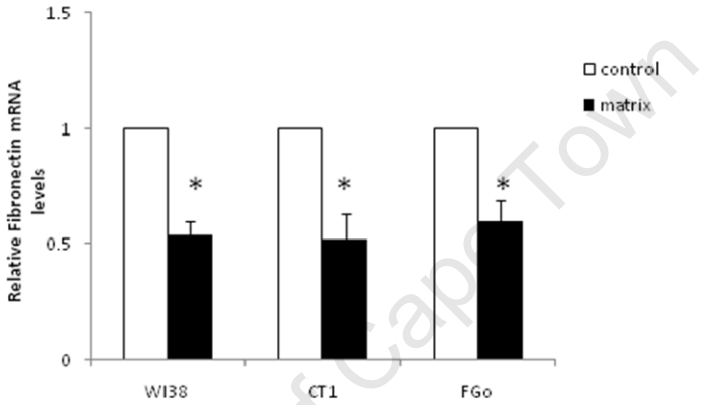

Figure 4.20 Effect of fibroblast-derived ECM on fibronectin mRNA levels.
WI38, CT-1 and FGo fibroblasts (A-C) were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then grown in DMEM media for 48 hrs. Cells were washed with PBS and RNA was extracted and five micrograms of total RNA were used in cDNA synthesis and qRT-PCR was performed to evaluate fibronectin mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

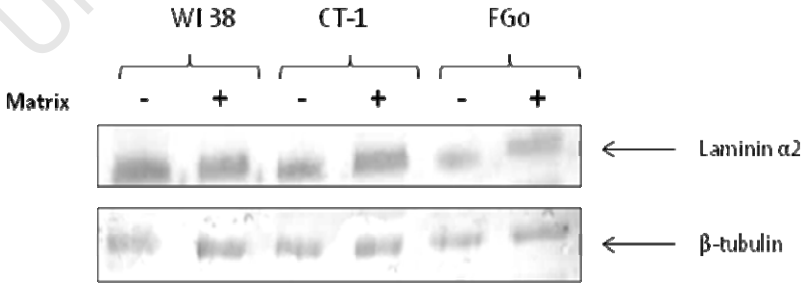

Figure 4.21 Effect of the fibroblast-derived ECM on laminin α2 protein levels.
WI38, CT-1 and FGo fibroblasts were seeded on dishes with or without fibroblast-derived ECM for 24 hrs and after changing media, were cultured for 48 hrs. Cell lysates (50 µg total protein) were separated by 7.5 % SDS PAGE and subjected to immunoblotting with mouse anti-laminin α2 antibody (1:1000). The secondary antibody used was goat anti-mouse IgG conjugated to horse radish peroxidase (1:1000). β-tubulin was used as a loading control to confirm equal loading of samples.

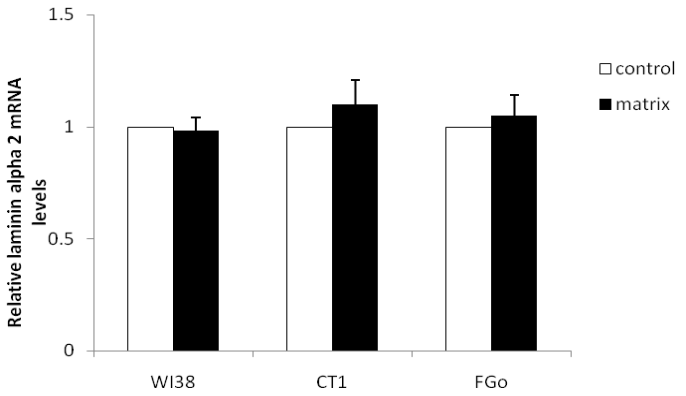

Figure 4.22 Effect of fibroblast-derived ECM on laminin α2 mRNA levels.
WI38, CT-1 and FGo fibroblasts (A-C) were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then grown in DMEM media for 48 hrs. Cells were washed with PBS and RNA was extracted and five micrograms of total RNA were used in cDNA synthesis and qRT-PCR was performed to evaluate laminin α2 mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments.

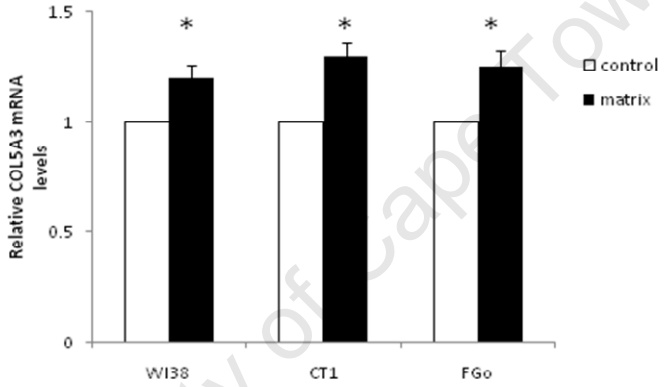

Figure 4.23 Effect of fibroblast-derived ECM on COL5A3 mRNA levels.
WI38, CT-1 and FGo fibroblasts (A-C) were seeded in dishes with or without fibroblast-derived ECM for 24 hrs and then grown in DMEM media for 48 hrs. Cells were washed with PBS and RNA was extracted and five micrograms of total RNA were used in cDNA synthesis and qRT-PCR was performed to evaluate COL5A3 mRNA levels using GAPDH as a control. The data are shown as the mean ± S.D. of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to fibroblasts plated on plastic.

4.2.7 Discussion

It has been suggested that growth factors or cytokines can adhere to the matrix in the stroma (Kao *et al*, 1983; Yamada and Cukierman, 2006). *In vitro*, growth factors such as PDGF and FGF have been shown to adhere to collagen-coated plates (Smith *et al*, 1999). When in contact with cells, these growth factors affect fibroblast proliferation and protein synthesis. In this study, the possibility that the matrix-mediated downregulation of type I collagen synthesis is caused by the presence of growth factors or other secreted factors such as cytokines was examined by using media, exposed to WI38 cells, and also by using the suspended matrix. When both the growth media exposed to fibroblasts and suspended/floating matrix were used, no effect was observed. The possibility that a structural component of the fibroblast-derived ECM was responsible for the results observed, was investigated, and only when fibroblasts were plated on purified type I collagen and on the fibroblast-derived ECM and was type I collagen synthesis downregulated. Thus direct contact between the specific matrix proteins and fibroblasts was necessary. It is reasonable to assume that the responsible factor be in insoluble form in the fibroblast-derived ECM, since the medium exposed to fibroblast and the suspended matrix, when introduced to fibroblasts plated on plastic did not affect type I collagen gene expression.

Differences in cell proliferation and thus cell density also did not account for the differences in type I collagen synthesis observed in this study. This suggests that the matrix effect on the fibroblasts type I collagen synthesis is not due to changes in cell proliferation and cell density, but is extracellular matrix-specific. Purified type I collagen and fibronectin have been used extensively as cell culture substrates that are intended to mimic the ECM environment found in tissues. It has been suggested that the use of thin collagen films may be useful in elucidating cellular remodelling of the matrix. Many studies have established that ECM components can influence gene expression (Kao *et al*, 1982; Streuli *et al*, 1993, 1995; Powell *et al*, 1996). Our results agree with results obtained by other investigators showing that the extracellular matrix composition strongly influences cells protein gene expression and organisation (Bissell *et al*, 2002; Elliott *et al*, 2003;

Langenbach *et al*, 2006). Bissell *et al* (2000) reported that the expression pattern of ECM genes in cells may vary depending on the type of matrices on which the cells are cultured. In the present study, a fibroblast-derived ECM and purified type I collagen inhibited collagen synthesis via a feedback mechanism. Procollagen mRNAs translation has been shown to be inhibited by cleaved collagen peptides. The N- and C-terminal peptides of type I collagen are known to inhibit collagen production by affecting polypeptide chain elongation and/or termination (Aycock *et al*, 1986; Wiestner *et al*, 1979). This suggests that collagen production is inhibited depending on the availability of specific proteases needed for the generation of the peptides. It is possible that the ECM downregulates COL1A2 transcription whilst its cleaved peptides affect COL1A1 mRNA translation. These data suggest that matrix proteins which normally reside in the stroma can inhibit fibroblast matrix synthesis.

The use of artificial matrices, purified type I collagen and fibronectin, implicated a structural component of the fibroblast-derived ECM as responsible for the downregulation of type I collagen synthesis. Since purified type I collagen matrix downregulated collagen synthesis, we focussed on known receptors for type I collagen. Receptors for soluble and ECM-bound type I collagen have been demonstrated to include integrins (Aplin *et al*, 1999; Barberis *et al*, 2000; Bork *et al*, 1999; Borrirukwanit *et al*, 2007). For comparison purposes, the receptor for another matrix protein, laminin, was also analysed. The mRNA levels of β1, α2 and α3 integrins were determined by real time RT PCR, after seeding fibroblasts on plastic and on matrix. There was an upregulation in the mRNA levels of both β1 and α2 integrins when fibroblasts were plated on a matrix. There was no change in mRNA levels of α3 integrin after plating fibroblasts on the matrix. The intracellular mechanism of substrate-dependent control on integrin gene expression is complex, is known to occur at both transcriptional and posttranscriptional levels and appears to be multifactorial (Delcommene and Streuli, 1994; Globus *et al*, 1995; Millward-Sadler and Salter 2004). Mammary epithelial cells and fibroblasts were shown to respond differently to the presence of ECM, with fibroblasts showing altered transcription of the β1 integrin gene, indicating that

the signaling pathways triggered in response to the ECM are cell type-dependent (Hynes, 1992, 2002; Giancotti, 2003; Brown, 2002; Schwartz and Assoian, 2001). It is possible that fibroblasts increase the levels of integrin receptors in order to maximise their chances of establishing contacts with the ECM, whilst those plated on plastic use other receptors, including other integrins, to make contact. Precisely why this should be so important to fibroblasts is not clear at this time, but it may reflect a generic wounding response that often occurs when cells are placed in tissue culture. There was no change in the levels of E-cadherin mRNA, matrix versus control, showing that cadherins are unlikely to be involved in the attachment to the fibroblast-derived ECM.

Furthermore, the use of blocking antibodies showed that integrins are involved and in particular α2β1 integrin is involved in the downregulation of type I collagen gene expression in the presence of the fibroblast-derived ECM. Collagen molecules contain RGD sequences in the helix-forming region, however a number of researchers have observed that attachment of integrins to several types of native collagens failed to be competed for by linear RGD containing peptides (Cardarelli *et al*, 1992; Davis, 1992; Kramer and Marks, 1989). Blocking the function of α3β1 integrin did not reverse the effect of the fibroblast-derived ECM on type I collagen synthesis. This implies that α3β1 integrin is not the receptor through which the stimulus, resulting in downregulation of type I collagen synthesis, is relayed to the nucleus. A general biologic principal can be recognised, that the ECM is informational to adjacent cell populations and also to cells that produce the ECM. The mechanism(s) by which fibroblast recognise the absence and presence of the ECM is presumably based on signalling through cell surface receptors for the appropriate matrix components. Occupation of the receptors would then result in a repressed transcriptional activity.

As a follow–up on matrix-mediated regulation of type I collagen synthesis, the study also investigated the effect of fibroblast-derived ECM on the expression of several ECM protein genes. There was a differential response to the presence of the ECM among the genes investigated. The expression of these

genes has never been studied in the presence of the fibroblast-derived ECM and therefore there is need to study more of the ECM genes to gain more insight into the expression of many proteins *in vivo*, which occur in the presence of the ECM. Further investigations, including a micro-array analysis, would be a necessity to elucidate the regulation of ECM proteins genes in the presence of a cell-derived matrix. In conclusion our data show that the gene expression of type I collagen is strongly affected by the presence of fibroblast-derived ECM and suggests a possible negative feedback loop *in vivo*, whereby the presence of the extracellular matrix downregulates type I collagen gene expression through integrins and in particular α2β1 integrin.

CHAPTER 5 GENERAL DISCUSSION

Previously viewed as a structural substrate for cells, it is now clear that the extracellular matrix, through specific receptors, induces biological responses from the cell surface and thus determines how cells interpret biochemical data from their surrounding stroma. To accurately determine how the properties of the ECM influence cellular behaviour, the experimental model system used must properly simulate the *in vivo* environment. This is because under *in vivo* conditions, the ECM components are synthesised in a specific ratio to each other, deposited and crosslinked in a specific sequence.

Studies on cell-matrix interactions have demonstrated the biological impact of the extracellular matrix on the structure and function of many cells (Sasser *et al*, 2007; Burgess, 2009; Rhee *et al*, 2007; Kudo *et al*, 2007; Che *et al*, 2006; Nho et al, 1990; Xia et al, 2008; Hadden and Henke, 2000).. Most of these studies involved cell culture models in which cells are plated on various extracellular matrix proteins and the expression of a tissue-specific function is assessed. There are reservations concerning the accuracy of these cell-culture models with respect to the intact tissue, in that the precise composition and structure of the ECM is not accurately simulated. The recent development of fibroblast-derived ECM provided a new and physiological assay system to study cell function (Serebriiskii *et al*, 2008). In this thesis therefore, a natural fibroblast-derived ECM was used to simulate *in vivo* environment, in the study of type I collagen gene regulation in human fibroblasts. Given the increasing prevalence of fibrotic diseases, which are characterised by excessive deposition of type I collagen within the stroma of tissue, understanding the mechanisms regulating type I collagen synthesis is imperative. As a component of the ECM, type I collagen provides essential support for maintaining tissue structure. Most of the type I collagen found in interstitial tissues is synthesised by fibroblasts which also synthesise factors that are involved in the regulation of collagen such as various growth factors and MMPs. Fibroblasts are therefore a useful model in the study of ECM regulation.

We report the downregulation of type I collagen gene expression when fibroblasts are plated on a fibroblast-derived ECM. This matrix-mediated downregulation of type I collagen synthesis occur at both transcriptional and post-transcriptional levels that is reflected in a significant decrease in both intracellular and secreted type I collagen protein levels. There is compelling evidence to suggest that when there is a large change in either mRNA or protein levels, multiple steps in the metabolism of the mRNA or the protein may have been altered (Stefanovic, 2005; Goldstein, 1991). The level of mRNA encoding a specific protein is determined by many factors including rate of transcription and the half-life of the mRNA. To determine whether gene transcription or mRNA stability, which may decrease α2(1) collagen mRNA expression level, was involved , an RNA stability study was performed using the transcription inhibitor actinomycin D. It was shown that there was no significant differences in α1(1) and α2(1) mRNA half lives between cells plated on plastic and matrix. Signal transduction pathways which trigger the changes in stability of collagen α1(1), mRNA are being elucidated. It is known that the activation of phophatidylinositol 3-kinase (PI3K) leads to stabilisation of collagen α1(1) mRNA in both liver, lung and skin fibroblast (Stefanovic, 2005; Friedman *et al*, 1992). In this study, however, there were no differences in the mRNA half lives and the possible involvement of any signaling transduction pathway in stabilising the mRNAs was not examined.

Remodeling of the ECM is primarily mediated by MMPs, which can collectively degrade all structural proteins of the ECM. MMPs are the driving force for degradation of ECM during remodeling and changes in MMP levels have been described in human cells in many pathological conditions. Differences in intracellular mRNA levels and not in protein levels may result from the secretion of MMPs into the media (Stefanovic, 2005). Correlation between the secretion of specific MMPs and the levels of type I collagen has been reported before (Laliberte *et al*, 2001). In this study we found that the fibroblast-derived ECM does not affect the gene expression levels of MMP-1, MMP-2, MMP-9, and MT1 MMP and the gelatinases activities. Work done by others show that when cells are plated on Matrigel, they increase the levels of at least the gelatinases activity (Ala-aho and Kahari, 2005; Ray and Stetler-

Stevenson, 1994). Differences in composition between the fibroblast-derived matrix and Matrigel might have contributed to the contrasting results obtained. The fibroblast-derived matrix is synthesised by human fibroblasts and some of its major components include type I collagen and type III collagen whereas Matrigel is made from EHS tumours (Kleinman *et al*, 1986), is comparable to the gel-like composition of the basement membrane and is rich in laminin, type IV collagen and other non-fibrous components of the matrix (Serebriiski *et al*, 2008).

Transient transfection with COL1A2 gene promoter constructs containing the sequences spanning -2300 to -107 base pairs driving the luciferase reporter gene revealed that the promoter activity was significantly decreased in response to the fibroblast-derived ECM. Deletion analysis of the 5'-flanking region of the COL1A2 gene promoter indicated that the response to the fibroblast-derived ECM depended on the region between -375 to -107 base pairs, where there are putative binding sites for AP1, Sp1, Sp3,Ets1 and Fli1 (Ihn, 2007) (Figure 5.1). Any one of these transcription factors and /or other still-to be identified factors could be responsible for the matrix-mediated downregulation of type I collagen as observed in this study. Amoung these

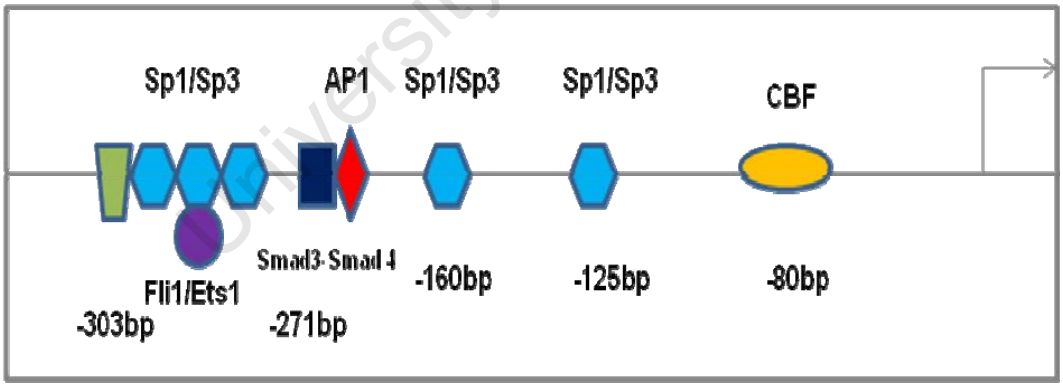

Figure 5.1. Transcriptional regulation of the human a2(I) collagen gene. The human a2(I) collagen promoter is regulated by transcription factors Sp1/Sp3, Ets1/Fli1, C/EBPb, AP-1, Smad3/Smad4, YB-1 and CCAAT-binding factor (CBF). (Adopted from Ihn, 2007).

transcription factors, the role of AP1 and Sp1 in the regulation of the COL1A2 gene expression has been extensively studied (Jinnin *et al*, 2006; Ihn *et al*, 2006). Electrophoretic mobility shift assays employing COL1A2 gene promoter constructs and the nuclear extracts from chick embryonic

chondrocytes indicated the involvement of an Sp1 factor in the cartilage-specific expression of the gene, since the addition of anti-Sp1 antibodies to the binding reaction resulted in a supershift of the DNA-protein complex. In addition, mutations in the Sp1 binding sites abolished the formation of the DNA-protein complex (Savagner *et al*, 1995).

We hypothesised that Sp1 may be involved in the matrix-mediated downregulation of type I collagen gene expression. Sp1 is has been reported to be involved in both upregulation and downregulation of type I collagen gene expression, depending on the other factors that it associates with. Interaction with smad3 was shown to increase type I collagen gene expression in scleroderma fibroblasts (Jinnin *et al*, 2006; Ihn *et al*, 2006) whilst interaction with Fli1 was shown to result in transcriptional repression of collagen genes including type I collagen (Jinnin *et al*, 2006). Indeed, deletion analysis showed that the longer constructs, -2300 to -107/ -721 to -107/ -375 to -107, resulted in the matrix-mediated downregulation of type I collagen whilst the short construct spanning the sequences -107/+54, displayed a negligible difference in activity, matrix versus control. Therefore the sequences from -375 to -107 seemed to be required for the decreased transcription of the COL1A2 gene, where there are putative binding sites for many Sp1 binding motifs. SiRNA is a conserved, genetic surveillance mechanism that allows the sequence-specific post transcriptional downregulation of target genes (Socker *et al*, 1998). We used siRNA technology to silence Sp1 gene expression and then determined the effect on type I collagen synthesis. Transfection of cells with Sp1 siRNA was not toxic to cells, and led to significant increases in the mRNA and protein levels of type I collagen. This pattern of change in mRNA and protein levels is consistent with the role of Sp1 as a mediator of the matrix-mediated downregulation of type I collagen in fibroblasts. The reason of the downstream type I collagen gene change after transfection of Sp1 siRNA, in the presence of the fibroblast-derived ECM, may arise from the interruption of the negative feedback loop in fibroblasts. Ghosh *et al* (2007) reported the involvement of TGF-β and Sp1 in the upregulation of type I collagen in tissue fibrosis whilst others report the involvement of Sp1 in the upregulation of type I collagen gene expression in scleroderma dermal fibroblasts (Jinnin *et al*,

2006). It has been reported before that Sp1 activates or represses different promoters depending on the other transcription factors it binds to or form complexes with (Santiago and Khachigian, 2004). Sp1 was found to be a repressor of COL1A2 promoter activity when it interacts with Fli-1 (Ramirez *et al*, 2006; Asano *et al*, 2009). Further investigation is therefore needed to clarify the other transcription factors involved and/or to verify whether Fli1 is involved.

Despite an increased understanding of intracellular signaling pathways that promote integrin-mediated function and differentiation in many cell types, mechanisms whereby specific integrins regulate fibroblast function and differentiation, and the role of MEK-ERK signaling in this process, remains unclear. Given the established role of MAPKs (JNK, MEK, p38) and PI3-kinase (Tian et al, 2002; Xia et al, 2008) in mediating many cellular processes, we investigated their role in mediating the effects of the fibroblast-derived ECM. It was observed that the MEK-ERK signaling cascade was involved in the matrix-mediated downregulation of type I collagen gene expression. The fact that inhibition of MEK-ERK signaling pathway and/or other pathways, can selectively reverse the ability of fibroblast-derived ECM to downregulate type I collagen gene expression, suggests a potential for pharmaceutical manipulation of the direct effects of the matrix on tissue type I collagen content.

In culture, cell substratum adhesion is mediated by at least two adhesion structures, weak interactions via close contacts and strong interactions via focal adhesions (Kim et al, 1997; Burridge *et al*, 1988). Focal adhesions are the points at which cells make their closest contacts with the substratum and provide the primary stabilising force for attachment of cultured cells. Integrin receptors are known to selectively localise to focal adhesions. Integrin-mediated interactions between cells and the ECM play a fundamental role in the development and function of a variety of tissues by triggering intracellular signals that regulate gene expression. Integrin-function blocking studies implicated the α2β1 integrin in the matrix-mediated downregulation of type I collagen and qRT PCR showed that only β1 and α2 integrin mRNA levels

increased significantly in the presence of the fibroblast-derived ECM. It is possible that when fibroblasts are plated on a fibroblast-derived ECM, they increase the levels of integrin subunit gene expression in order to increase their chances of establishing further contacts with the ECM. Others have shown that increased integrin expression is not important in cell adhesion to the substratum but integrin avidity (Kudo *et al*, 2007; Putnins *et al*, 1999). However, substratum-dependent control on integrin gene expression has been noted previously in other cell systems (Abraham *et al*, 2006; Chung *et al*, 2007; Hornebeck and Maquart, 2003; Tian et al, 2002; Xia et al, 2008).

Collagen-binding integrins can contribute to disease processes by mediating some of the aberrant cell-matrix interactions responsible for such disease. The presence of α1β1 and α2β1 integrins on myofibroblasts indicates that they might modulate ECM synthesis in fibrotic conditions (Zutter and Edelson, 2007). Excessive fibrosis is characterised by altered collagen production in diseases such as hepatic cirrhosis, pulmonary fibrosis, sclerosis, scarring and arterial restenosis (Varga *et al*, 1995). Available data suggest that α2β1 integrin might be one integrin candidate involved in lung and liver fibrosis. Cooperativity between integrins and growth factor receptors in the regulation of MAPK signaling pathways has already been demonstrated (Giancotti and Ruoslahti, 1999). Growth factor dependent induction of ERK signaling in NIH 3T3 cells is strongly dependent on integrin-mediated cell adhesion (Aplin and Juliano, 1999; Renshaw *et al*, 1997). Based on our results, our proposed model suggests a mechanism whereby integrins can cooperate with other receptors to induce MEK-ERK-dependent gene regulation. Specifically our results in fibroblasts showed that α2β1 integrin and not α3β1 integrin, was required for ras-mediated MEK-ERK activation in the presence of the fibroblast-derived ECM.

These results led us to propose a model to explain the role of the fibroblast-derived ECM in the regulation of type I collagen gene expression in fibroblasts. Key components of this model are as follows: (1) Fibroblasts must be in contact with a collagen-containing ECM (2) Fibroblasts bind to the matrix via interactions between type I collagen and the α2β1 integrins, possibly

among others (3) Integrin liganding activates the Ras/Raf-1/MEK-ERK signaling pathway that transduce signals to the nucleus, which might lead to the phosphorylation of Sp1 transcription factor and the subsequent downregulation of type I collagen synthesis. This proposed model is shown in Fig 5.2 and is supported by results obtained in this study. In summary, this study showed that the fibroblast-derived ECM-mediated downregulation of type I collagen is via receptor-meditated activation of the MEK-ERK signaling pathway that requires the activity of Ras. Lastly, this study showed that the expressions of several genes are affected differently by the presence of the ECM. Whilst most studies are done on plastic, the results obtained from this study call for caution when interpreting results obtained from such studies.

These findings provide new insights into the mechanisms of signaling in fibroblasts and as such may have an impact on the regulation of fibroblast proliferation and differentiation, and fibroproliferative diseases.

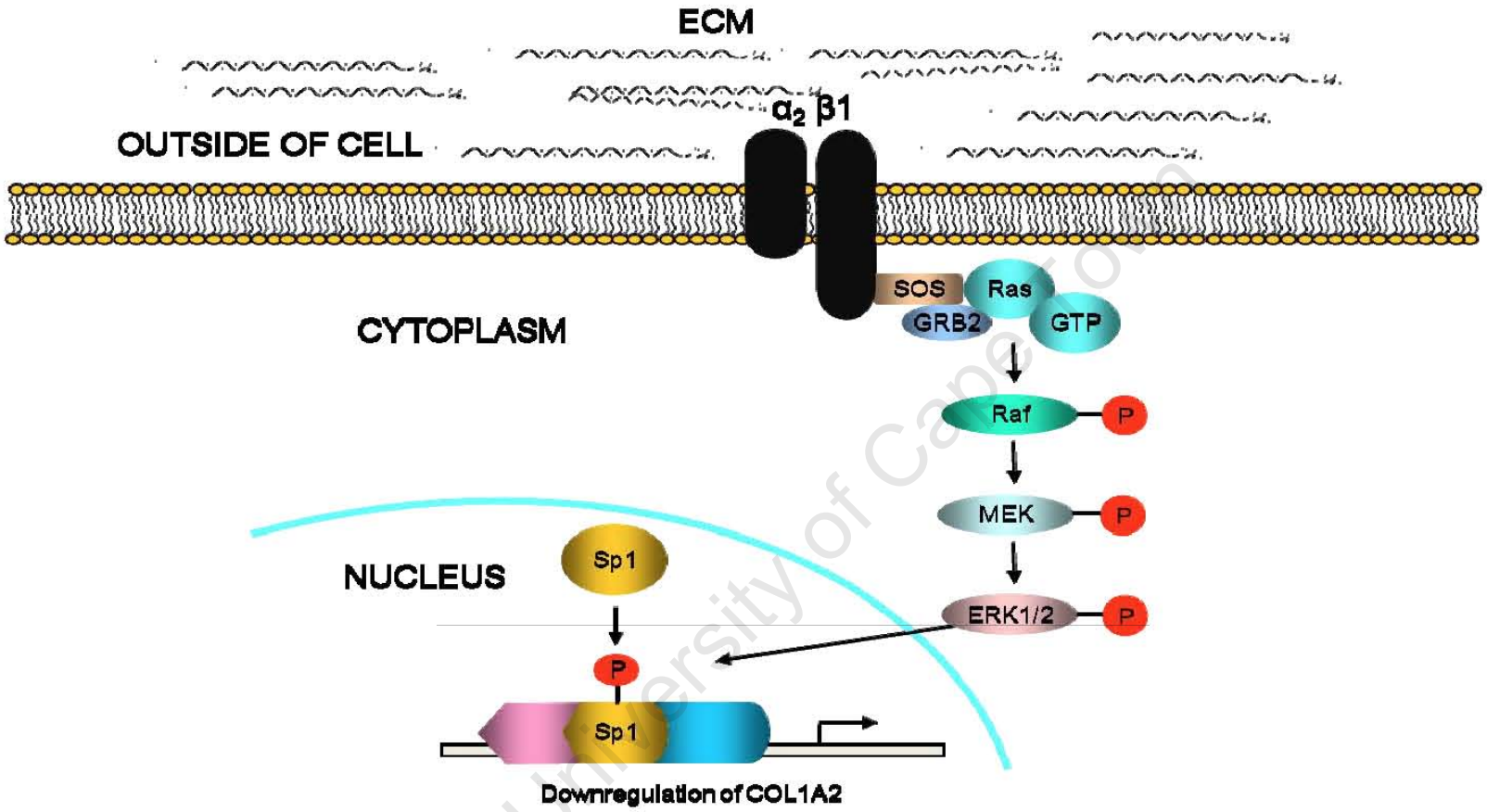

Figure 5.2 Model proposed for ECM-mediated downregulation of type I collagen expression through activation of MEK-ERK signaling pathway in fibroblasts. Fibroblasts must be in contact with a collagen-containing ECM and this interaction between type I collagen and its cell receptor,α2β1, Activates the Ras/Raf-1/MEK-ERK signaling pathway that transduce signals to the nucleus leading to the downregulation of type I collagen synthesis.

CHAPTER 6 CONCLUSION

This study extends our understanding of ECM-dependent feedback regulation of type I collagen gene expression. We have shown that gene expression of ECM proteins, in particular type I collagen, and their integrin receptors, can all be regulated by cell-matrix interaction. During pathological conditions such as in cancer invasion and metastasis and in fibrosis, changes in type I collagen content are typically observed. The results of the present study provide evidence that the fibroblast-derived ECM downregulates type I collagen gene expression through activation of the MEK-ERK signaling cascade and that there is need for direct contact between the fibroblasts and the matrix for this to occur. These findings, to the best of our knowledge, represents the first study to shed light on feedback regulation of type I collagen gene expression by a fibroblast-derived ECM.

This study strongly supports the critical role of Sp1 and its associated factors such as Fli1 in the matrix-mediated downregulation of type I collagen synthesis. Given the complexity of collagen gene regulation, other factors are likely to contribute to this process, either as part of the Sp1/Fli1 complex or through independent pathways. Future studies should be directed at the generation of a mouse model such as transgenic mice with fibroblast-specific deletion of Sp1 or Fli1 which will allow better characterisation of the genes regulated by these two transcription factors in fibroblasts. The results of this study support the notion that the absence of Sp1 and its associated factors might play a pathological role in conditions where type I collagen gene expression is altered such as in scleroderma. In addition, our data suggest that there is a reciprocal relationship between integrin signaling and some key transcription factors. Our data also show that ligation of integrins by plating fibroblasts on the fibroblast-derived ECM, generates Sp1 transcripts, independent of the presence of growth factors and cytokines. The findings of this study strongly suggest that the significant number of signaling studies published to date using cells grown on artificial substrates or plastic should be re-evaluated under arguably more physiological conditions such as the fibroblast-derived ECM. While the principles of signaling established using

regular tissue culture are likely to remain valid, the extent, nature and regulation of specific signaling responses of cells may differ when using cell-derived matrices such as the fibroblast-derived ECM.

It is unlikely that any single aspect of remodeling itself, in this case changes in type I collagen gene expression, will adequately explain the changes observed in pathological conditions such as in cancer invasion and metastasis and in fibrosis. However, understanding the potential contributions of the extracellular matrix in the regulation of type I collagen synthesis may aid in the development of therapeutic strategies for the treatment of the many diseased conditions where type I collagen regulation is altered.

CHAPTER 7: MATERIALS AND METHODS

7. 1 Materials

7.1.1 Reagents

Cell culture reagents were obtained from GIBCO BRL Life Technologies (Gaithersburg, MD). Pepsin was obtained from Southern Biotech (Birmingham, AL). U0126 (ERK 1, 2 inhibitor) and SB203580 (p38 MAPK inhibitor) were from Calbiochem (La Jolla, CA), while PD98059 (ERK 1, 2 inhibitor), PI-3 kinase inhibitor (Wortmanin) and SP600125 (JNK inhibitor) were from Cell Signaling (Upstate, Lake Placid, NY, USA). Transfectin and the SDS-PAGE molecular weight standards were from BioRad (Cape Town). The pGL2 basic vector, renilla pRL-HSV-TK vector, a dual-luciferase detection system was obtained from Promega (Madison, WI). Purified type I collagen was purchased as a solution of acid-stabilized monomer (Vitrogen; Cohesion Technologies, Inc., Palo Alto, CA). Purified fibronectin was from Sigma (St Louis, MO). The dominant negative mutant MEK and its parent vector, pCMV5, were a gift from Prof. R.T Franceschi (University of Michigan, USA) (Roca and Franceschi, 2008). Plasmid N17Ras.cmv, a pCMV5 plasmid encoding the S17 -N dominant negative mutant Ras protein was a gift from Prof. Michael Birrer (National Cancer Institute, USA) (Leaner *et al*, 2005). Other chemicals and solvents if not otherwise specified were purchased from either Sigma (St Louis, MO) or Merck Biosciences (Darmstadt, Germany).

7.1.2 Cell Lines

The cell lines used in this study were the following:

Human skin fibroblasts (FGo) were originally established from surgical biopsies of human skin in South Africa (van der westhuysen *et al*, 1984)

Human embryonic lung fibroblasts, (WI38) (ATCC CCL-75) (Manassas, VA, USA)

γ-radiation transformed WI-38 human lung fibroblast, CT-1 fibroblasts (a gift from Dr M. Namba (Tokyo, Japan) (Namba *et al*, 1980)

Simian virus-40 transformed WI38 fibroblasts, (SVWI38) (de Haan *et al*, 1986)

Human fibrosarcoma cells, HT-1080 (ATCC CCL-121) (Manassas, VA, USA).

7.1.3 Antibodies

The anti-type I collagen antibody (Catalogue No: 1310-01, Lot No: D3405-Y318) was purchased from Southern Biotech (Birmingham, AL). The phospho-p44/42 mouse polyclonal antibody was from Cell Signalling (Catalogue No: 9106S, Lot No: 27) (Upstate, USA). The anti-fibronectin antibody (Catalogue No: CP13L, Lot No: D37065) was from Calbiochem (La Jolla, CA). The monoclonal anti-α2β1 integrin antibody (Catalogue No: MAB1998, Lot No: LV1430235) was from Chemicon (Temecula, CA, now Millipore, USA). The anti-tubulin antibody (Catalogue No: sc-9104, Lot No: E2308), monoclonal mouse antibodies to human integrin α2β1 (P1E6, Lot No: 0083a) and to human integrin α3β1(P1B5, Lot No: 0083b) were from Dako Corporation (Carpinteria, CA, USA), anti-MT1 MMP (Catalogue No: sc-12367, Lot No: 15), anti-NFk-β (Catalogue No: sc-109, Lot No: L1007), p44/42 ERK (Catalogue No: sc-153, Lot No: D1007) and the anti-Sp1 (Catalogue No: sc-14027, Lot No: E2808) antibodies were obtained from Santa Cruz (USA). Secondary antibodies were HRP-conjugated and together with other primary antibodies not mentioned here, were from either Santa Cruz or Pierce.

7.2 Methods

7.2.1 Maintenance of cells in culture

Cells were cultured at 37 ^{0}C in a humidified atmosphere of 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. For experiments, confluent fibroblasts (70 %) (WI38, CT-1 and FGo) were split using 0.5 % trypsin-0.53 mM EDTA and plated in culture dishes with or without the matrices used in the study i.e fibroblast-derived ECM, purified type I collagen and fibronectin. The media were changed three times a week. Only early passage cells were used in the studies.

7.2.2 Thawing and Freezing down of cells

To grow cells, vials containing the cells were removed from nitrogen tanks and rapidly thawed in a 37^{0}C water bath. The vials were then wiped with 70 % ethanol and the cells were mixed with fresh DMEM media and spun at 1000

r.p.m for 4 minutes. The media were decanted and the cells were then suspended in fresh DMEM media in a culture dish or flask.

In order to store cells for long periods, freezing was done. Freezing of cells was done after removing the media by aspiration. Cells were then trypsinised with 0.5 % trypsin in PBS containing 10 mM EDTA for 2-3 minutes in the incubator at 37^0C. An equal volume of serum-containing DMEM was then added to stop the trypsinisation. Cells were counted and the media was removed through centrifugation at 1000 r.p.m for 5 minutes. The cells were resuspended with freezing down media (70 % DMEM, 20 % FCS and 10 % DMSO) by gently pippetting twice. The cells were aliqouted into 2 ml ampoules at about 1 x 10^6 cells/ml and stored at -80 ^{0}C for 48 hrs before being transferred to a liquid nitrogen tank. Every two months the cells were checked for mycoplasma contamination.

7.2.3 Preparation of fibroblast-derived ECM

Confluent WI38 cells were split using 0.5 % trypsin in PBS containing 10 mM EDTA and cells were seeded in 35 mm or 100 mm tissue culture dishes. The cells were grown in DMEM supplemented with 10 % heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin. The temperature was maintained at 37 ^{0}C in a humidified incubator (70 %) and CO_2 was be maintained at 5 %. Fresh ascorbic acid (50 µg/ml) was added every alternate day based on established protocols (Serebriiskii *et al*, 2008). Cells were grown to 8 days post confluence after which the medium was drained off, the cells were rinsed with PBS and lysed by adding 20 mM freshly prepared ammonium hydroxide for about one minute (Green and Yamada, 2008; Serebriiskii *et al*, 2008). The matrix was washed three times with sterile PBS, fixed to the dish with a 50 % ethanol rinse, air dried and sterilised overnight under UV light in a tissue culture cabinet. The matrices were checked using an inverted phase contrast microscope. This treatment removed cells but left a cell-free *in vivo*-like 3D matrix that remained attached to the culture dishes. Matrix-coated dishes were stored at 4^0C and used within two weeks of preparation. Extracellular matrix protein integrity is stable under these storage conditions (Serebriiskii *et al*, 2008). Before use, the matrix was rinsed with sterilised PBS followed by one rinse with tissue

culture media. The quality of the matrix was checked by staining with Coomassie Brilliant Blue. Artificial matrices were made from purified type I collagen (Cohesion Technologies) and fibronectin (Sigma). Purified Type I collagen and fibronectin were used at a concentration of 0.1 mg/ml and incubated in culture dishes at 37^0C for at least 1 hr. Gelation of the matrices occurred in less than 30 minutes.

7.2.4 Cell Proliferation Assay

Cell proliferation was determined by either counting cells after trypsinisation or by the MTT assay. At the end of each experiment, cell were trypsinised then counted using a haemocytometer. The MTT assay was carried out using the Cell Proliferation Kit I (Roche Diagnostics, South Africa) as described by the manufacturer. Briefly, cells (1.5×10^3) were plated in 96-well plates with or without matrix, in a final volume of 90 µl DMEM per well. After incubating cells for the indicated periods, the cells were washed with phosphate buffered saline (PBS) and incubated in fresh medium containing 10 µl (final concentration of 0.5mg/ml) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 h at 37 ^{0}C. The formazan produced in the reaction, was dissolved by 200 µl solvent solution (0.1 N HCl in isopropanol) overnight. The spectrophotometric absorbances of samples were measured at 595 nm using a microtiter plate reader (Model 680, Biorad). The MTT data are presented as the mean (±S.D.) of triplicate experiments. The statistical analysis was performed with GraphPad Prism 5.0 software (2006).

7.2.5 Cell Attachment Assay

To determine if the rate of cell attachment to the different matrices (fibroblast-derived ECM, type I collagen and fibronectin) was the same, the adhesion assay was performed. Type I collagen and fibronectin were diluted at 100 µg/ml in DMEM containing 0.1% BSA and 200 µl of each was added in triplicate to 6-well plates. The coated-plates were incubated at 37 ^{0} C for 1hr. Control wells or those coated with ECM were then rinsed with PBS containing 3% BSA for 30 mins at 37 ^{0}C. Cells were detached using trypsin/EDTA, washed twice with DMEM/BSA and equal number of cells were seeded on the matrices. Incubation was continued for 90 minutes after which the media

containing unattached cells was removed and then attached cells were trypsinised and counted. Each experiment was performed in triplicate and three independent experiments were performed.

7.2.6 Quantitative Real Time RT-PCR

7.2.6.1 RNA Isolation

Total RNA was extracted according to Chomczynski and Sacchi (1983). Fibroblasts were seeded in 35 mm or 100 mm dishes with or without ECM. After 24 hrs the medium was changed to fresh DMEM and incubation was continued for the different time points used in the experiments. The medium was aspirated, the cell surface rinsed with ice cold PBS and Trizol reagent (Invitrogen Corporation, California, USA) was used to obtain the RNA according to the manufacturer's instructions. In brief cells were lysed by adding 1 ml of Trizol reagent to the culture dishes and passing the homogenate several times through a pipette. The homogenised samples were incubated for 5 minutes at room temperature to permit a complete dissociation of the nucleoprotein complexes. Chloroform (0.2 ml per 1ml of Trizol reagent) was added and the tubes were shaken vigorously by hand for 15 seconds and incubated at room temperature for 3 minutes. The samples were centrifuged at 12 000 r.p.m for 15 minutes at 4^0C. The upper phase was transferred to a fresh tube and the RNA precipitated by mixing with isopropyl alcohol (0.5 ml per 1 ml of Trizol reagent for the initial homogenisation). The tubes were incubated at room temperature for 10 minutes and centrifuged at 12 000 r.p.m for 10 minutes at 4^0C to pellet the RNA. RNA was rinsed once with 75 % ethanol. The RNA sample was vortexed and centrifuged at 7500 r.p.m for 5 minutes at 4^0C. The RNA was air-dried for 10 minutes and finally dissolved in 50 µl RNase-free water by heating at 55 ^{0}C for 10 minutes and stored at -80^0C. The concentration of RNA was determined spectrophotometrically (DU650 spectrophotometer, Beckman Instrument Inc, Fullerton, California, USA) by measuring absorbance (OD) at 260 nm and 280 nm. The formula: RNA concentration (µg/ml) = OD_{260} x 40 µg/ml x dilution factor, was used to calculate the RNA yield. RNA quality was checked by electrophoresis on 1% agarose formaldehyde gels containing 0.5 µg/ml ethidium bromide.

7.2.6.2 RNA Electrophoresis

7.2.6.2.1 Preparation of a 1% formaldehyde agarose gel

Agarose formaldehyde mini gels were prepared by boiling 0.5 g high-gelling agarose in 45 ml distilled water mixed with 5 ml of 10 x MOPS. After the solution has cooled down to approximately 65^{o}C, 2.7 ml of 27 % formaldehyde was added. After cooling, ethidium bromide was added to the solution and mixed properly. The gel was poured into the gel tank, combs were inserted and allowed to set in the fume hood. MOPS (1x) was added to the tank and used as the running buffer. Electrophoresis was at 60V for 45 minutes.

7.2.6.2.2 RNA sample preparation

Two micrograms of the RNA were analysed by gel electrophoresis. Volumes equivalent to 2 µg of RNA were mixed with sterile distilled water and RNA loading buffer. The mixture was then added to the wells. Running standards were loaded beside the samples. The gel was run at 60 V until the bromophenol blue dye has migrated two-third the length of the gel. The gel was destained three times by placing it in water for about 30 minutes each and then examined under UV transilluminator to visualise the RNA. The integrity of the RNA was ascertained by the intactness of the 18S and 28S ribosomal RNA.

7.2.6.3 Synthesis of cDNA

Complementary DNA (cDNA) was generated from 5 µg of RNA and oligo $(dT)_{15}$ primer by using Impromp II reverse transcriptase (Promega Inc, Madison, WI, USA) in the presence of dNTPs and DTT according to the manufacturers' instructions. Briefly, the following were added to a nuclease-free microcentrifuge tube:

50 µM oligo $(dT)_{15}$	1 ul
5 µg total RNA	X ul
10 mM dNTPs mix	1 ul
Distilled water	Y µl
Total volume	9 ul

(X = depends on the concentration of the RNA and Y = add up to a final volume of 9 µl).

The mixture was heated at 70 ^{0}C for 10 minutes and placed on ice immediately after removal from the heating block. The mixture was spun by brief centrifugation for 4 seconds. A mixture of the following was made:

5 x First strand buffer	5 µl
0.1 M DTT	1 µl
dNTPs mix	1 µl
RNase inhibitor	1 µl
$MgCl_2$	1 µl
Impromp II reverse transcriptase	1 µl
Water	1 µl
Total volume	11 µl

This mixture was mixed with the one above to give a final volume of 20 µl. The resultant mixture was properly mixed and incubated at 42 ^{0}C for 2hrs. The resulting cDNA was now ready to be used as a template for PCR amplification.

7.2.6.4 Quantitative real time RT- PCR

Quantitative RT PCR was performed using the Mini Opticon Monitor 3 (Biorad) with the KAPA SYBR qPCR Universal Kit (KAPA BIOSYSTEMS, S.A). The PCR reaction was done in a final volume of 25 µl, containing 0.5 µmol of each primer and 12.5 µl KAPA SYBR qPCR Universal Kit.

PCR reaction set up

Kapa Biosystem mix	12.5 µl
Primer forward	1.0 µl
Primer reverse	1.0 µl
Water	8.5 µl
cDNA or water (control)	2.0 µl
Total volume	25 µl

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primers and conditions used are shown below:

GAPDH

Primer pair	qRT PCR Condition		Product Size
Forward: 5'-GGCTCTCCAGA	95 ^{0}C: 10 min		
ACATCATCC-3'	95 ^{0}C: 10 sec		
Reverse: 5'-GCCTGCTTCACC	55 ^{0}C: 8 sec	40 cycles	192 bp
ACCTTC-3'	72 ^{0}C: 15 sec		

COL1A1

Primer pair	qRT PCR Condition		Product Size
Forward primer: 5'-CAGCCGCTTCA	95 ^{0}C: 10 min		
CCTACAGC-3'	95 ^{0}C: 20 sec		
Reverse primer: 5'-TTTTGTATTCAATC	60 ^{0}C: 20 sec	40 cycles	83 bp
ACTGTCTTGCC-3'	72 ^{0}C: 20 sec		

primer sequences were taken over from Martin *et al.*, 2001

COL1A2

Primer pair	qRT PCR Condition		Product Size
Forward primer: 5'-GATTGAGACCCTT	95 ^{0}C: 10 min		
CTTACTCCTGAA-3'	95 ^{0}C: 20 sec		
reverse primer: 5'-GGGTGGCTGAGTC	60 ^{0}C: 20 sec	40 cycles	78 bp
TCAAGTCA-3'	72 ^{0}C: 20 sec		

primer sequences were taken over from Sengupta *et al.*, 2003

COL5A3

Primer pair	qRT PCR Condition		Product Size
Forward primer: 5'-CAGCCGCTTCA	95 ^{0}C: 10 min		
CCTACAGC-3'	95 ^{0}C: 20 sec		
Reverse primer: 5'-TTTTGTATTCAATC	60 ^{0}C: 20 sec	40 cycles	179 bp
ACTGTCTTGCC-3'	72 ^{0}C: 20 sec		

Fibronectin

Primer pair	qRT PCR Condition		Product Size
Forward: 5'-GAAGGAATATC	95 ^{0}C: 10 min		
TCGGTGCCA-3'	95 ^{0}C: 30 sec		
Reverse: 5'-CGGGAATCTTCTC	60 ^{0}C: 30 sec	35 cycles	228 bp
TGTCAGC-3'	72 ^{0}C: 60 sec		

primer sequences were taken over from Tripathi *et al.*, 2004

Human β1 Integrin

Primer pair	qRT PCR Condition		Product Size
Forward: 5'-GACTGATCAGTTCA	95 ^{0}C: 10 min		
GTTTGCTGTGTGTTT-3'	95 ^{0}C: 15 sec		
Reverse: 5'-CCCTGCTTGTATACAT	58 ^{0}C: 15 sec	45 cycles	174
TCTCCACATGATTT-3'	72 ^{0}C: 15 sec		

primer sequences were taken over from Borrirukwanit *et al.*, 2006

Human α2 Integrin

Primer pair	qRT PCR Condition		Product Size
Forward: 5'-GACCTATCCACTGCCA	95 ^{0}C: 10 min		
CATGTGAAAAA-3'	95 ^{0}C: 15 sec		
Reverse: 5'-CCACAGAGGACCACAT	58 ^{0}C: 15 sec	45 cycles	160
GTGAGAAAA-3'	72 ^{0}C: 15 sec		

primer sequences were taken over from Borrirukwanit *et al.*, 2006

Human α3 Integrin

Primer pair	qRT PCR Condition		Product Size
Forward: 5′-GCTGCCAAAAAAGCCA	95 ^{0}C: 10 min		
AGTCTG-3'	95 ^{0}C: 10 sec		
Reverse: 5'-TGCTGGTTCGGAGGAA	58 ^{0}C: 10 sec	45 cycles	160
TAGGGTAG-3′	72 ^{0}C: 09 sec		

primer sequences were taken over from Kudo *et al*, 2007

E-cadherin

Primer pair	qRT PCR Condition		Product Size
Forward: 5'-GGCTCTCCAGA	95 ^{0}C: 10 min		
ACATCATCC-3'	95 ^{0}C: 10 sec		
Reverse: 5'-GCCTGCTTCACC	55 ^{0}C: 10 sec	45 cycles	133 bp
ACCTTC-3'	72 ^{0}C: 10 sec		

Sp1

Primer pair	qRT PCR Condition	Product Size
Forward: 5'-AATTTGCCTGC	95 ^{0}C: 5 min	
CCTGAGTGC-3'	95 ^{0}C: 20 sec	
Reverse: 5'-TTGGACCCATG	57 ^{0}C: 30 sec	50 cycles 169 bp
CTACCTTGC-3'	72 ^{0}C: 30 sec	

Sp3

Primer pair	qRT PCR Condition	Product Size
Forward: 5'-ACAGCTTGGCTA	95 ^{0}C: 10 min	
GGCCTGCTC-3'	95 ^{0}C: 10 sec	
Reverse: 5'-TGGCAGCGTAGGT	60 ^{0}C: 15 sec	45 cycles 141 bp
TTTCGAGAGC-3'	72 ^{0}C: 10 sec	

MMP 1

Primer pair	qRT PCR Condition	Product Size
Forward: 5'-GCTGGGAGCAAAC	95 ^{0}C: 10 min	
ACATCTGACCT-3'	95 ^{0}C: 30 sec	
Reverse: 5'-TGAGCCGCAACACG	60 ^{0}C: 10 sec	40 cycles 146 bp
ATGTAAGTTG-3'	72 ^{0}C: 30 sec	

primer sequences were taken over from Qu *et al.*, 2006

MMP 2

Primer pair	qRT PCR Condition	Product Size
Forward: 5'-TGTGTCTTCCCCTTAC	95 ^{0}C: 10 min	
CTTT-3'	95 ^{0}C: 30 sec	
Reverse: 5'-GATCTGAGCGATGC	60 ^{0}C: 10 sec	40 cycles 146 bp
CATCAA-3'	72 ^{0}C: 30 sec	

primer sequences were taken over from Madani *et al*, 2006.

MMP 9

Primer pair	qRT PCR Condition	Product Size
Forward: 5'- GAGACAGCATGGCCAA	95 ^{0}C: 10 min	
ATTA-3'	95 ^{0}C: 30 sec	
Reverse: 5'-CTCTAGAAACTGCTGAGG	60 ^{0}C: 10 sec	40 cycles 146 bp
GC-3'	72 ^{0}C: 30 sec	

primer sequences were taken over from Qu *et al.*, 2006

7.2.6.5 Comparative Ct Method

Real-time RT PCR data were analysed using a comparative method based on the critical threshold, (C_t), previously called the crossing point. In this method the amount of the gene of interest was normalised against the housekeeping gene, GAPDH, and expressed as fold induction using the $2^{-\Delta\Delta Ct}$ **(**Hundley *et al*., 2006). The experiments were done three times in triplicate.

Fold induction = $2^{-\Delta\Delta Ct}$

Where $\Delta\Delta Ct$ = {Ct GI (unknown sample) – Ct (GAPDH) (unknown sample)} - {Ct GI (calibrator) – Ct (GAPDH) (calibrator)}

The calibrator sample is any sample chosen to represent 1 x expression of the gene of interest. Results are shown as fold induction of mRNA compared to control.

7.2.7 Gelatin Zymography

Zymographic analysis of protease activity was performed as described by Helary *et al* (2005). Polyacrylamide gels contained 1 mg/ml gelatin in a buffered solution consisting of 5 ml 1.5 M Tris-HCl, pH 8.8, 200 µl 10 % SDS, 8 ml 30% Acrylamide/bis Acrylamide (29:1) and 5 ml distilled water. Stacking gels contained 4% polyacrylamide in 0.125 M Tris-HCl, pH 6.8. Gels were polymerised by adding 50 µl of 10 % ammonium persulfate and 10 µl of 0.1 % TEMED. Samples (the loading volume was normalised for protein concentrations as measured by the Bradford method (Biorad) were added to 2x loading buffer (non-reducing) and electrophoresis was done at 25 mA for 2hrs. After electrophoresis gels were washed twice (30 mins each) in 200 ml of 2.5 % Triton X-100 with constant stirring and incubated in incubation buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 0.2 M NaCl and 0.02 % Brij-35) for 18 hrs at 37^0C. Gels were then stained with Coomassie brilliant blue RR-250 (20 % methanol) and destained in 15% acetic acid, 10 % methanol. Gelatinolytic activity was evident as transparent zones in the blue gels. The area of the cleared zones was analysed with the Fluor-S Multimager (Biorad).

7.2.8 Transient Transfections and Promoter Assays

Fibroblasts were transiently transfected with the indicated COL1A2/Luciferase constructs, MMP-1/LUC construct, the dominant negative mutant MEK expression vector, the dominant negative mutant Ras expression vector, N17Ras, and the necessary parental vectors. In all experiments 50 ng of renilla luciferase construct (pRL-HSV-TK) was co-transfected as an internal control. Fibroblasts were seeded in 35 mm or 100 mm dishes and incubated with DNA/transfectin reagent for 6 hrs after which the media were changed and incubation continued for 24 hrs. Fibroblasts were then trypsinised and seeded in 35 mm or 100 mm dishes with or without matrices. Cell lysates were prepared using a passive lysis buffer (Promega) and luciferase activities were measured using a dual Luciferase Assay System (Promega, Madison, USA). Reporter gene activities (firefly luciferase) were normalised to the co-transfected renilla activity. Matrix-mediated change was determined as the fold change in the luciferase activity relative to that in cells cultured in the absence of matrix.

7.2.9 Propagation of plasmid DNA

7.2.9.1 Preparation of competent *E.coli* (XL1-blue) cells

The method used is a modification of that described by Sambrook (Sambrook *et al*., 1989). Fifty microlitres of *E.coli* (XL1-blue) cells from a frozen glycerol stock were inoculated into 3 ml of Luria broth and grown overnight at 37^0C with shaking. The overnight culture was inoculated into 300 ml of Luria broth in a 2 L conical flask and incubated at 37^0C with vigorous shaking until the cultures reached an OD_{650nm} of 0.4 to 0.6. The cells were pelleted by centrifugation at 5000 rpm at 4^0C in a Beckman JA10 rotor for 10 mins. The media was decanted and the pellet was resuspended in 40 ml of ice cold 60 mM $CaCl_2$, 10 mM Pipes pH 7.2. This suspension was left on ice for 30 mins, transferred to Corex glass centrifuge tubes and centrifuged at 5000 rpm at 4^0C for 5 mins in a Beckman JA-20 rotor. The media was decanted and the tubes were inverted for one minute to allow any trace of the fluid to drain away. Four ml of a solution of 60 mM $CaCl_2$, 10 mM Pipes and 15% glycerol were added to the pellet and gently mixed. The cell suspension was stored in aliquots of 200 µl at -80^0C.

7.2.9.2 Transformation of Competent Cells with plasmid DNA

The transformation of the *E. coli* (XL1-blue) cells was done using the different plasmids used in the study (Sambrook *et al*., 1989). An aliquot of competent cells were thawed on ice and 100 µl transferred to a 15 ml sterile tube. To the cells, was added 1 ng of the required plasmid DNA, mixed gently by swirling and incubated on ice for 30 mins, followed by heat shock at 42 ^{0}C for 2 mins before the addition of 1 ml of pre-warmed (37^{0}C) Luria Broth (LB) and incubation at 37^{0}C for 1hr. Aliquots of 50, 100 and 200 µl aliquots were plated out on Luria agar plates containing 50 µg/ml ampicillin and incubated overnight at 37^{0}C. A successful batch of competent cells usually formed between 10^7 and 10^8 colonies per µg of plasmid DNA after overnight incubation.

7.2.9.3 Preparation of plasmid DNA

A 500 ml overnight culture of E.coli containing the desired plasmid was harvested at 5000 rpm for 10 mins in a Beckman JA-20 rotor at 4^{0}C. The Qiagen plasmid DNA extraction kit was used to prepare plasmid DNA as described by the manufacturers. After precipitation the DNA was washed with 70% ethanol and resuspended in sterile TE or sterile water depending on the final use of the DNA. The concentration of DNA was determined spectrophotometrically (DU650 spectrophotometer, Beckman Instrument Inc, Fullerton, California, USA) by measuring absorbance (OD) at 260 nm and 280 nm. The formula: DNA concentration (µg/ml) = OD_{260} x 50 µg/ml x dilution factor, was used to calculate the DNA yield.

7.2.9.4 Isolation of DNA fragments

A known amount of plasmid DNA was digested with the required restriction enzymes for at least 1 hr, depending on the enzyme being used, and the digested material resolved on 1% agarose gels containing 0.5 µg/ml ethidium bromide. DNA molecular weight markers were routinely included in all agarose gels to determine the approximate sizes of the DNA bands. The DNA fragment of interest was excised from the gel, excess agarose was removed and gel pieces transferred to a microfuge tube containing 500 µl of Promega

DNA binding solution. The DNA was extracted using the Promega gel extraction kit (Promega) according to the manufacturer's instructions.

7.2.10 Nuclear Protein Extraction

Nuclear proteins were extracted from cells using the method of Lee and Green (1990). Briefly, cells grown in 35 mm or 100 mm diameter tissue culture dishes were rinsed and harvested in ice cold PBS using a rubber policeman and pelleted in a Beckman TJ 6 centrifuge at 2000 rpm at 4^0C for 10 minutes. The cells were pelleted and resuspended in 5 packed cell volumes of buffer A and allowed to swell on ice for 10 minutes, pelleted again and resuspended in 2 original packed cell volumes of buffer A. The suspended cells were lysed through five cycles of slow aspiration and rapid ejection using a syringe with a 26-gauge needle. Cell lysis was checked by microscopy. The nuclei were pelleted by centrifugation at 13000 rpm at 4^0C for 30 minutes. Pelleted nuclei were resuspended in buffer C, containing 1 µg/ml aprotinin, 1 µg/ml pepstatin and 1 µg/ml leupeptin. The homogenised nuclei were stirred on ice for 30 minutes. The salt soluble proteins were separated from nuclei debris by centrifugation in a Beckman JA-20 rotor for 30 minutes, at 13000 rpm at 4^0C. The supernatant was dialysed against buffer D for 2 hrs at 4^0C. The dialysate was centrifuged at 13000 rpm for 30 minutes in a Beckman JA20 rotor at 4^0C, and the supernatant stored at -80^0C in aliquots of 50 µl.

7.2.11 Preparation of medium for determining the presence of secreted factors in medium

To determine if secreted factors are released into the media, media exposed to WI38 fibroblasts was used. WI38 fibroblasts were used since these are the fibroblasts used in the preparation of the fibroblast-derived ECM. WI38 fibroblasts were grown in DMEM medium containing 10 % fetal calf serum supplemented with 100 µg/ml penicillin and 100 units /ml streptomycin. After 2 days of incubation, the fibroblasts were rinsed with PBS and fresh media was added. After 3 days of incubation the medium was harvested and centrifuged at 5 000 rpm for 1 hr at 4 ^{0}C remove any cellular debris. In one set of experiments the media was used just after harvesting and in another the

media was treated as follows: The medium was then dialysed against distilled water in dialysis tubing which had been boiled in 1mM EDTA and water. The tubing was then allowed to cool to 4^0C. Dialysis was done overnight at 4^0C. Two separate experiments were done. The medium was used either as unlyophilised, mixed with fresh DMEM or was lyophilised and reconstituted in $1/10^{th}$ the original volume of sterile water. Before use both unlyophilised and lyophilised media were filter sterilised through 0.2 µm Millipore filters. For experiments half of the unlyophilised medium was mixed with half fresh DMEM media and then added to plated fibroblasts. In the same manner half of the reconstituted lyophilised media was mixed with fresh DMEM media and added to fibroblasts. After incubation, RNA and proteins were harvested for real time RT PCR and western blot analysis as described elsewhere.

7.3 Protein Analysis

7.3.1 Isolation of secreted type I collagen in media

Cells (1.5 x 10^6) were seeded in 100 mm culture dishes with or without matrices in DMEM containing 10 % fetal calf serum (FCS) for 24 hrs before being changed to serum-free DMEM media containing ascorbic acid (50 µg/ ml) and 50 µg/ml β-aminopropionitrile for 48 hrs. The media was harvested and total proteins in the medium were quantified using the Bradford protein determination assay (Biorad) using bovine serum albumin as a standard. The samples were incubated overnight with pepsin (100 µg/ml, pH 2.5, 4^0C) to cleave all proteins present in the sample except type I collagen. Subsequently the proteins were TCA precipitated (10 % TCA) and the samples resuspended in SDS loading buffer and electrophoresed on a 7.5 % polyacrylamide gel. Gels were fixed, stained with Coomassie Brilliant Blue and destained. Cells were counted before and after the experimental period to exclude the possibility that any change in type I collagen levels may have resulted from a change in cell numbers.

7.3.2 Western Blot Analysis

7.3.2.1 Harvesting proteins

Cells (1.5 x 10^6) were seeded in 100 mm culture dishes with or without matrices in DMEM containing 10 % fetal calf serum (FCS) for 24 hrs before

addition of fresh media and incubation continued for 48 hrs. At the end of each experiment cells were washed with cold PBS and treated as follows: cells cultured on plastic were scraped with a rubber scraper and lysed using lysis buffer for 30 mins on ice; cells cultured on matrices were liberated from the matrices by trypsinisation and washed with cold PBS, centrifuged at low speed (1000 rpm) and lysed with lysis buffer for 30 mins on ice. The lysis buffer used contained 10 mM Tris-HCl pH 7.6, 10 mM NaCl, 3 mM $MgCl_2$ and 1 % (v/v) Nonidet P-40 with pepstatin, leupeptin and aprotinin. The cell lysates were sonicated for 10 seconds on ice (Heat System-Ultrasonics, Inc, Plainview, New York), centrifuged at 10 000 rpm for 10 mins at 4^0C and the clear supernatant was used in subsequent experiments. Total proteins in the cell lysates were quantified as described in section 7.3.1 above.

7.3.2.2 SDS-Polyacrylamide gel electrophoresis

7.3.2.2.1 Preparation and electrophoresis of gels

Glass plates were cleaned with distilled water and 70 % ethanol. The plates were assembled, ensuring the plates are even at the bottom. The plates were locked into the casting frame and clipped into the stand. The resolving and the stacking gels were prepared in 10 ml Falcon tubes and 10 % APS and TEMED were added when ready to pour (see Table 7.1). Separate gels were poured into a Biorad Miniprotean system and their compositions are shown in Table 7.1. Once the resolving gel has been poured either water or saturated butanol was added on top to remove air bubbles and to obtain an evenly level interface with the stacking gel. After the resolving gel has set, the water or the butanol was decanted and the stacking gel was added on top. Combs were inserted and once the gel had polymerised, the combs are removed. The wells are then rinsed with 1 x electrophoresis buffer. Equal amounts of total protein lysates were mixed with the loading buffer and water up to the same volume. The samples (20-50 µg/lane) were heated for 5 minutes at 95^0C before loading and the gel was run at 150 V for 70 minutes to separate the proteins.

Table 7.1 Different concentrations of SDS-PAGE used in the study

	Stacking gel	Resolving gels		
	4 %	12%	10%	7.5%
Distilled water	3.65 ml	2.15	2.72	3.70
Buffer	0.625	3.75	3.7	3.75
30 % acrylamide	0.650	4	3.3	2.5
10 % SDS	50 ul	50	50	50
10 % APS	25 ul	50	50	50
TEMED	10 ul	10	10	10

N.B Add the 10 % APS and TEMED last and when ready to pour as polymerisation will be fast.

7.3.2.2.2 Transfer of proteins to nitrocellulose membranes

Transfer of proteins to Hybond C nitrocellulose membranes was done using the Biorad minigel transfer system. Transfer buffer (1x) was prepared and cooled to 4^{o}C. Nitrocellulose membrane and Whatman 3M filter paper were cut to the size of the gel, about 6 cm x 7 cm, and soaked in transfer buffer for 15 minutes. A sandwich of black side of cassette, sponge, filter paper, gel, nitrocellulose membrane, filter paper and sponge was prepared and placed in the transfer apparatus. Transfer was carried out at 100 V for 1.5 hours at 4^{0}C. After proteins were transferred to a membrane, the protein side of blot was marked. The membrane was rinsed twice with TBS containing 0.1% Tween 20 (TBS-T). In order to check if equal loading was done, the gel was placed in fixing solution for 1 hr, immersed in Coomassie blue soln and shaken for 1hr. The gel was destained until the bands were visible and then dried on the gel drier for about an hour at 75 ^{0}C.

7.3.2.2.3 Immunoblotting

The membrane was blocked with 5 % fat-free milk in TBS-T. The membranes were then incubated overnight at 4^{0}C with diluted primary antibodies, most of which are shown in Table 7.2 below. After two washes in TBS-T buffer, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hr at room temperature. After two TBS-T washes,

detection was done using the Lumiglo substrate (KPL, USA), and the blot was exposed to photographic film.

Table 7.2 Primary and secondary antibodies used for western blot analysis

Ag	Blocking Soln	Primary antibodies	Secondary Antibodies
Type I collagen	5% milk	Southern Biotech 1:1000 dilution in 5 % milk	Donkey anti-goat (Santa Cruz) 1:1000 dilution in 5% milk
phospho-ERK 1,2	5% milk + 3 % BSA	Cell Signaling 1:500 dilution in 1 % BSA	Goat anti-mouse (Pierce) 1:1000 dilution in 1% BSA
ERK 2	5% milk	Santa Cruz 1:1000 dilution in 5 % milk	Goat anti-rabbit (Pierce) 1:1000 dilution in 5% milk
β- Tubulin	5% milk	Santa Cruz 1:1000 dilution in 5 % milk	Goat anti-rabbit (Pierce) 1:1000 dilution in 5 % milk
Fibronectin	5% milk	Calbiochem 1:1000 dilution in 5 % milk	Goat anti-rabbit (Pierce) 1:2000 dilution in 5% milk
Laminin α2	5% milk+ 3 % BSA	Santa Cruz 1:1000 dilution in 5 % milk + 3 % BSA	Goat anti-mouse (Pierce) 1:1000 dilution in 5% milk
MT1 MMP (MMP 14)	5% milk	Santa Cruz 1:1000 in 5 % milk	Goat anti-rabbit (Pierce) 1:3000 dilution in 5% milk
p-p38	5% milk	Santa Cruz 1:1000 dilution in 5 % milk + 1 % BSA	Goat anti-rabbit (Pierce) 1:1000 dilution in 5% milk
Sp1	5% milk	Santa Cruz 1:500 dilution in 5 % milk	Goat anti-rabbit (Pierce) 1:1000 dilution in 5% milk

7.3.2.2.4 Stripping a blot

For subsequent analysis of the same blot with a second antibody, the blot was immersed in 1M glycine, pH 2.5 for 10 minutes at room temperature with

shaking and then neutralised by the addition of 1/10th volume of 1M Tris, pH 7.5 for 5 minutes. The blot was then washed twice in TBS-T, followed by blocking as described above.

7.4 Treatment with kinase inhibitors

For the analysis of signaling pathways, fibroblasts were seeded in 35 mm or 100 mm dishes with or without matrices. Fibroblasts were treated with the indicated doses of U0126 or PD98059 (MEK inhibitors) for 24 hrs in DMEM (Ihn *et al*, 2002; Ihn and Tamaki, 2000; Ihn and Tamaki, 2002). Fibroblasts were also treated with the indicated doses of p38 MAPK inhibitor, SB203580, the JNK inhibitor, SP600125, and the PI3-K inhibitor, wortmanin. Dose-dependent studies were done to determine the concentrations of inhibitors required for the inhibition of the different signaling pathways. Cells were also treated with 0.1 % DMSO alone, as the solvent control. The levels of type I collagen in cell lysates and in the media, mRNA and promoter activity were determined as described above after treatment with the various inhibitors.

7.5 Blocking of α2β1 and α3β1 integrins functions in fibroblasts

Treatment of fibroblasts treatment with integrin-blocking antibodies was perfomed according to a published protocol with minor changes (Langholz *et al*, 1995). Briefly cells cultured for 24 hrs were trypsinised, washed in PBS and resuspended in fresh DMEM. Equal number of cells were incubated with either 10 µg/ml or 20 µg/ml of the well characterised blocking monoclonal mouse anti-human integrin α2β1 antibodies (MAB1998, clone BHA2.1, Chemicon, USA; P1E6, Lot No: 0083a, Dako Corporation (Carpinteria, CA, USA), to human integrin α3β1(P1B5, Lot No: 0083b, Dako Corporation, Carpinteria, CA, USA), or with PBS (control) for 30 minutes at 37^{0}C. Cells incubated with non-specific mouse IgG showed similar results to cells incubated in PBS, the latter were therefore used as controls throughout. Integrin-blocked fibroblasts were seeded on control dishes or on matrices and the experiments were stopped after 24 hrs for mRNA and protein analysis.

7.6 Sp1 siRNA transfection

To study the influence of Sp1 gene expression on matrix-mediated downregulation of type I collagen gene expression, a direct loss of function approach of silencing the endogenous Sp1 by RNAi was used (Lee *et al*, 2006; Tatenhorst *et al*, 2006). 5 x 10^5 fibroblasts were seeded in 35 mm culture dishes with or without the fibroblast-derived ECM a day before transfection. The transfection of siRNA was carried out using Transfectin (BioRad) according to the manufacturer's instructions. Transfectin (6 µl) was mixed with 4 µl (0.5 µg) of 10 µM siRNA double strands and incubated 20 mins at room temperature. The siRNA mixtures were added to the cells dropwise and incubated for 6 hrs. After 6 hrs the medium was changed and transfected cells were grown for 48 hrs at 37^0C in 5 % CO_2. After incubation, the proteins and RNA were harvested for western blot and real time RT PCR analysis respectively. The siRNA used was the small interfering RNA (siRNA; Santa Cruz) targeting endogenous human Sp1. A negative control siRNA molecule of identical size was used in all experiments, consists of a non-targeting 20-25 nucleotide siRNA. The negative control is a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA.

7.7 Cell Microscopy

Cultured cells were viewed with an inverted microscope (Carl Zeiss Microimaging, Inc., Germany) equipped with phase contrast optics and photographed using a SPOT digital microscope camera and corresponding software, SPOT advanced , version 3.5.6 for Windows (Diagnostic Instruments Incorporated, Michigan, USA).

7.8 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 3.02. All data were expressed as means +-SEM. Paired Student's t test was used to evaluate the statistically differences between control fibroblasts (no matrix) and fibroblasts seeded on matrix. Values of $P < 0.05$ were considered significant. All experiments were repeated at least three times.

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Appendix A: Buffers and Solutions

Acrylamide/Bisacrylamide Solution (30%) (29:1)

29 g Acrylamide

1 g Bisacrylamide

Made to 100 ml with distilled water and stored in dark at 4 ^{0}C

Acrylamide Stock Solution (40%) (38:2)

38 g Acrylamide

2 g Bisacrylamide

Made to 100 ml with distilled water and stored in dark at 4 ^{0}C

7.5 M Ammonium acetate, pH 7.4

Dissolve 57.81 g ammonium acetate in 60 ml of distilled water, adjust pH to 7.4 with ammonia solution, make up to 100 ml with distilled water and sterilise by filtration using a filter of 0.45 µm pore size

2 M Ammonium acetate, pH 7.4

Dissolve 15.416 g ammonium acetate in 60 ml of distilled water, adjust pH to 7.4 with ammonia solution, make up to 100 ml with distilled water and sterilise by filtration using a filter of 0.45 µm pore size

10 % Ammonium persulfate (APS)

Dissolve 100 mg of APS in 1 ml of distilled water, store at 4^{0}C

Ampicillin Stock Soln (10 mg/ml)

Dissolve 500 mg Ampicillin in 50 ml of distilled water. Sterilise by filtration through a filter of pore size 0.22 µm and store at -80 ^{0}C. Dilute to a working solution of 50 µg/ml.

Aprotinin (10 mg/ml)

Dissolve 100 mg aprotinin in 100 ml of autoclaved distilled water. Aliquot the solution and store at -20 ^{0}C.

0.1 M $CaCl_2$

Dissolve 2.94 g of $CaCl_2$ in 200 ml of distilled water and autoclave.

Coomassie blue staining solution

0.5 g Coomassie BB

500 ml methanol

100 ml acetic acid

400 ml distilled water

DEPC-Treated water (0.01 %)

Add 10 µl of DEPC to distilled water (100 ml) and stir at room temperature. Leave overnight and then autoclave

Destaining Solution (10 % Acetic acid (v/v))

100 ml acetic acid

100 ml methanol

800 ml distilled water

1 M Dithiothreitol (DTT)

Add 3.09 g DTT to 20 ml of 10 mM sodium acetate (pH 5.2) and mix. Filter sterilise using a 0.22 µm pore size filter. Store aliquots at -20 ^{0}C.

0.5 M EDTA, pH 8.0

Add 37.22 g of $Na_2EDTA.2H_2O$ to 140 ml of distilled water. Adjust the pH to 8.0 with 10 N NaOH then add distilled water to a final volume of 200 ml. Autoclave and store at room temperature.

0.5 M EDTA (DEPC-treated), pH 8.0

Add 37.22 g of $Na_2EDTA.2H_2O$ to 140 ml of distilled water.. Adjust the pH to 8.0 with 10 N NaOH then add DEPC-treated water to a final volume of 200 ml. Autoclave and store at room temperature

DNA Elution Buffer

0.5 mM ammonium Acetate

10 mM Magnesium Acetate

1 mM EDTA

70 % Ethanol

70 ml of absolute ethanol

Add 30 ml of distilled water

75 % DEPC- Ethanol

75 ml absolute ethanol

25 ml DEPC-treated water

Fixing Solution

100 ml acetic acid

500 ml methanol

400 ml distilled water

Freezing down media

70 % Fetal bovine serum

20 % DMEM media

10 % Dimethylsulphoxide (DMSO)

Store at 4 ^{0}C

Glycerol Storage Buffer

50 mM Tris-HCl (pH 8.3)

40% Glycerol

5 mM $MgCl_2$

0.1 mM EDTA

10 x HBS for transfection (per 100 ml of distilled water)

5.0 g Hepes

8.0 g NaCl

0.37 g KCl

1.0 g Dextrose

0.103 g Na_2HPO_4 (anhydrous -1.45 mM)

HSB Buffer for nuclear extraction

0.5 M NaCl

50 mM $MgCl_2$

2 mM $CaCl_2$

10 mM Tris-HCl (pH 7.4)

250 mM KCl

Dissolve 1.86 g KCl in distilled water up to 100 ml. Autoclave

L-agar (per litre**)**

10 g Tryptone

5 g Yeast extract

5 g NaCl

15 g Bacto-agar. Autoclave

L-Broth (per litre**)**

10 g Tryptone

5 g Yeast Extract

5 g NaCl

Add distilled water to 1L and adjust pH to 7.0 with 10 N NaOH. Autoclave

LB plates (with Ampicillin)

Weigh 15 g agar and add LB medium to 1L. Autoclave and allow to cool to about 40 ^{0}C then add Ampicillin to a final concentration of 100 µg/ml. Pour into 100 mm dishes and let the agar harden. Store at 4 ^{0}C

10 X Loading Buffer

0.25 % bromophenol blue in 40 % glycerol (v/v)

Lysogen Extract Buffer

50 mM Tris- HCl (pH 7.5)
1 mM EDTA
1 mM DTT
1 mM PMSF

2 M $MgCl_2$

Dissolve 40.66 g $MgCl_2$. $6H_2O$ in 80 ml of distilled water and then make up to 100 ml and autoclave.

10 x MOPS Buffer

0.4 M MOPS, pH 7.0
0.1 M Sodium acetate
0.01 M EDTA , pH 8.0
Dissolve 20.6 g MOPS in 400 ml 50 mM sodium acetate, adjust pH to 7.0 with 10 N NaOH , add 10 ml 0.5 M EDTA , make up to 500 ml with autoclaved DEPC-treated water

1.5 M NaCl

Dissolve 8.75g NaCl in 80 ml of distilled water, make up to 100 ml.

2,5 % non-fat milk blocking solution

Dissolve 1.25 g non-fat milk powder in 30 ml TBS-T, make up to 50 ml with TBS-T

5 % non-fat milk blocking solution

Dissolve 2.5 g non-fat milk powder in 30 ml TBS-T, make up to 50 ml with TBS-T

PBS (pH 7.4)

137 mM NaCl
2.7 mM KCl
4.3 mM $Na_2HPO_4.2H_2O$, 1.4 mM KH_2 PO_4
Made to 1 litre with distilled water. Autoclave

1 mg/ml Pepstatin A

Dissolve Pepstatin A in absolute ethanol to a final concentration of 1 mg/ml and freeze aliquots at -20^{0}C.

Phenol: Chloroform: Isoamyl alcohol (25:24:1)

Mix one part of phenol and one part of chloroform: isoamyl alcohol (24:1)

100 mM PMSF

Dissolve PMSF in absolute ethanol to a final concentration of 100 mM and freeze aliquots at -20^{0}C.

5 x Protein loading buffer

Dissolve 1.75 g Tris in 10 ml distilled water and add 30 ml glycerol. Adjust the pH to 6.8 with concentrated HCl and add 5g SDS, make up to 50 ml with distilled water.

5 x Protein loading dye

200 µl 5 x protein loading buffer

50 µl of β-mercaptoethanol, 10 µl of bromophenol blue

2 x Reaction Buffer without nucleotides

10 mM Tris-HCl (pH 8.0)

5 mM $MgCl_2$

0.3 M KCl

RIPA Buffer

200 µl RIPA solution

2 µl of 2 mg/ml pepstatin

2 µl of 10 mg/ml aprotinin

2 µl of 100 µM PMSF

RIPA Solution

150 mM NaCl

1 % Triton X-100

0.1 % SDS

25 mM Tris, pH 7.5

1 % sodium deoxycholate

RNA Loading Buffer (25 ml)

1 mM EDTA, pH 8.0

0.25 % Bromophenol Blue (w/v)

0.25% Xylene Cyanol

50% Glycerol (v/v)

50 µl DEPC

Add distilled water to a final volume of 25 ml, Stir overnight and autoclave

RNA Running Buffer (10 x)

200 mM Morpholinopropanesulfonic acid (MOPS) (pH 7.0)

50 mM Sodium Acetate

10 mM EDTA

RNA Sample Buffer

10 ml deionised formamide

3.5 ml 37 % formaldehyde

1.0 ml 10 x MOPS running buffer

Prepare in nuclease-free water, use high grade glycerol to avoid ribonuclease activity. Dispense into 200 µl aliquots and store at -20^{0}C.

Screening Binding Buffer (10 x)

200 mM Hepes

30 mM $MgCl_2$

400 mM KCl

10 mM DTT

10 % SDS

Dissolve 10 g sodium dodecyl sulphate in 80 ml distilled water (heat to 80^{0}C) , make up to 100 ml with distilled water

10 × Running buffer

29 g Tris

144 g Glycine

10 g SDS

Made to 1 Litre with distilled water.

SDS-PAGE Running Buffer (5 x)

0.25 M Tris-HCl (pH 8.3)

1.92 M Glycine

0.5 % SDS

SDS/Tris Buffer

5% SDS (w/v)

0.5 M Tris-HCl (pH 7.4)

0.125 M EDTA

Solution 1 (Rapid plasmid prep)

25 mM Tris-HCl (pH 8.0)

10 mM EDTA

50 mM Glucose

Solution 2 (Rapid plasmid prep)

200 mM NaOH

1% SDS

Solution 3 (Rapid plasmid prep)

3 M Potassium Acetate (pH 4.8)

Stop Buffer

0.05% Bromophenol Blue

0.5% SDS

1 mM EDTA, 50% Glycerol (v/v)

Stripping Solution

1.4 ml β-mercaptoethanol

40 ml 10 % SDS

12.5 ml Tris-HCl, pH 6.7

Make up to 200 ml with distilled water

Alternate stripping solution

1 M Glycine pH 2.5

T4 Polynucleotide kinase buffer (10 x)

500 mM Tris-HCl (pH 7.6)

100 mM $MgCl_2$

50 mM DTT

1 mM Spermidine

1 mM EDTA (pH 8.0)

TAE Electrophoresis Buffer (1 x)

40 mM Tris

5 mM Sodium Acetate

1 mM EDTA

Adjust pH to 7.8 with glacial acetic acid

TAE Electrophoresis Buffer (1 x) (pH 8.3)

90 mM Tris

90 mM Boric Acid, 2.5 mM EDTA. Adjust pH to 8.3.

TBS-Tween

50 ml 1M Tris base

30 ml 5M NaCl

250 µl Tween-20

Make up to 1litre with distilled water

TE buffer (10x) (pH 8.0)

100 mM Tris-Cl

10 mM EDTA

Adjust pH to 8.0

TES Solution

10 mM N-tris (hydroxymethyl-2-aminoethanesulforic acid (TES) pH 7.4

10 mM EDTA

0.2% SDS

TES/NaCl Solution

10 mM TES (pH 7.4)

10 mM EDTA

0.2% SDS

0.6 M NaCl

Top agar (per litre)

10 g Tryptone

5 g Yeast extract

5 g NaCl

7 g Bacto-agar

10×Transfer buffer

144 g glycine

38 g Tris

Make up to 1 litre with distilled water.

Tris Buffered Saline (TBS)

25 mM Tris (pH 7.5)

0.9% NaCl (w/v)

TTE (transfections)

1 mM Tris-HCl (pH 8.0)

0.025 mM EDTA

1M Tris pH 6.8 (per litre)

Add 121 g Tris base to 800 ml of distilled water

Adjust pH with concentrated HCl to pH 6.8 and then add distilled water up to one litre. Autoclave

1M Tris pH 7.5 (per litre)

Add 121 g Tris base to 800 ml of distilled water

Adjust pH with concentrated HCl to pH 7.5 and add distilled water up to one litre. Autoclave

1M Tris pH 8.8 (per litre)

Add 121 g Tris base to 800 ml of distilled water

Adjust pH with concentrated HCl to pH 8.8 and then add distilled water up to one litre. Autoclave

10 x Transfer Buffer (per litre)

25 mM Tris

192 mM Glycine

20 % methanol

Add 29.0 g of Tris and 144 g of Glycine to 800 ml of distilled water.

Mix properly and add distilled water to 1 litre. When preparing 1 x transfer buffer add methanol to a final concentration of 20 % (v/v).

TBS-T (per litre)

50 mM Tris pH 7.5

150 mM NaCl

0.05 % Tween-20

1 X Trypsin-EDTA solution

0.05% (w/v) trypsin, 0.53 mM EDTA

Prepare in a calcium and magnesium-free 1 x PBS

Water-saturated phenol

Dissolve 100 g phenol crystals in 100 ml of autoclaved DEPC-treated water at 60 to 65ºC, let stand in the fumehood overnight, aspirate the upper water phase and store for up to 1 month at 4ºC.