PART A

NOVEL ASPECTS OF RENAL TUBULOINTERSTITIAL FIBROSIS

by

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DECLARATION

I declare that except where due acknowledgement has been made, the work described within is mine. This work has not been submitted previously for any other academic award and has been carried out after the official commencement date. Any person that has contributed to the editing of this thesis has been accordingly acknowledged. This thesis is in total less than 90,000 words.

Catherine Winbanks

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PUBLICATIONS

Original Articles

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Role of the phosphatidylinositol 3-kinase and mTOR pathways in the regulation of renal fibroblast function and differentiation
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Manuscripts in preparation

Winbanks C, Kelynack KJ, Grimwood L, Darby, IA, Hewitson TD, Becker GJ
Explanting is a useful *ex vivo* model of renal epithelial-mesenchymal transition

Winbanks C, Grimwood L, Darby, IA, Hewitson TD, Becker GJ
Constituents of the coagulation cascade are spatially and functionally related to experimental tubulointerstitial fibrogenesis

Conference Presentations

Winbanks C, Hewitson TD, Kelynack KJ, Grimwood L, Becker GJ
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Winbanks C, Hewitson TD, Grimwood L, Gasser A, Darby IA, Becker GJ
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Winbanks C, Hewitson TD, Grimwood L, Gasser A, Darby IA, Becker GJ
PI3K and mTOR regulate rat renal fibroblast function and differentiation
Australian and New Zealand Society of Nephrology, 2006

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Renal explanting provides a useful *ex vivo* model of epithelial-mesenchymal transition
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Constituents of the coagulation cascade are spatially and functionally related to experimental tubulointerstitial fibrogenesis
American Society of Nephrology, San Diego, 2006

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[2,3-^{3}H Pro]-Pro ^{32}P	Deoxycytidine-5'-[α^{32}P]-triphosphate
4E-BP1	4E binding protein-1
αSMA	Alpha smooth muscle actin
ABC	Avidin-biotin complex
abl	Abelson non-receptor tyrosine kinase
AGEs	Advanced glycation end products
Akt	Acute transforming retrovirus thymoma
AMPK	Adenosine monophosphate-activated kinase
APAAP	Alkaline phosphatase- anti alkaline phosphatase
Apx.	Appendix
βigH3	Transforming growth factor beta inducible gene H3
bFGF	(Basic) Fibroblast growth factor
bME	2-Mercaptoethanol
BMP-7	Bone morphogenic protein-7
BSL	Bandeiraea Simplicifolia
$CaCl_2$	Calcium chloride
CPM	Counts per minute
CTGF	Connective tissue growth factor
DAB	3,3'-diaminobenzidine
dH_2O	Distilled, de-ionised water
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
dNTP	Deoxyribonucleotide triphosphate
DPM	Disintegration per min
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylene-diamine-tetra-acetic acid
eEF2K	Eukaryotic elongation factor 2 kinase
EGF	Epidermal growth factor
eIF4E	Eukaryotic initiation factor 4E
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal regulated kinase

ESRD	End stage renal disease
ESRF	End stage renal failure
FA	Fractional area
FCS	Fetal calf serum
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FKBP12	FK506 binding protein-12
FKHR	Forkhead homologue of rhabdomyosarcomma
FRB	FKBP12 rapamycin binding (domain)
FSP-1	Fibroblast specific protein-1
g	Gram
G-protein	GTP-binding protein
GPCR	G-protein coupled receptor
GSK-3	Glycogen synthase kinase-3
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HEAT	Huntingtin, EF3, A subunit of PP2A, TOR1
HEPES	N-2-hydroxethypiperazine-N'-2-ethansulfonic acid
HGF	Hepatocyte growth factor
hr	Hours
HRP	Horse radish peroxidase
IgAN	IgA nephropathy
IGF	Insulin-like growth factor
ILK	Integrin linked kinase
JAKs	Janus family of tyrosine kinases
LEF	Lymphoid enhancer-binding factor
Kg	kilogram
L	Litre
LY	LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one)
µg/ml	Microgram per millilitre
µl	Microlitre
M	Molar
MAPK	Mitogen activated pathway kinase
MCP-1	Monocyte chemoattractant protein-1

MEK	MAPK-ERK kinase
MeOH	Methanol
Min	minutes
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Millilitre
MMP	Matrix metalloproteinase
MOPS	4-morpholine-propane-sulphonic acid
mSin	mammalian stress activated protein kinase interacting protein
mTOR	Mammalian target of TOR
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
MTT	3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
nM	Nanomole
O/N	Overnight
p-	Phosphorylated
P	Passage
PAR	Proteinase activated receptor
PAI-1	Plasminogen activator inhibitor-1
PBS	1x phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD	PD98059
PDGF	Platelet derived growth factor
PDK1	Phosphoinositide-dependent kinase 1
PDK2	Phosphoinositide-dependent kinase 2
pg	picogram
PH	Pleckstrin homology
Pha-E	Phaseolus vulgaris erythroagglutinin
Pha-L	Phaseolus vulgaris leukoagglutinin
PI3K	Phosphatidylinositol 3-kinase
PIKK	PI kinase related-kinase
PIP_2	Phosphatidylinositol-(4,5)-biphosphate

PIP_3	Phosphatidylinositol-(3,4,5)-triphosphate
PMSF	Phenylmethylsulfonyl fluoride
PNA	Arachis Hypogaca
PTEN	Phosphatase and tensin homologue
PVP	Polyvinylpyrolidone
RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted
RAPTOR	Regulatory associated protein of TOR
RECA	Rat endothelial cell antigen
REDD1	Regulated in development and DNA damage
Rheb	Rapamycin homologue enriched in brain
RICTOR	Rapamycin insensitive companion of mTOR
rpm	Revolutions per minute
RTK	Receptor tyrosine kinase
S-	Serine
S6K1	S6 kinase1
S6RP	S6 ribosomal protein
SD	Standard deviation
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
Smad	SMA mothers against decapentaplegic
ssDNA	Sheared salmon sperm DNA
Stat	Signal transducer and activator of transcription
5´TOP	5´ terminal oligopyrimidine tract
T-	Threonine
TβR I	Transforming growth factor receptor I
TβR II	Transforming growth factor receptor II
TBST	Tris buffered solution-Tween-20
TCA	Trichloroacetic acid
TEMED	Tetraethylmethalenediamide
TGFβ1	Transforming growth factor beta-1
TIMP	Tissue inhibitor metalloproteinase
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor alpha
TOS	TOR signalling

tPA	Tissue plasminogen activator
TRAP	Thrombin receptor agonist peptide
TRIS	Tris-hydroxy-methyl-aminomethane
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP nick end labelling assay
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
UUO	Unilateral ureteral obstruction
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
ZO-1	Zona occludens-1

ABSTRACT

Regardless of aetiology, the accumulation of scar tissue is the final common pathway of progressive kidney disease. This so-called fibrosis or sclerosis manifests itself in three forms: glomerulosclerosis, vascular sclerosis and tubulointerstitial fibrosis, affecting the glomerulus, vessels and tubules/interstitium respectively. Morphological studies have repeatedly shown that tubulointerstitial fibrosis is the key histological predictor of the progression of declining renal function. However, despite its significance there are currently no treatments available to abrogate this process. Those that suffer from the progressive accumulation of scar tissue therefore eventually succumb to renal failure.

Tubulointerstitial fibrosis is largely mediated by fibroblasts and myofibroblasts present in the interstitium. Whilst quiescent and sparse in the normal interstitium of the kidney, in response to injury fibroblasts become activated by a complex array of inflammatory and pro-fibrotic mediators. The subsequent differentiation of these cells into myofibroblasts is a histological hallmark of fibrosis. Myofibroblasts share phenotypic properties with both smooth muscle cells and fibroblasts and express the phenotypic marker αSMA. These cells are characterised as the key synthesisers of extracellular matrix and their presence ultimately leads to the relentless accumulation of extracellular matrix proteins and loss of renal function.

The pathological entities leading to fibrosis inextricably depend on complex signalling pathways that dictate the cell response by integrating a number of extracellular and intracellular cues. Our current understanding of the mechanisms of tubulointerstitial fibrosis have largely focussed on the TGFβ signalling axis, primarily because this pathway is a central mediator in the regulation of an enormous number of pro-fibrotic events such as chemotaxis, regulation of the extracellular matrix, the autocrine activation of various growth factors and in the recently characterised phenomena of EMT. This signalling axis therefore plays a major role in the regulation of interstitial fibroblast proliferation, differentiation and matrix

synthesis. However, as we have come to understand that a large number of factors are capable of influencing fibroblasts (such as mechanical stress, proteases and various growth factors), it can be appreciated that the TGFβ axis is unlikely to mediate all of these responses. This is reflected in the current research, which increasingly highlights the involvement of other signalling pathways in fibrosis.

Many of the well-known growth factors that exert effects on renal fibroblasts (such as FGF, EGF and PDGF) involve the activation of receptor tyrosine kinases. However, beyond activation of these receptors, the signalling events dictating the response of fibroblasts are relatively undefined. It is well known that the serine/threonine kinase PI3K, most well known for its role in cell survival, is capable of mediating the responses of these growth factors. Moreover, mTOR, responsible for integrating stress and amino acids and controlling cell growth through downstream substrates, is increasingly known for its ability to integrate growth factor signals mediated through the upstream PI3K. This is corroborated by a number of studies in VSMC and lung fibroblasts which have highlighted the potential role of PI3K and mTOR in the regulation of key events relevant to fibrosis. This information has served as a basis for *Chapter 3: The role of PI3K and mTOR in the regulation of fibroblast proliferation and collagen synthesis,* and the first part of *Chapter 5: The role of PI3K and mTOR in the regulation of myofibroblast differentiation.* These studies have identified a key role for PI3K and mTOR in the regulation of fibroblast proliferation, differentiation and collagen synthesis.

The work described within has also attempted to more fully elucidate the derivation of myofibroblasts during fibrosis. EMT is a process that is integral to embryogenesis and cancer and is becoming characterised as an important source of myofibroblast derivation. The proteome to engage EMT has been shown to involve a number of cellular cues, the surrounding microenvironment including the extracellular matrix as well as numerous

cytokines, vaso-active peptides and non-cytokine-mediated pathways. In light of this and after encountering problems using an *in vitro* cell line to study EMT, work described within also includes *Chapter 4: Development and validation of an ex vivo model of EMT*. This model aims to better represent the *in vivo* environment and allows for easy tracking of transitioning cells, and overcomes some of the difficulties with *in vivo* experiments. Whilst previous studies have largely focused on the role of TGFβ and Smads in EMT, the signalling circuitry involved to fully engage the EMT proteome is not fully clear. This model has therefore been used to identify novel regulators involved in EMT and was utilised in the second part of *Chapter 5: The role of PI3K and mTOR in EMT*.

Although cytokines and growth factor mediators are thought to be chiefly responsible for mechanisms of tubulointerstitial fibrosis, we now know that non-cytokine mediated pathways such as AGEs and PAI-1 can regulate mechanisms of fibrosis, some of which are independent of traditional cytokine agonists. Serine proteases of the coagulation cascade may also play roles in renal disease in addition to their traditional role in haemostasis. Members of the coagulation cascade primarily carry out cell-mediated effects by acting through specific cell-surface receptors called PARs. A number of coagulation factors have been implicated in the formation of crescents in glomerular diseases however, the role of coagulation in tubulointerstitial fibrosis is less well-known. The work described in *Chapter 6: Constituents of the coagulation cascade are spatially and functionally related to experimental tubulointerstitial fibrosis* has examined temporal and spatial relationships of coagulation factors, their specific cell-surface receptors and myofibroblasts *in vivo* to establish potential roles of non-cytokine-mediated pathways that aid our understanding of mechanisms of fibrosis.

The renal fibroblast plays an indispensable role in tubulointerstitial fibrosis and the aim of this thesis was to examine those facets of renal fibroblast function that are most devastating to renal function and culminate in an expansion of the renal interstitium during fibrosis. This

work hopes to provide useful information to aid the understanding of the multifaceted mechanisms involved in renal tubulointerstitial fibrosis.

Chapter 1

Introduction

1.1 INTRODUCTION

Within Australia, as elsewhere, chronic kidney disease is a major public health problem with enormous social and economic cost to the community. Approximately 60,000 Australians have some degree of renal impairment, with almost 5,000 of these receiving ongoing dialysis for end stage renal failure (ESRF) (Australian Kidney Foundation, 1999). On a global scale, it is estimated that the population with end stage renal disease (ESRD) will exceed 2 million patients by the year 2010 (Lysaght, 2002). Even more worrying is the realisation that the population of patients with kidney failure is increasing at approximately 5% per annum. United States National Health Examination Surveys indicate that this is due in a large part to obesity and diabetes which are associated with an increased risk of developing chronic kidney disease (Fox et al., 2004). Chronic kidney disease is therefore increasingly recognised as an epidemic and has been described as a critical health problem by those at the forefront of this area of research (Eddy and Neilson, 2006; Kiberd, 2006).

For people who currently suffer with ESRF, the only options are dialysis and transplantation. These therapies are forms of renal replacement and do not treat the cause of ESRF. They present themselves with inherent difficulties such as undefined waiting periods, increased mortality and decreased quality of life. Because accurate predictors of those at risk and those with established progressive renal disease are not available, there is an increasing emphasis on fundamental research that can identify new therapeutic targets to combat ESRD (Eddy and Neilson, 2006).

Regardless of aetiology, chronic kidney disease culminates in the accumulation of excess extracellular matrix (ECM), characterised as glomerulosclerosis, tubulointerstitial fibrosis and vascular sclerosis. Unfortunately, the best available therapies, primarily targeting the renin-angiotensin system (RAS), only delay progression by months and ultimately patients succumb to renal failure. It is therefore apparent that despite the underlying significance of

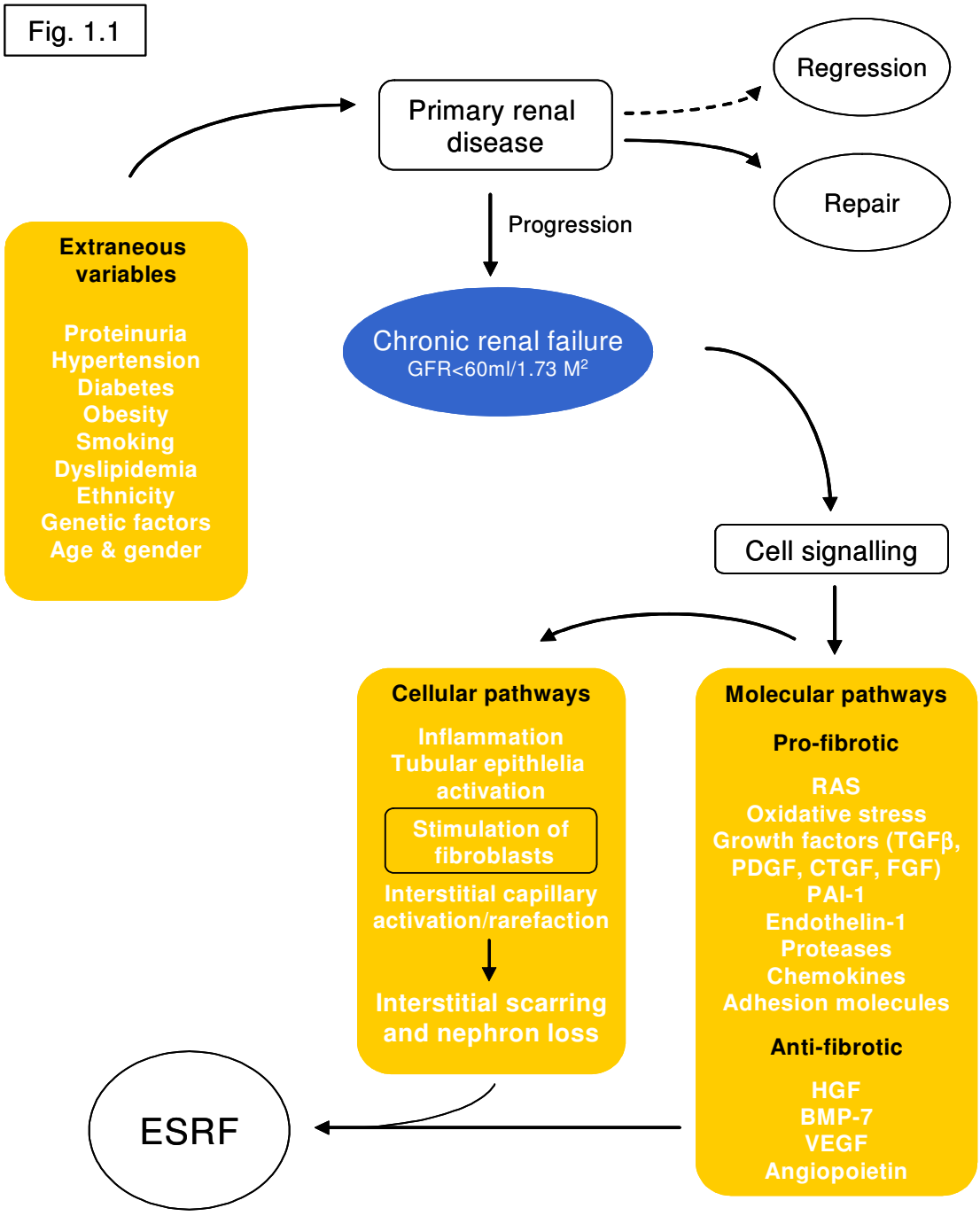
sclerosis and fibrosis, there is no current treatment available to abrogate the progression of renal fibrosis.

In order to formulate viable treatment options, the current focus is on enhancing our understanding of the mechanisms of tubulointerstitial fibrosis at a molecular level. Over recent years, research has identified key regulators that dictate the progression of renal disease. These factors may be extraneous variables, or the molecular and cellular pathways that lead to ESRF *(Fig. 1.1)*. Investigators in this field now strongly recognise the key roles of interstitial fibroblasts in mediating fibrosis. These cells acquire features of smooth muscle cell differentiation in injury (Hewitson and Becker, 1995; Hewitson et al., 1998; Hewitson et al., 1995). Recognised from *de novo* expression of α-smooth muscle actin (αSMA), the so-called myofibroblast is responsible for scarring through its proliferation, increased expression of ECM proteins and contraction of the surrounding matrix (Hewitson et al., 1998).

Although seemingly a simple connective tissue cell, fibroblasts represent highly heterogenous cell populations with complex mechanisms of derivation and function. Although the presence of fibroblasts and their differentiated counterparts is a key indicator of the severity of tubulointerstitial fibrosis, our understanding of this cell during fibrosis lacks the type of detail that is needed to formulate viable therapeutic treatments to ameliorate fibrosis. Central to this, although the transforming growth factor β (TGFβ) signalling axis is a well-characterised mediator of fibroblast function, the complex array of fibroblast regulators in the interstitial milieu highlight the likely involvement of other signal transduction pathways.

Indeed, given that the like of fibroblast growth factor (FGF), epidermal growth factor (EGF) and platelet derived growth factor (PDGF) act through receptor tyrosine kinases (RTK) and play roles in fibroblast regulation, this suggests that fibroblast signalling is not solely dependent on the TGFβ axis. It is well known that the serine/threonine kinase phosphatidylinositol 3-kinase (PI3K) is a key integrator of these growth factors through RTK

Fig. 1.1



Overview of factors involved in inducing and regulating pathways that lead to ESFR. The progression or recovery from renal disease is influenced by a number of extraneous variables including obesity and type II diabetes. Progressive renal disease involves complex cellular and molecular pathways of which activation of the interstitial fibroblast is a key feature. Currently, the most well-established pro-fibrotic molecular pathways in tubulointerstitial fibrosis are TGFβ1 and RAS. Although regression of fibrosis is not well-established in the kidney (dashed line), HGF and BMP-7 amongst others, currently hold promise.

Adapted from Eddy and Neilson, 2006.

and studies in lung fibroblasts and vascular smooth muscle cells (VSMC) highlight the role in this kinase in controlling proliferation, differentiation and ECM synthesis in these cells. Mammalian target of rapamycin (mTOR), most well-known for its role in immunosuppression, is increasingly recognised for its ability to integrate growth factor signals from PI3K by acting as a downstream mediator and is now tagged as a central regulator of cell cycle progression and differentiation. This suggests that these serine/threonine kinases may play roles in the regulation of fibroblast function.

Studies have also highlighted roles of non-cytokine mediated pathways in tubulointerstitial fibrosis that can mediate effects through a GTP-binding (G-protein) protein coupled receptor (GPCR) family called protease activated receptors (PARs). Whilst the roles of proteases of the coagulation cascade are well outlined in glomerular diseases, the role of this cascade in tubulointerstitial fibrosis is less well known. The most insightful information of their significance however can be ascertained from the serine protease thrombin which has been shown to mediate a number of fibrotic events *in vitro*. However, the roles of the coagulation cascade in the interstitium are relatively unknown.

It has become increasingly recognised that myofibroblasts may be derived from a number of sources, including resident fibroblasts, migrating perivascular (adventitial cells), recruitment of circulating progenitor cells, and injured tubular cells through a process of epithelial-mesenchymal transition (EMT). *In vivo* studies have underscored the importance of tubular EMT in renal fibrosis and this has changed the concept that fibroblasts are only locally derived. EMT is a highly complex process and whilst TGFβ is a key regulator of this process and the likes of mitogen-activated protein kinase (MAPK) and Rho have also been implicated in renal EMT, the signalling circuitry involved in engaging the EMT proteome is not well established.

This review therefore primarily focuses on facets of fibroblast function and regulation during fibrosis. Accordingly, it draws focus to the known cellular mechanisms of

tubulointerstitial fibrosis, the roles of the renal fibroblast in fibrosis, the role of the coagulation cascade in tubulointerstitial fibrosis, myofibroblast derivation including EMT, and mechanisms of fibroblast signalling in fibrosis.

1.2 FIBROSIS

Fibrosis can be defined as the deposition of excessive amounts of connective tissue in an organ. Whilst repair and healing through fibrosis constitute an essential response to tissue injury in order to restore normal function, in some cases the inflammatory process becomes prolonged and fibrosis will ensue independently of the initial stimulus. This type of response is maladaptive and can have devastating consequences on the parenchymal tissue of organs including the liver (Desmouliere et al., 2003), lung (Nagler et al., 1996), heart (Lim and Zhu, 2006), and kidney (Strutz and Zeisberg, 2006) ultimately leading to organ failure.

1.3 RENAL TUBULOINTERSTITIAL FIBROSIS

The tubulointerstitium makes up more than 90% of the renal volume and comprises the tubules, renal parenchymal space between the tubules, glomeruli and vessels; the normal interstitium being made up of a loose hydrated matrix of collagens (especially types I and III), proteoglycans, and fluid, in which matrix-producing fibroblasts, macrophages, and dendritic cells are distributed. The interstitial matrix is therefore important for both the structural and the functional integrity of the kidney. Diffusion of oxygen from the peri-tubular capillaries to the tubules and the transfer of solute and water will largely be influenced by the width and the constituents within the interstitium. Any alteration to the interstitial matrix has profound effects on both fluid and electrolyte balance and renal function.

Primarily for this reason, fibrosis of the interstitium is now the key correlate to the decline in renal function, often reflected by reductions in glomerular filtration rate (GFR) to less than $60ml/1.73M^2$ (Kiberd, 2006) (Rodriguez-Iturbe et al., 2005). Furthermore,

tubulointerstitial fibrosis occurs regardless of whether the initial pathology occurs in the glomeruli, tubules or vessels. This was first noted by Spühler and Zollinger in 1953 (Spuhler and Zollinger, 1953) and it is now established that in most patients with chronic renal disease, a decline in renal function correlates with the severity of lesions of the tubular and interstitial compartments of the kidney more closely than with glomerular injury (Bohle et al., 1994; D'Amico, 1999; Nath, 1992; Risdon et al., 1968).

Given the relative significance of the tubulointerstitium, renal tubulointerstitial fibrosis is the final common pathway leading to ESRF and may ultimately proceed independently of the initial stimulus (Kriz and LeHir, 2005). Furthermore, it is a key feature of progressive renal disease regardless of the underlying aetiology. This may include hypertension, glomerulonephritis or vasculitis, diabetes, infection or kidney stones (Chatziantoniou and Dussaule, 2005).

Similar to fibrosis in other solid organs in the body, renal tubulointerstitial fibrosis is characterised by continuous tissue injury, inflammation and ECM deposition. However, unlike fibrosis in the liver (Farci et al., 2004), heart (Diez et al., 2002) and skin (Delanian et al., 2003), the regression of kidney fibrosis is not as well-established. Whilst the process of tubulointerstitial fibrosis involves a number of pathological entities including persistent proteinuria, hyperfiltration, cellular inflammation and the release of cytokines (Eddy and Neilson, 2006), the most significant feature of tubulointerstitial fibrosis and indeed the likely reason that this pathological process is linked to declining renal function is the unyielding expansion of the interstitial volume. During progressive renal disease it is characterized by thickened tubular basement membranes and an expanded interstitial compartment with an increase in collagens I, III, V, VII, laminin (Eddy, 1996). This relentless increase in interstitial volume is a complex process that is regulated by matrix degrading enzymes and their inhibitors, likely to be dictated by the interstitial milieu, and ultimately the expansion of interstitial components encroach the surrounding parenchymal tissue. Tubulointerstitial

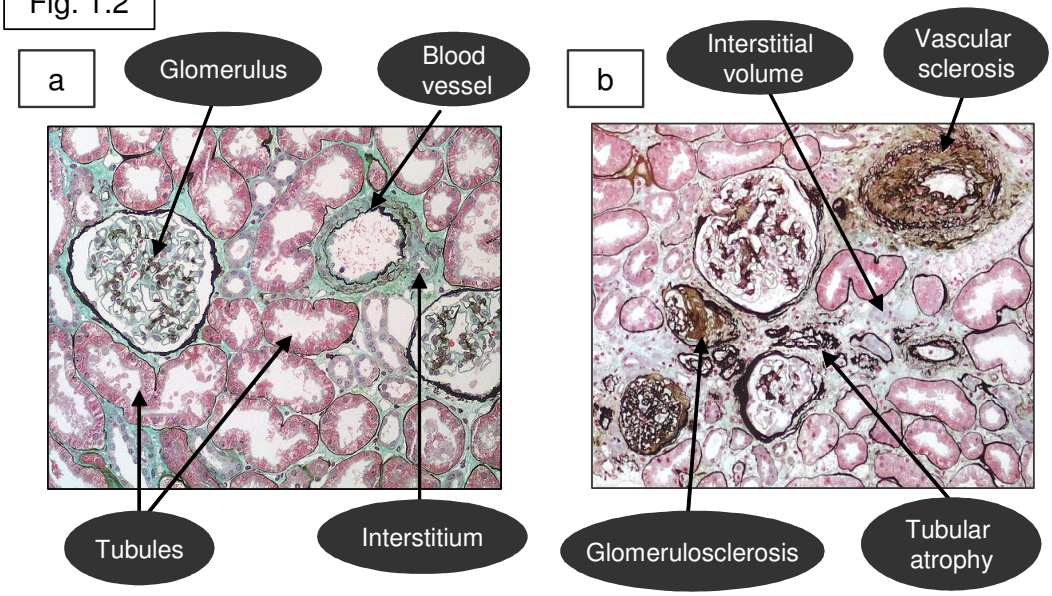
fibrosis can therefore be characterised by glomerulosclerosis, tubular atrophy including obliteration of peri-tubular capillaries, and vascular sclerosis (el Nahas et al., 1997). This facilitates the release of more cytokines, and inflammatory mediators, aggravating the repetitive cycle of tissue injury and ECM deposition, and this will often lead to ESRF *(Fig. 1.2).*

1.3.1 Causes of tubulointerstitial fibrosis

Tubulointerstitial fibrosis can be induced as a result of damage to glomerular structures or can occur due to primary interstitial disease. In humans and animal models, the majority of renal diseases which progress onto renal failure are a result of glomerular-based insults. Consistent with the notion that renal tubulointerstitial fibrosis is the final common pathway of nearly all types of progressive renal disease, it is thought to be induced by a diverse range of pathogenetic stimuli. These include toxins (eg. heavy metals) (Madrenas et al., 1991), drugs (eg. analgesics), crystals (eg. calcium phosphate, uric acid), infections, obstruction (Nagle et al., 1973), lipid deposition, immunologic mechanisms, acute elevations in capillary pressure and by ischemia. Many of these factors activate tubular epithelial cells.

Glomerular disease can also injure the tubulointerstitium through mechanisms that may involve glomerular cytokine release, complement activation (Camussi et al., 1983), effects of proteinuria (Eddy, 1989), ischaemia (Nagaoka et al., 2000), or cross-reactive immunity (Mendrick et al., 1980). Although it is thought that many of these injurious mediators induce interstitial fibrosis through activation of the tubular segments of the nephron, the exact mechanisms are controversial. As outlined by Kriz and LeHir (Kriz and LeHir, 2005), tubular damage may be brought about by mis-directed glomerular ultrafiltrate, present in sclerotic diseases, or by glomerular inflammation. Whilst the former process is thought to lead to obstruction or atrophy, and degeneration of the tubule, in inflammatory diseases crescents form within the glomerulus leading to encroachment of the

Fig. 1.2



Histological comparison of normal kidney structure and ESRF. Masson's Trichrome staining demonstrating (a) normal kidney histology including the renal interstitium, the tubules, vessels and glomeruli, and (b) tubulointerstitial fibrosis as a feature of ESRF, which is characterised by variable degrees of glomerulosclerosis, vascular sclerosis and tubular atrophy.

glomerulotubular junction. Tubular injury may also result from glomerular damage through leakage of protein. Tubular absorption of high concentrations of protein is sufficient to elicit an inflammatory response in a number of animal models (Abbate et al., 1998; Eddy and Giachelli, 1995; Eddy et al., 2000). However, whilst proteinuria has been demonstrated in a number of studies to be a correlate of the degree of interstitial fibrosis (Burton and Harris, 1996; Remuzzi, 1999; Zoja et al., 2003), there is little evidence to suggest the damage is irreversible (Hoffmann et al., 2004; Kriz et al., 2003; Le Hir and Besse-Eschmann, 2003). For an outline of the various causes of tubulointerstitial fibrosis, see *Fig. 1.3.*

1.3.2 Pathogenesis of tubulointerstitial fibrosis

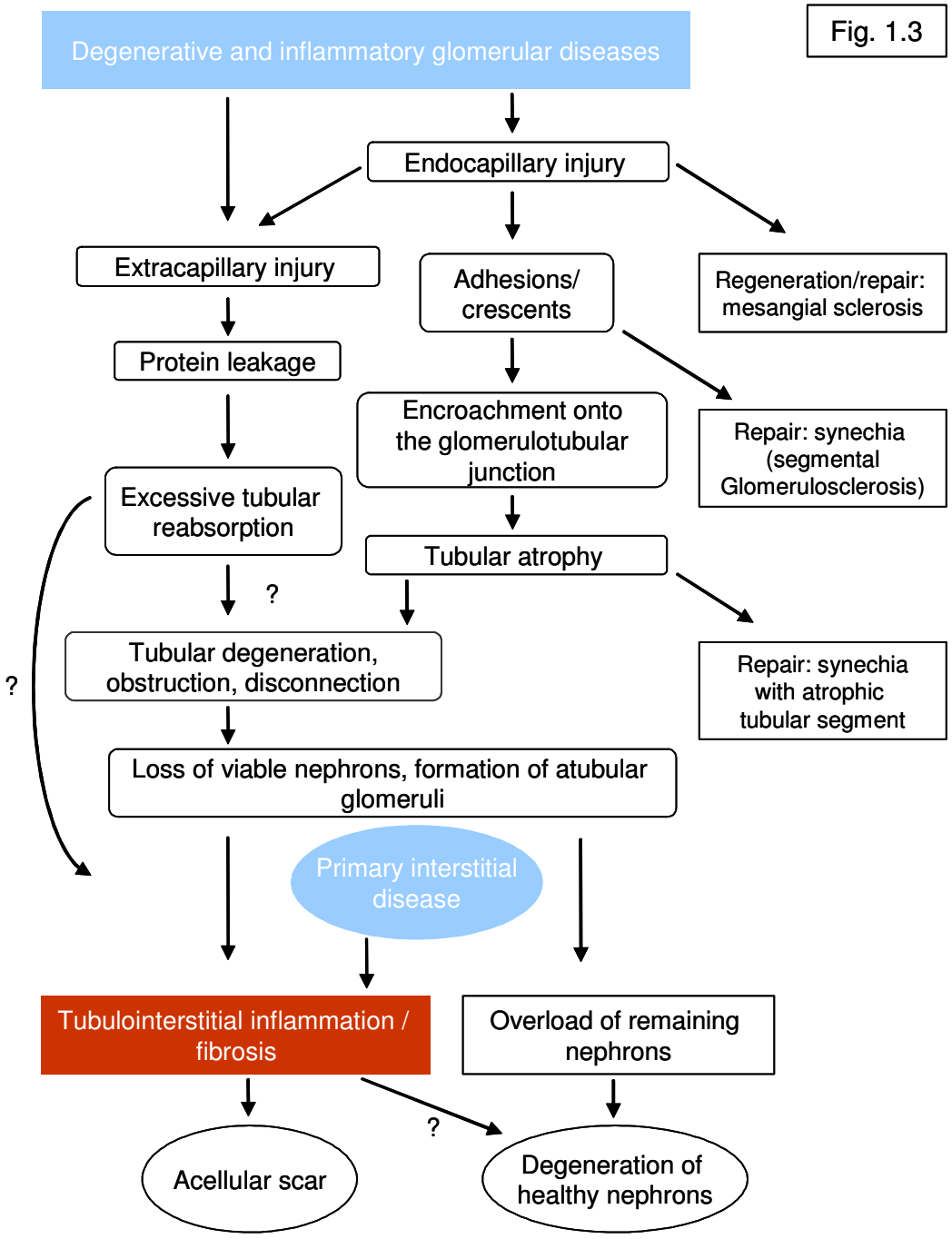
The evolution of fibrosis involves several discrete but overlapping events: inflammation, fibrogenesis, remodelling and resolution.

1.3.2.1 Inflammation

1.3.2.1.1 Chemical signals in tubulointerstitial fibrosis

Epithelial and endothelial cells can be damaged and consequently activated in a variety of ways as outlined above. The response of these cells, particularly that of epithelial cells, includes degeneration and apoptosis (Yang et al., 2006c), proliferation (Bonventre, 2003), the secretion of ECM components (Grande et al., 2002a; Shirato et al., 2003), or EMT (Iwano et al., 2002). However, the primary cause of tubulointerstitial inflammation and indeed the key response of tubular epithelial cells is substantial release of inflammatory cytokines. As outlined in *Fig. 1.4*, the activation of tubular epithelial cells and the subsequent induction of tubulointerstitial inflammation occurs in response to release of chemoattractants including monocyte chemoattractant protein-1 (MCP-1) and RANTES (regulated on activation, normal T-cell expressed and secreted), a process that is mediated by the up-regulation of nuclear factor κB (NFκB) (Rodriguez-Iturbe et al., 2005). This leads to the

Fig. 1.3

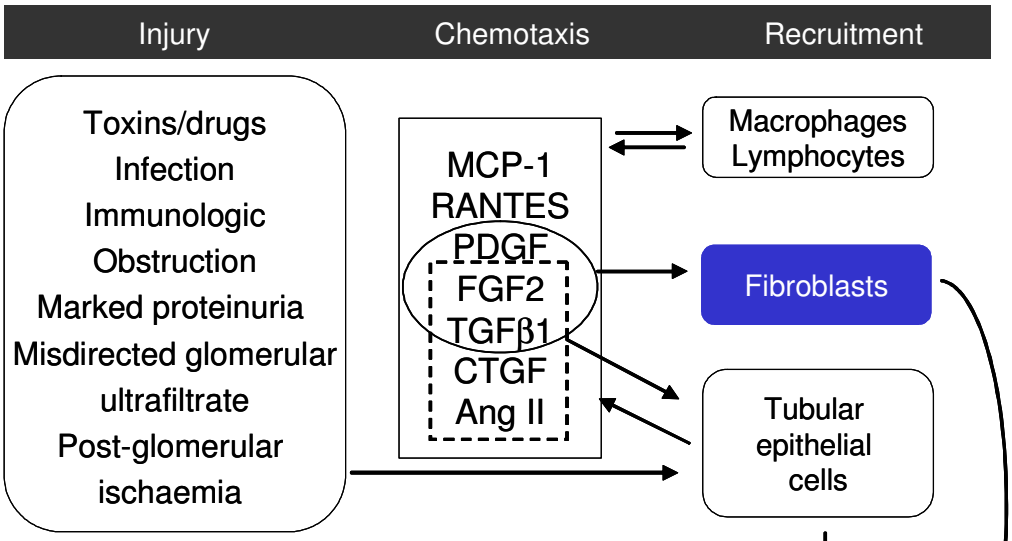


Mechanisms of induction of tubulointerstitial fibrosis. Glomerular based injury which elicits endocapillary damage can heal by repair and regeneration without any downstream effects, however severe endocapillary injury can result in formation of crescents, encroachment of the renal tubule, and tubular atrophy. Extracapillary injury can result in proteinuria and excessive compensatory tubular reabsorption. Both pathogenetic mechanisms lead to tubular degeneration and loss of viable nephrons. This places extra work on remaining nephrons (overload hypothesis) and can lead to tubulointerstitial inflammation and fibrosis.

Question mark indicates not firmly established.

Adapted from Kriz and LeHir, 2005.

Fig. 1.4



Cellular mechanisms of tubulointerstitial fibrosis. Tubulointerstitial fibrosis can be induced by diverse stimuli including toxic or drug insult, obstruction, proteinuria, misdirected glomerular ultra-filtrate and post-glomerular ischaemia. This activates tubular epithelial cells which can respond by secreting various cytokines including MCP-1 and RANTES leading to the infiltration of mononuclear inflammatory cells including macrophages and lymphocytes. During this phase we also see the recruitment and activation of the interstitial fibroblast (regulated mainly by TGFβ1, PDGF and FGF) which responds in an autocrine or paracrine fashion by proliferating and differentiating into myofibroblasts. Myofibroblasts represent a key effector cell in fibrosis and may also be derived via EMT, a process which is mainly mediated by TGFβ amongst other cytokines. These molecular events culminate in renal fibrosis.

Adapted from Rodriguez-Iturbe et al, 2005.

infiltration of mononuclear inflammatory cells including monocytes, macrophages and T lymphocytes into the tubulointerstitial space (Strutz and Neilson, 2003). These cells secrete various cytokines, which leads to a further increment in the local cytokine pool.

Consistent with the complexity of fibrosis, an array of mediators become up-regulated in the interstitium including TGFβ1, PDGF, connective tissue growth factor (CTGF), FGF, EGF, and vasoactive mediators such as angiotensin II and endothelin. The increase in the concentration of this pro-fibrotic milieu acts to stimulate the chemokine-mediated recruitment of more interstitial mononuclear cells.

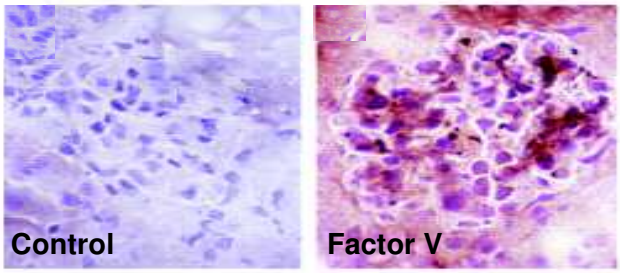
1.3.2.1.2 Non-chemical mediators: Coagulation cascade in renal fibrosis

Although cytokines and growth factors are recognized as key mediators of renal tubulointerstitial fibrosis, a number of non-cytokine mediated pathways are likely to be involved including tissue plasminogen activator (tPA) (Yang et al., 2002b), plasminogen activator inhibitor-1 (PAI-1) (Eddy, 2002; Matsuo et al., 2005), advanced glycation end products (AGEs) (Oldfield et al., 2001) and serine proteases of the coagulation cascade.

Given that (a) the coagulation cascade can be directly activated during injury and inflammation (Grandaliano et al., 2001; Sekiya et al., 1994) and (b) that a number of studies have localized specific surface receptors of the family of coagulation factors to a number of renal cells including tubular epithelial cells (Sower et al., 1995; Vesey et al., 2005; Xiong et al., 2005; Xu, 1995), endothelial cells (Xu, 1995) fibroblasts (Hewitson et al., 2005; Sower et al., 1995; Xiong et al., 2005) and mesangial cells (Grandaliano et al., 2000a; Xu, 1995), a growing number of studies have demonstrated the presence of coagulation factors in renal diseases of varying etiology. Although many experimental models have confirmed the involvement of the coagulation/fibrinolysis system during glomerular inflammation and repair in the kidney *(Fig. 1.5a-b)* (Grandaliano et al., 2000a; Hertig and Rondeau, 2004; Liu et al.,

Fig. 1.5

a

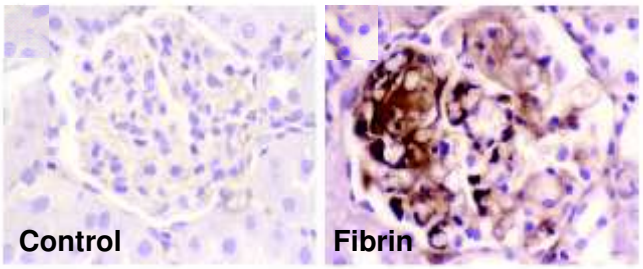


Demonstration of the coagulation cascade in glomerular-related pathologies. Factor V deposition in MsPGN in the mesangium and capillary loops.

MsPGN = mesangioproliferative glomerulonephritis.

Liu et al, 2004

b



Fibrin can also be demonstrated in the glomerulus of a kidney with MsPGN.

Liu et al, 2004.

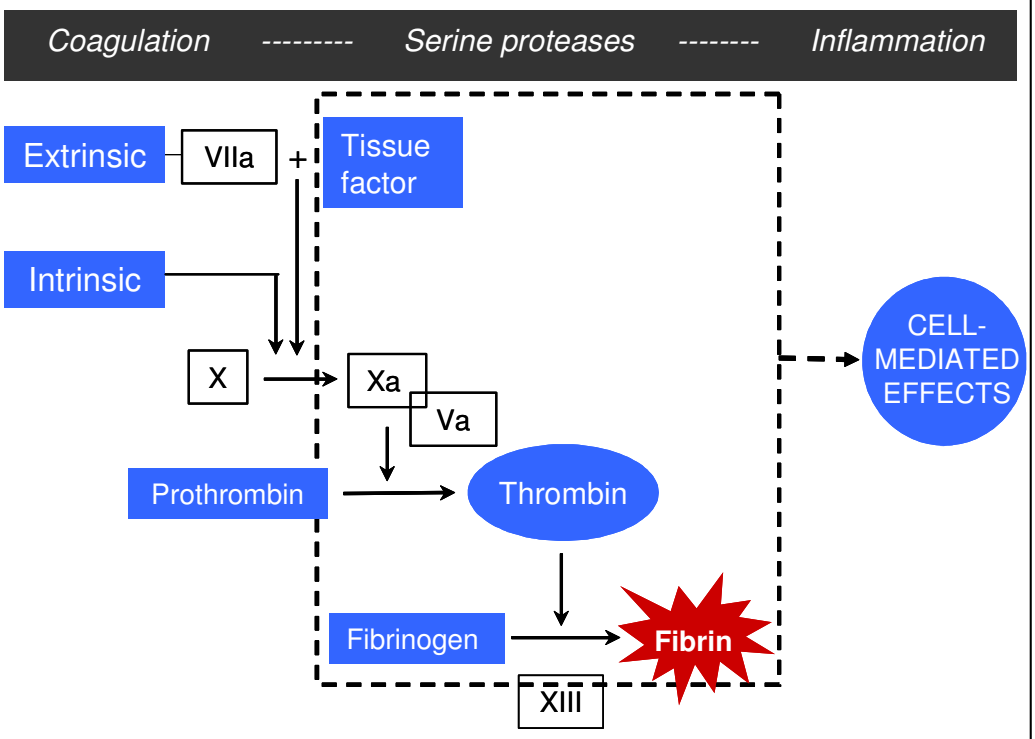
2004; Liu et al., 2000; Ono et al., 2001), less is known about serine proteases, and the role they play in tubulointerstitial fibrosis.

The traditional function of the coagulation cascade is to ensure that there is minimal blood loss through the formation of a mesh clot consisting of fibrin and platelets. Coagulation comprises a complex series of event that can be activated through either extrinsic or intrinsic pathways. During the initial stages, tissue factor (thromboplastin; a membranous glycoprotein) acts in concert with factor VII to catalyse the formation of active factor Xa from factor X (Matsuyama et al., 2003). Stimulation of the intrinsic pathway on the other hand directly activates factor Xa. Linking of factor Xa to factor Va then facilitates the generation of thrombin from prothrombin. This ultimately leads to the formation of fibrin, and the deposition of fibrin clots around injured vessels *(Fig. 1.6)*. This is the classical interpretation of the role that the coagulation cascade plays in physiological circumstances.

Growing evidence however, suggests that members of the coagulation cascade may facilitate inflammatory processes and fibrosis (Coughlin, 2000; Ossovskaya and Bunnett, 2004; Rondeau, 2001). These studies suggest that, although historically, cytokines have been shown to mediate key events in fibrosis, non-cytokine mediators can have pro-fibrotic effects independently, or working in cooperation with cytokine mediators. In this theme, studies have demonstrated the concurrent activation of the coagulation cascade during disorders associated with inflammation and excessive deposition of ECM in the kidney (Bogatkevich et al., 2001).

Fibrin, the end-product of coagulation, has become a persistent hallmark of the inflammatory response (Smiley et al., 2001) and correlates with the severity of renal tubulointerstitial damage in a number of studies (Cunningham et al., 1999; Liu et al., 2000; Ono et al., 2001). This has been documented in the tubulointerstitium of renal diseases including experimental unilateral ureteral obstruction (UUO), human allograft rejection and acute ischaemic renal failure (Enestrom et al., 1988; Faulk et al., 1989; Grandaliano et al.,

Fig. 1.6



The dual role of the coagulation cascade in haemostasis and inflammation.

2000b; Wang et al., 1997; Wang et al., 1996; Wendt et al., 1995; Yamamoto and Loskutoff, 1997). Other studies have also identified the presence of factor Xa (Blanc-Brude et al., 2001), tissue factor (Grandaliano et al., 2000a), and factor V (Liu et al., 2000) during tissue damage and inflammation. Most work to date however has focused on the serine protease thrombin which can be found in the interstitium as a consequence of increased capillary permeability (Plante et al., 1996) and misdirected filtration from the glomerulus to the interstitium (Kriz et al., 2001), both features of interstitial pathology.

The serine protease thrombin plays regulatory roles in vascular tone, wound healing and inflammatory reactions through the stimulation of platelets, leukocytes, endothelial cells, smooth muscle cells and fibroblasts (Grand et al., 1996; Ludwicka-Bradley et al., 2000; Sekiya et al., 1994), consistent with its ability to be generated independently of the coagulation cascade (Cole et al., 1985; Sekiya et al., 1994). The potential pro-fibrotic cell-mediated effects of thrombin have existed for over 30 years. Thrombin's ability to induce cell mitogenesis was initially noted in chick embryo fibroblasts in 1975 (Chen and Buchanan, 1975). Since then thrombin has been found to have a plethora of pro-fibrotic effects including acting as a chemo-attractant for fibroblast populations (Bogatkevich et al., 2001; Hewitson et al., 2005), up-regulating CTGF (Chambers and Laurent, 2002; Chambers et al., 2000; Howell et al., 2001), interleukin-6 (IL-6) (Sower et al., 1995), MCP-1 (Vesey et al., 2005), TGFβ1 (Bachhuber et al., 1997; Grandaliano et al., 2001; Vesey et al., 2005), PDGF (Ohba et al., 1994), tissue inhibitor of metalloproteinase-1 (TIMP-1) (Shirato et al., 2003), and up-regulating ECM components including fibronectin (Shirato et al., 2003; Vesey et al., 2005) and procollagen mRNA (Chambers et al., 1998; Hewitson et al., 2005; Howell et al., 2001). Furthermore, it has been documented that thrombin and αSMA can co-exist in the inflammatory and early fibrotic stages in a number of pathological situations (Coughlin, 2000), and accordingly, thrombin has also been shown to be an activator of fibroblasts *in vitro* in the lung (Bogatkevich et al., 2001; Tani et al., 1991), skin (Dawes et al., 1993) and kidney

(Hewitson et al., 2005) and can also mediate TGFβ1-independent differentiation of fibroblasts into myofibroblasts (Bogatkevich et al., 2001).

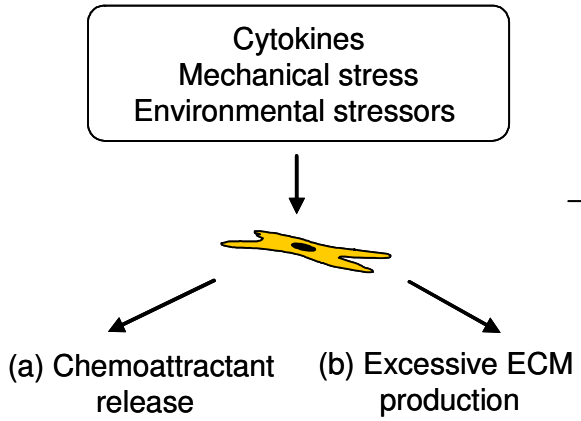
1.3.2.2 Fibrogenesis

Whilst a process like wound healing leads to relatively normal functioning with minimal scar tissue, the process of tubulointerstitial fibrosis is generally a relentless process that occurs when the inflammatory response is prolonged, leading to the continued inflammatory cell release of pro-fibrotic moieties and mesenchymal cell stimulation (Bitterman and Henke, 1991). During this phase we see the activation and recruitment of interstitial fibroblasts, which hyperproliferate in response to cytokines such as FGF, PDGF and EGF, migrate to areas of damage in response to chemotactic substances, and differentiate into αSMA-positive myofibroblasts. As mentioned, this phenotype facilitates the deposition of abundant ECM proteins and indeed, these cells are the principal generators of ECM proteins in fibrosis *(Fig. 1.4)*. Other cells may also become activated during interstitial inflammation and it has been shown that both mesangial cells and epithelial cells can contribute to the accumulation of ECM proteins (Liu, 2006).

Although the reasons of why tubulointerstitial fibrosis does not resolve are somewhat elusive, there are a number of likely mechanisms to explain the prolonged nature of the response in the kidney. It is believed that the fibrotic response is enhanced because of the presence of continuous fibrogenic signals which may become increasingly amplified (Liu, 2006). Integral to the amplification of fibrogenic signals is the perpetuation of the activated fibroblast (Zeisberg et al., 2000) independent of the initial stimulus and the subsequent generation of exuberant amounts of ECM proteins *(Fig. 1.7a-c)*.

Furthermore, in fibrosis the accumulation of ECM proteins is believed to develop as a result of disruption to the turn-over and production and of its constituents, potentially due to respective down regulation of metalloproteinase (MMP) activity, and increases in TIMPs

Fig. 1.7



(c)

Myofibroblast Formation

Lack of apoptosis

MMPs activity ?
TIMPs and PAI-1 ?

Autonomous proliferation and
? ECM deposition

EMT

Classic response of the fibroblast (a-b), and the roles of the fibroblast in tubulointerstitial fibrosis (c).

Adapted from Qi et al, 2006.

(Jones et al., 1992) and PAI-1 (Johnson et al., 2002). This may also result from prolonged autocrine and paracrine stimulation from local cells. Lastly, the termination of fibroblast activity by apoptosis, unlike wound healing (Darby et al., 1990; Desmouliere et al., 1995), has not been convincingly shown in renal fibrosis (Hewitson et al., 2000a; Strutz and Muller, 1999).

In cases where inflammation subsides, matrix synthesis may also continue due to prolonged autocrine stimulation from activated resident fibroblasts (Lonnemann et al., 1995). Furthermore, it has been noted that whilst myofibroblast differentiation from resident fibroblasts may be an early event in fibrosis, EMT may be a delayed event (Yang and Liu, 2001). This provides a mechanism whereby the fibroblast pool may be replenished with ongoing fibrosis and provides a driving force for the sustenance and aggravation of tissue injury and inflammation during progressive renal disease.

1.3.2.3 Remodelling

The composition of interstitial scars primarily consist of collagen types I, III and IV, fibronectin and tenascin as well as other glycoproteins such as proteoglycan, thrombospondin and osteopontin (Harris and Neilson, 2006). Newly synthesised matrix is progressively remodelled by various proteases (secreted by fibroblasts and macrophages), while contraction of matrix increases its density leading to the loss of blood supply rendering the tissue acellular and fibrotic. This distorts the parenchymal architecture of the kidney leading to its collapse and loss of kidney function.

1.3.2.4 Resolution

The resolution of tubulointerstitial nephritis represents an important step in limiting the extent of renal scarring (Kuncio et al., 1991). How fibroblasts are removed at the completion of scarring remains unclear, with phenotypic regression, cell death and ex-

migration all possible mechanisms however they have not been convincingly proven. Studies in skin wound healing (Darby et al., 1990; Desmouliere et al., 1995) suggest that fibroblastic cells are removed by apoptosis at the completion of wound healing. Importantly apoptosis leads to the safe removal of cells by phagocytosis, whereas in contrast, necrosis provokes tissue injury and inflammation (Savill, 1994). To date evidence that renal fibroblasts are removed efficiently by apoptosis is inconclusive (Lane et al., 2002; Tang et al., 1996).

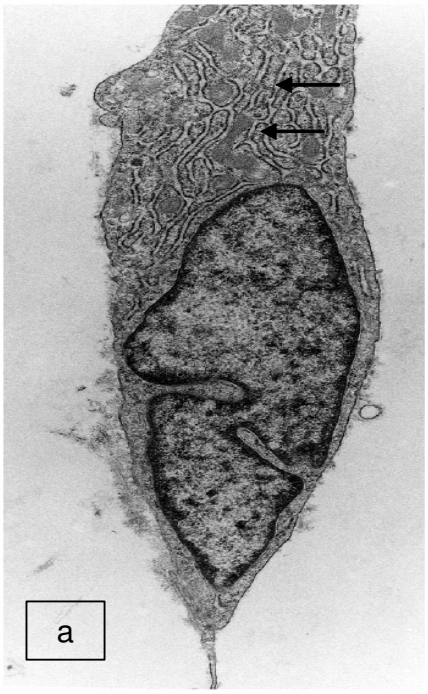
1.4 ROLE OF THE FIBROBLAST IN TUBULOINTERSTITIAL FIBROSIS

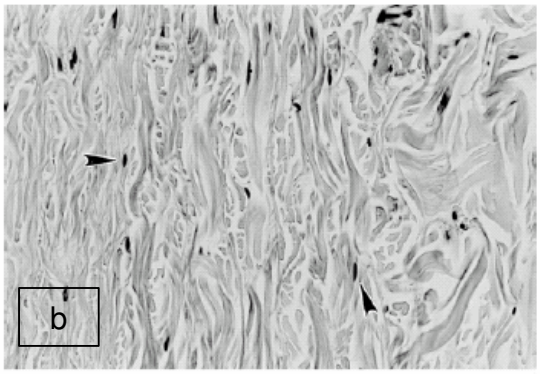
In the normal kidney, fibroblasts are typically found surrounding arterioles but their distribution is relatively sparse and they have a low turn over rate. However, in fibrosis these cells become activated, and are responsible for the generation of scar tissue through hyperproliferation, increased synthesis of ECM proteins, and contracture of the surrounding tissue. These cells are influenced by a variety of cytokines, growth factors, and non-cytokine agonists, however many studies indicate that fibrosis is dictated by a hierarchy of mediators, the most important arguably being TGFβ1, PDGF and FGF.

1.4.1 Fibroblast structure and function

Fibroblasts are the prototypical mesenchymal cell (Qi et al., 2006). Renal fibroblasts were originally classified into 'fibroblast-like' cells in the cortex and 'lipid-laden' cells in the medulla (Bohman and Jensen, 1976; Bohman and Jensen, 1978). Since then, a number of fibroblasts have been classified into different groups based on mitotic and matrix synthesising activities (Grupp and Muller, 1999; Muller et al., 1992; Zeisberg et al., 2000). The fibroblast displays a typical elongated spindle phenotype with cortical actins (γ and β) in their cytoplasm. Intracellularly, they have a large nucleus, dense endoplasmic reticulum and an extensive golgi apparatus however mitochondria are sparse (Qi et al., 2006) *(Fig. 1.8a).* Fibroblasts have abundant microtubules allowing them to contact tubules of adjacent

Fig. 1.8

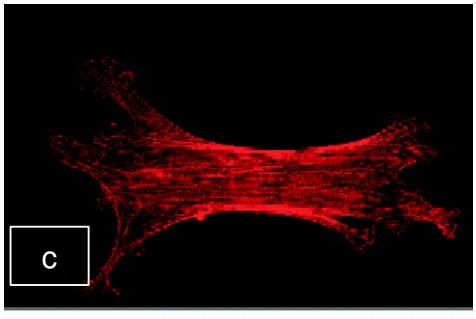


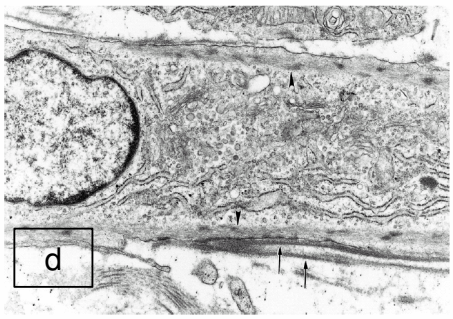


Fibroblasts (black arrow heads) surrounded by wisps of collagen.

As demonstrated by EM, fibroblasts have a spindle shape morphology with slender polar extensions and prominent rER (arrows).

EM = electron microscopy.





(c) αSMA microfilaments in myofibroblasts demonstrated by Immunofluorescence (Hinz et al., 2001) and EM (d); a fully differentiated myofibroblast displays characteristic features including a prominent rER, a large golgi apparatus, myofilaments (arrow heads) and a substantial fibronexus (arrows).
Images (a)-(b), (d) from Eyden, 2004.

fibroblasts, capillaries, parietal cells, other interstitial cells and nerve terminals through focal adhesions within their cytoplasm (Qi et al., 2006). Functionally, these cells are therefore an integral part of the interstitium providing structural support and integrity to the ECM.

Fibroblasts are also a major regulator of the ECM. ECM is normally maintained by controlling the amount of matrix synthesis in balance with that which is degraded and this is chiefly regulated by MMPs and TIMPs. Fibroblasts are a major source of these proteases and their inhibitors (Valle and Bauer, 1979) as well as PAI-1 and interstitial collagens *(Fig. 1.8b)*. These cells are therefore a key regulator of ECM balance. Moreover, fibroblasts express many growth factor receptors, integrins such as $\alpha 1$, $\alpha 4$, $\alpha 5$ and $\beta 1$ (Norman and Fine, 1999), cell adhesion molecules and non-integrin matrix receptors such as discoidin domain receptors (Qi et al., 2006). This allows them to respond to a variety of stimuli and efficiently regulate the ECM through communication inter-cellularly and with the surrounding matrix. Although active fibroblasts are heterogeneous in their biochemical synthesis and are capable of secreting many types of proteins, increased synthesis of fibronectin is a universal event and acts as an important mediator of collagen deposition by acting as a scaffold on which subsequent interstitial collagen is fixed (Goyal and Wiggins, 1991) (Clark, 1989). Once fibronectin has been deposited in the ECM, this facilitates the deposition of a variety of interstitial collagens including collagen types I, III and V (Rodemann and Muller, 1991). *In vitro* studies with non-renal fibroblasts suggest that they are also capable of synthesising several other ECM components including chrondroitin sulphate (David et al., 1989) and hyaluronan (Heldin et al., 1989).

1.4.2 Myofibroblast structure and function

The term myofibroblast is given to differentiated fibroblasts that express the VSMC associated protein αSMA (Hewitson and Becker, 1995; Hewitson et al., 1998; Hewitson et al., 1995). Accordingly, these cells share phenotypic similarities between VSCM and fibroblasts. These cells were originally identified as the cells responsible for wound healing by Gabbiani et al (Gabbiani et al., 1971), and are characterised in the kidney as the major ECM generating cells in tubulointerstitial fibrosis (Neilson, 2006; Qi et al., 2006). They have been demonstrated in a wide variety of renal diseases associated with renal interstitial fibrosis in humans (Goumenos et al., 1994; Yang et al., 1998)(Hewitson and Becker, 1995) and experimental models (Diamond et al., 1995; Hewitson and Becker, 1995). It is the acquisition of these cells that is therefore a key indicator of the severity of tubulointerstitial fibrosis (Qi et al., 2006).

In contrast to the reduced and spindle shaped cytoplasm of an inactive fibroblast, myofibroblasts have a much larger cytoplasm to facilitate the synthesis and secretion of collagen, elastin and reticulin precursors as well as the glycosaminoglycans found in ground substance. Their cytoplasm also contains an extensive rough endoplasmic reticulum (rER) and golgi apparatus, and hemidesmosomes *(Fig. 1.8d).* Myofibroblasts also have structural features similar to the smooth muscle cell including longitudinal cytoplasmic bundles of microfilaments and nuclear membrane folds (Sappino et al., 1990). These differentiated cells also express vimentin, desmin (Eyden, 2001) and αSMA bundles *(Fig. 1.8c).* These actin bundles terminate at the myofibroblast surface in the fibronexus. The fibronexus is an adhesion complex that uses transmembrane integrins to connect with extracellular fibronectin and therefore provides a mechano-transduction system where myofibroblasts can transmit force generated by stress fibres into the surrounding matrix (Tomasek et al., 2002). Myofibroblasts are also connected to each other through Gap junctions potentially allowing them to form multi-cellular contractile apparatuses during healing.

1.4.3 Phenotypic markers of the fibroblast and myofibroblast

Because fibroblasts are a heterogenous cell population with poor immunogenicity, reliable markers have been difficult to identify. *In vitro* studies of cultured renal fibroblasts suggest that they undergo a one-way terminal differentiation through 3 mitotically active progenitor fibroblasts, then after mitosis differentiate to 3 post-mitotic cell types (PM; PMF IV, PMF V, PMF VI), with increasing biosynthetic activity for various fibroblast proteins, including collagen (Muller et al., 1992). The characterisation of fibroblasts can therefore be ambiguous when differentiating these cells from other mesenchymal cells like VSCM or pericytes. Recently fibroblast specific protein (FSP1) has become a widely used marker of fibroblasts (Iwano et al., 2001; Strutz et al., 1995), however this marker has come into question as it has been shown *in vivo* that the human homologue s100A4 is expressed by mononuclear inflammatory cells (Le Hir et al., 2005). Myofibroblast-specific markers are also lacking and to complicate matters, it is thought that only a subset of activated myofibroblasts express the most widely used marker of these cells, αSMA (Okada et al., 2000a). Furthermore, αSMA is also found in numerous other cells including smooth muscle cells, pericytes, myoepithelial and interstitial cells of Cajal (Eyden, 2004). *Table 1.1* outlines the various putative markers of fibroblasts and myofibroblasts.

Cell	Marker	Reference
Interstitial fibroblast	5' ectonucleotidase, CD44, PDGF α and β receptors, FSP1, ICAM, DDR2, HSP47, NGF	(Iwano et al., 2002; Ohba et al., 2005; Qi et al., 2006; Strutz et al., 1995)
Myofibroblast	αSMA	(Darby et al., 1990)

Table 1.1: Putative markers of fibroblasts and myofibroblasts.
Intracellular adhesion molecule (ICAM), discoidin domain receptor (DDR), heat shock protein (HSP), nerve growth factor (NGF).
Adapted from Strutz and Zeisberg (Strutz and Zeisberg, 2006).

1.4.4 Activation of the fibroblast in tubulointerstitial fibrosis

Fibroblasts from fibrotic kidneys display a higher mitogenic potential and a 3-5 fold increase in the rate of collagen synthesis as compared to their quiescent counterparts (Rodemann and Muller, 1991), consistent with the concept that fibroblasts from normal kidneys differ from fibroblasts found in fibrotic kidneys. The activation of fibroblasts can occur through four distinct mechanisms: (a) paracrine or autocrine stimulation by growth factors (TGFβ, PDGF, FGF and CTGF); (b) direct cell-cell contacts (leukocytes and macrophages); (c) through the ECM though integrin signalling (α and β), and (d) by changed environmental conditions in renal disease (hypoxia, AGEs, ROS and high glucose) (Qi et al., 2006; Zeisberg et al., 2000). Once activated, fibroblasts can maintain their activated phenotype after the initial stimulus has been long removed (Strutz and Muller, 1995), presumably through autocrine stimulation (Strutz et al., 2000). It is these activated fibroblasts that are good predictors of the development of tubulointerstitial fibrosis (el Nahas et al., 1997) and animal studies that have incorporated a suicide transgene have been shown to conditionally minimise fibrosis through the depletion of fibroblasts (Iwano et al., 2001).

Activated fibroblasts become motile and migrate through the surrounding interstitial matrix to sites of damage, aided by the secretion of stromelysins and collagenases (Kuncio et al., 1991) and chemotactic substances (Wiggins et al., 1993). They are also capable of secreting collagens type I, II and V (Rodemann and Muller, 1991) and fibroblasts in anchored lattices also increase expression of genes coding for collagens I, III, VI, XII and tenascin-C, (Chiquet, 1999; Chiquet-Ehrismann et al., 1994; Lambert et al., 1992). It is likely that the migration to the site of injury precedes the phenotypic change to αSMA expressing myofibroblasts, since it has been shown *in vitro* that the fully differentiated myofibroblast is not motile (Ehrlich and Rajaratnam, 1990). The increase in fibroblast number doesn't only occur through migration but also local proliferation. Early during chronic renal disease, fibroblasts rapidly proliferate to occupy the interstitium. Anchored collagen lattice studies

show that fibroblasts become elongated, align in a plane of force offering least resistance and hyperproliferate (Lambert et al., 1992; Nakagawa et al., 1989). This proliferation is mainly mediated through the direct or indirect actions of local cytokines and other factors (Kovacs and DiPietro, 1994). For a complete list of such factors that regulate fibroblast proliferation, differentiation (outlined below) and ECM synthesis (outlined below), see *Table 1.2*.

1.4.5 Fibroblast differentiation in tubulointerstitial fibrosis

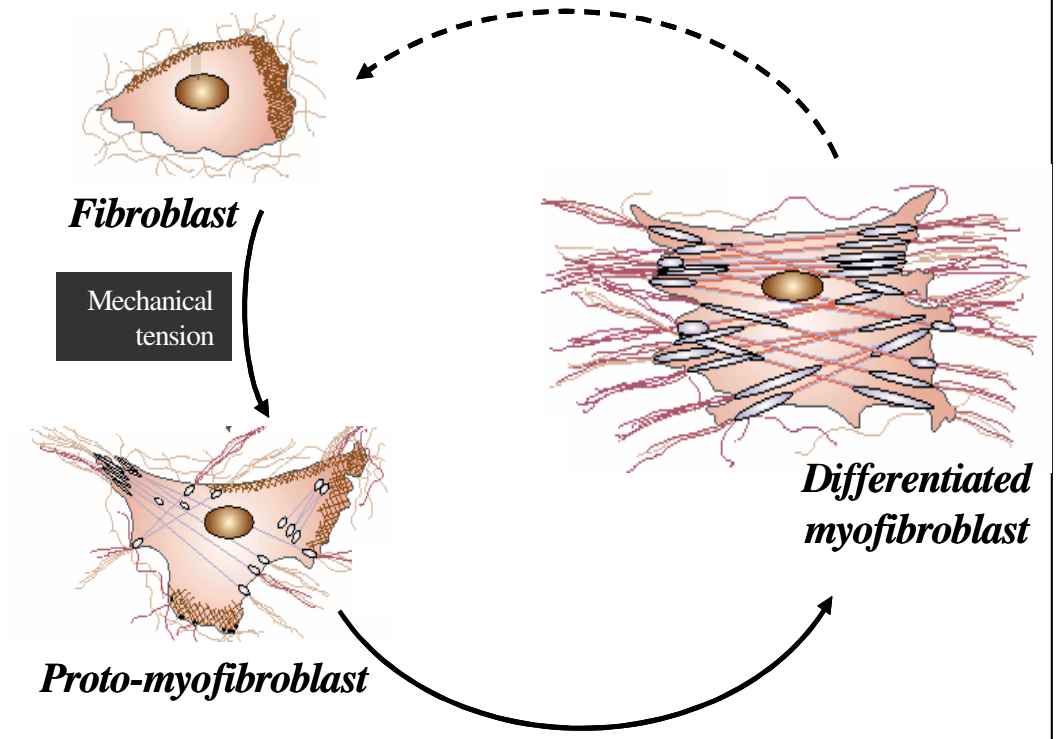
As previously mentioned myofibroblasts, are usually recognized by their *de novo* expression of αSMA. The differentiation of these cells is a fundamental event in fibrosis and is thought to be chiefly regulated by TGFβ1 (Desmouliere et al., 1993). Given that it has been noted that only a subset of these cells express αSMA (Desmouliere et al., 1992a), the term 'proto-myofibroblast' has been given to an intermediate form of a differentiated fibroblast that does not express this contractile protein (Tomasek et al., 2002) *(Fig. 1.9).* It is believed that mechanical tension and PDGF (Desmouliere et al., 1992b) are the main inducers of the proto-myofibroblast phenotype. These cells express cytoplasmic actins (but not αSMA) and the ED-A splice variant of fibronectin which is not expressed by fibroblasts, and they are capable of generating contractile force.

Myofibroblasts are distinguished from proto-myofibroblasts by the presence of αSMA microfilaments and also by increased expression of ED-A fibronectin, focal adhesions and more stress fibres. For a proto-myofibroblast to differentiate into an αSMA-positive cell, it is believed that TGFβ1 is an essential mediator of this process, which can be released by platelets, macrophages and parenchymal cells (Desmouliere et al., 1993; Tomasek et al., 2002). TGFβ1 can facilitate differentiation by enhancing the assembly of stress fibres and the formation of fibronexus adhesion complexes (Tomasek et al., 2002). Differentiation is also facilitated by the expression of fibronectin when it is expressed in the ED-A splice variant form (Serini et al., 1998) and by sustained mechanical stress *(Fig. 1.9).*

Effector	*Fibroblast specific effects*	*Signalling mechanism*	*References*
TGFβ1	+/-Proliferation, ECM synthesis, differentiation, CTGF expression, decreases MMP expression, increases TIMP expression	TGFβ-TβR-Smad 2/3 axis, abl	(Ignotz and Massague, 1986), (Igarashi et al., 1993), (Edwards et al., 1987; Frazier et al., 1996), (Reisdorf et al., 2001), (Strutz et al., 2001), (Desmouliere et al., 2003), (Wang et al., 2005)
PDGF-BB	Proliferation, differentiation and ECM deposition	PDGFR, PI3K, Ras	(Bonner, 2004), (Tang et al., 1996), (Tang et al., 1997).
***CTGF**	+/-Proliferation, differentiation, ECM deposition	Smad axis	(Duncan et al., 1999) (Yokoi et al., 2001), (Frazier et al., 1996).
bFGF	Proliferation and differentiation, inhibition of ECM synthesis	FGFR1	(Ichiki et al., 1997), (Strutz et al., 2000), (Strutz et al., 2001).
EGF	Proliferation	EGFR	(Alvarez et al., 1992), (Kikuchi et al., 1995), (Heldin et al., 1989).
AGEs	Proliferation, CTGF expression, ECM synthesis, apoptosis	RAGE, JAK/Stat5	(Peterszegi et al., 2006), (Huang et al., 2005), (Alikhani et al., 2005), (Lee et al., 2004), (Twigg et al., 2002), (Guh et al., 2001).
Thrombin	Proliferation, differentiation, ECM synthesis, CTGF synthesis	PAR-1, MAPK, JNK	(Hewitson et al, 2005), (Bogatkevich et al., 2001), (Chambers et al., 2000), (Chambers et al., 1998), (Remillard and Yuan, 2005).
Angiotensin II	TGFβ synthesis, proliferation, ECM synthesis, differentiation	Smad axis, RhoA, p38, ERK, JNK, PI3K	(Kellner et al., 2006), (Huang et al., 2006), (Lijnen et al., 2006), (Liu et al., 2006b), (Pokharel et al., 2002).
Endothelin	Differentiation, contraction, migration	ETA, PI3K, [#]MAPK	(Shi-Wen et al., 2004), (Wang et al., 1994)

Table 1.2 Major regulators of fibroblast function.
abl = abelson non-receptor tyrosine kinase, FGFR = fibroblast growth factor receptor, EGFR = epidermal growth factor receptor, ETA = endothelin A (receptor).
*CTGF primarily acts as a downstream mediator of TGFβ.
[#] In cells similar to myofibroblasts (mesangial cells).

Fig. 1.9



The process of myofibroblast differentiation. Fibroblasts are thought to be able to transdifferentiate into proto-myofibroblasts in response to mechanical stress. Proto-myofibroblasts contain cytoplasmic stress fibers that terminate in fibronectin adhesion complexes and express ED-A fibronectin at their cell surface. For the full differentiation of these cells into myofibroblasts, TGFβ in the presence of ED-A fibronectin and mechanical tension is required. Myofibroblasts secrete large amounts of the ED-A splice variant of fibronectin and express αSMA. These cells contain large fibronexus adhesion complexes and are capable of generating more contractile force than proto-myofibroblasts. (Dashed line indicates not strongly supported).

Adapted from Tomasek et al, 2002.

The sequence of events of fibroblast activation, proliferation, migration, differentiation and ECM synthesis is not entirely clear. It is generally thought that fibroblasts are activated and proliferate locally or migrate to the site of damage to then proliferate in response to chemotactic mediators. These cells can then differentiate into their contractile counterparts. The myofibroblast is thought to be a less motile cell whose primary role is the secretion of matrix proteins and contracture. It is unclear whether myofibroblasts undergo significant proliferation, however animal studies have demonstrated exponential increases in myofibroblast mitosis and incorporation of bromo-deoxyuridine, a marker of cells that have entered the S phase of mitosis (Hewitson et al., 1995).

1.4.6 Myofibroblast contraction and ECM synthesis in tubulointerstitial fibrosis

Once significant numbers of myofibroblasts have formed in the area of tissue damage, they begin to actively secrete various collagens into the interstitium and are stimulated to contract. It is believed that matrix accumulation results from a combination of excessive production of ECM, and an inhibition of matrix breakdown, however these mechanisms are not clear. TGFβ1 is a key mediator of the synthesis of ECM proteins such as fibronectin and collagen type I (Strutz and Neilson, 2003), however a number of factors in the fibrotic milieu are capable of also inducing ECM synthesis. The myofibroblast is likely to play a major role in the dis-regulation of ECM homeostasis during fibrosis, and when taken with the ability of myofibroblasts to contract the surrounding matrix, activation of these cells can result in the compression of the surrounding parenchyma.

The contraction of myofibroblasts has been shown to be largely mediated by integrins through collagen lattice studies (which mimic the *in vivo* condition) (Kelynack et al., 2000; Klein et al., 1991). The intracellular stress filaments in myofibroblasts are bound to collagen fibrils through focal adhesions and fibronexus junctions which signal through integrin

transmembrane receptors. Stress-fibre contraction leads to force generation and local matrix contraction causing a shortening and bundling of the surrounding collagen network. Myofibroblasts then secrete new matrix components to stabilise the new collagen organization increasing the density of collagen in the ECM (Tomasek et al., 2002). This type of response is magnified when the myofibroblast is continuously stimulated to contract, which may lead to an increase in interstitial volume and contracture of the matrix.

1.5 MYOFIBROBLAST DERIVATION IN TUBULOINTERSTITIAL FIBROSIS

1.5.1 Resident fibroblasts

Local proliferation and migration from adjacent tissues, in particular the perivascular region, have been generally accepted as the mechanisms by which tissue myofibroblast numbers increase (Ross et al., 1970; Wiggins et al., 1993). The close association with fibroblasts in wound healing systems (Clark, 1989) supports the view that the myofibroblast phenotype is derived from fibroblasts. Much of this rationale is based upon *in vitro* studies where after stimulation, previously quiescent fibroblasts express αSMA (Ronnov-Jessen and Petersen, 1993). Sequential ultrastructural studies in renal biopsies also imply that myofibroblasts are derived from resting fibroblasts (Yang et al., 1998). It has also been suggested that renal interstitial myofibroblasts are mitotically active, suggesting that they may also be derived from local proliferation of other myofibroblasts (Hewitson et al., 1995). Other possibilities including a pericyte origin (Nehls and Drenckhahn, 1993) and even a macrophage derivation have been proposed (Bhawan and Majno, 1989). In some fibrogenic tissues, the myofibroblast may be derived from a specialised cell type, as in hepatic fibrosis where myofibroblasts represent activated perisinusoidal or hepatic stellate cells (Hines et al., 1993). To complicate matters further, the pathogenesis of atheroma involves invasion of migrating smooth muscle cells, phenotypically similar to the myofibroblast, into the intima of

vessels (Desmouliere and Gabbiani, 1992; Hosenpud, 1993) which probably represents a partial loss of smooth muscle cell phenotype to resemble myofibroblasts.

Moreover, mesangial cells can also differentiate into myofibroblasts. Mesangial cells create the ECM supporting network of the glomerular apparatus, secrete growth factors controlling normal cell turnover, and modulate filtration via contraction / relaxation. Upon stimulation by glomerular injury, mesangial cells acquire phenotypic characteristics of myofibroblasts. These activated myofibroblasts have the ability to proliferate at the site of injury, acquire smooth muscle cell properties, express αSMA and secrete collagens I and III as well as coagulation factors (Hertig and Rondeau, 2004).

These and other studies have confirmed that the derivation of the myofibroblast is diverse and heterogenous pools of myofibroblasts are likely play a role in fibrosis. Whilst the derivation of myofibroblasts from the sources listed above are likely to contribute to the population of myofibroblasts, it is now suggested that the majority of interstitial myofibroblasts are derived from interstitial fibroblasts, bone-marrow derived pluripotential cells and through renal EMT (Iwano et al., 2002).

1.5.2 Bone-marrow-derived pluripotential cells

There is evidence that bone-marrow derived fibrocytes exist within the interstitium during fibrosis (Grimm et al., 2001; Iwano et al., 2002) and that these cells are recruited to sites of injury by undergoing mesenchymal-epithelial transition (MET). In an experimental model of sex mismatched bone marrow transplants, they demonstrated mismatched deoxyribonucleic acid (DNA) in these cells, showing they were of donor origin, and that these donor cells were detectable in host skin wound chambers suggesting a fibroblast-like role in tissue repair. These cells have been shown to express ECM proteins such as procollagen I and procollagen III (Phillips et al., 2004) suggesting a role for these cells in the formation of connective tissue. Moreover, studies using bone marrow chimeras, have established that 12%

of resident interstitial FSP1^{+}, CD34^{-} fibroblasts may be derived from bone marrow (Iwano et al., 2002).

1.5.3 Renal epithelial mesenchmyal transition

EMT is a phenomenon that occurs in various biologic processes such as embryonic and post-natal development, carcinogenesis and chronic diseases (Kalluri and Neilson, 2003). EMT can be defined as the acquisition of phenotypic as well as functional properties of mesenchymal cells by epithelial cells (Zeisberg et al., 2001). In this process epithelial cells lose their markers and phenotypic characteristics, acquire a mesenchymal phenotype, degrade the basement membrane and migrate into the surrounding matrix. During embryogenesis, EMT and MET give rise to an array of fully differentiated adult cell types derived from pluripotential cells present in the developing embryo (Khew-Goodall and Wadham, 2005). In the adult such transitions have been thought to generally be confined to those seen in wound healing and angiogenesis (Khew-Goodall and Wadham, 2005). Of late it has however been noted that embryonic EMT can be recapitulated during certain adult disease states such as cancer (Grunert et al., 2003; Lee et al., 2006) and fibrosis (Iwano et al., 2002; Thiery and Sleeman, 2006) whereby dramatic morphological and functional changes take place to allow cells to develop a migratory and invasive capacity. In fact, the potential for EMT in adult cell types has been documented for nearly twenty years and it is possible that this phenomena may be a relatively ubiquitous phenomenon in labile cell types (Boyer et al., 1989; Gilles et al., 1994; Miettinen et al., 1994; Pagan et al., 1997). Accordingly, accumulating evidence suggests that cellular plasticity in the adult may have been underestimated (Grunert et al., 2003; Lee et al., 2006; Thiery and Sleeman, 2006).

Furthermore, given that renal tubular epithelial cells (apart from the collecting duct) develop from metanephric mesenchyme during embryogenesis through MET (Liu, 2004a; Okada et al., 2000b), the existence of correct genetic cues and biological stimuli such as organ

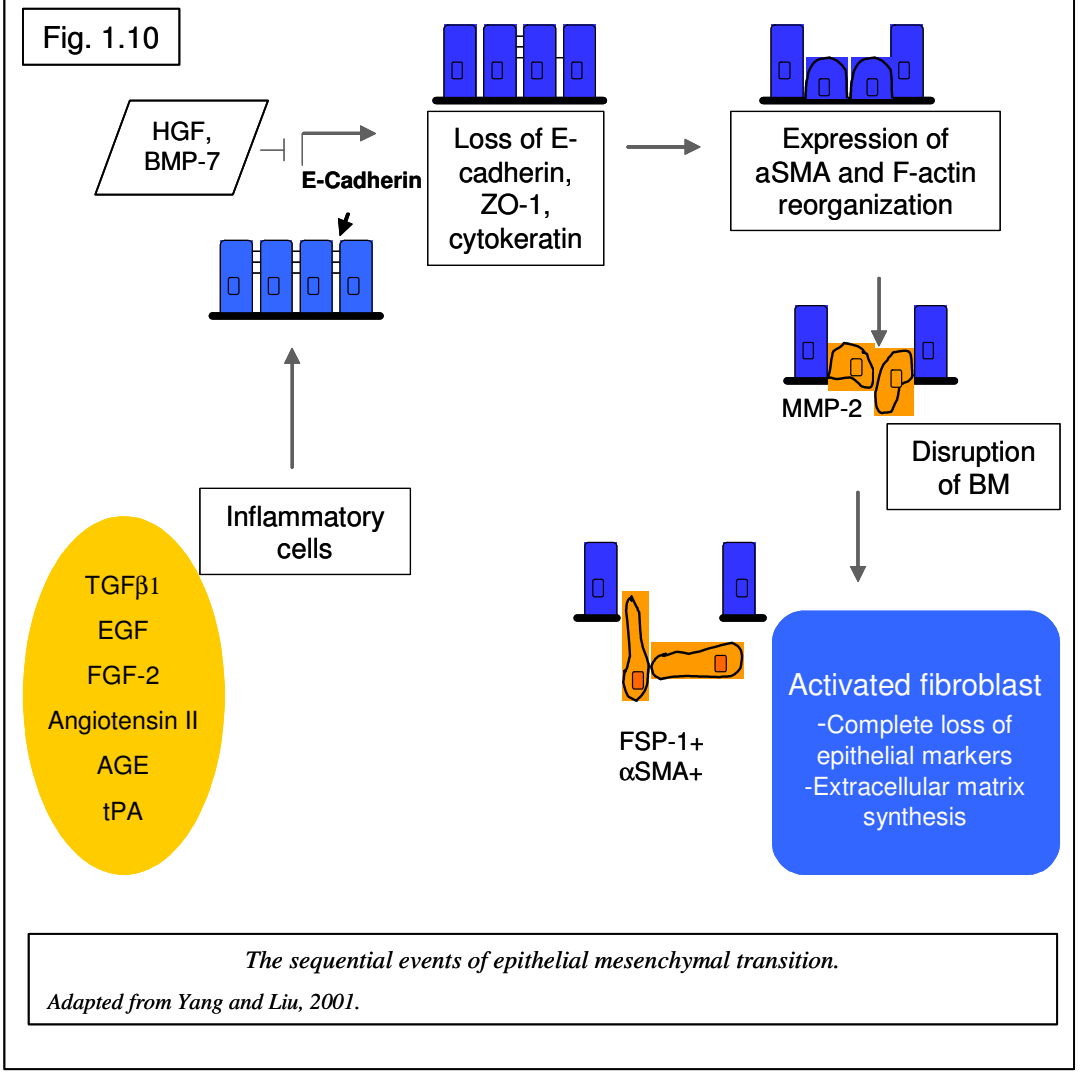
remodelling (Hay, 1995) may underlie the potential for tubular epithelia to undergo a reversal of their embryonic development pattern (Okada et al., 2000b). Indeed, the frequent observation of EMT in human Immuoglobulin A (IgA) nephropathy, rapidly progressive glomerulonephritis (RPGN)(Jinde et al., 2001), marked tubulointerstitial fibrosis (Vongwiwatana et al., 2005), diabetic glomerulosclerosis, lupus nephritis (Rastaldi et al., 2002), and in animal models of renal disease (Burns et al., 2006; Ng et al., 1998; Strutz et al., 1995; Yang and Liu, 2001) implicates tubular epithelial cells as a source of interstitial myofibroblasts. Amongst the animal models used to demonstrate EMT, the most convincing and widely acknowledged study was that by Iwano et al (Iwano et al., 2002). In a model of UUO, genetically tagged Lac Z tubular epithelial cells were shown to express both Lac Z and FSP-1. This group came to the conclusion that during fibrosis induced by UUO, approximately 36% of renal fibroblasts were derived from EMT. This suggests that a process that was once thought to be confined to embryogenesis may in hindsight be a fundamentally important process in the pathogenesis of renal scarring.

1.5.3.1 Sequential events involved in EMT

Although it has been established that EMT does occur in a wide variety of renal diseases, we are only beginning to learn about the mechanisms involved in EMT from *in vitro* studies. EMT has been shown to be a highly complex process which relies on an interplay of different cytokine and non-cytokine mediators that in turn influence the integrity of the tubular epithelial cell, its basement membrane, expression of cell adhesion proteins, and the nearby interstitium (Iwano et al., 2002; Okada et al., 2000b). Renal EMT is therefore inherently dependent on the surrounding microenvironment, the interstitium and a cocktail of mediators, which can fully induce this process or various events involved in EMT.

As outlined in *Fig. 1.10*, EMT can be induced by the release of inflammatory mediators in the surrounding environment, including those released by the disrupted epithelia

Epithelial-mesenchymal transition



The sequential events of epithelial mesenchymal transition.

Adapted from Yang and Liu, 2001.

and fibroblasts (Iwano et al., 2002; Kalluri and Neilson, 2003). The majority of research to date has focused on elucidating agonists of EMT and these include TGFβ1 (the principle inducer of EMT) (Fan et al., 1999; Strutz and Neilson, 2003; Yang and Liu, 2001), FGF (Strutz et al., 2002), EGF (Okada et al., 1997; Strutz et al., 2002; Zeisberg et al., 2002), interleukin 1 (IL-1) (Fan et al., 2001), IGF (Morali et al., 2001) and CTGF (Burns et al., 2006). Many of these factors do not appear to be able to induce EMT independently but rather act in concert with TGFβ1 or other mediators. Given the complexity of EMT, it can also be induced by a number of other factors including components of the ECM (collagen type I (Fan et al., 1999), non-cytokine mediated pathways such as AGEs (Li et al., 2004b; Oldfield et al., 2001) and tPA (Yang et al., 2002b), and vasoactive peptides such as angiotensin II (Chen et al., 2006; Liu, 2004a). Furthermore, endogenous negative regulators of EMT have been identified in HGF and bone morphogenic protein (BMP-7). These two mediators are the most notable inhibitors of EMT both *in vitro* and *in vivo* (Liu, 2004a; Mizuno et al., 1998; Zeisberg et al., 2003) and they normally act by counteracting the actions of TGFβ1 and restoring E-cadherin (Yang and Liu, 2002; Zeisberg et al., 2003).

In response to the local up-regulation of one or more of these cytokines, the initial event is thought to be the loss of epithelial cell adhesive proteins such as E-cadherin and tight junction proteins such as zona occludens-1 (ZO-1) (Okada et al., 1997; Yang and Liu, 2001). This leads to the de-stabilisation and a compromised epithelial cell integrity including loss of apical-basal cell polarity (Yang and Liu, 2001). This type of dissociation from neighbour counterparts promotes cytoskeletal reorganisation involving loss of cytokeratin and gain of vimentin (Strutz et al., 2002; Yang and Liu, 2001), *de novo* αSMA expression and F-actin reorganization (Alpers et al., 1994; Boukhalfa et al., 1996; Hewitson et al., 1995; Muchaneta-Kubara and el Nahas, 1997; Ng et al., 1998; Yang and Liu, 2001). Even though αSMA expression has been shown previously to negatively correlate with migration in myofibroblasts (Hinz et al., 2001), this seems to precede and promote migration and invasion

of these transitioning cells into the interstitium (Liu, 2004a; Yang and Liu, 2001). This is primarily initiated by degradation of the type IV collagen and fibronectin basement membrane (Ng et al., 1998; Zeisberg et al., 2001), facilitated by gelatinase enzymes MMP-2 (Yang and Liu, 2001) and MMP-9 (Zeisberg et al., 2002). These cells then acquire synthetic capability which culminates in an increased deposition of ECM which can lead to destruction of normal renal architecture and organ failure (Khew-Goodall and Wadham, 2005).

1.5.3.2 Signal transduction pathways involved in renal EMT

Epithelial cells and mesenchymal cells represent two extreme cell types in the spectrum of cell classes and contain a number of distinguishable features. Epithelial cells can be characterised by: (a) forming continuous cell layers; (b) apical, lateral and basal membrane domains; (c) tight junctions; (d) intracellular organelle polarization; and (e) lack of mobility. This is in contrast to mesenchymal cells which (a) do not form continuous cell layers; (b) have no clearly demarcated membranes; (c) lack polarisation and (d); are motile (Larue and Bellacosa, 2005). For an epithelial cell to change its phenotype into that of a mesenchymal cell, as seen in the phenomenon of EMT, drastic molecular alterations are therefore required. This involves the binding of growth factors to epithelial ligand receptors which stimulate intrinsic kinase activity (Boyer et al., 2000; Fan et al., 1999; Morali et al., 2001; Okada et al., 1997; Strutz et al., 2002) and the subsequent recruitment of various signal transduction pathways.

TGFβ1 has been shown to be the key inducer of renal EMT, and has been shown to be involved in the sequential events of EMT including expression of mesenchymal markers, the down regulation of epithelial markers, induction of F-actin reorganization, up-regulation of basement membrane proteases and enhancement of cell migration and invasion (Fan et al., 1999; Strutz and Neilson, 2003; Strutz et al., 2002; Yang and Liu, 2001; Zeisberg et al., 2002). It is therefore clear that the majority of research to date has focused on elucidating the

role of the TGFβ-Smad signalling axis. However, given (a) the complexity of EMT, (b) the involvement of a plethora of cytokine and non-cytokine mediators and (c) that EMT has been shown to be induced independently of TGFβ1 (Burns et al., 2006; Li et al., 2004a; Li et al., 2004b), a number of signalling transduction pathways are likely to be involved in this process. To date, it is believed that in addition to Smads, members of the MAPK cascade and the Rho kinases play key roles in renal EMT.

The TGFβ signalling axis involves the Smad family which act by transducing signals of TGFβ from the cell surface to the nucleus. Of the Smads, a number of studies strongly suggest that Smad2/3 play key roles in TGFβ-induced EMT. The roles of these Smads are demonstrated in studies that have over-expressed Smad7, which is an endogenous inhibitor of Smad2/Smad3 phosphorylation. Over expression of Smad7 in tubular epithelial cells, inhibits EMT induced by TGFβ1 (Li et al., 2002) and αSMA induction (Valcourt et al., 2005). Conversely, over expression of Smad2/3 also leads to EMT, whereas, as expected, the BMP-specific Smads1/5 do not (Valcourt et al., 2005).

A number of studies have demonstrated the cross-talk between TGFβ signalling and members of the MAP kinase family in EMT including Ras, RhoA, MAPK-ERK kinase (MEK1/2) and extracellular signal related kinase (Erk1/2) (Bhowmick et al., 2001; Healy et al., 1999; Li et al., 2004a; Schramek et al., 1997; Xie et al., 2003). It is believed that these signalling pathways are involved mainly in cytoskeletal remodelling and the dissociation of epithelial cell adhesion during EMT.

GTPases of the Rho family have been implicated in the induction of stress fibre formation (Bhowmick et al., 2001), focal adhesions (Ridley and Hall, 1992), actin polymerisation associated with filopodia (Ridley and Hall, 1992) and E-cadherin clustering (Fukata and Kaibuchi, 2001; Ozdamar et al., 2005). Furthermore, loss of cell adhesion may also be facilitated by a change in GTPase activity (Zondag et al., 2000) and also by activation of Ras and Src pathways (Boyer et al., 2000) leading to the downstream recruitment of the

Raf/MAP kinases and the engagement of the EMT transcriptome. Furthermore, ERK has also been implicated in a number of studies and is thought to be involved in migration, conversion to an invasive phenotype and repression of E-cadherin (Grande et al., 2002b; Irie et al., 2005; Janda et al., 2002; Xie et al., 2004; Zavadil et al., 2001). The MAPK cascade may also induce EMT independently of TGFβ et al (Li et al., 2004b). In this study, AGEs induced receptor for AGEs (RAGE)-mediated EMT with expression of αSMA and loss of E-cadherin directly through the MEK1-ERK1/2 pathway. Furthermore, inhibition of ERK inhibits TGFβ induced Smad2 phosphorylation and the inhibition of p38MAP kinase and ERK abolishes TGFβ-induced EMT (Rhyu et al., 2005).

1.5.3.3 Transcription factors involved in EMT

The culminating effect of signalling pathways is to regulate the transcriptional activity of specific genes within the nucleus (Boyer et al., 2000). To date a number of factors have been identified including the integrin-linked kinase (ILK)-associated transcription factors lymphoid enhancer-binding factor (LEF) and β-catenin, and members of the *Snail/Slug* family of transcription factors. It is believed that these factors play roles in the suppression of E-cadherin expression (Arias, 2001; Zeisberg et al., 2003).

The importance of ILK in TGFβ-induced EMT has been shown by Li et al (Li et al., 2003) where over-expression of kinase-dead ILK abolished epithelial transition in a Smad dependent manner. ILK has also been shown to regulate the transcription factors, β-catenin and LEF. β-catenin normally mediates the contact of E-cadherin with the cytoskeleton (Zavadil and Bottinger, 2005) and stabilisation or degradation of this cytoplasmic protein can lead to repression of E-cadherin expression as demonstrated in TGFβ/Smad –mediated or integrin-mediated ILK activation inhibition of β-catenin/LEF translocation to the nucleus (Kim et al., 2002b; Labbe et al., 2000; Li et al., 2003; Tan et al., 2001).

This process is thought to involve the transcription factor *Snail* (Batlle et al., 2000; Grille et al., 2003), whose expression is ablated in Smad3 knock-out mice and in cells neutralised with antibodies to TGFβ (Sato et al., 2003). This transcription factor has also been shown to increase the expression of mesenchymal genes (Comijn et al., 2001). Furthermore, other transcription factors have been identified in EMT including *Hey1*, *Jag1* and *Notch*. TGFβ-induced EMT has been shown to be blocked by ribonucleic acid (RNA) silencing of *Hey1* or *Jag1*, and by chemical inactivation of *Notch* (Zavadil et al., 2004). Moreover, the transcription factor *Slug* induces the first phase of growth factor-induced EMT, including desmosome dissociation, and the initiation of cell separation (Savagner et al., 1997).

1.6 SIGNALLING IN FIBROSIS

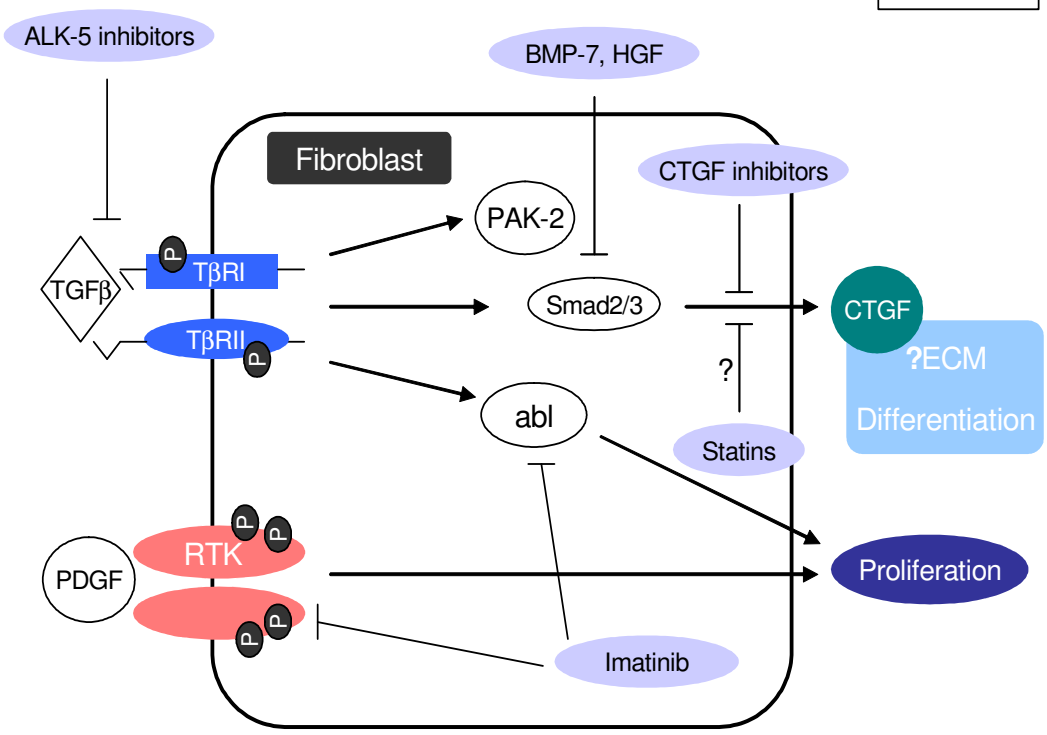
Because the signalling pathways that regulate fibroblast function are indispensable to renal fibrosis and the culminating effects of proliferation, differentiation and ECM synthesis can result in loss of kidney architecture and function, it is important to characterise the signalling pathways involved in the regulation of this key pro-fibrotic cell. Cell signalling entails those events at the membrane level where growth factors/cytokines bind membrane receptors, and the subsequent recruitment of intracellular signalling cascades which allow membrane bound receptors to dictate changes to nuclear gene expression patterns.

1.6.1 Membrane-bound receptors

Apart from those molecules that are lipid soluble, most regulatory molecules exert their effects on cells by binding to specific membrane receptors and stimulating intracellular signal transduction cascades. TGFβ, the most well-known agonist of renal fibrosis exerts its effects through pairs of type I and type II receptors (known respectively as TβR I and TβR II) and can be classified as serine/threonine kinase receptors. These receptors have been the target of small molecule inhibitors including activin receptor like kinase-5 (ALK-5) used to

down-regulate fibroblast function (Callahan et al., 2002)(*Fig. 1.11*). A number of other membrane-bound receptors may also dictate responses in fibrosis including (a) growth hormone receptors (eg. Jak/Stat pathway) (b) GPCR which can activate or inactivate plasma-bound membrane proteins or ion channels and can ultimately activate downstream cascades such as the MAPK cascade, and (c) RTK which act by phosphorylating tyrosine residues within the receptor domain and mediate a number of effects in fibrosis by responding to signals from agonists such as FGF, PDGF and EGF, and linking key downstream pathways such as Ras and PI3K. The latter two mechanisms are particularly interesting because (a) although GPCR are not well characterised in fibrosis, thrombin has been shown to activate members of GPCRs known as PARs on a number of renal cells and (b) although it is well-known that RTK can be activated in response to extracellular ligands, the intracellular signalling cascades are less well understood. Those receptors most well characterised in renal fibroblasts are outlined in *Fig 1.11.*

Fig. 1.11



Cell signalling mechanisms in renal fibroblasts and current intervention strategies. TGFβ signalling has been most well characterised in fibroblasts and is stimulated when the catalytically inactive TβRI is assembled with the TβRII upon ligand binding. This leads to activation of Smads 2/3, PAK-2 or abl, and subsequent differentiation, proliferation and ECM synthesis as well as the synthesis of CTGF. The other key membrane receptor family, RTK's, integrate a number of growth factor signals that are involved in fibroblast function including FGF, HGF, PDGF, EGF and IGF.

PAK-2 = p21 activated kinase-2.

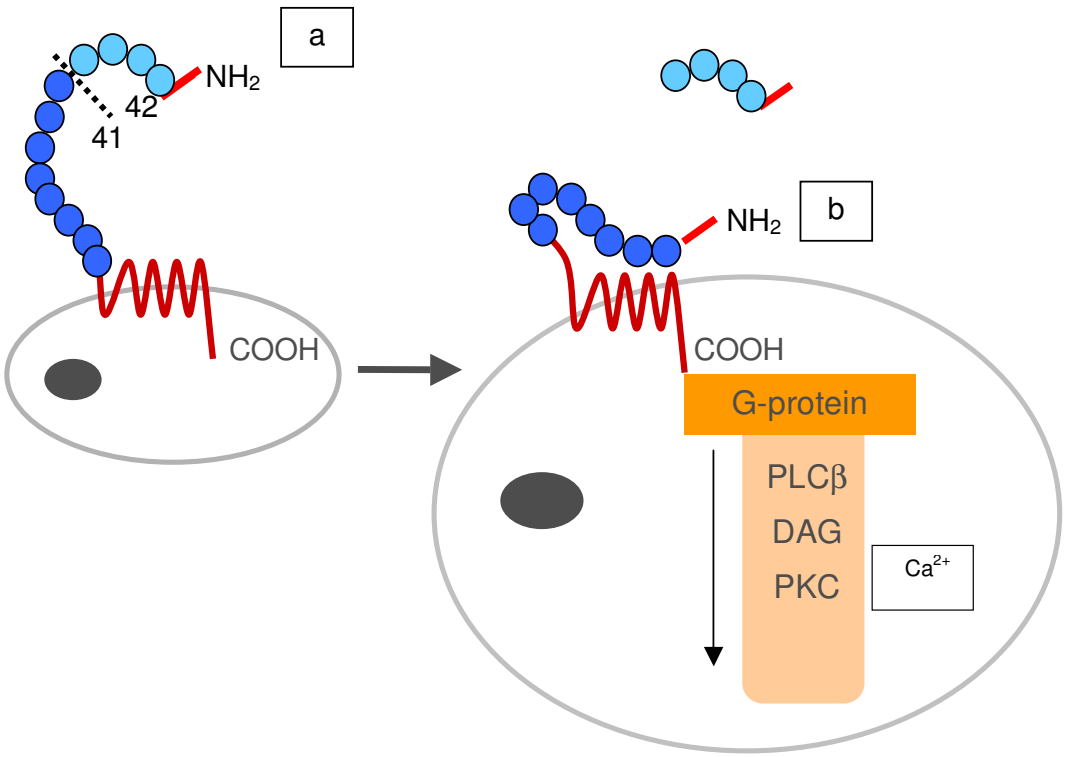
Adapted from Hirschberg and Wang, 2005.

1.6.1.1 Protease activated receptors

Thrombin and other serine proteases activate GPCRs belonging to the PAR family through a unique mechanism of proteolytic cleavage (Chambers and Laurent, 2002). To date, four PARs have been identified, PAR-1 to PAR-4, which can be distinguished by a unique N-terminal cleavage site. Thrombin can activate PAR-1, -3 and -4 (Chambers and Laurent, 2002) but not PAR-2 (specific for trypsin and other proteases) (Bradshaw, 2004b). PAR-1 and PAR-2 have been identified in the kidney (Grandaliano et al., 2003; Xu et al., 1995) with PAR-1 being found on endothelial cells, mesangial cells, epithelial cells and fibroblasts (Grandaliano et al., 2000b; Hewitson et al., 2005; Xu et al., 1995), and PAR-2 found in tubular epithelial regions (Grandaliano et al., 2003), specifically on the basolateral membrane of collecting ducts (Bertog et al., 1999). Although less is known about the respective roles and distribution of PAR-3 and PAR-4 (Vesey et al., 2005), it is thought that PAR-3 acts as a thrombin binding site rather than a fully activated receptor whilst higher concentrations of thrombin are required to stimulate PAR-4. The current literature has heavily focused on the role of thrombin acting through PAR-1 and therefore suggests that thrombin carries out most of its cell-mediated effects through this receptor.

PARs have evolved to respond through a common mechanism which has been most thoroughly elucidated by examining the action of thrombin through PAR-1. Once the protease binds to PAR-1, it cleaves the amino terminal exo-domain exposing the amino acid sequence SFLLRN *(Fig. 1.12a)*. This newly exposed N-terminus then acts as the tethered peptide ligand and binds to the heptahelical portion of the receptor to activate transmembrane signalling (Coughlin, 2000) *(Fig. 1.12b)*. When thrombin proteolytically activates PAR-1, downstream signalling is mediated through GPCRs. PAR-1 is capable of coupling a number of G proteins including members of the $G_{12/13}$, G_q and G_i families (Coughlin, 2000; Dery et al., 1998).

Fig. 1.12



Mechanism of action of PAR-1. Chiefly through Gα, PAR-1 can activate PLC-β leading to the formation of DAG with subsequent Ca^{2+} mobilisation and activation of PKC. This can activate members of the MAPK cascade and lead to mitogenesis, growth and transcription (Ossovskaya and Bunnett, 2004, Dery et al, 1998).
PLC = phospholipase C; DAG = diacylglycerol; PKC =protein kinase C; Ca^{2+} = calcium.

Studies have examined PAR-1 in renal disease. It has been demonstrated in crescentic glomerulonephritis that PAR-1 knock-out mice show greatly reduced glomerular lesions as compared to mice treated with thrombin receptor agonist peptide (TRAP) (Cunningham et al., 2000). Grandaliano has shown *in vivo* in human allograft nephropathy and *in vitro* that proximal tubular epithelial cells express PAR-1 in response to exogenous thrombin (Grandaliano et al., 2000b). Furthermore, the up-regulation of PAR-1 correlates with interstitial fibrin deposition, the severity of tubulointerstitial fibrosis and urinary excretion of TGFβ1 (Grandaliano et al., 2001), suggesting a possible role for PAR-1 in mediating fibrosis. However, when examined *in vitro* by utilising synthetic agonist peptides of PAR 1 and 2, it was shown that key fibrotic responses of tubular epithelial cell including the secretion of fibronectin, TGFβ1 and MCP-1 in response to thrombin was only reproduced by the PAR-2 synthetic peptide and not by PAR-1 (Vesey et al., 2005). This makes the role of up-regulated PAR-1 during fibrosis unclear, however recently it has been demonstrated that elevated expression of PAR-1 in the presence of thrombin, mediated the spread and migration of endothelial cells in association with the acquisition of mesenchymal characteristics (Archiniegas et al., 2004). This provides a possible unidentified role for PAR-1 in the regulation of thrombin-induced EMT.

1.6.1.2 Receptor tyrosine kinases

RTK's integrate a number of growth factor signals that are involved in fibrosis including FGF, HGF, PDGF, EGF and IGF. The commonality shared by each of these subgroups is the presence a protein tyrosine kinase domain in their intracellular portion. RTK also have a glycosylated extracellular domain, to which the appropriate growth factor binds, and a short transmembrane domain. Activation of RTKs leads to diverse biological responses including proliferation, differentiation, cell motility, changes in cell morphology, ECM

production and gene transcription, and many of these responses are central to fibrosis. When a growth factor binds to its receptor, this promotes dimerisation (formation of two stable receptor molecules) and auto-phosphorylation. The receptor then undergoes a conformational change or there may be the creation of binding sites for signalling molecules that interact specifically with phosphotyrosine residues. These new protein domains are often tyrosine kinase substrates and include Src homology 2 (SH2), phosphotyrosine, SH3 and pleckstrin-homology (PH) domains. Once the creation of signalling complexes has occurred, they often serve to recruit cytoplasmic enzymes to the plasma membrane and into close proximity to their membrane-related substrates.

A number of studies have highlighted the involvement of RTK and their ligands in fibrosis (Bonner, 2004; Chevalier et al., 2000; Rossini et al., 2005; Strutz et al., 2001; Terzi et al., 2000; Wang et al., 2005). Studies utilising imatinib, an inhibitor of the PDGF receptor (PDGFR) and the abelson non-receptor tyrosine kinase (abl), significantly reduce renal fibrosis *in vivo* (Wang et al., 2005). PDGF has also been shown to stimulate fibroblast proliferation independently of Smad2/3 (Wang et al., 2005).

1.6.2 Intracellular signalling cascades

Cell surface receptors do not directly regulate gene expression but rather they relay signals through intracellular signalling cascades. These cascades provide the link between extracellular signals and transcription/translation activation. The most studied intracellular signal transduction pathways in renal fibrosis are the TGFβ-Smad signalling axis, the ERK1/2MAPK cascade, and the Ras and Rho kinases.

In addition to membrane-bound serine/threonine kinase receptors, the TGFβ axis also includes the Smads, a family of conserved transcription factors that are intimately involved with mediating TGFβ1 responses. Formation of the heterodimer receptor complex leads to activation of receptor Smads (R-smads; Smads 2/3) and their subsequent translocation to the

nucleus with the cofactor of R-Smad (co-Smad; Smad 4). Smad2/3 have been shown to play key roles in fibrosis in a number of fibrotic models (Fujimoto et al., 2003; Ma et al., 2003; Sato et al., 2003) and in fibroblasts (Piek et al., 2001; Verrecchia et al., 2001; Yang et al., 2003). This axis is the central mediator of a number of key processes in fibrosis including fibroblast regulation and ECM synthesis, EMT, tubular apoptosis and chemotaxis (Border and Noble, 1997; Ma et al., 2003; Roberts, 1998; Roberts, 2002; Sato et al., 2003). Because TGFβ plays a key immuno-modulatory role, the Smad family have been a much more viable therapeutic target and a number of endogenous and exogenous inhibitors have been used to target this axis including HGF/BMP-7 (Liu, 2004b; Morrissey et al., 2002), CTGF inhibitors (Yokoi et al., 2004) and statins (Watts and Spiteri, 2004) *(Fig.1.11)*. There are also many other known regulators of key events in fibrosis which can mediate these effects independently of TGFβ (Burns et al., 2006; Hocevar et al., 1999; Li et al., 2004a; Ma et al., 2003; Piek et al., 2001), independently of Smads (Wilkes et al., 2005; Wilkes et al., 2003) or in concert with TGFβ signalling (Massague, 2000; Massague and Chen, 2000). Indeed, the induction of TGFβ signalling has been noted as a point of convergence through which the integration of many other pathways is likely to occur (Liu, 2006), and this has been demonstrated in a number of studies (Bakin et al., 2000; Bhowmick et al., 2001; Chen et al., 2002; Daniels et al., 2004; Fukami et al., 2004).

The ERK1/2MAP kinase family consists of the ERK pathway (Tian et al., 2000), and the stress activated pathways; the c-jun N-terminal kinase and the p38 pathways. This pathway is not only a likely point of cross-talk with the TGFβ axis (Engel et al., 1999; Funaba et al., 2002; Furukawa et al., 2003; Kretzschmar et al., 1999), but is also involved in fibronectin synthesis (Furukawa et al., 2003; Suzuki et al., 2004), proliferation and survival (Pat et al., 2003).

Ras/Rho are kinases that are involved in proliferation, growth and inflammation. Recent work suggests an extensive role for the small monomeric GTPases of the Ras

superfamily in fibroblast proliferation (Sharpe and Hendry, 2003) and also myofibroblast contraction (Hendry and Sharpe, 2003). In human fibroblasts, various Ras isoforms, including Ki-Ras, are involved in serum- and EGF-stimulated proliferation *in vitro* (Sharpe et al., 2000) and are therefore likely to be involved during the early stages of fibrosis as fibroblasts occupying the interstitium rapidly increase in number. Ras is also involved in inducing growth arrest and differentiation (Bar-Sagi and Feramisco, 1985).

Rho-associated coiled-coil forming protein kinase (ROCK) has been implicated in the progression of renal interstitial fibrosis. Inhibition of ROCK has been shown to reduce renal fibrosis *in vitro* and *in vivo* by suppressing αSMA expression, macrophage infiltration, TGFβ1 and α1(I) collagen mRNA expression in UUO (Nagatoya et al., 2002). Moreover, Rho has been shown to be necessary for TGFβ–mediated up-regulation of CTGF (Heusinger-Ribeiro et al., 2001), a key downstream mediator of TGFβ signalling, and is also involved in the regulation of αSMA microfilament contraction (Han et al., 2002).

The Janus family of tyrosine kinases (JAK) / Signal transducer and activator of transcription (Stat) pathway, a ubiquitous signalling cascade that is activated by growth factors and cytokines involved in proliferation and growth, has also been implicated in the regulation of fibroblast function. Huang et al (Huang et al., 1999) found that JAK and Stat1/3 mediate AGE-induced mitogenesis in kidney fibroblasts and collagen production (Huang et al., 2001). JAK2/Stat5 signalling has also been implicated in similar experiments involving AGE-induced fibroblast proliferation (Guh et al., 2001). Finally, Stat6-/- mice subjected to UUO exhibit decreased collagen synthesis (Yukawa et al., 2005).

1.6.2.1 Phosphatidylinositol 3-kinase

RTK provide the link between a number of key effectors in fibrosis and the intracellular signalling cascades that ultimately regulate gene expression. This receptor family can recruit the involvement of a number of downstream kinases including Ras and PI3K. Interestingly, there are a growing number of studies to suggest that PI3K and its downstream liaison mTOR act as central controllers of cell growth and proliferation. This suggests that these pathways may play roles in the regulation of fibroblasts during fibrosis, by either acting independently or in concert with TGFβ signalling.

PI3Ks are a family of serine/threonine kinases that play key roles in the regulation of growth factor signals involved in proliferation, cell survival, glucose metabolism and insulin signalling. These kinases phosphorylate the D3 position of the inositol ring of phosphoinositides. There are three classes of PI3K which are organised according to substrate preference and sequence homology (Engelman et al., 2006). The first class of PI3K's have been the most extensively studied. This class is divided into class IA and class IB, and whilst class IB is thought to be exclusively activated by GPCRs, class 1A is predominantly activated by RTKs. Class II PI3K can be activated by RTK, cytokine receptors and integrins however their specific functions are not well understood. The role of class III is also relatively unknown however the yeast homologue vacuolar protein-sorting defective 34 (Vps34) has been shown to regulate mTOR suggesting a role in the control of cell growth. Given the sparse availability on the latter two classes of PI3K, this review will focus on class I PI3Ks.

1.6.2.1.1 Structure of Class I PI3K

Class I PI3K is composed of families of homologous proteins containing regulatory (p85) and catalytic (p110) subunits, of which there are various isoforms of each subunit. The p85 regulatory subunit consists of a core p110 binding domain which is flanked by two SH2

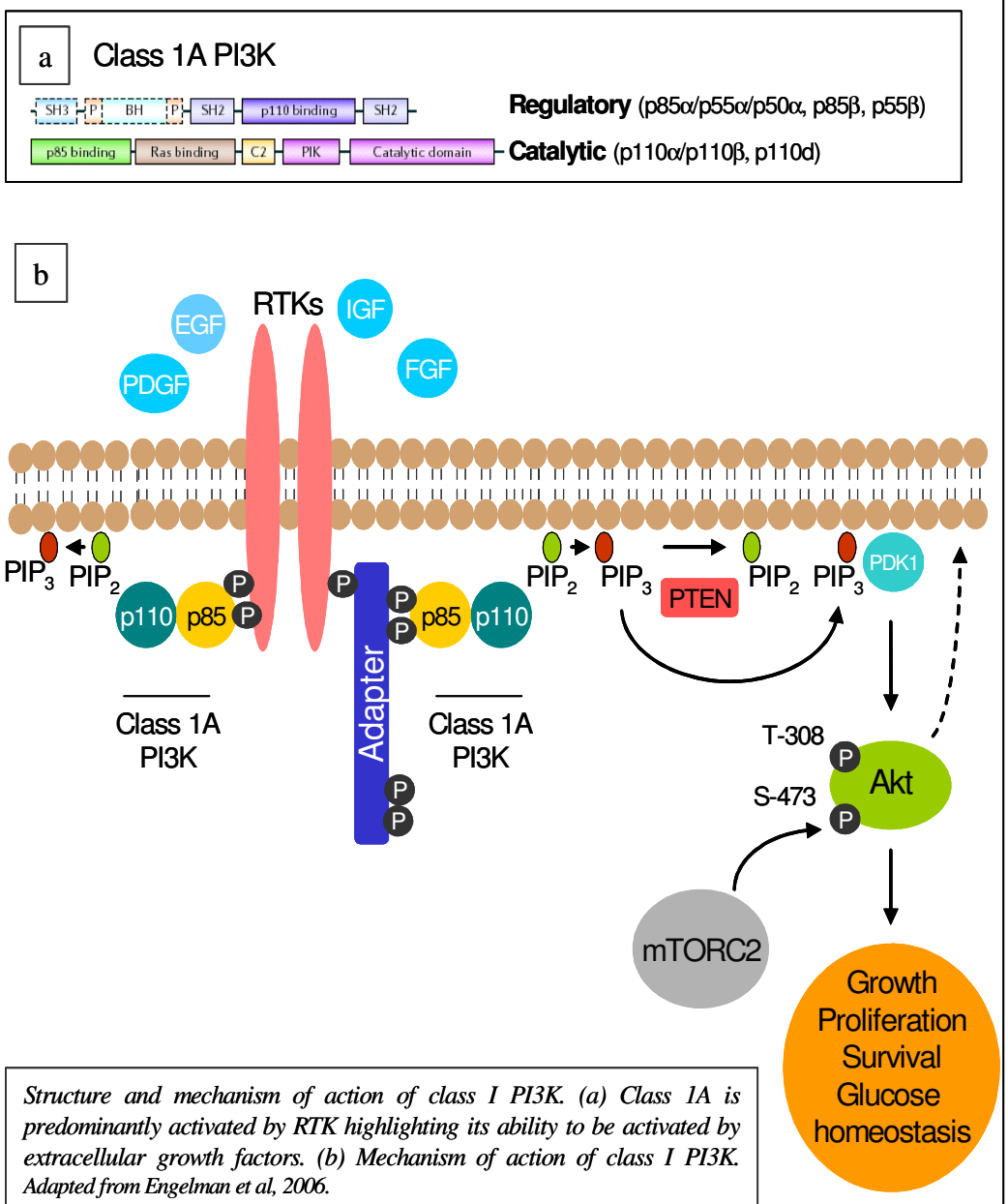
domains. The longer isoforms also have an extended N-terminal region (Engelman et al., 2006) (dashed line, *Fig. 1.13a).* The p110 catalytic subunit contains an N-terminal p85-binding domain, a Ras-binding domain (RBD), a C2 domain, a phosphatidylinositol kinase homology domain (PIK) and a C-terminal catalytic domain *(Fig. 1.13a)* (Engelman et al., 2006). In mammals, this class of PI3K controls glucose homeostasis, cell migration, growth and proliferation (Cantley, 2002).

1.6.2.1.2 Mechanism of action of PI3K

Class I PI3K primarily play the role of converting phosphatidylinositol-(4,5)-biphosphate (PIP_2) to phosphatidylinositol-(3,4,5)-triphosphate (PIP_3). As outlined in *Fig. 1.13b,* extracellular ligands such as PDGF, EGF, FGF or IGF, RTK bind to the glycosylated extracellular domain of RTKs which then become activated and dimerise (formation of two stable receptor molecules). Activation of RTK leads to the creation of signalling complexes with domains which often serve to recruit cytoplasmic enzymes to the plasma membrane into close proximity to their membrane-related substrates [231]. In particular, it allows for the recruitment of the regulatory p85 subunit, or adapter proteins such as insulin receptor substrate (IRS) to bind to phosphotyrosine residues of the SH2 domain of RTK. Binding in this manner relieves the basal inhibition that p85 normally exerts over the catalytic subunit, allowing the p110 catalytic subunit to become activated. This leads to the recruitment of PIP_2 to the plasma membrane and the generation of PIP_3 (negatively regulated by the phosphatase and tensin homologue tumour suppressor; PTEN). The generation of PIP_3 leads to the recruitment of molecules with a PH to the cell membrane including the serine/threonine acute transforming retrovirus thymoma (Akt) and phosphoinositide-dependent kinase 1 (PDK1).

Akt is a protein serine/threonine kinase that exists in mammalian cells as three different isoform proteins (Akt1, Akt2, Akt3) encoded by different genes (Hay and Sonenberg, 2004), and is composed of three functional domains: an N-terminal PH domain, a

Fig. 1.13



Structure and mechanism of action of class I PI3K. (a) Class 1A is predominantly activated by RTK highlighting its ability to be activated by extracellular growth factors. (b) Mechanism of action of class I PI3K. Adapted from Engelman et al, 2006.

kinase domain and a C-terminal hydrophobic motif (Scheid and Woodgett, 2003). When recruited to the plasma membrane, Akt is phosphorylated at serine-473 (S-473) within its hydrophobic motif by phosphoinositide-dependent kinase 2 (PDK2), recently identified as mTOR complex 2 (mTORC2) (Sarbassov et al., 2005). This preceded and facilitates the phosphorylation of threonine-308 (T-308) within its activation loop by PDK1. Once Akt is activated, it plays a role in the inhibition of apoptosis through the phosphorylation (and inhibition) of pro-apoptotic proteins such as Bad and the forkhead transcription factors, regulation of metabolism by phosphorylating glycogen synthase kinase 3 (GSK-3) (Downward, 2004), glucose metabolism, cell cycle progression and growth through a number of downstream effectors (Shaw and Cantley, 2006).

1.6.2.1.3 Roles of PI3K

LY294002 and wortmannin are the most widely used inhibitors to examine the role of PI3K in growth factor mediated signalling. Using these inhibitors, genetic deletion or through the knock out of various isoforms, PI3K has known to regulate several biological functions in addition to survival including proliferation, differentiation and ECM synthesis (Brennan et al., 1999; Gerasimovskaya et al., 2005a; Kanayasu-Toyoda et al., 2002; Krymskaya et al., 1999; Runyan et al., 2004; Shao et al., 2004). Studies have implicated PI3K in the regulation of T cell (Breslin et al., 2005), muscle cell (Derossi et al., 1998) and endothelial cell (Kanda et al., 1997) proliferation. More importantly, PI3K regulates fibroblast proliferation in *in vitro* studies on cultured human cardiac fibroblasts (Hafizi et al., 1999) and pulmonary artery adventitial fibroblasts (Gerasimovskaya et al., 2005b). Furthermore, PI3K can be activated by the pro-fibrotic molecules EGF, FGF and PDGF via RTKs to induce ECM deposition (Burgering and Coffer, 1995; Floege et al., 1999; Strutz et al., 2001; Tang et al., 1996) and has been shown to be involved in mesangial cell collagen synthesis (Runyan et al., 2004).

This suggests that this kinase may play a previously unrecognised role in fibroblast regulation.

1.6.2.2 Mammalian target of rapamycin

Signalling through the phosphorylation of Akt, PI3K is able to activate multiple effectors including mTOR (Raught et al., 2001; Scott et al., 1998). TOR is the founding member of the PI3K related protein (Ser/Thr) kinase family and was discovered fortuitously when the mechanism of action of rapamycin, a natural product of a strain of the soil bacterium, *Streptomyces Hygroscopicas*, was being investigated (Bradshaw, 2004b). mTOR regulates ribosome biosynthesis and cap-dependent translation through the downstream ribosomal protein S6 kinases (S6Ks) and the eukaryotic initiation factor 4E (eIF4E) binding proteins (4EBP1) respectively (Fingar and Blenis, 2004; Ponticelli, 2004), and is heavily involved in the regulation of cell proliferation, growth, glucose homeostasis and cell survival. This pathway contains a number of kinases that when mutated give rise to various cancers including lymphangioleiomyomatosis (LAM), tuberous sclerosis complex (TSC) (Ma et al., 2005), and Peutz-Jeghers syndrome (Inoki et al., 2005a). However, in the kidney this kinase is currently most-well known for as the key controller of T cell proliferation and hence is the target of the immunosuppressive drug rapamycin after transplantation.

1.6.2.2.1 Structure of mTOR

mTOR exhibits a 42% amino acid sequence identity to the yeast TOR protein and contains 2549 amino acids comprised of several conserved structural domains. The N-terminus contains 20 tandem HEAT (Huntingtin, EF3, A subunit of PP2A, TOR1) repeats at its NH_2 end, which are implicated in protein-protein interactions. Its C- terminal consists of a FAT domain and an FKBP12 receptor binding (FRB) domain (site of rapamycin-FKBP12 complex binding), a PI3K-like kinase domain (Hay and Sonenberg, 2004), a repressor domain

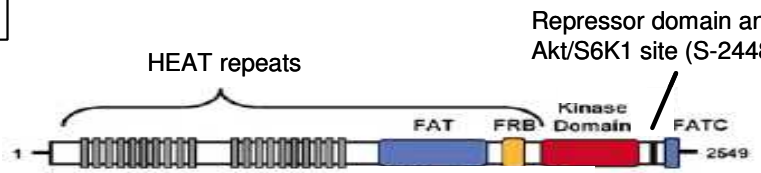
(S-2448) and a FATC domain (FAT and FATC may be involved in the regulation of kinase activity (Peterson et al., 2000) *(Fig. 1.14)*. The kinase domain of mTOR constitutes only approximately 10% of the total protein, and the *in vivo* function of the remainder is essentially unknown (Oldham et al., 2000).

1.6.2.2.2 mTOR forms two active complexes

Over the past five years, studies on mTOR signalling have distinguished two active mTOR complexes; mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is the complex originally identified as the target of rapamycin and has been more extensively studied. Relatively less in known about the functions of mTORC2 and its upstream inputs, however the regulatory proteins that form both complexes are becoming increasingly characterised. mTORC1 is formed by mTOR, regulatory associated protein of TOR (raptor) and GβL/mLST8 and is responsible for mediating mTOR's downstream effects *(Fig. 1.15a)*. The mTOR interacting protein raptor is required for mTOR mediated phosphorylation of 4EBP1 and S6K (Hara et al., 2002) and has been shown to be indispensable to mTOR function (Hara et al., 2002; Oshiro et al., 2004).

mTORC2 is formed by mTOR, rapamycin insensitive companion of mTOR (rictor) and GβL/mLST8 (Sarbassov dos et al., 2006) and a very recently characterised protein; mSin (mammalian stress activated protein kinase interacting protein) (Frias et al., 2006; Jacinto et al., 2006; Yang et al., 2006b) *(Fig. 1.15b)*. mTORC2 has been identified to date to play two key roles: (a) directly phosphorylating Akt S-473 (Sarbassov et al., 2005) and (b) in the regulation of cytoskeletal organization (Inoki et al., 2005b). This complex, in contrast to mTORC1, is insensitive to rapamycin (Sarbassov et al., 2004).

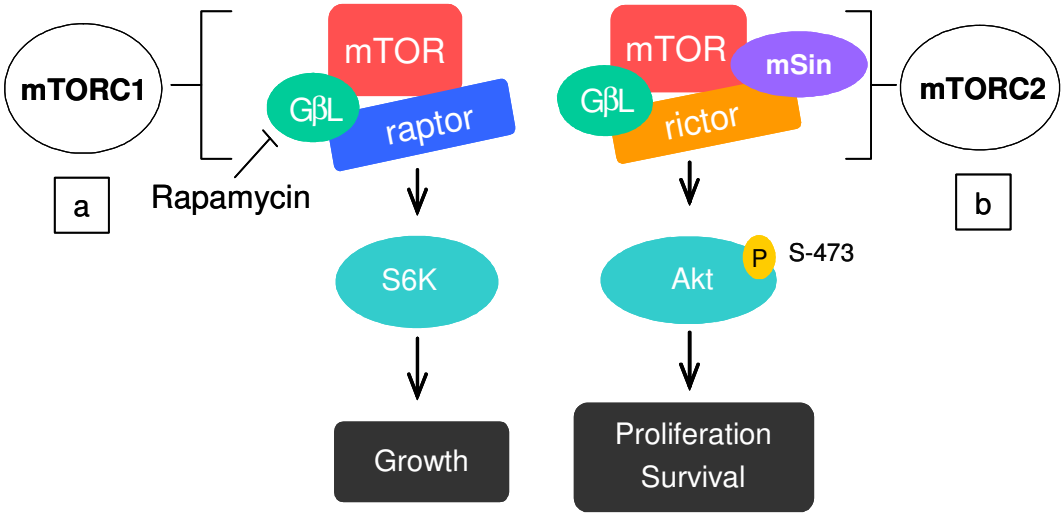
Fig. 1.14



Structure of mTOR. The repressor domain (S-2448) may be involved in a negative feedback loop with S6K (Chiang et al, 2005) and has also been characterised as an Akt substrate (Sekulic et al, 2000).

Adapted from Fingar and Blenis, 2004.

Fig. 1.15



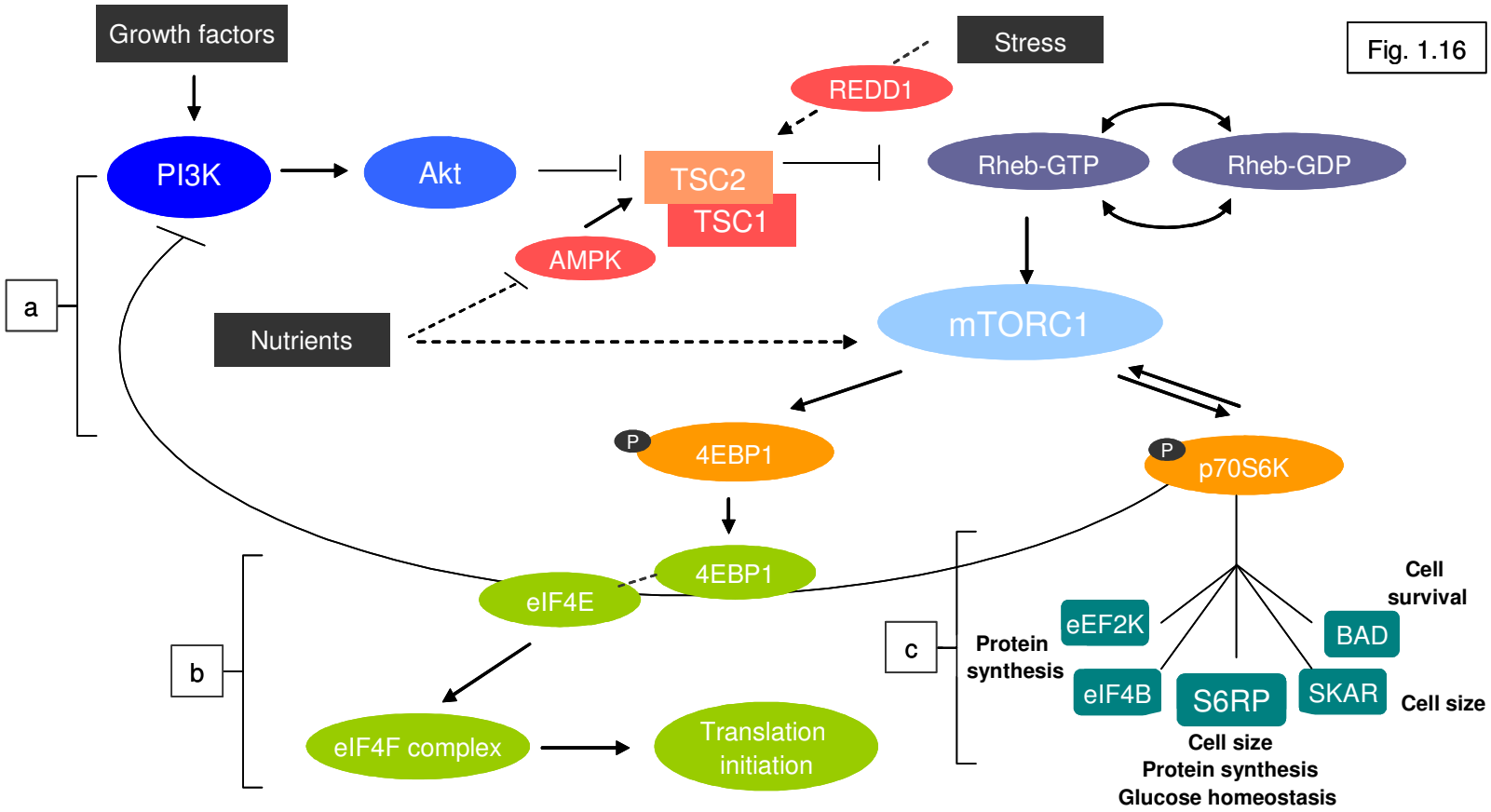
Composition of mTORC1 and mTORC2.

Adapted from Sarbassov et al, 2005.

1.6.2.2.3 mTOR signalling and key upstream regulators

mTORC1 signalling, and the activity of its downstream targets is dependent on the integration of intracellular stress (eg. osmotic stress; (Inoki et al., 2005b) or hypoxia (Arsham et al., 2003)), nutrients (eg. nitrogen and carbon sources; (Beretta et al., 1996)), energy (Hahn-Windgassen et al., 2005) and amino acid levels (eg. glutamine; (Crespo et al., 2002)). The loss of *d*TOR in *Drosophilia* results in phenotypes characteristic of amino-acid deprivation, including reduced nucleolar size, lipid vesicle aggregation, and cell cycle arrest (Zhang et al., 2000), and the withdrawal of amino acids within the media of mammalian cells leads to deactivation of S6K and de-phosphorylation of 4EBP1 (Hara et al., 1998). Similarly, glucose-deprived cells demonstrate severe inhibition of phosphorylation of T-36/45 of 4EBP1 (Patel et al., 2001). Whilst it has been established for many years that mTOR integrates these factors to control cell growth and proliferation, the underlying signalling mechanism is only beginning to be elucidated. It is now believed that the two tumour suppressor proteins, TSC1 (hamartin) and TSC2 (tuberin) (TSC1/2) are key integrators of converging mTOR signals (Inoki et al., 2002) *(Fig. 1.16a)*.

Originally identified as negative regulators of TOR signalling in *Drosophila*, (Gao et al., 2002), TSC1/2 is now thought to serve as a point of integration between growth-stimulatory and growth-suppressive signalling by modulating the activity of a small GTPase, Rheb (rapamycin homologue enriched in brain) (Saucedo et al., 2003; Stocker et al., 2003). Rheb acts as a means for integration of a variety of intracellular signals as well as growth factors (Inoki et al., 2005b), and cycles between a GTP-bound active state and a GDP-bound inactive state. When activated, Rheb phosphorylates mTOR and consequently activates downstream signalling. The state of Rheb is dependent on signalling upstream of TSC1/2 which receives a number of positive and negative input signals and in turn this regulates the GTPase activating protein (GAP) activity that is exerted over Rheb.



Mechanism of mTOR signalling and downstream effectors
(a) mTOR integrates stress, nutrients and amino acids with growth factors directly or through the tuberous sclerosis complex (TSC1/2) which plays a role in integrating converging mTOR signals. TSC1/2 controls mTOR activity by exerting GAP activity over Rheb which cycles between an active GTP-bound state and an inactive GDP-bound state. In cases of increased cellular stress (hypoxia) or decreased energy (?AMP:ATP ratio) these signals can be integrated via Redd1 and AMPK respectively ultimately activating the GAP activity of TSC2 leading to an accumulation of GDP-bound Rheb and inactivation of mTOR. mTOR also integrates growth factors through PI3K and Akt leading to inactivation of TSC2's GAP activity and ultimately to mTOR activation. mTOR carries out its effects through a number of downstream effectors including (b) 4EBP1 and (c) S6K (see text for details).

For instance, in cases of decreased availability of nutrients, amino acids, energy (↑AMP:ATP), or intracellular stress, these signals can converge onto the TSC2 complex and act to phosphorylate and activate TSC2. Recent studies have identified upstream signalling pathways involved in the transference of these signals to TSC2 including REDD1 (regulated in development and DNA damage 1; responsive to stress via hypoxia inducible factor; HIF) (Corradetti et al., 2005), and AMPK (adenosine monophosphate-activated kinase; responsive to decreased ATP) (Hahn-Windgassen et al., 2005). This allows TSC2 to exert its GAP activity over Rheb, leading to an accumulation of GDP-bound Rheb and inhibition of the downstream effectors of mTOR. In these cases, the net effect is the negative regulation of protein synthesis by mTOR and the up-regulation of autophagy (Dennis et al., 1999). In contrast, in response to growth factor signalling, mediated predominantly through PI3K and Akt, although this similarly phosphorylates TSC2, the phosphorylation inactivates its GAP activity leading to an accumulation of GTP-bound Rheb and activation of its downstream effectors *(Fig. 1.16a).*

Whilst TSC1/2 is likely to be a key integrator of converging mTOR signals, it should be noted that because TSC1 and TSC2 do not exist in budding yeast, these complexes including Rheb are unlikely to mediate all signals converging on mTOR (Sarbassov dos et al., 2005) and it is postulated that a number of direct inputs also control mTOR activity *(Fig. 1.16a).*

1.6.2.2.4 The relationship between PI3K and mTOR

In addition to the integration of stress, growth factors, amino acids and energy by mTOR, mTOR is also increasingly known for its ability to regulate growth factors through PI3K and Akt (Bradshaw, 2004b; Fingar and Blenis, 2004). These responses are similarly mediated through mTOR downstream effectors S6K and 4EBP1 (Bradshaw, 2004b). This is supported by the following findings: (a) Akt has been shown to directly phosphorylate mTOR at T-2446 and S-2448 (Sekulic et al., 2000); (b) Chung et al (Chung et al., 1994) has shown that mutated PDGF receptors that inhibit the activation of PI3K also inhibit the downstream phosphorylation of S6K1 and the use of PI3K pharmacological inhibitors also inhibit S6K and 4E-BP1 (Brunn et al., 1996; Cheatham et al., 1994); (c) if Akt is over-expressed, it promotes rapamcyin sensitive 4EBP1 phosphorylation (Gingras et al., 1998) and (d) over-expression of a dominant-negative form of Akt or deletion of Akt1 inhibits 4EBP1 and S6K1 phosphorylation respectively (Peng et al., 2003). Lastly, PTEN deficient cells (the negative regulator of PI3K signalling) have a high level of 4EBP1 and S6K1 phosphorylation (Neshat et al., 2001). This suggests that *d*TOR is at least is epistatic to *d*PTEN (Oldham et al., 2000). These and other studies strongly implicate PI3K as a growth factor-mediated regulator of mTOR and its downstream kinases.

1.6.2.2.5 Downstream targets

mTOR regulates ribosome biosynthesis and translation through the downstream ribosomal protein S6Ks and the eIF 4EBPs (Fingar and Blenis, 2004; Ponticelli, 2004). It is thought that raptor facilitates this process by acting as a scaffold protein and physically linking S6K and 4EBP1 to mTOR allowing these substrates to be phosphorylated within their TOR signalling (TOS) motif (Inoki et al., 2005b).

Mammalian cells express two forms of S6K called S6K1 and S6K2. These kinases are thought to be major regulators of the S6 ribosomal protein (S6RP) substrate. These

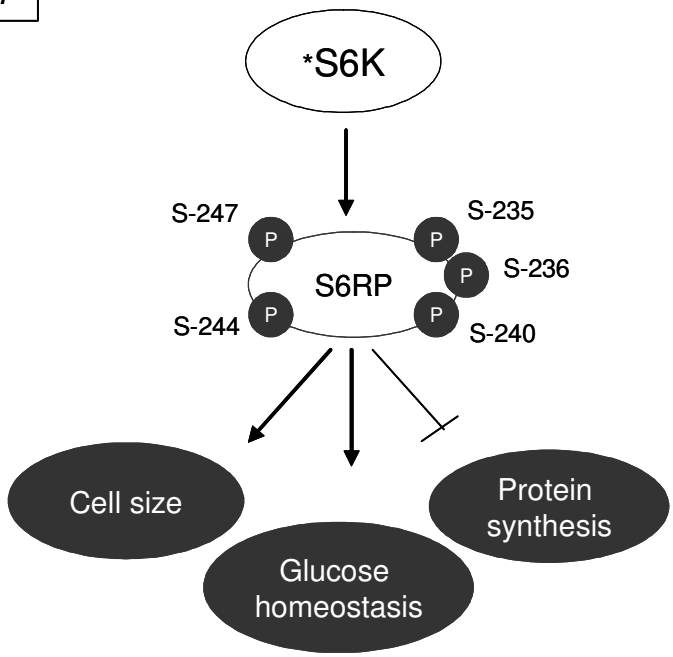
isoforms share a high degree of homology and the second form of this kinase was originally identified in mice which displayed a sustained S6RP phosphorylation in response to deletion of the S6K gene (Shima et al., 1998). Full phosphorylation of S6RP is thought to be carried out by this more recently identified kinase (Ruvinsky and Meyuhas, 2006).

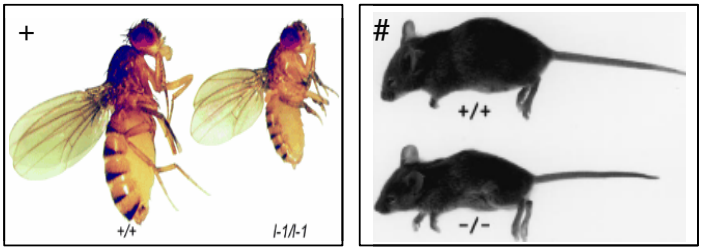
Kim and colleagues have shown that through the knockdown of mTOR, the activation of S6K is repressed (Kim et al., 2002a), and over-expression of mTOR increases the phosphorylation of S6K (Brown et al., 1995). Studies such as these have therefore characterised S6K as a key downstream effector of mTOR (Harrington et al., 2004) and have implicated it as a key regulator of cell size. *Drosophila* deficient in the *d*S6K gene exhibit a large delay in development and a severe reduction in body size without affecting cell number (Montagne et al., 1999), whilst the growth of mice with homozygous disruption of S6K is also largely retarded (Ruvinsky et al., 2005) *(Fig. 1.17)*.

This kinase is also involved in the regulation of a number of downstream effectors of mTOR including BAD (Harada et al., 2001), SKAR (S6K1 Aly/Ref-like target) (Richardson et al., 2004), eukaryotic elongation factor 2 kinase (eEF2K) (Wang et al., 2001b), eIF4B (Raught et al., 2004), IRS (Harrington et al., 2004) and mTOR itself (Chiang and Abraham, 2005). These substrates are involved in regulating cell survival (BAD), cell size (SCAR) and protein synthesis (eEF2K and eIF4B), whilst IRS and mTOR are thought to be part of reciprocal negative feedback loops, allowing S6K to regulate upstream signalling (Sekulic et al., 2000) (Manning, 2004)(refer to *Fig. 1.16c*). The main substrate of S6K however is S6RP which is often used as a read-out of mTOR activity and is thought to contribute up to 30% of total cellular protein (Toker, 2000).

S6K and S6RP have traditionally thought to be involved in the control over mRNA transcripts with a 5' tract of pyrimidine (TOP) motif which encode various proteins associated with the function or the assembly of the translational apparatus (Jefferies et al., 1997; Krymskaya et al., 1999; Lane et al., 1993). This has been largely based on the temporal

Fig. 1.17





Roles of S6RP as a downstream effector of mTOR. S6RP contains 5 phosphorylation sites of which Ser235/236 are major mTOR substrates. S6RP is thought to play key roles in the regulation of glucose homeostasis, protein synthesis and cell size. Knock-out of the dS6K gene leads to a marked reduction in the size of Drosophilia (+Montagne et al, 1999) and mice with homozygous disruption of S6K retards growth by approximately 20% (#Shima et al, 1999). These growth defects are attributed to a reduction in cell size not cell number.

**Adapted from Ruvinsky and Meyuhas, 2006.*

activation between these effectors and the translational activation of TOP mRNAs, leading to the formation of a model that related translational efficiency of TOP mRNAs to S6RP phosphorylation (Jefferies et al., 1994).

Recently it has been shown that both S6K and S6RP are disposable for these roles (Ruvinsky and Meyuhas, 2006; Ruvinsky et al., 2005). By knocking in alanine substitutions at all five phosphorylatable serine residues of S6RP (Ruvinsky et al., 2005), and knocking out S6K1 and S6K2 in mice (Pende et al., 2004), it provides compelling evidence that the primary role of these downstream effectors is not in the control over mRNA transcripts. It is now believed that the key roles of S6RP include the negative regulation of protein synthesis (because the global rate of protein synthesis is higher in S6RP-/- knockout mice than in wild type mice (Ruvinsky et al., 2005), in the modulation of glucose metabolism and in the regulation of cell size (Ruvinsky et al., 2005) (*Fig. 1.17*).

4EBP1 is the other main downstream effector of mTOR. The initiation of translation of nuclear transcribed eukaryotic mRNAs is dependent on the cap binding protein, eIF4E, and the eIF4 initiation factors. When mTOR is activated directly or through Rheb, it primarily controls these initiation factors by regulating 4EBP1 (Brunn et al., 1997). The phosphorylation of this downstream effector is inhibited by rapamycin, preventing cap-dependent translation by the eIF4E family of translation factors (Beretta et al., 1996; Gingras et al., 1998; Graves et al., 1995), whilst over-expression of FK506 (a molecule that competes for binding with rapamycin to FKBP12) reverses the rapamycin-mediated inhibition of 4E-BP1 phosphorylation (Beretta et al., 1996).

The regulation of the eIF family of translation factors depends on 4EBP1's phosphorylation state. Phosphorylation of 4E-BP1 prohibits its association with eIF4E, allowing eIF4G to bind to eIF4E and form the active eIF4F complex (during translation eIF4G functions as a complex with eIF4A and eIF4E; called eIF4F) *(*refer to *Fig. 1.16b)* (Bradshaw, 2004a). Hypophosphorylation of 4EBP1 however promotes the binding of 4E-

BP1 with eIF4E, preventing the initiation factor from forming the translational complex and in these cases 4EBP1 acts as a translational repressor (Inoki et al., 2005b). The role of 4E-BPs during translation is therefore to regulate this cap-dependent translation by competing with eIF4G for binding with eIF4E.

1.6.2.2.6 Functional roles of mTOR - Rapamycin

Studies of mTOR have heavily relied on rapamycin and its derivatives (CCl779, RAD001, AP23573), as TOR mutations have not yet been reported in multicellular organisms (Zhang et al., 2000). These pharmacological inhibitors have been shown to be highly specific and bind mTOR by forming a complex with intracellular FKBP12. This complex then binds to the region upstream of the C terminus called the FRB region and acts to inhibit the activity of mTOR. However, the exact mechanism of inhibition is unclear.

mTOR plays a chief role in cell cycle progression from the G1-to-S phase (Abraham, 2005) predominantly by regulating levels of cyclin D and $p27^{kip}$ polypeptides. mTOR has therefore been implicated in the control of IL-2 stimulated T-cell proliferation as shown by p27 knockout mice (Cohen, 2002; Nourse et al., 1994), VSMC, endothelial cell (Mohacsi et al., 1997), hepatic stellate cell (Zhu et al., 1999) and human renal epithelial cell proliferation (Pallet et al., 2005), as well as bile duct hyperplasia (Biecker et al., 2005). Accordingly rapamycin induces cell-cycle arrest and is prescribed as an effective immunosuppressant after kidney transplantation (Breslin et al., 2005). The potential roles for rapamycin have however grown over the past decade. Rapamycin is now also used to inhibit vascular stent occlusion (Marx and Marks, 2001), and mTOR inhibitors are also being widely tested in clinical trials for the treatment of cancer (Cully et al., 2006; Hidalgo and Rowinsky, 2000; Panwalkar et al., 2004; Sawyers, 2003). Moreover, very recent studies have established the potential benefit of rapamycin in insulin resistance (Shah and Hunter, 2006; Um et al., 2004), and renal (Chen et al., 2005; Sakaguchi et al., 2006) and heart (Boluyt et al., 2004) hypertrophy.

Emerging studies have also highlighted its potential use as a therapeutic agent in fibrosis. mTOR is involved in the regulation of mesangial cell proliferation (Daniel et al., 2000), PDGF-induced cardiac fibroblast proliferation (Simm et al., 1997) and lung fibroblast proliferation after transplantation (Azzola et al., 2004). In addition, it has been established that differentiation and ECM synthesis is regulated by mTOR and S6K in VSMC (Martin et al., 2004). *In vivo* studies have shown that rapamycin inhibits liver fibrosis (in bile-duct ligation (Biecker et al., 2005) and carbon tetrachloride models (Zhu et al., 1999) by decreasing key mediators of fibrosis including TGFβ1. The potential for rapamycin to inhibit renal fibrosis has been demonstrated in a model of renal ischaemia reperfusion injury where a reduction in the expression of fibrosis-associated genes was demonstrated (Jain et al., 2001). Together, these studies underscore the importance of fully elucidating all dimensions of mTOR and PI3K function in key fibrogenic mediators.

Given that rapamycin and its derivatives have been shown to inhibit a variety of functions potentially relevant to fibrosis including proliferation and differentiation, ECM synthesis, and TGFβ1 expression in a number of studies, an investigation into the role of mTOR and PI3K in the regulation of renal fibroblast function, a key mediator of fibrosis is now warranted.

1.7 AIMS AND RATIONALE

Tubulointerstitial fibrosis is the final common pathway all of types of progressive renal disease. As evident from the previous passages, the interstitial fibroblast is a key effector cell in fibrosis responsible for the secretion of exuberant amounts of ECM proteins that can eventually lead to the demise of renal structure and function. This thesis therefore contends to further elucidate the molecular mechanisms encompassing the derivation and regulation of the renal fibroblast.

In summary, specific aims of this study include:

1.7.1 Are PI3K and mTOR involved in the regulation of renal fibroblasts?

Whilst it has been established that the TGFβ signalling axis is a key regulator of fibroblast activation and ECM synthesis, it is likely that other signalling pathways are involved consistent with the complexity of fibrosis. Given that the involvement of other signalling pathways are not well characterised, the aim of this study is to examine the potential of PI3K and mTOR to regulate renal fibroblast function.

1.7.2 Do PI3K and mTOR regulate myofibroblast differentiation and EMT?

The differentiation of fibroblasts into myofibroblasts is a key indicator of the severity of tubulointerstitial fibrosis. It is therefore important to identify the regulatory mechanisms involved in differentiation, in addition to the TGFβ signalling axis. Given that PI3K and mTOR are implicated in the differentiation of VSMC (which share inherent similarities to fibroblasts), this study aimed to investigate the role of PI3K and mTOR in myofibroblast differentiation.

The importance of EMT in renal tubulointerstitial fibrosis is becoming universally accepted underscoring the need to delineate the regulatory and signalling mechanisms involved in this process. The aim of this study is also to use a model of EMT to study

regulatory mechanisms of EMT including the elucidating the role of PI3K and mTOR in renal EMT.

1.7.3 Is there a relationship between the coagulation cascade and tubulointerstitial fibrosis?

In addition to their role in clotting, the coagulation cascade has been implicated in renal disease. Whilst more heavily elucidated in glomerular-based lesions, less is known about their roles in tubulointerstitial fibrosis. The aim of this work is to understand the role that members of the coagulation cascade may play in tubulointerstitial fibrosis.

Chapter 2

General Methods

2.1 INTRODUCTION

This chapter describes those general methods which have been used across experimental chapters[*]. Outlined in this chapter are (a) a description of the procedure of UUO, (b) the generic procedure of explanting to obtain cells for experimental purposes, (c) cell culture protocols, (d) immunohistochemistry protocols and (e) protocols for biochemistry studies (Northern blotting, Western blotting and Real time PCR).

Details of the specific experimental design are included after the introduction of each experimental chapter whilst those methods which were specific to the chapter are included in a section labelled *Specific Method Protocols,* located at the back of each experimental chapter. A detailed description of material derivation, reagents and stock solution protocols, as well as antibody specifications used throughout this thesis can be found in the relevant Appendices: *Appendix 1: Materials and Suppliers; Appendix 2: Reagents*, *Buffers and Stock Solutions and Appendix 3: Primary and Secondary Antisera.*

2.2 EXPERIMENTAL MODELS

Experiments in this thesis utilised cells and tissue from UUO *(2.2.1)* and explant tissue from the rat normal renal cortex *(2.2.2)* of Sprague-Dawley rats.

2.2.1 Experimental tubulointerstitial fibrosis

All experimental chapters apart from *Chapter 4* have utilised cells or tissue derived from UUO at 3 days post-ligation. UUO was performed in rats in order to mimic the pathological changes that occur in progressive kidney disease in humans. UUO is amongst the most common experimental models of interstitial fibrosis (Bascands and Schanstra, 2005) (Klahr and Morrissey, 2002). This model was chosen because as a result of stress-induced injury, it encompasses the various stages of renal injury leading to an accelerated form of

[*] To avoid long descriptions in experimental chapters, Northern blotting is an exception and has been included within this chapter.

tubulointerstitial fibrosis without the complications of glomerular disease. This includes cellular infiltration, tubular proliferation and apoptosis, EMT, myofibroblast accumulation, increased ECM deposition, and tubular atrophy *(Fig. 2.1)* (Bascands and Schanstra, 2005). Accordingly, fibroblasts derived from this model are activated in a similar manner of the *in vivo* equivalent (Muller and Rodemann, 1991).

Under general anaesthesia, the left ureter of male Sprague-Dawley rats (250-300g) was ligated in two places using 5/0 gauge surgical silk and cut in-between. The contralateral ureter was left intact. Animals were sacrificed after 3 days for the retrieval of tissue for cells, and at various intervals between 3-21 days for histological studies (*Chapter 6*). The procedure was approved by *The Royal Melbourne Hospital Animal Ethics Committee* and was conducted in accordance with the NHMRC *Code of Practice for the Care and Use of Animals in Research.*

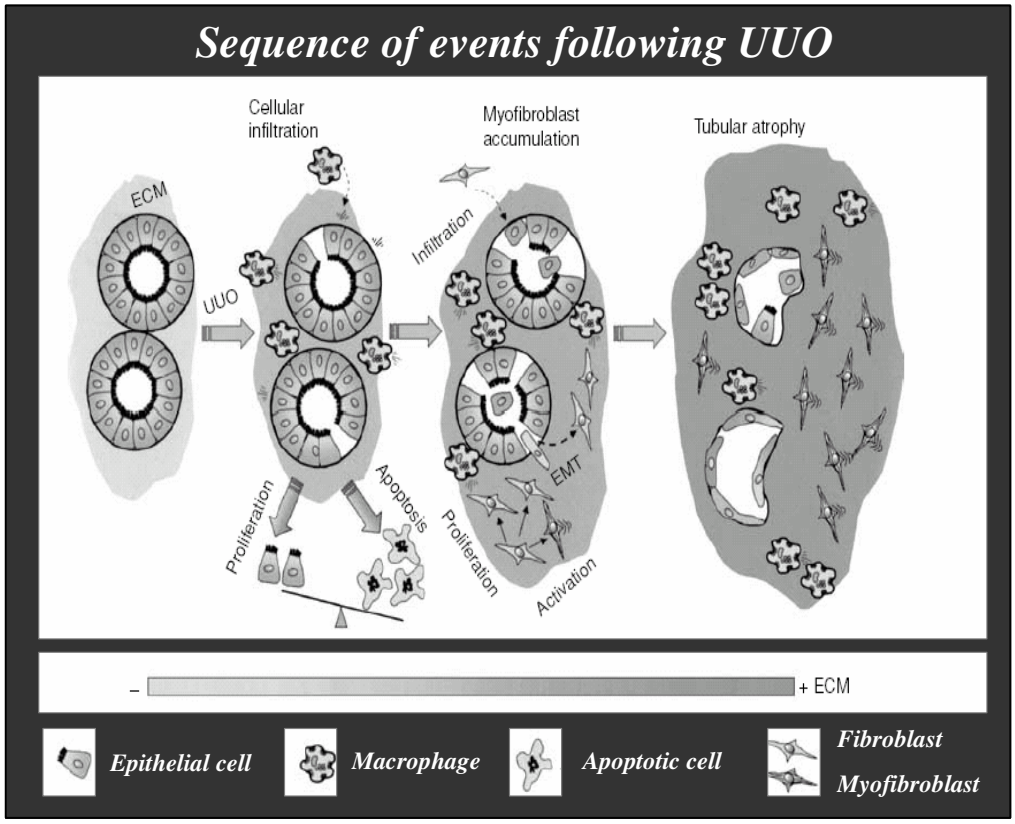
2.2.2 Explants from Sprague-Dawley rats

Kidney cortical tissue for explants was derived from normal kidneys of Sprague-Dawley rats killed by asphyxiation with an 80%:20% mixture of CO_2:O_2. Tissue was collected in ice-cold Hanks' salt solution containing gentamycin. Tissue and cells obtained from these kidneys were the basis for studies on EMT (used in *Chapter 4-6*). The procedure was approved by *The Royal Melbourne Hospital Animal Ethics Committee* and was conducted in accordance with the NHMRC C*ode of Practice for the Care and Use of Animals in Research.*

2.3 EXPLANTING OF THE RENAL CORTEX

Explanting served a dual purpose in this thesis: (a) to obtain cells and tissue from fibrotic kidneys and (b) to examine cells grown from normal rat renal kidneys in studies of EMT. In each case the same methodology was used, albeit using different tissue. The following passages outline the general technique of tissue explanting and any subsequent adaptations.

Fig. 2.1



UUO as an experimental model of tubulointerstitial fibrosis. UUO represents a form of obstructive nephropathy and although rarely seen in adults, it is one of the rare experimental models of primary tubulointerstitial disease.

Adapted from Bascands et al, 2005.

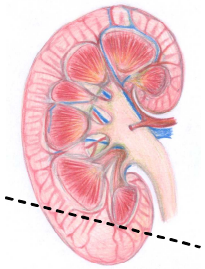
2.3.1 Explanting procedure

Petri dishes were prepared by coating each dish with warm 1% gelatin matrix (diluted at a ratio of 1:1 in phosphate buffered saline; PBS). 1ml of gelatin solution was delivered into each petri covering the entire surface evenly, and washed with 2-3ml of PBS. Petris were then incubated at 37°C for at least 30min. After the preparation of Hanks buffer solution with gentamycin, a sterile dressing pack containing sterile gauze pieces, forceps and tray was used to explant the tissue.

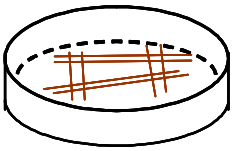
Using sterile gauze, the kidney was orientated to expose the cortex and a midline incision using a scalpel blade was made. The capsule was then pinched back. The cortex was removed and placed into the gelatinised petri dishes where it was then minced and embedded into the gelatin in a criss-cross fashion. Approximately 2ml of Dulbecco's modified eagle's medium (DMEM) plus fetal calf serum (FCS) was then added to the explanted tissue and the petris were placed into a 37°C 5%CO_2/95%O_2 incubator overnight (O/N). The next day, an additional 2ml of medium was added to the tissue preparations and petris were left at 37°C. Explant outgrowths to be used for the *ex vivo* model of EMT were fixed in cold methanol at 3-17 days post-explanting *(section 2.3.2)* by flooding petris with cold methanol for 10min and then air-drying. Cells obtained from UUO to be used for *in vitro* studies were propagated for 17-21 days in DMEM + 10%FCS before being passaged and transferred to 25cm^2 flasks cells, to be used for subsequent experiments *(section 2.4).* The explanting methodology is summarised schematically in *Fig. 2.2.*

Fig. 2.2

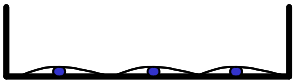
Explanting procedure of the renal cortex



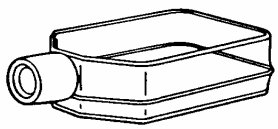
Remove cortex

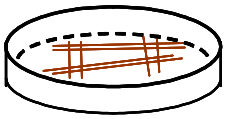


Mince and embed tissue



Primary cell outgrowth





Subculture cells (UUO)

Fix in cold methanol (ex-vivo)

Explanting procedure of the renal cortex. Those cells obtained for in vitro studies from UUO were sub-cultured (left) whilst cells grown out of explants in the ex-vivo model of EMT were fixed at various time points post-explanting (right).

2.4 CELL CHARACTERISATION

2.4.1 Characterisation of cells obtained from UUO

Sub-cultured cells grown from fibrotic kidneys subjected to UUO were routinely characterised by immuno-cytochemistry to confirm the propagation of fibroblasts. Standard staining techniques (*section 2.6.1*) were used to identify positive and negative staining for phenotypic markers (*Table 2.1*), as described previously (Hewitson et al., 2000b).

Antisera	**Specificity**
Vimentin	Mesenchymal cells (smooth muscle, fibroblasts, de-differentiated epithelium)
αSMA	Smooth muscle cells, myofibroblasts
Desmin	Mesangial cells
Collagen III	Mesenchymal
Pan-keratin	Epithelial cells
RECA	Endothelial cells

Table 2.1: Specificity of antisera used**.** RECA = Rat endothelial cell antigen

Cells consistently stained for vimentin and collagen III, 40-50% for αSMA, approximately 10% for desmin. Only very occasional cells stained for pan-cytokeratin or rat-endothelial cell antigen (RECA) (<1%). On this basis, cells were classified as fibroblasts, a proportion of which showed myofibroblast differentiation.

2.4.2 Characterisation of cells derived from normal Sprague-Dawley rats

Cells from normal kidney explants were phenotyped by immunocytochemistry for αSMA, FSP1, desmin, RECA, E-cadherin and cytokeratin (*Table Appendix; Apx. 3.1*) to determine the composition of cells that grow out of explants. Standard staining techniques (*2.6.1*) were used to identify positive and negative staining for phenotypic markers.

2.5 PASSAGING, FREEZING, THAWING and COUNTING of FIBROBLASTS DERIVED from UUO

2.5.1 Passaging

For cells undergoing the first passage, media was discarded and the explant layer washed with 2ml sterile PBS. 1ml of trypsin was added and left for 15min at 37^{o}C. After 15min, 2ml of medium was added to each explant and the remaining cells that did not dislodge were gently removed with a sterile cell scraper. The cell suspension was then transferred to a 25cm^2 flask and approximately 7ml of fresh medium was added. For all other passages, 2ml of trypsin was added to confluent flasks and left for 15min at 37 ^{o}C. Five ml of medium was then added and cells were centrifuged at 5000rpm for 10min. Cell supernatant was then removed and cells were resuspended in 10ml DMEM. Cells were then either seeded for experiments or placed into new flasks and left at 37 ^{o}C.

2.5.2 Freezing and thawing

Nine ml of medium was aliquoted into a sterile 10ml tube with 1ml of dimethyl sulphoxide (DMSO). This was placed in a freezer or on ice until ready to use. Cells were passaged as per normal and slowly resuspended in the prepared DMSO/medium mixture. The cells were then transferred to sterile nunc tubes, wrapped in tissue paper and placed at -80°C, to be later transferred to liquid nitrogen. When cells required thawing, 9 ml of medium was aliquoted into a falcon tube and warmed in the water bath. The frozen cells were then thawed at 37°C and the contents were then placed into the sterile tube. The pre-heated medium was then slowly added with gentle agitation to resuspend the cell solution. The cells were then transferred to a flask and left for at least 4-6hr for cell attachment. The next day, fresh medium was added to remove any remaining traces of DMSO.

2.5.3 Cell counting

Cells were quantified with a 1/400mm haemocytometer in order to seed cells at a specific density. 100µl of cell solution was placed into a test tube and diluted 1:1 with 100µl trypan blue (to exclude dead cells).

Cell number was found by:

$$\text{Cell no.} = x / Sq \times 10^4 \times D \times V$$

where

x = number of cells

Sq = number of large squares counted

D = dilution factor

V = Volume remaining (ml)

The total number of cells to seed per well was found by:

$$x = \frac{\text{Density} \times \text{volume remaining}}{\text{Total cell number}}$$

The appropriate number of cells were seeded into petri dishes / six well plates and the desired medium added to each well. Cells were then left in the incubator O/N at 37°C to adhere.

2.6 IMMUNOHISTOCHEMISTRY and CYTOCHEMISTRY

2.6.1 Cytochemistry (Immunoperoxidase)

Cells were grown on coverslips and fixed in methanol. Standard Avdin-biotin-complex (ABC) techniques were used to stain cells using the antisera in *Table Apx. 3.1* (Hewitson et al., 2001). In each case, cells were washed in PBS, blocked with normal species relevant serum, and incubated with primary antisera for 1hr. Following incubation, coverslips were washed in PBS, incubated with the appropriate biotinylated secondary antibody *(Table Apx. 3.2)*, washed in PBS, incubated with ABC and exposed to diaminobenzidine (DAB)

chromagen for 2-5min. Finally, cells were washed in water, counterstained with Harris' haematoxylin and mounted with Gurr Aquamount.

2.6.1.1 Enumeration of cells

In cases where cells were enumerated based on staining patterns, the cells within a number of random fields were counted. A real representation of the staining pattern was gained by counting between 800-1000 cells per slide throughout a number of randomly chosen fields. Slides were counted blindly. In the majority of cases, results are expressed as a percentage of control of total cells counted.

2.6.2 Immunohistochemistry

2.6.2.1 Paraffin-embedded rat tissue sections

Embedded sections were placed at -20°C prior to cutting and were then cut using a microtome. Tissue was then placed into water, alcohol and immersed in water at 40°C. Sections were then removed, placed on silane-coated glass slides and air-dried. Slides were then placed in an incubator O/N at 42°C, baked at 60°C for a further 45min, and then stored for future use.

2.6.2.2 Immunohistochemistry of tissue sections

Immunohistochemistry was carried out on paraffin embedded sections by using antibodies against fibrinogen/fibrin, factor V and αSMA (*Table Apx. 3.1-3.2)* as described previously (Hewitson et al., 1998).

2.7 BIOCHEMISTRY STUDIES: NORTHERN BLOTTING, WESTERN BLOTTING, REAL TIME PCR

2.7.1 Extraction of cellular protein, DNA and RNA

To obtain cellular RNA, DNA and protein from fibroblasts for Northern blotting, real time PCR and Western blotting, TRIZOL reagent was utilised according to the manufactuer's instructions. An RNA clean-up kit was also used to remove proteinaceous material or DNA for the preparation of samples for real time PCR as per the guidelines.

2.7.2 NORTHERN BLOTTING

Samples were centrifuged at 12000 rpm for 20min at 4°C and the pellets washed in 1ml cold 75% ethanol. Pellets were then vortexed, centrifuged at 7500 rpm for 5min and air dried for 15min.

The gel apparatus was washed with 0.1%SDS and two changes of dH_2O and the tray was then set up. An appropriate agarose gel was prepared by heating the agarose, MOPS and water until the agarose had dissolved. The formaldehyde was then added. The agarose gel buffer was then made up and each test sample was prepared with 20ul RNA, 10ul sample buffer, and 5µl ethidium bromide. The samples were loaded and the gel was run at 115V for about 1hr. If mRNA bands were even northern blotting was carried out. In this case the gel was placed in 10% SSC into a pyrex dish and placed O/N at 4°C.

The next day, two large gel tanks and four glass plates were washed with 0.1%SDS and rinsed with sterile dH_2O. Two paper wicks were then cut using 3M chromatography paper. Whilst resting glass plates on a gel tank, the wick was set across it and wet and 500ml 10×SSC was poured into the tank. The gel was placed upside down onto the middle of the wick and the nylon membrane placed on top. This was covered with two small pieces of 3M chromatography paper, a stack of blotting paper and secured with second glass plate and a

weight. The apparatus was left O/N at room temperature. The following day the membrane was removed and soaked in two changes of 2xSSC for 15min (10ml 20xSSC: 90ml dH_2O). The membrane was then wrapped in glad wrap and the RNA fixed to the membrane by UV by placing wrapped membrane onto the transilluminator for 10min. The UV fixed membrane was stored at -70°C.

To carry out the pre-hybridisation reaction, the membranes were placed into sterile roller bottles. The pre-hybridisation solution was then prepared and added to each roller bottle. After denaturing 10mg/ml ssDNA by heating above 95°C for 2min, 50µl per filter was added to each bottle and left rotating in the hybridisation oven for 4hr at 42°C.

In sterile tubes the hybridisation reaction mixture was made up consisting of 5µl DNA, 10µl labelling buffer, 5µl primers and 17µl dH_2O. This was then denatured at 95°C for 2min, followed by the addition of 10µl labelling buffer, 5µl ^{32}P and 3µl Klenow enzyme and subsequent incubation for 2hr at 42°C. Unincorporated ^{32}P was removed by spinning the labelling mixture through a sephadex-G50 column. The syringe was filled with sephadex G-50 saturated tris-EDTA-NaCl (TEN) buffer and centrifuged at 1600rpm for 4min. A sterile tube was placed into the bottom of 10ml tube to collect the purified radiolabelled insert. This was centrifuged at 1600rpm for 4 min and the purified probe was transferred to a sterile tube. The probe was then denatured by heating to 95°C for 2min and added the to filters in the prehybridisation solution. This was left rotating O/N at 42°C.

The following washes of increasing stringency were prepared in sterile 100ml bottles: 10×SSPE, 0.1% sodium dodecyl sulphate (SDS), 5×SSPE, 0.1% SDS and 2×SSPE, 0.1% SDS. The pre-hybridisation solution containing the probe was removed and 10-20ml of wash solution added to each roller bottle. Each wash was performed for about 1hr at 42°C with rotation. Filters were then removed from filter bottles and placed between two pieces of gladwrap. Membranes were then exposed to x-ray film for 2hr or O/N, developed and

photographed. Lastly, procollagen mRNA was quantified using densitometry software (Kodak 1D image analysis).

2.7.3 WESTERN BLOTTING: SDS-PAGE

The Western blotting apparatus was initially washed with de-ionised distilled water and 100% alcohol and dried. Plates were assembled together with stackers in a pouring chamber. An appropriate polyacrylamide gel according to the molecular weight of the desired protein was then prepared *(Apx. 2.5)*. Contents were mixed in a small conical flask, pipetted between glass plates and overlayed with distilled water. Gels were left to set for approximately 45min. The stacking gel was then prepared and once water was poured off from the base gel, the stacking gel was pipetted on top, well combs arranged in the stacker, and left to set for 20min. Combs were removed and wells washed with de-ionised distilled water. Protein samples that were extracted with Trizol were prepared according to the following protocol:

1. Protein samples were thawed and heated at 60ºC for 10min and samples with equal amounts of 2×SDS buffer with or without 2-Mercaptoethanol (bME) were prepared.
2. A Rainbow Standard and Biotinylated protein ladder/HRP-conjugated anti-biotin antibody were also prepared according to the manufacturer's instructions. Both the standards and the samples were heated at 90ºC for 10min and pulse centrifuged.
3. Glass plates were then transferred to the electrode apparatus, snapped into place and inserted into the miniprotein gel tank. 500ml of tank buffer (1%SDS) was prepared and poured into the apparatus to cover the wells in the upper gel tank.
4. Standards and samples were then loaded using a Hamilton pipette and the lower gel tank was filled with the rest of the 1%SDS buffer. Gels were run at 80V for 10min and once samples had passed through stacking gel, the voltage was increased to 200V for 40min.

5. Gels were stained with Coomassie blue or prepared for transfer to nitrocellulose membrane. If staining for Coomassie blue, gels were immersed in the dye for 30-60min and destained in 50% MeOH and 10% acetic acid. The destainer was changed until protein bands were evident. The gel was then stored in acetic acid or photographed.

Alternatively, gels were pre-treated according to the following:

1. Gels that were transferred were initially soaked in prepared cold transfer buffer for at least 30min.
2. To pre-treat the PVDF membrane, membranes were cut to size and immersed in methanol for 15sec, dH_2O for 5min, and transfer buffer for at least 15min.
3. The gel sandwich apparatus was then set up consisting of fibrous pads soaked in transfer buffer, 3M paper (cut to pad size) also soaked in buffer and placed on top of pad, followed by the addition of the gel.
4. The apparatus was completed by adding another piece of 3M paper and finally another fibrous pad. The apparatus was clipped together and placed in the transfer holder. The holder was placed in the tank and an ice-pack was added.
5. Cold transfer buffer was added and transfers were run at 100V for 1hr. Upon completion, the sandwich was disassembled and membranes were soaked in cold 5% skim milk/PBS-Tween O/N at 4ºC.

Antibody labelling was then carried out according to the following:

1. The next day, skim milk solution was removed and membranes were rinsed in PBS-Tween for 45min while shaking. The primary antibody, diluted in PBS-Tween, was then added for 1-2hr at room temperature with shaking, or O/N at 4ºC.

2. Membranes were then washed in PBS-Tween for 45min and the secondary horse-radish peroxidase (HRP)-conjugated secondary antibody, diluted in PBS-Tween, was added for 1hr with shaking (*Appendix 4; Table 3.3*).
3. Membranes were then washed in PBS-Tween for 1hr with shaking.
4. Using a chemiluminescence (ECL) detection kit, the reagent was prepared according to the manufacturer's instructions and membranes were incubated for the specified amount of time.
5. Excess liquid was rinsed off and membranes placed between two sheets of acetate paper. Membranes were placed inside an x-ray cassette and exposed to x-ray film.
6. X-ray film was then developed for 2min, washed in water and fixed for 2min in fixative.
7. If the membranes were to be stripped and re-probed, western blotter stripper was added for 15min with shaking. They were then exposed to a new film to ensure removal of the previous probe. Membranes could then be re-probed, as described above.
8. Results were quantified (in the case of αSMA) using densitometry software.

2.7.4 REAL TIME PCR

The concentration of RNA in the samples was analysed by (a) spectrophotometry or (b) SYBR Green RNA quantitative analysis.

(a) Using sterile tubes, 119μl of water and 1μl of test RNA were placed into each. 120μl of water was used in the control sample. Using a crystal quartz, the spectrophotometry machine was zeroed with the control, and samples were then analysed at a wavelength of 260nm. This was repeated three times for each sample and the results averaged.

(b) The SYBR Green assay was carried out according to the manufacturer's instructions. Fluorescence values were plotted against a standard curve to find the RNA concentration of each sample.

Once the concentration of values were attained, DNA was removed from samples:

1ug/µl of RNA was made up to a total of 10µl with appropriate volumes of DNase, DNase buffer and dH_2O. Samples were then vortexed, centrifuged and left at room temperature for 30min. 1µl of stop buffer was then added and samples were left for 15min at 65°C and then placed on ice.

Samples were then reverse transcribed according to the following:

1. The RNA concentration was adjusted to 100ng by taking placing 1µl into thin walled PCR tubes and adding dH_2O, random hexamers and RNAse out to a total volume of 7µl. After briefly centrifuging, samples were heated to 65°C for 5min and cooled on ice.
2. A master mix was then prepared using the superscript III reverse transcription kit consisting of sample buffer, $MgCl_2$, Dithiothreitol (DTT), deoxyribonucleotide triphosphate (dNTP), dH_2O and superscript III enzyme.
3. 13µl was added per tube and briefly centrifuged. Samples were then heated at 25°C for 10min, 50°C for 50min, 85°C for 5min and cooled at 4°C before placing on ice.
4. Finally, 15µl of dH_2O was added, and samples were pulse centrifuged and stored at -20°C.
5. Stock primers were thawed and heated to 75°C for 5min and primers and housekeeper gene were prepared according to the optimal running temperature. At a concentration of 0.5µM, 20µl of each of the forward and reverse primers were added to dH_2O.
6. A master mix of each primer set was prepared which consisted of Jump Start, forward primer, reverse primer and dH_2O. A total of 25µl was loaded as either duplicates or triplicates into 96 well PCR plates with 23µl of the primer master mix and 2µl of the DNA of interest.
7. Negative controls containing primer only and water only were included and samples were run according to the set up below. The melt curve of each sample was analysed to verify a single product according to the peak melting temperature time.

Cycle	Repeats	Step	Dwell time (min)	Hold	Set point (°C)	Melt curve	Temp (°C)
1	3	1	5		95.0		
2	60	1	1		94.0		
		2	30sec		60.0		
		3	1		72.0		
3	1	1	1		55.0		
4	85	1	10sec		55.0	#	0.5
5	1	1	-	#	4.0		

Table 2.2: Biorad real time PCR program

2.8 STATISTICAL ANALYSIS

All data is represented as mean ± standard deviation (SD), unless indicated. Treatment groups were compared using one-way analysis of variance (ANOVA) with the TUKEY test correction for multiple comparisons. $p<0.05$ was considered statistically significant in all studies.

Chapter 3

The Role of Phosphatidylinositol 3-Kinase and mTOR in the Regulation of Renal Fibroblast Proliferation and Collagen Synthesis

3.1 INTRODUCTION

The interstitial fibroblast is a key mediator of tubulointerstitial fibrosis and in response to a cocktail of circulating cytokine and non-cytokine mediators, these cells can (a) proliferate autonomously, (b) secrete chemotactic substances which may recruit more fibroblasts, inflammatory cells or activate other interstitial/glomerular cells and (c) differentiate into more potent counterparts that are capable of secreting ECM proteins and mediating the contracture of parenchmyal and matrix tissue. Whilst the cytokine agonists that mediate these processes have been well characterised, the intracellular signalling axes that mediate fibroblast proliferation and synthetic activity are less well-known.

Of those pathways that regulate fibroblast activity, the TGFβ signalling axis is most well characterised. This signalling axis integrates a variety of signals including those from angiotensin II (Fukami et al., 2004), CTGF (Grotendorst, 1997), AGEs (Li et al., 2004a), PDGF (Daniels et al., 2004), FGF (Strutz et al., 2001) and TGFβ1 itself (Runyan et al., 2004). However, given the complexity of cell-signalling pathways, the control of fibroblast function is likely to involve other signalling pathways. This study therefore aimed to identify other signalling pathways involved in regulating two important responses of the fibroblast; proliferation and ECM synthesis.

Proliferation and ECM synthesis can be regulated through integrin signalling (Chiquet, 1999) or local environmental stressors (Zeisberg et al., 2000) however, key mediators of these processes are growth factors. Although typically associated with cancer, cell survival and glucose metabolism (Inoki et al., 2005b), phosphatidylinositol 3-kinase (PI3K) also regulates proliferation, differentiation and ECM synthesis (Asano et al., 2004; Brennan et al., 1999; Breslin et al., 2005; Gerasimovskaya et al., 2005a; Krymskaya et al., 1999) and is increasingly known for its ability to integrate growth factor signals through receptor tyrosine kinases (RTKs). In response to extracellular ligands such as PDGF, FGF, EGF or IGF, RTK's dimerise and autophosphorylate leading to the generation of

phosphatidylinositol-(3,4,5)-triphosphate (PIP_3) from phosphatidylinositol-(4,5)-biphosphate (PIP_2) and the recruitment of molecules to the plasma membrane with a pleckstrin homology (PH) (Engelman et al., 2006). One such PH containing kinase is acute transforming retrovirus thymoma (Akt), which once translocated, can be phosphorylated by mammalian target of rapamycin complex 2 (mTORC2) at S-473 (Sarbassov et al., 2005). This precedes and facilitates the phosphorylation of Akt T-308 by phosphoinositide dependent kinase 1 (PDK1) (Alessi et al., 1997; Balendran et al., 1999a). Once activated, Akt is capable of activating the downstream serine/threonine kinase mTOR. This founding member of the PI3K related protein kinase family is involved in cell cycle progression and cell proliferation (Inoki et al., 2005b), primarily by regulating the G1-S phase of the cell cycle.

mTOR is a key integrator of stress, nutrient and growth factor signals and many of these converging signals are integrated through the tuberous sclerosis complex (TSC1/2) (Inoki et al., 2002). TSC1/2 exerts GTPase activating protein (GAP) activity over the positive regulator of mTOR Rheb. This latter protein cycles between an active GTP-bound state and an inactive GDP-bound state. mTOR is increasingly recognized for its ability to integrate growth factor signals such as IGF, FGF, PDGF and insulin via PI3K. In response to these and other growth factors, Akt directly phosphorylates TSC2 inactivating its GAP activity, leading to an accumulation of GTP-bound Rheb. This leads to the activation of downstream effectors S6 ribosomal protein S6 kinases (S6Ks) and the eukaryotic initiation factor (eIF) 4E binding proteins (4EBPs) (Fingar and Blenis, 2004; Ponticelli, 2004) *(Fig. 1.16a-c).* Accordingly, it is likely that mTOR may play a key role in integrating a number of growth factors that are directly relevant to the regulation of fibroblasts in fibrosis.

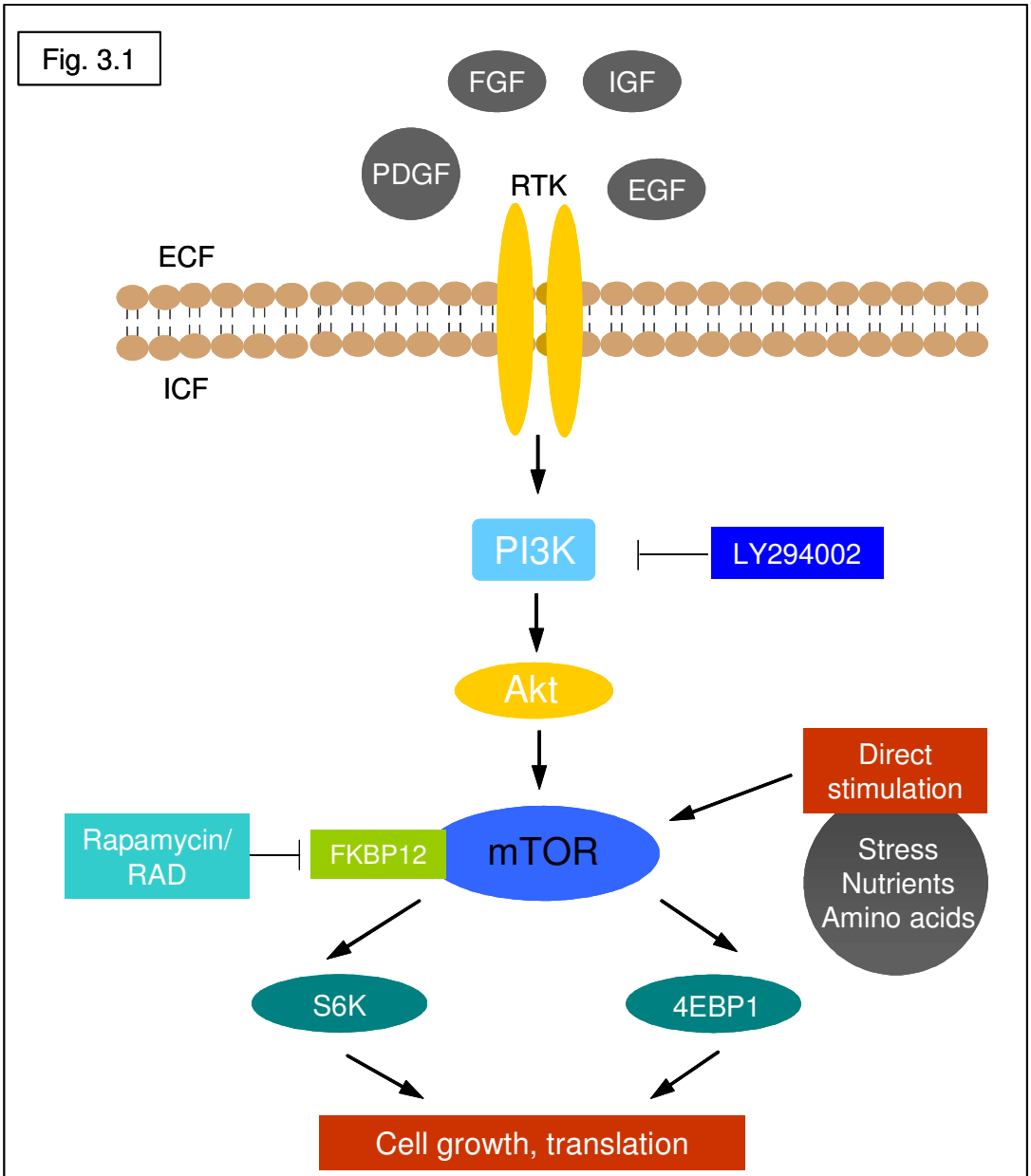
Given that autonomous fibroblast proliferation and aberrant collagen synthesis are key roles of activated fibroblasts in fibrosis, this study was aimed at elucidating the roles of these kinases in the regulation of rat fibroblasts derived from fibrotic rat kidneys. This study has investigated the role of mTOR and PI3K in the regulation of fibroblast function by using the

rapamycin derivative RAD and the PI3K inhibitor LY294002 (LY). Rapamycin and its derivatives, best known for their immunosuppressive properties (Breslin et al., 2005), have been shown to inhibit mTOR by binding to the intracellular binding protein FKBP12 (Meric-Bernstam and Mills, 2004) which binds to the FKBP12 rapamycin binding (FRB) domain of mTOR inhibiting its activity. LY binds to the ATP binding site of PI3K inhibiting all classes of PI3K (with less affinity for class II PI3K) *(Fig. 3.1).*

3.2 EXPERIMENTAL DESIGN

This study utilised cells derived from kidneys at 3 days post-UUO. Cells were treated for 48hr with (a) various doses of RAD (10, 100, 200nM), LY (10μM), or the Mek1 inhibitor PD98059 (PD; 20μM), (b) treated with combinations of these drugs, or (c) maintained in control medium, as indicated. Drugs were added to standard medium (1xDMEM supplemented with 10%FCS, 1M N-2-hydroxethypiperazine-N'-2-ethansulfonic acid; HEPES, 2mM glutamine and 500Units/ml penicillin/streptomycin), unless specified otherwise. These studies were based on the response of fibroblasts to serum-based growth factor/cytokine stimulation. Using these standard procedures, this chapter examined the signalling pathways encompassing PI3K and mTOR including Akt S-473 and S6RP, and then carried out a number of functional studies assessing the role of these kinases in cell proliferation and collagen synthesis.

Fig. 3.1



Mechanism of action of rapamycin derivative RAD and LY

ECF = extracellular fluid, ICF = intracellular fluid.

3.3 RESULTS

3.3.1 Cell signalling mechanisms in renal fibroblasts

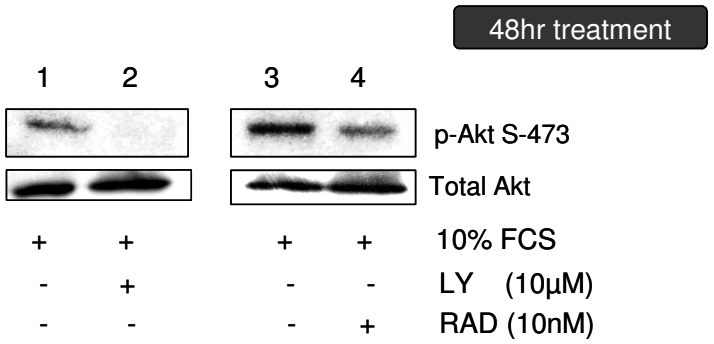
3.3.1.1 LY inhibits the phosphorylation of Akt S-473

Akt is a serine/threonine kinase with an N-terminal pleckstrin homology (PH) domain. When the formation of PIP_3 is catalysed by the p110 catalytic subunit of PI3K, Akt is translocated to the plasma membrane to bind PIP3 at its PH domain. Akt can then be phosphorylated at S-473 by PDK2 and this precedes and facilitates the phosphorylation of T-308 by PDK1. Activation of Akt can lead to the downstream phosphorylation of mTOR of which S-2448 and T-2446 have been identified as the likely phosphorylation sites targets for Akt (Sekulic et al., 2000). In order to confirm that Akt is a downstream mediator of PI3K in renal fibroblasts, Akt phosphorylation at S-473 was examined. Western blotting indicated that 10μM LY completely inhibited the serum-induced phosphorylation of Akt S-473, as compared to total Akt (lane 2 vs. lane 1; *Fig. 3.2*). This is consistent with LY mediated inhibition of downstream PI3K signalling and is representative of duplicated experiments (independent experiments).

3.3.1.2 RAD down-regulates the phosphorylation of Akt S-473

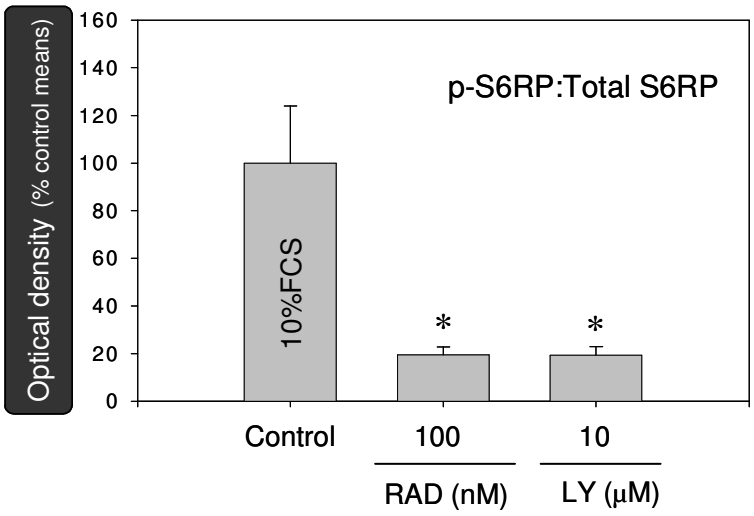
Western blot analysis also indicated that the phosphorylation of S-473 Akt was down-regulated in response to 10nM RAD (*Fig. 3.2*, lane 4 vs. lane 3). This provides a novel finding into the regulation of Akt in renal fibroblasts.

Fig. 3.2



Western blotting was performed on equal volumes of cell protein using a polyclonal antibody against phosphorylated-Akt (p-Akt S-473). Akt phosphorylation was completely inhibited by 10μM LY in response to serum-based mitogen stimulation for 48hr (lane 2 vs. lane 1). This is an expected and established result. More interestingly, Akt S-473 phosphorylation was partially inhibited by 100nM RAD (lane 4 vs. lane 3). Even loading was confirmed by examining total Akt phosphorylation levels.

Fig. 3.3



*The effect of the mTOR and PI3K inhibitors on downstream mTOR effectors was examined by measuring the ratio of phosphorylated S6RP (S-235/236) to total S6RP. Relative to the control group, 100nM RAD and 10μM LY inhibited phosphorylation of S6RP by approximately 80% (*p<0.001, n=3). This result is a representative of duplicate experiments.*

3.3.1.3 S6RP phosphorylation is inhibited by both RAD and LY but not by the MEK1 inhibitor, PD

To confirm that both RAD and LY inhibit downstream targets of mTOR, the effect of these kinase inhibitors on the phosphorylation of S6 ribosomal protein (S6RP) was examined. The ratio of p-S6RP to total S6RP was measured using enzyme-linked immunosorbent assay (ELISA). *Fig. 3.3* demonstrates that S6RP (S-235/236) is a downstream target of mTOR. Relative to the control group, 100nM RAD and 10μM LY reduced phosphorylation of S6RP by approximately 80% ($p<0.001$, $n=3$). *Fig. 3.3* is representative of a duplicate set of experiments.

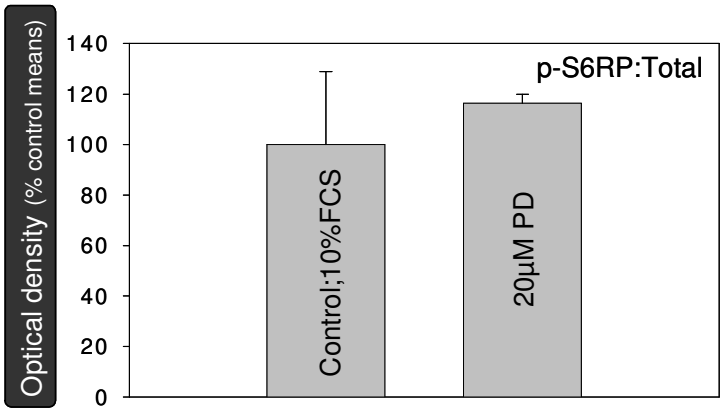
Given that the literature indicates that PI3K and mTOR can cross-talk with other substrates, the potential for the MAPK pathway to phosphorylate S6RP was also investigated. In contrast to LY and RAD, 20μM of the MEK1 inhibitor PD did not affect p-S6RP as a ratio compared to total S6RP levels (*Fig. 3.4*). Again, *Fig. 3.4* is representative of a duplicate experiment.

3.3.1.4 mTOR can be constitutively activated independent of serum-based factors

It is believed that the mTOR pathway contains a constitutively active circuitry and recently it has been shown that smooth muscle cells exhibit a proliferative phenotype independent of growth factors (Mourani et al., 2004). In light of the ability of mTOR to be constitutively activated by amino acids and nutrients, this experiment was carried out to determine if, as in smooth muscle cells, mTOR can be constitutively activated in the absence of FCS in fibroblasts by using S6RP as a read-out.

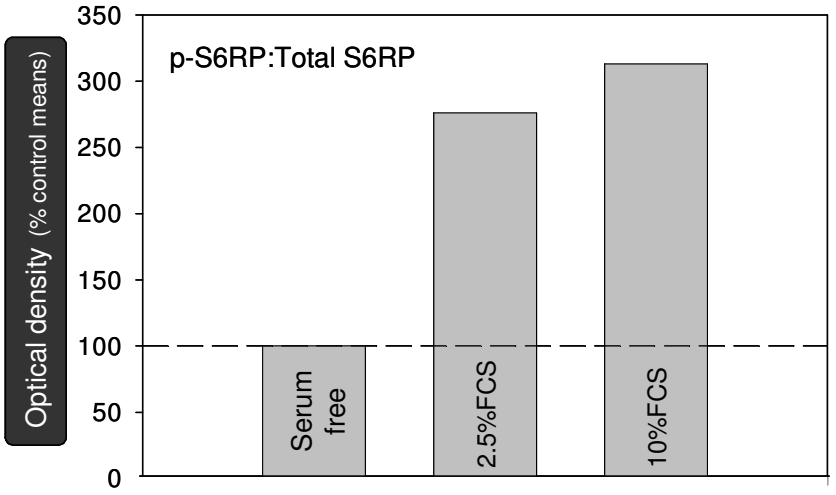
To examine this, fibroblasts were exposed to either serum free medium or medium supplemented with 2.5% or 10% FCS. *Fig. 3.5* demonstrates that compared to the control, cells grown in 2.5% and 10%FCS displayed an increase in S6RP phosphorylation by 176%

Fig. 3.4



In response to FCS-based mitogen stimulation, MEK1 inhibition did not change the level of S6RP phosphorylation significantly from the control.

Fig. 3.5



S6RP phosphorylation was examined as a ratio of p-S6RP:total S6RP. Compared to the serum free medium, cells grown in 2.5% and 10% FCS displayed an increase in S6RP phosphorylation by 176% and 212% respectively (illustrative of a duplicate experiment). This indicates that, consistent with its ability to be activated by amino acids and nutrients, mTOR maintains a constitutively active circuitry in the absence of growth factors within FCS.

and 212% respectively (illustrative of a duplicate experiment). Very little change in phosphorylation was evident in response to the varying degrees of FCS. This confirms that mTOR can maintain a constitutively active circuitry independently of growth factors.

3.3.2 LY and RAD down-regulate fibroblast mitogenesis

Dose response studies were undertaken to establish if mTOR and PI3K are involved in regulation of renal fibroblast kinetics.

3.3.2.1 LY and RAD reduce the number of renal fibroblasts

RAD (10, 100, 200nM) decreased proliferation over 5 days by 64±5, 59±8 and 66±6% respectively (*Fig. 3.6*; all $p<0.001$ vs. control). Similarly, LY reduced proliferation by 68±11% (*Fig. 3.7*; $p<0.001$ vs. control).

3.3.2.2 LY and RAD reduce DNA synthesis in renal fibroblasts

Changes in cell number were reflected by a reduction in DNA synthesis, as measured by incorporation of tritiated [^{3}H] thymidine. RAD (10, 100, 200nM) inhibited serum induced DNA synthesis by 53±7%, 50±9% and 42±7% respectively (*Fig. 3.8*; all $p<0.001$ vs. control). 10μM LY also decreased thymidine incorporation by 68±2.3% (*Fig. 3.9*; $p<0.001$ vs. control). This indicates the involvement of both mTOR and PI3K in the regulation of the cell cycle and supports rapamycin's well-known inhibitory effect on cell cycle arrest.

Fig. 3.6

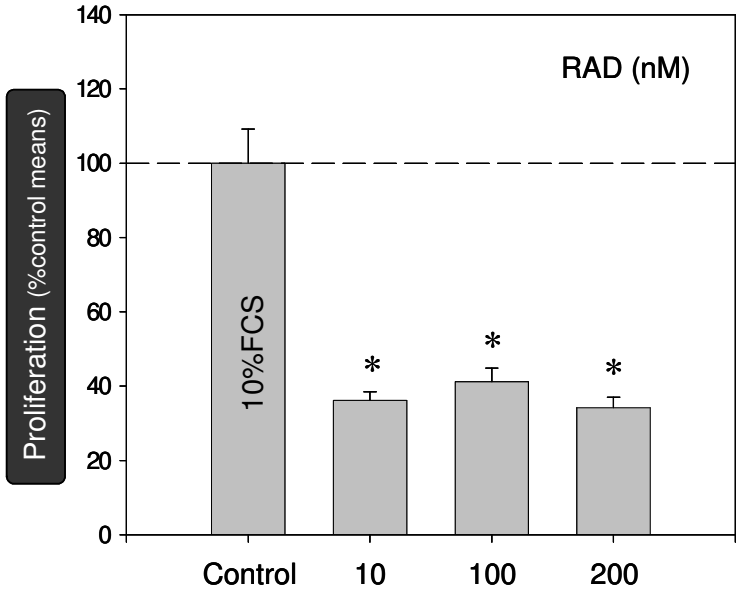
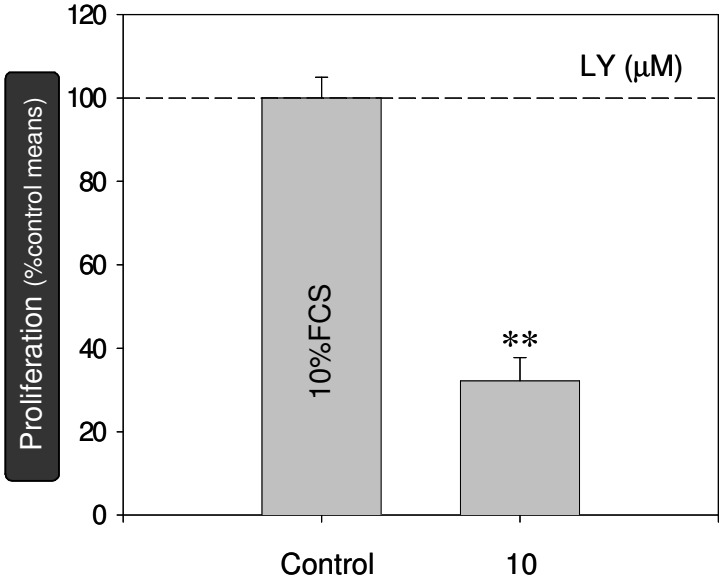


Fig. 3.7



*Both RAD (Fig. 3.6) and LY (Fig. 3.7) decreased cell number over 5 days. RAD (10, 100 and 200nM) decreased proliferation by 64±5, 59±8 and 66±6% respectively (*all p<0.001 vs. control) while LY (10μM) reduced growth by 68±2.3% (**p < 0.001 vs. control). Results are expressed as mean ± S.D.% of a duplicate experiment (n=4) and are presented as a percentage of control means.*

Fig. 3.8

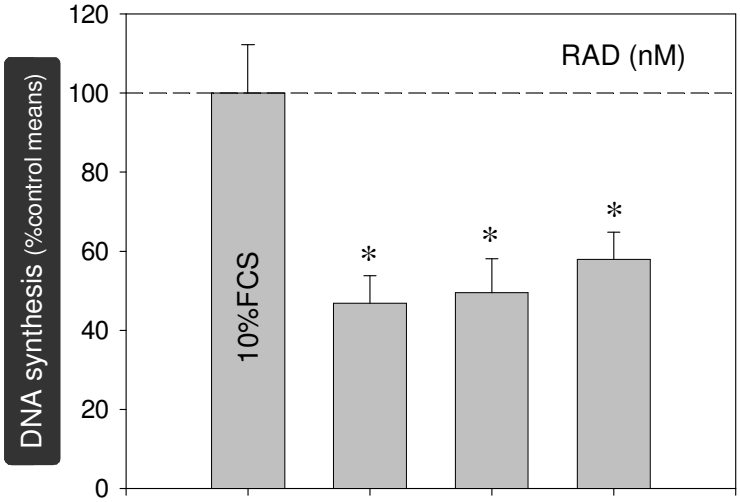
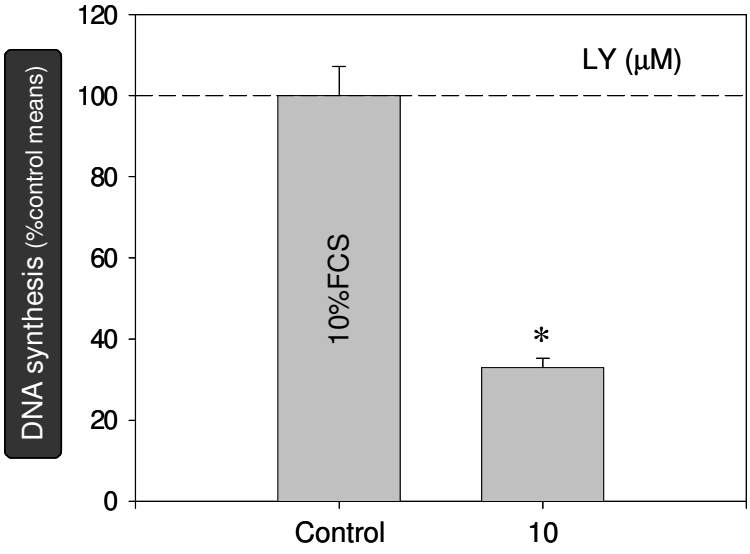


Fig. 3.9



*Changes in cell proliferation were paralleled by RAD and LY-mediated reductions in DNA synthesis, as measured by tritiated [^{3}H] thymidine incorporation. RAD (10, 100 and 200nM) decreased thymidine incorporation by 53±7, 50±9 and 42±7% (Fig. 3.8, *all $p < 0.001$ vs. control). LY (10µM) reduced DNA synthesis by 67.1 ± 2.3% (Fig. 3.9, $p < 0.001$ vs. control). Results are expressed as mean ± S.D.% of a representative duplicate experiment (n=4) and are presented as a percentage of control means.*

3.3.2.3 Combined LY and RAD treatment further decreases fibroblast mitogenesis

In addition to their individual effects, treatment with both RAD and LY further decreased serum-induced fibroblast mitogenesis. Co-inhibition with 10μM LY and 100μM RAD decreased cell number by 97±1.6%, significantly more than either drug alone (*Fig. 3.10;* $p<0.05$ vs. control, $p<0.05$ vs. 100nM RAD). Similarly, the 78±4% reduction in DNA synthesis with both drugs was more than that seen individually (*Fig. 3.11;* $p<0.001$ vs. control, $p<0.05$ vs. 100nM RAD;) suggesting that these pathways may work in cooperation to regulate the cell cycle.

3.3.2.4 The ERK1/2 MAPK pathway and PI3K may act cooperatively to regulate fibroblast proliferation

The role of the ERK1/2MAPK cascade in fibroblast proliferation was examined by using the Mek-1 inhibitor PD which inhibits the ERK1/2MAPK cascade (Dudley et al., 1995). As shown by *Fig. 3.12*, PD, a known inhibitor of proliferation, inhibited fibroblast proliferation by 43.4±6% ($p<0.001$ vs. control means) whilst individually RAD and LY inhibited proliferation by 58.9±8% and 67±2.5% respectively ($p<0.001$ vs. control). In response to co-inhibition with LY and PD, a further decrease in proliferation by 15.9±3% was noted as compared to LY ($p<0.05$ vs. LY, $n=4$), whilst no further significant decrease resulted from co-inhibition with PD and RAD. It has been noted that PI3K can stimulate the MAPK pathway, and these results, representative of a duplicate experiment, suggest that the ERK1/2MAPK cascade may act in parallel or cross talk with PI3K in the regulation of fibroblast proliferation.

Fig. 3.10

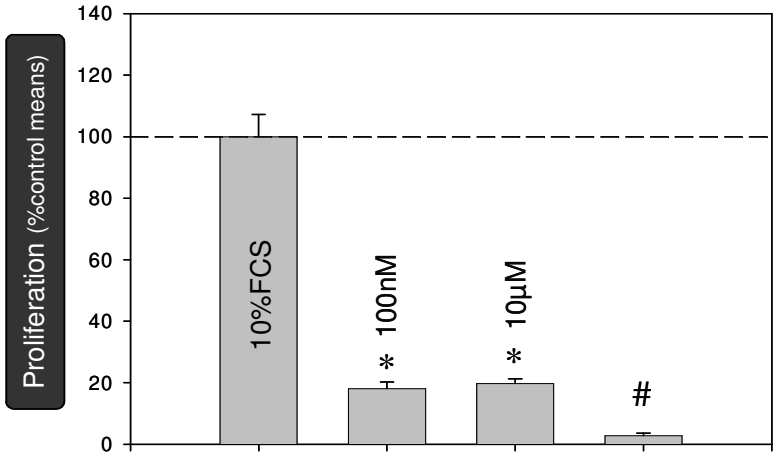
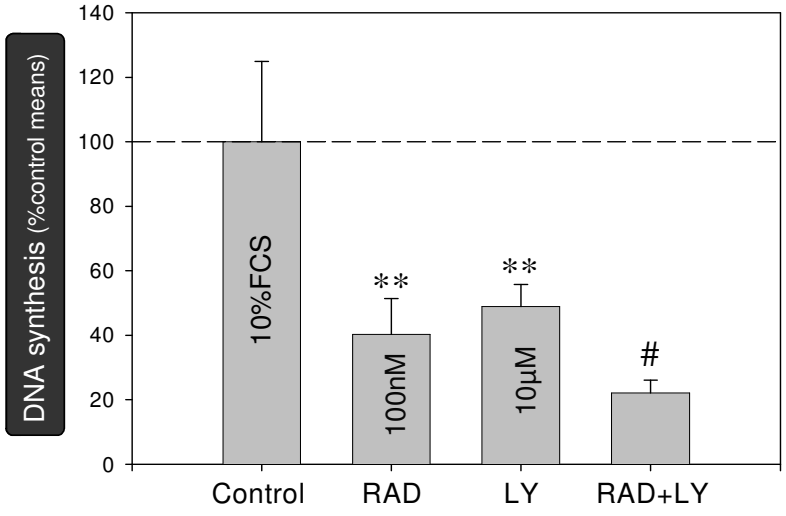
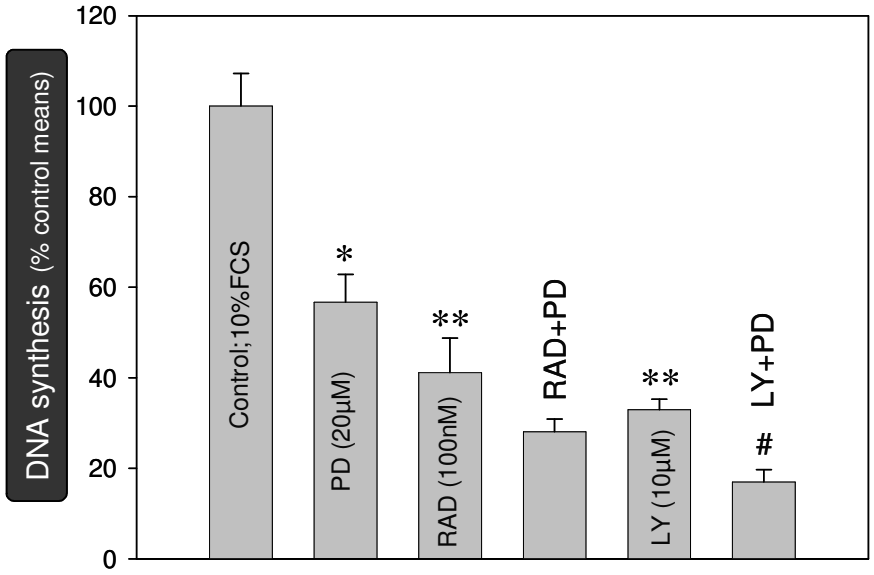


Fig. 3.11



Combined treatment of cells with RAD (100nM) and LY (10µM) further decreased serum-induced fibroblast proliferation by 97±1.6%, significantly more than either drug alone (Fig. 3.10). Similarly, the reduction in DNA synthesis (78±4%) with both drugs was more than that seen individually (Fig. 3.11). Results illustrate a duplicate experiment. Data is expressed as mean ± S.D.%, with findings presented as a percentage of control means ($^{*}p < 0.001$ vs. control, $^{**}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. 100nM RAD & 10µM LY, n=4).

Fig. 3.12



*PD inhibited fibroblast proliferation by 43.3±6% (*p<0.001 vs. control) whilst individually, RAD and LY inhibited proliferation by 58.9±8% and 67±2.5% (**p<0.001 vs. control). Co-inhibition of PD with LY mediated a further decrease in proliferation by 15.9±3% as compared to LY (#p<0.05 vs. LY, n=4). No significant difference was noted in response to co-inhibition with RAD and PD.*

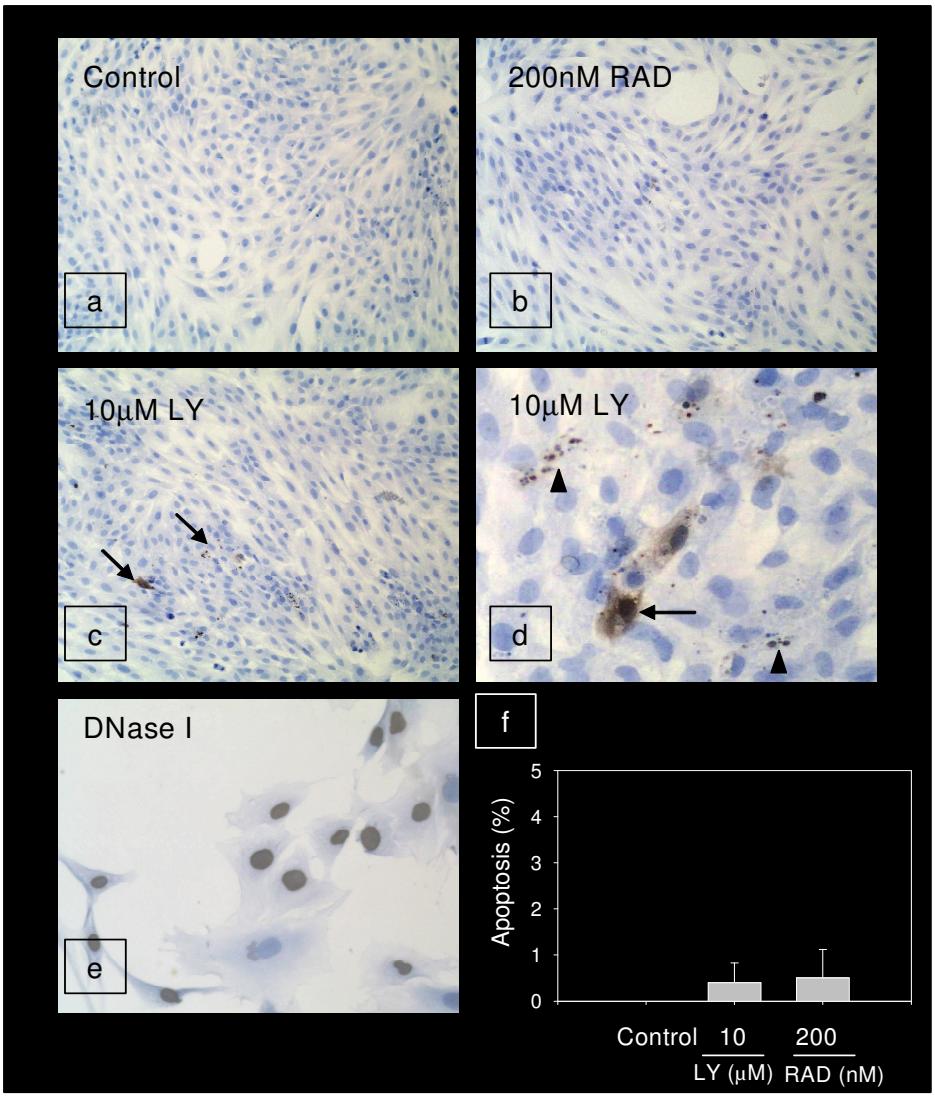
3.3.3 Changes in proliferation are independent of apoptosis or changes to cell viability

Previous studies of PI3K have implicated it as a major regulator of cell survival. To determine if the demonstrated effects of RAD and LY on proliferation were due to changes in the incidence of cell apoptosis, apoptosis and cell viability testing was carried out. LY and RAD treated cells were assessed with TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labelling assay), nuclear morphology was quantified (propidium iodide), and mitochondrial activity (MTT; dimethylthiazol-yl-diphenyl tetrazolium bromide) was examined.

3.3.3.1 TUNEL

The role of apoptosis was specifically examined by comparing results of TUNEL in control, LY (10μM) and RAD (200nM) treated cells (*Fig. 3.13*). This technique identifies the presence of DNA fragmentation in apoptotic cells caused by activation of endonuclease activity during cell death (Hewitson et al., 2000a). While TUNEL detected almost universal labelling of DNase digested cells (*Fig. 3.13e*), labelling in 200nM RAD (*Fig. 3.13b*) and 10μM LY (*Fig. 3.13c*) treated groups was not qualitatively different from cells grown in control medium (*Fig. 3.13a*). Only the occasional cell labelled positive for TUNEL (*Fig. 3.13d*, arrow). Quantifying these results confirmed that apoptosis was not significantly increased in any of the treated groups *(Fig. 3.13f).*

Fig. 3.13



The incidence of apoptosis, as assessed by TUNEL, was not significantly affected by 200nM RAD or 10μM LY. Cells were exposed to control medium, RAD or LY supplemented medium. Photomicrographs of cells grown in (a) control, (b) RAD and (c) LY were not qualitatively different, and only occasional TUNEL positive cells could be found (arrows). (d) At higher magnification, these cells show the characteristic condensation of chromatin (arrow) and formation of apoptotic bodies (arrowheads). (e) In the positive control, most DNase treated cells were labelled with TUNEL. (f) Quantitative assessment showed no significance difference between groups in the incidence of TUNEL positive cells. The graph represents pooled observations (n=4) from two independent experiments. Results are represented as a percentage of total cells counted.

3.3.3.2 Propidium Iodide

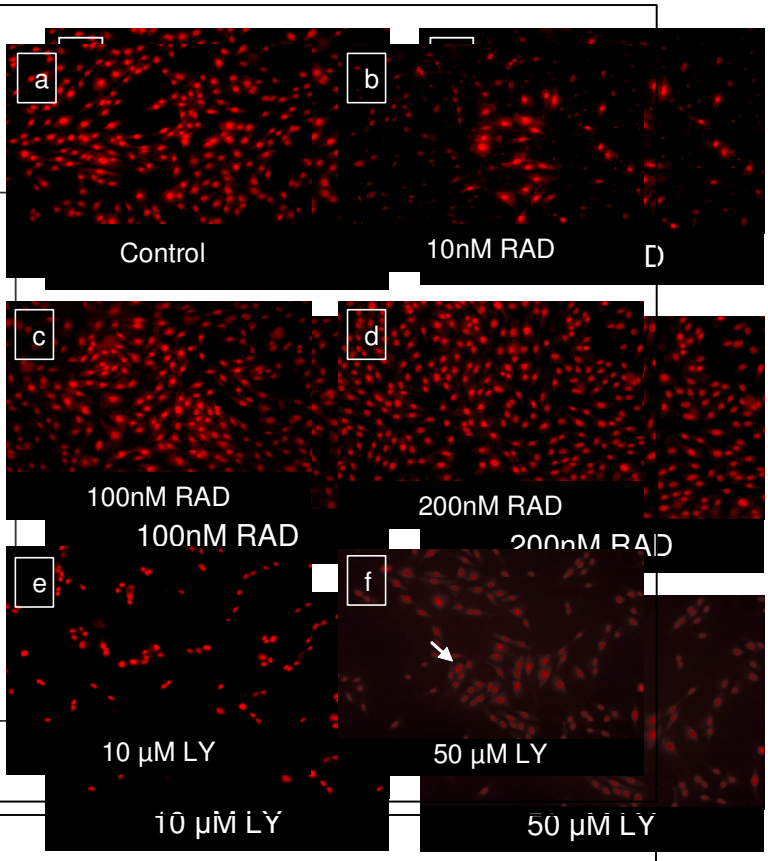
Qualitative assessment of cell morphology using propidium iodide staining only identified very rare instances of nuclear fragmentation, condensation or cytological changes associated with cell apoptosis in all RAD (10, 100, 200nM) (*Fig. 3.14b-d*) and LY (10μM) (*Fig. 3.14e*) treated groups compared to the control (*Fig. 3.14a*). A higher incidence of apoptotic bodies was noted in response to 50μM LY (*Fig. 3.14f*, arrow).

3.3.3.3 MTT Assay

The experimental doses of 10, 100, 200nM RAD or 10μM LY did not effect cell viability, as measured by the ability of cell mitochondria to reduce MTT to a formazan salt (*Fig. 3.15*). It was therefore concluded that mitochondrial viability was not affected by any of these doses over the standards time-course of these experiments. *Fig. 3.15* represents an average of three independent experiments.

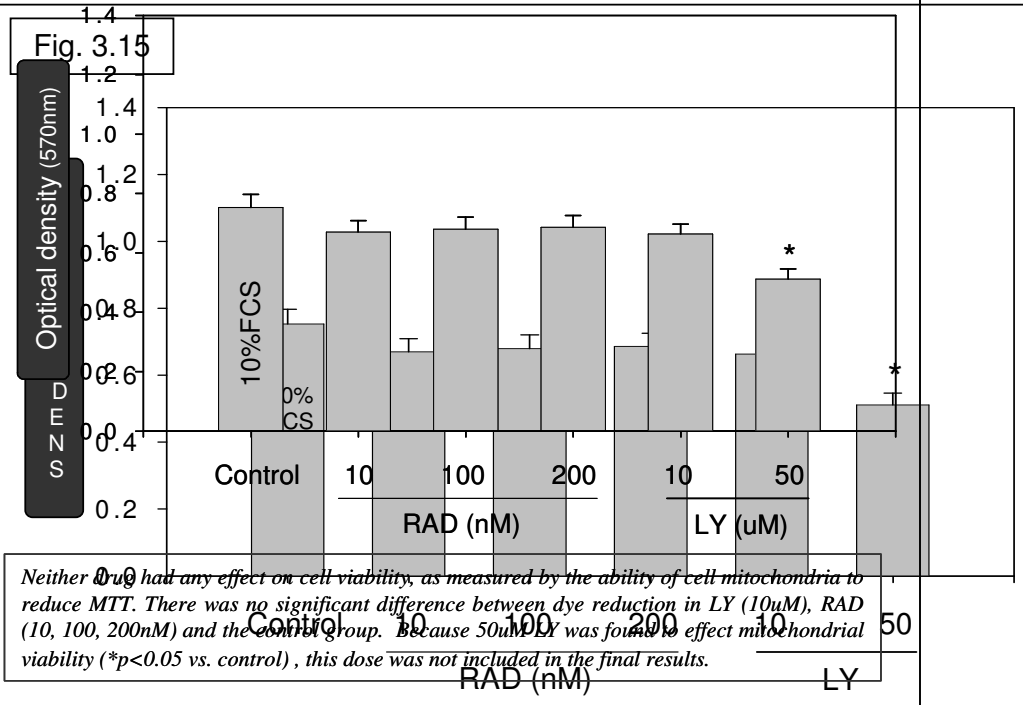
Preliminary experiments incorporated the use of 50μM LY. Propidium iodide staining *(3.3.3.2)* indicated that a higher incidence of apoptotic bodies were present in response to this dose. The MTT viability test also indicated that mitochondrial activity was compromised in cells treated with 50μMLY (0.510±0.158) compared to the control group (0.75±0.197) (*Fig. 3.15*, $p<0.05$ vs. control, n=4). This dose was therefore excluded from the final results.

Fig. 3.14



Qualitative assessment of cell morphology in two separate experiments using propidium iodide staining to identify very occasional instances of nuclear fragmentation or cytological changes associated with apoptosis in the RAD (10, 100, 200nM; b-d) and LY (10μM; e) treated groups compared to the control (a). A higher incidence was however noted in the higher dose of LY (50μM; f, arrow).

Fig. 3.15



*Neither drug had any effect on cell viability, as measured by the ability of cell mitochondria to reduce MTT. There was no significant difference between dye reduction in LY (10uM), RAD (10, 100, 200nM) and the control group. Because 50uM LY was found to effect mitochondrial viability (*p<0.05 vs. control) , this dose was not included in the final results.*

*Neither drug had any effect on cell viability, as measured by the ability of cell mitochondria to reduce MTT. There was no significant difference between dye reduction in LY (10uM), RAD (10, 100, 200nM) and the control group. Because 50uM LY was found to effect mitochondrial viability (*p<0.05 vs. control) , this dose was not included in the final results.*

3.3.4 PI3K and mTOR regulate the synthesis of total collagen and α1(I) mRNA

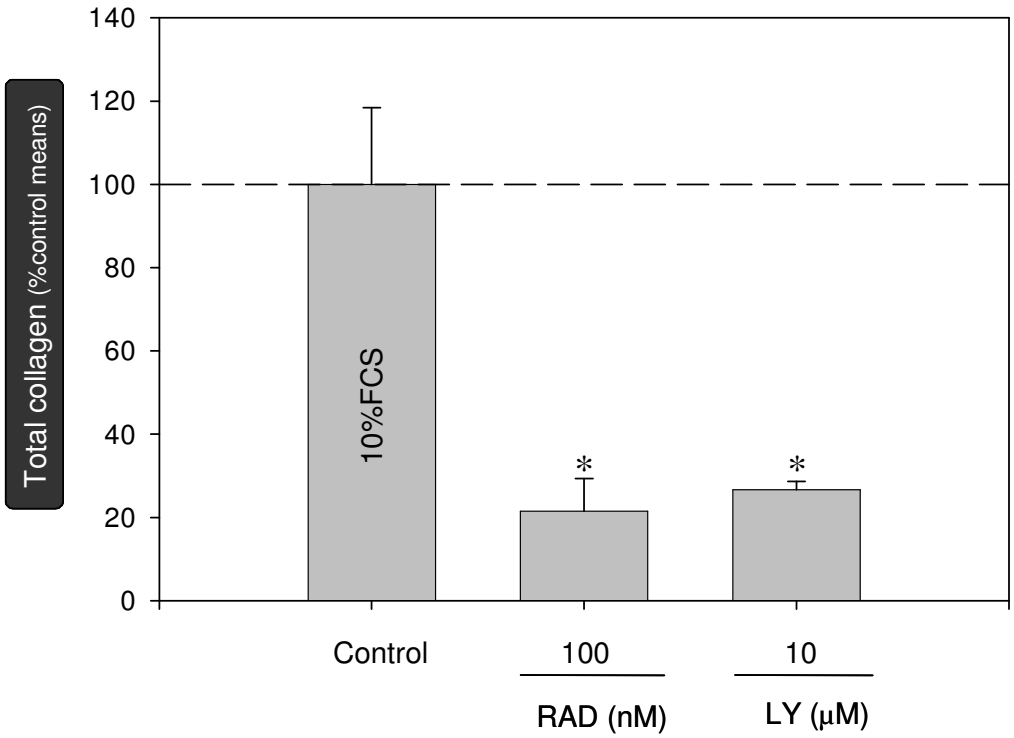
3.3.4.1 Inhibition of PI3K and mTOR decrease total collagen synthesis

To determine whether PI3K or mTOR were involved in regulating collagen synthesis, further implicating their involvement in fibrosis, incorporation of radioactively labelled hydroxyproline was analysed. Incorporation of hydroxyproline by cells treated with RAD (100nM) and LY (10μM) was significantly reduced by 78.5±7.8% and 73.3±2% (*Fig. 3.16*; both $p<0.001$ vs. control, n=4). These results are representative of duplicate experiments.

3.3.4.2 Procollagen mRNA synthesis is regulated by mTOR

The hydroxyproline incorporation assay has established that total collagen synthesis is decreased in response to both RAD and LY. To determine whether α1(I) procollagen mRNA levels were also regulated by mTOR Northern blotting analysis was carried out on fibroblasts treated with 100nM RAD. Loading was standardised using densitometry software according to 18S levels (*Fig. 3.17a*). Representative of duplicate experiments, *Fig. 3.17b* illustrates that α1(I) procollagen mRNA synthesis is decreased by 29.2%, 53.8% and 66.5% in response to respective doses of 10, 100, and 200nM RAD (n=4).

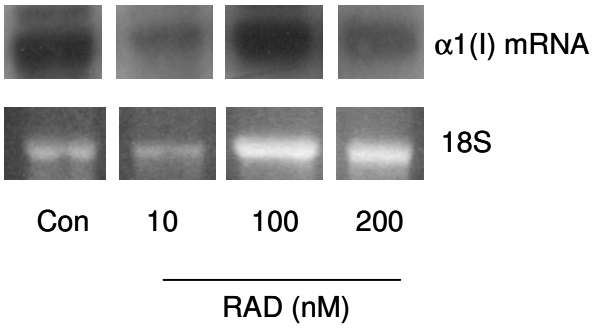
Fig. 3.16



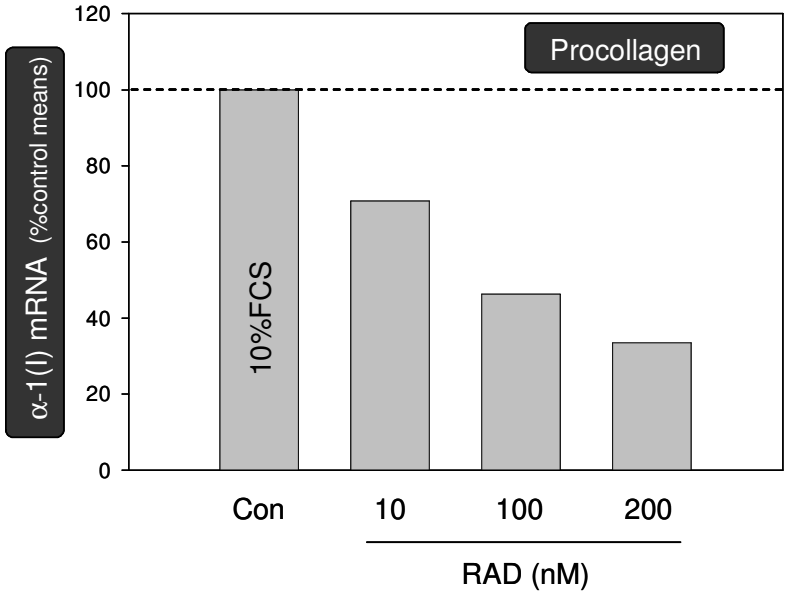
*A reduction in total collagen synthesis via hydroxyproline incorporation can be demonstrated in renal fibroblasts treated with RAD and LY. Total collagen synthesis was reduced 79±8% and 73±2% by 100nM RAD and 10μM LY respectively (*p < 0.001 vs. control). Results are presented as a percentage of control means and illustrate a duplicate experiment.*

Fig. 3.17

a



b



These results highlight the potential for mTOR to control transcription in addition to its well known role in translation. Northern blotting was carried out to determine the role of mTOR in a(I)1 procollagen synthesis. (a) Loading was standardised to 18S levels using densitometry software. (b) Treatment of fibroblasts with all doses of RAD mediated decreases in a1(I) mRNA levels by 29.2% (10nM), 53.8% (100nM) and 66.5% (200nM). These results are representative of a duplicate experiment.

Con = control

3.4 DISCUSSION

Defining the components of a signalling pathway that control fibrosis is integral to developing rational treatment strategies. The studies described within have attempted to identify novel signalling axes through which fibroblasts are regulated. Indeed, given that pathways governing fibroblast function have not been well documented in the literature, a number of novel findings have resulted from this work.

Initial studies were aimed at elucidating the signalling pathways encompassing mTOR and PI3K by examining the phosphorylation of Akt S-473 and S6RP S-235/236 in response to RAD and LY. This study supports previous studies by demonstrating that inhibition of PI3K with LY completely inhibits the serum-stimulated phosphorylation of S-473 Akt after 48hr. A novel finding however, was the ability of RAD to modestly down-regulate Akt S-473 phosphorylation in renal fibroblasts. Downstream of mTOR, phosphorylation of the S6RP S-235/236 was inhibited in response to both mTOR and PI3K inhibition. Functional studies were then carried out to ascertain the role of mTOR and PI3K in activated renal fibroblasts derived from UUO. Antagonism of mTOR and PI3K decreased mitogenesis, which also occurred in an additive fashion when both inhibitors were used in combination. Inhibition of PI3K and mTOR also lead to decreases in total collagen synthesis with the latter kinase playing a regulatory role in the synthesis of α(I)1 procollagen mRNA. Given the complexity of cell signalling pathways and their likelihood to interact, the role of the ERK1/2MAPK pathway in mTOR and PI3K-mediated proliferation was investigated. Whilst the MEK1 inhibitor (PD) did not affect the phosphorylation status of S6RP, when used in combination with the PI3K inhibitor, it led to a further decrease in cell proliferation. Lastly, mTOR is increasingly known for its ability to integrate growth factors via PI3K however this study highlights that its constitutive activation is not dependent on growth factor signalling.

3.4.1 Akt S-473 and S6RP: Respective substrates of PI3K and mTOR

To better understand the intracellular signal transduction pathways encompassing PI3K and mTOR, the relationship between these kinases and the phosphorylation status of respective downstream targets Akt S-473 and S6RP S-235/236 was ascertained.

These studies indicated that in the presence of LY, the phosphorylation of the S-473 Akt was completely inhibited, which supports previous findings (Le Pabic et al., 2005), and this confirms that PI3K activation leads to the downstream recruitment of Akt in renal fibroblasts. Akt S-473 is phosphorylated by PDK2 and this is believed to precede and facilitate phosphorylation of Akt T-308 by PDK1 (Sarbassov et al., 2005; Yang et al., 2002a) leading to full activation of Akt and increasing its activity 4-5 fold compared to PDK1-mediated T-308 phosphorylation alone.

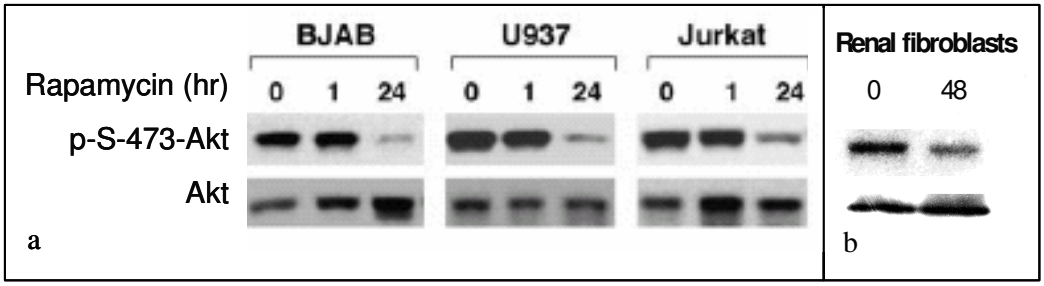
More interestingly, a modest RAD-mediated down regulation of Akt S-473 phosphorylation was also evident. At the time, although this finding was unexpected, it is however consistent with the very recent *in vivo* findings in mouse embryonic fibroblasts (MEF) (Sarbassov dos et al., 2006). This group has demonstrated that the rictor-mTOR complex (mTORC2) directly phosphorylates Akt S-473 in numerous different human cell lines. The real identity of the kinase responsible for phosphorylation (named phosphoinositide dependent kinase 2; PDK2) of Akt S-473 has been controversial for many years with PDK1 (Balendran et al., 1999b) ILK (Persad et al., 2001), autophosphorylation (Toker and Newton, 2000), PKCβ (Kawakami et al., 2004) and DNA-dependent protein kinase (DNA-PK) (Feng et al., 2004) all postulated as possible mediators. However, recently Sabatini's group has shown convincingly that mTORC2 is the bona fide PDK2 (Sarbassov et al., 2005). Furthermore, they have also shown that prolonged exposure to rapamycin can lead to inhibition of the assembly of mTORC2 by interfering with the ability of rictor to bind mTOR (Sarbassov dos et al., 2006). This occurs because although mTORC2 is rapamycin insensitive (Sarbassov et al., 2005), rapamycin can still bind free mTOR and inhibit the

assembly of mTORC2. Consequently, this mechanism prevents mTORC2 from phosphorylating Akt S-473. This has been demonstrated in numerous transformed or primary human cell lines *(Fig. 3.18a)* and is consistent with the findings in this study *(Fig. 3.18b)* which also supports the discovery that the actual identity of PDK2 is the rictor-mTOR complex (Sarbassov et al., 2005).

S6RP is a substrate of the upstream S6K isoforms and is believed to be involved in the control of glucose homeostasis, cell size and the negative regulation of protein synthesis (Ruvinsky et al., 2005). Numerous studies have established a strong correlation between S6K activity and S6RP phosphorylation (Gressner and Wool, 1974; Nemenoff et al., 1988; Proud, 2002), and based on the premise that phosphorylation of S6K should demonstrate a proportional increase in the phosphorylation of its ribosomal protein target, this experiment examined S6RP phosphorylation in renal fibroblasts. Similar to previous findings, this experiment showed that S6RP S-235/236 phosphorylation was inhibited by both RAD (Calvo et al., 1992; Chen et al., 2005; Chung et al., 1992; Fingar and Blenis, 2004; Gerasimovskaya et al., 2005b; Jefferies et al., 1997; Le Pabic et al., 2005; Price et al., 1992), and LY (Adi et al., 2001; Cheatham et al., 1994; Monfar et al., 1995; Weng et al., 1995). The present results therefore verify S6RP as a downstream effector of mTOR and PI3K in renal fibroblasts, however its exact role in these cells is unknown.

Fig. 3.18

Akt S-473 phosphorylation after 24hr rapamycin treatment



Rapamycin can inhibit the phosphorylation of Akt S-473 by mTORC2 in a number of cell lines (a) and in renal fibroblasts (b) in response to prolonged treatment.

BJAB = B cell lymphoma cell line; U937 = human leukemic monocyte lymphoma cell line; Jurkat = human T-cell leukaemia cell line.

(a) Sarbassov et al (2006).

Increasing evidence suggests that S6RP can be regulated by mechanisms in addition to mTOR and PI3K including the MEK pathway (Herbert et al., 2000). In fact it has been shown that S6RP phosphorylation at S-235/236 in S6K1 and S6K2 deficient cells still occurs (Pende et al., 2004). This was found to be due to a MAP-dependent kinase, most likely p90rsk (Pende et al., 2004). Whilst studies using this inhibitor have found that ERK1/2 activation is important in the phosphorylation of S6K in HEK293 cells (Herbert et al., 2000), haematopoeitic cells (Blalock et al., 2003) and hepatocytes (Okano et al., 2003), S6RP S-235/236 phosphorylation levels were not significantly changed in response to treatment with the MEK1 inhibitor, PD. Whilst it may therefore be possible that cross-talk of mTOR with MEK is a cell-specific mechanism as indicated by Lin et al (Lin et al., 1995) who demonstrated that the rapamycin sensitive activation of downstream effectors has been shown to be independent of MAPK, it is also likely that the upstream S6K's may compensate for any lack of S6RP phosphorylation by a MAP-dependent kinase.

3.4.2 Constitutive activation of mTOR

mTOR is an evolutionary conserved kinase whose signalling depends on the integration of signals from nutrients and amino acids to mediate its downstream effects. Withdrawal of amino acids or glucose within the medium of cultured mammalian cells leads to deactivation of S6K and de-phosphorylation of 4E-BP (Hara et al., 1998; Patel et al., 2001). To determine whether mTOR maintains a constitutively active circuitry independent of growth factors, the phosphorylation of S6RP was examined in the absence of FCS, and compared to cells exposed to increasing gradations of FCS. This work indicated that S6RP can be phosphorylated independently of serum-based growth factors in renal fibroblasts, presumably by intracellular amino acids and nutrients found within components of the media. These results concur with the ability of mTOR to act as a constitutively activated pathway, as previously demonstrated in VSMC (Mourani et al., 2004). Even though LY or mutated

PDGF receptors (which are responsive to extracellular growth factors) inhibit the downstream phosphorylation of S6K1 (Brunn et al., 1996; Cheatham et al., 1994; Chung et al., 1994), it appears that mTOR does not solely depend on growth factor-mediated signalling to be constitutively activated. However, activation of mTOR may be facilitated in the presence of growth factors, as indicated by phosphorylation levels in cells maintained in higher concentrations of FCS. Given that renal fibrosis involves a marked up-regulation of growth factors and cytokines, many of which are capable of signalling through PI3K and mTOR, it is therefore possible to hypothesise that during fibrosis, mTOR activity may be significantly up-regulated.

3.4.3 Regulation of fibroblast mitogenesis

Proliferation of fibroblasts is a fundamental response during chronic renal disease (Hewitson et al., 1995). As a result of proliferation, these cells can facilitate fibrosis through the secretion of chemotactic substances, differentiation and secretion of abundant ECM proteins. Regulation of the cell cycle by mTOR can be best demonstrated by its ability to regulate levels of cyclin D, $p21^{cip}$ $p27^{kip}$ polypeptides and hence regulate the G1-S phase of the cell cycle. mTOR inhibition therefore causes G1-phase cell cycle arrest. Both rapamycin and its derivatives, and more general PI3K inhibitors, are known to inhibit proliferation in a variety of mesenchymal cells including cardiac fibroblasts (Hafizi et al., 1999), glomerular mesangial cells (Wang et al., 2001a) and VSMC (Mohacsi et al., 1997; Moon et al., 2000). This work therefore expands upon previous studies by showing that both RAD and LY inhibit proliferation of renal fibroblasts.

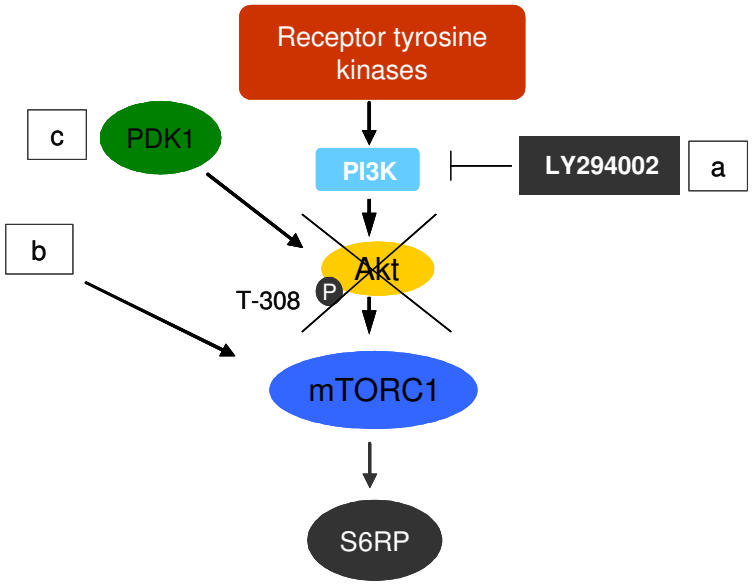
RAD has also been shown to inhibit the proliferation of fibroblasts from lung transplant recipients (Azzola et al., 2004). Studies such as these have direct clinical implications in the treatment of renal transplant recipients and provide a good premise to assess the likely *in vivo* responses of human fibroblasts in response to rapamycin and its

derivatives. Although RAD and LY mediated decreases in S6RP S-235/236 phosphorylation, knock-out of its upstream kinases leads to a decrease in cell size not cell number (Montagne et al., 1999). It is therefore possible that proliferation is not mediated through this downstream effector. Because phosphorylation of 4EBP1 facilitates translation, it may be possible that this substrate is a key mediator of cell proliferation.

The combined effect of RAD and LY on proliferation has been highlighted by Breslin et al (Breslin et al., 2005) who showed that treatment of T cells with both rapamycin and LY results in a cooperative inhibition of T cell proliferation. Similarly, this study has also shown that when RAD and LY are used in combination, it mediates a further decrease in fibroblast proliferation. Since LY completely inhibited the phosphorylation of Akt S-473 *(Fig. 3.19a)*, the further decrease in proliferation seen with a combination of RAD and LY is consistent with other studies showing that mTOR can activated by pathways other than PI3K (*Fig. 3.19b).* For instance, studies in *Drosophilia* show that mTOR can be activated by *d*PDK1 independent of *d*Akt/*d*PI3K to mediate *d*S6K activation (Radimerski et al., 2002). Further studies are needed to establish this mechanism in renal fibroblasts. However, it is worthy to note that because the other phosphorylation site T-308 (phosphorylated by PDK1) has not been examined, complete inactivation of Akt activity cannot be assumed *(Fig. 3.19c).*

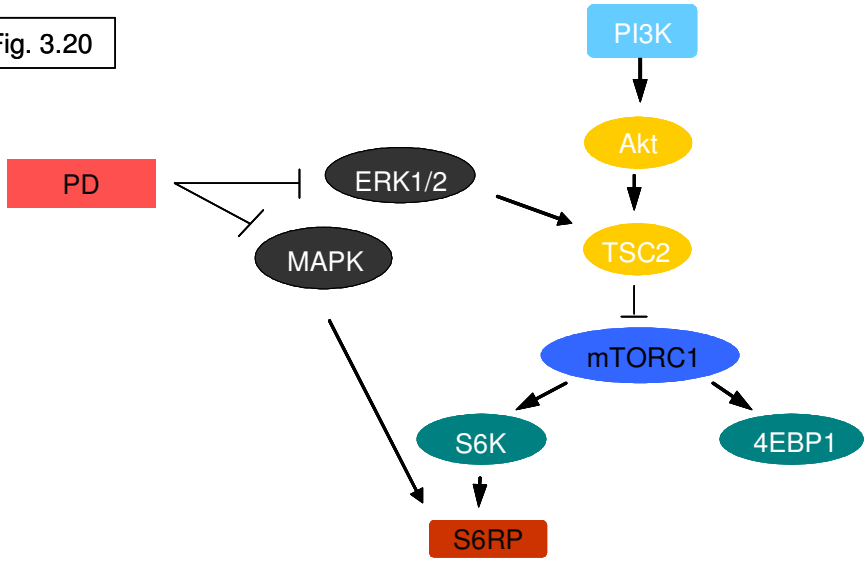
Furthermore, the results contained herein have shown that co-incubating PD with LY resulted in a further reduction in cell proliferation. The MAPK pathway, well known for the regulation of proliferation and differentiation, has also been increasingly acknowledged for its ability to signal through mTOR, corroborated by TSC2's ability to act as a direct substrate of ERK and to activate mTOR (Shaw and Cantley, 2006) *(Fig. 3.20).* This study indicates that the ERK1/2MAPK cascade and PI3K may act cooperatively to regulate fibroblast proliferation. Once again, because Akt phosphorylation was completely inhibited by LY, PI3K and the ERK1/2MAPK cascade may regulate proliferation independently of Akt. This

Fig. 3.19



Potential for mTOR to be activated by alternate pathways in the regulation of fibroblast proliferation.

Fig. 3.20



Rationale for investigating the potential for cross-talk between ERK/MAPK and PI3K/mTOR. The ERK1/2/MAPK pathway targets two substrates within the mTOR pathway (TSC2 and S6RP) suggesting that PI3K and mTOR may cross-talk with this kinase cascade. The Mek inhibitor PD was therefore used to examine S6RP and its role in proliferation.

has indeed been shown Bondeva et al (Bondeva et al., 1998), who demonstrated that PI3K regulates the MAPK pathway independently of Akt.

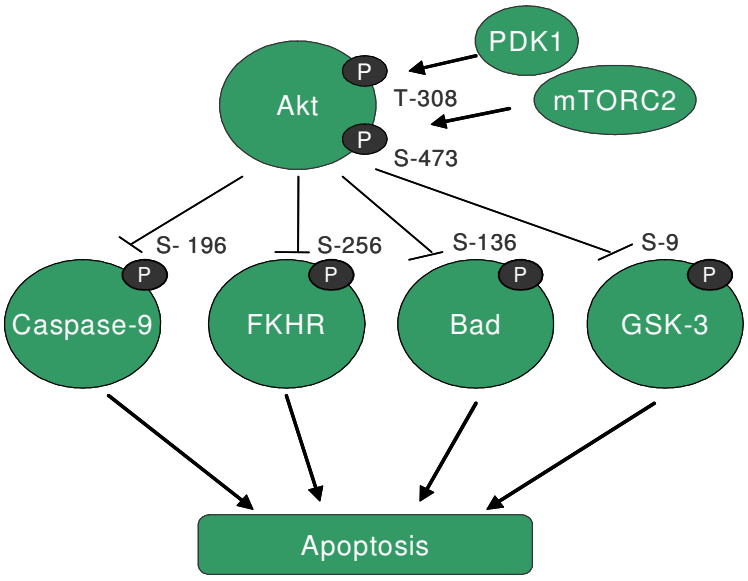
3.4.4 Apoptosis

The assessment of cell viability was particularly important in functional studies because LY completely inhibited the phosphorylation of Akt S-473. Shutting down PI3K/Akt is associated with an increased incidence of apoptosis in many studies primarily because Akt lacks the regulatory capability to phosphorylate (and inactivate) a number of pro-apoptotic molecules including Bad and GSK-3 (Downward, 2004) *(Fig. 3.21)*. Given the significance of PI3K in cell survival, a number of cell viability tests including tests for apoptosis and mitochondrial viability were carried out which indicated that cell survival and viability were not affected by treatment with RAD or LY. This confirms that the results of functional studies were not due to apoptosis or changes to cell viability.

3.4.5 Regulation of collagen synthesis

Aberrant collagen deposition is the histological hallmark of fibrosis and its destructive consequences. mTOR plays a key role in the regulation of protein synthesis through downstream effectors S6K and eEF2 and eIF4B *(Fig. 3.22*) (Ruvinsky and Meyuhas, 2006). As noted by Martin et al (Martin et al., 2004), given that mTOR plays a key role in the regulation of protein synthesis, inhibition of this pathway would lead to a decrease in total protein synthesis (Boluyt et al., 2004; Chen et al., 2005), a process that is likely to have ramifications in the regulation of collagen synthesis (Martin et al., 2004). In this study, treatment with RAD and LY demonstrated a significant inhibition in total collagen, as measured by hydroxyproline incorporation. These results were not due to a decrease in cell number because cells were grown to confluency after treatment. In agreement, a number of studies have also highlighted that an additional benefit of rapamycin treatment is the

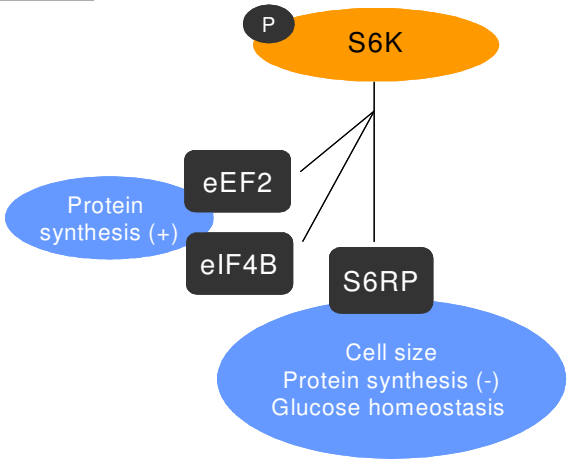
Fig. 3.21



Akt as a key anti-apoptotic mediator. Akt can phosphorylate and inactivate a number of downstream pro-apoptotic factors including Caspase-9, FKHR, Bad and GSK-3.

Adapted from www.cellsignal.com

Fig. 3.22



Key roles of S6K and S6RP in the regulation of protein synthesis

eEF2 = Eukaryotic elongation factor 2.

inhibition of collagen production. Rapamycin significantly decreases collagen secretion in rat mesangial cells *in vitro* (Kim et al., 2004), and activation of the PI3K-PDK1-Akt axis by leads to increased collagen I expression (Runyan et al., 2004). Furthermore, PI3K has been shown to be involved in the regulation of ECM proteins in keloids (Lim et al., 2003) and in the liver by regulating MMP-13 expression (Lechuga et al., 2004). However, other studies make the *in vivo* relevance of my findings are unclear. Rapamycin has variously been shown to inhibit collagen synthesis in UUO (Wu et al., 2006) and hepatic fibrosis (Zhu et al., 1999), leave collagen III expression unchanged (Jain et al., 2001) and even up-regulate collagen deposition (Shihab et al., 2004a; Shihab et al., 2004b).

Interestingly, studies that found that rapamycin increases collagen, also found that TGFβ1 levels were increased in renal transplant biopsies (Shihab et al., 2004a; Shihab et al., 2004b). Similarly, those studies that found that rapamycin-mediated decreases in collagen (Runyan et al., 2004; Wu et al., 2006; Zhu et al., 1999), also found that TGFβ1 levels were significantly decreased, as was the activity of the downstream Smads (Runyan et al., 2004). Whether there is a relationship between TGFβ1 and collagen in these studies has not been determined. However, it is increasingly acknowledged that components of the PI3K and mTOR pathways cross-talk with the TGFβ-Smad signalling axis.

3.4.6 Regulation of collagen transcription

Whilst mTOR is most well known for its role in translation, increasing evidence suggests mTOR plays a role in the control of transcription and rapamycin has been shown to inhibit members of the forkhead family of transcription factors including forkhead homologue of rhabdomyosarcomma (FKHR; Foxo1) and Foxo4a (Sarbassov dos et al., 2006). This is consistent with the findings described within which suggest that mTOR may regulate transcription. This is of novel importance in renal fibroblasts because, dictated by what are seemingly cell-dependent or stimulus-dependent effects, studies have shown that the

regulation of collagen gene expression varies. Whilst collagen mRNA inhibition has been shown to be mediated by changes at the transcriptional level in (a) amino acid-starved fibroblasts (Krupsky et al., 1997), (b) dermal fibroblasts in response to LY (Shegogue and Trojanowska, 2004), and (c) in response to mTOR inhibition using small interfering RNA (siRNA) (Shegogue and Trojanowska, 2004), the stability of α1(I) collagen mRNA may change without changes to the rate of transcription, as shown in lung fibroblasts due to inhibition of PI3K (Ricupero et al., 2001), and in activated hepatic stellate cells (myofibroblast-like cells) (Stefanovic et al., 1997).

3.4.7 Summary

In summary, this study has provided novel insights into the cell signalling mechanisms of mTOR and PI3K with regard to their role in renal fibroblasts. Whilst mTOR can function in a constitutively activated manner, PI3K and Akt function upstream of mTOR. RAD is a universal inhibitor of the mTORC1 complex, and this study has shown that with prolonged RAD treatment (48hr), it can also inhibit mTORC2, therefore regulating signalling both upstream and downstream of mTOR in these cells. S6RP functions as a downstream effector of mTOR, however whether this kinase mediates the functional effects noted in response to mTOR and PI3K inhibition awaits further delineation.

Functional studies have provided important insights into the mechanisms of renal fibroblast regulation during a number of key pro-fibrotic events and highlight rapamycin's therapeutic potential in addition to its roles in immunosuppression. mTOR and PI3K play roles in mediating fibroblast proliferation and collagen synthesis and co-inhibition of these pathways propagates additive decreases to fibroblast mitogenesis. It is likely that PI3K and mTOR cross-talk with a number of other intracellular substrates and this study has demonstrated the potential for the ERK1/2MAPK pathway to interact with the PI3K axis during fibroblast proliferation. By identifying the components of signalling pathways that

play key roles in the mediation of fibroblast proliferation and ECM synthesis, major features of fibrosis, this study hopes to contribute toward the development of potential intervention strategies that may be used to ameliorate fibrosis.

3.5 SPECIFIC METHOD PROTOCOLS

The end of each experimental chapter contains a version of *Table 3.1* for reference to methods used in this chapter that are described within *Chapter 2*.

Method	Section
UUO	2.2.1
Protein extraction	2.7.1
Western blotting	2.7.3
Cell counting	2.6.1.1
Northern blotting	2.7.2

Table 3.1: References for methods described in *Chapter 2* used in this chapter.

3.5.1 Western blotting for Akt S-473

Cellular protein was extracted from fibroblasts treated with either RAD (10nM), LY (10μM) or from those cells maintained in control medium. Western blotting was used to determine the ratio of total Akt to phosphorylated Akt with polyclonal antibodies to the phosphorylated S-473 residue of Akt according to *2.7.3*. This antibody recognizes all isoforms of Akt at S-473. Membranes were incubated with either a 1:500 dilution of anti-phosphorylated Akt or a 1:1000 dilution of anti-Akt O/N, followed by a 1:2000 incubation with HRPconjugated anti-rabbit IgG *(Table Apx. 3.2).*

3.5.2 S6RP phosphorylation (S-235/236)

The effect of serum-starvation, and the PI3K, mTOR and MEK inhibitors on S6RP was determined by measuring the ratio of phosphorylated S6RP S-235/236 to total S6RP. Cells were grown to confluency in 6-well plates in 10%FCS + DMEM and starved O/N in 1%FCS + DMEM. RAD (100nM), LY (10μM), PD (20μM) or differing concentrations of FCS (0%, 2.5%, 10%) were then administered for 120min and incubated at 37ºC. The p-S6RP ELISA was then carried out according to the manufacture's protocol.

3.5.3 Cell kinetics

3.5.3.1 Cell number assay

Cellular proliferation was determined by measuring total cell number over a 5-day period. Fibroblasts were trypsinised and seeded into 6cm diameter petri dishes at a density of $2x10^4$ cells per dish in DMEM+10% FCS and left O/N to adhere at 37°C. Following cell adhesion, control medium or medium containing RAD (10, 100, 200nM), LY (10μM), or combinations of these drugs were added and cells were left at 37°C. On day 5, cells were trypsinised, resuspended and counted with a haemocytometer. Non-viable cells were excluded by adding trypan blue in a 1:1 ratio to each aliquot.

3.5.3.2 DNA synthesis

Fibroblast DNA synthesis was estimated by measuring incorporation of tritiated [^{3}H] labelled thymidine in FCS stimulated cells as described previously (Hewitson et al., 2001). Thymidine incorporation was used to determine the effects of various drugs on DNA synthesis. Equal numbers of cells were seeded in DMEM + 10%FCS in 36 mm^2 multiwell plates at $2x10^4$ per well and left O/N at 37 ^oC. On day 2, cells were then grown in DMEM + 5% FCS for 24hr to arrest cell cycle progression. Cells were incubated for 24hr in DMEM+10%FCS only or medium containing RAD (10, 100, 200nM), LY (10μM), PD (20μM), or combinations of these drugs at 37°C. On day 3, media was removed and 0.25μCi (250μCi/250μls) of tritiated thymidine was added with or without test drug to each well and left O/N at 37°C. At the end of the incubation period, medium was removed, cells were washed in 2-5ml PBS×2, and 200μl of 5% trichloroacetic acid (TCA) was added to each well. Cells were then incubated at 4^{o}C for 60min. TCA supernatant was removed, and cells were scraped in the presence of 200μl 0.25M NaOH and transferred to scintillation vials. Approximately 10ml of scintillation fluid was added and radioactivity was counted in a scintillation counter as counts per minute (cpm).

3.5.4 Cell viability

3.5.4.1 TUNEL

Apoptotic nuclei were detected using TUNEL (Hewitson et al., 2000a). Cells were passaged as described previously and an equal number of cells were seeded onto sterile coverslips within petris and grown in DMEM + 10% FCS. Once cells were subconfluent, they were treated with RAD (200nM) or LY (10μM) or maintained in control medium at 37°C. Cells were then fixed in 4% paraformaldehyde in PBS for 5min and the assay was carried out according to the manufacturer's instructions. A number of coverslips were also included as positive controls by treating them with DNase to digest cells (1000U/ml). Apoptotic cells were identified by a combination of nuclear morphology and brown nuclear staining, as described previously (Hewitson et al., 2000a).

3.5.4.2 Propidium iodide

Propidium iodide staining was used to examine nuclear morphology after treatment with RAD (10, 100, 200nM) or LY (10, 50μM). Cells were fixed in cold methanol, washed in PBSx2 and incubated with propidium iodide diluted in PBS (1mg/ml). Cells were then washed in PBSx2 and analysed with a fluorescent microscope. Apoptotic cells were identified as containing clusters of small round nuclear bodies.

3.5.4.3 MTT ELISA

Cell viability was estimated using an MTT assay. This assay is based on the ability of the mitochondria to reduce colourless MTT to a purple formazan salt. Fibroblasts were trypsinised as normal and an equal number of cells seeded into 96 well plates with DMEM + 5% FCS in low HEPES (10mM). Once cells were confluent, they were incubated for 48hr in DMEM + 5%FCS in 10mM HEPES with RAD (10, 100, 200nM), LY (10, 50μM), or maintained in control medium. Following this, media was removed and 5mg/ml MTT was

added with DMEM + 5%FCS in 10mM HEPES at a 1:4 ratio. Plates were placed in foil and incubated at 37ºC for 4hr. Cells were then lysed by the addition of DMSO and Sorensen's glycine buffer was added at a 1:8 ratio. Reaction product was estimated from the optical density at 570nm on a Sunrise ELISA plate reader.

3.5.5 Collagen synthesis

Total collagen synthesis was estimated form hydroxyproline incorporation, as described previously (Fullerton and Funder, 1994) to determine the role of mTOR and PI3K in total collagen synthesis. The method was carried out according to the following passages.

1. Cells were trypsinised and an equal number of cells were then seeded and grown to confluency. Cells were then treated with standard media supplemented with 1ml of 100mM ascorbic acid and RAD (100nM) or LY (10μM), or maintained in control medium with 100mM ascorbic acid. This treatment regime was repeated every 24hr over a 72hr period.
2. On the fourth day, 3.3ml of incubation medium per well was added containing the drug being tested for 4hr at 37ºC. After 4hr, 33μl of the labelling isotope [2,3-^{3}H Pro]-Pro was added to the incubation medium for 18hr at 37ºC.
3. After the 18hr exposure, plates were placed on ice and the medium fraction was collected in a 10ml tube. The volume collected was used to calculate how much 100% TCA, 10% proline and 10% bovine serum albumin (BSA) to add to each sample.
4. Each sample received appropriate concentrations of 10% TCA, 0.04% proline and 0.1% BSA and were then placed at 4ºC O/N to precipitate the protein.
5. The next day medium fractions were centrifuged down for 20min at 13000rpm at 4^{o}C. On ice, supernatant was removed and the pellet resuspended with 1ml 10%TCA /1mM proline.

6. Samples were then centrifuged at 6-7000rpm for 5min at 4°C. On ice, the pellet was resuspended in 1ml 5% TCA/1mM proline and centrifuged at 6-7000rpm for 5min at 4°C.
7. Removal of the supernatant and resuspension of the pellet in 1ml 5%TCA/1mM proline was repeated and samples were centrifuged at 6-7000rpm for 5min at 4°C again. The pellet was then dissolved in 500µl 0.2M NaOH.
8. From each sample dissolved in 0.2M NaOH, duplicate aliquots of 200µl were made in clean tubes. The NaOH in each sample was neutralised by adding 100µl of 1M HEPES buffer and using pH strips, the pH was adjusted with HCl or NaOH to 7.2-7.4.
9. Samples were then left O/N at 4°C.
10. The next day, equal numbers of samples were treated with either 40µls of positive collagenase mix or 40µl of negative collagenase mix. Samples were then incubated at 37°C for 90min.
11. Samples were immediately placed on ice and 0.5ml of 20% TCA/0.5% tannic acid was added and leave for 60min. Samples were then centrifuged in tubes at 6-7000rpm at 4°C for 5min.
12. Then the supernatant was removed and placed in labelled scintillation vials. Pellets were washed with 0.5ml 5% TCA, centrifuged at 6-7000rpm at 4°C for 5min and the remaining supernatant was combined with the original in scintillation vials.
13. 10ml of scintillation fluid was then added to each sample, and radioactive counts measured as disintegration per minute (dpm) was determined using a beta counter. The non-collagenase digested sample ('-') counts were subtracted from the '+' sample in order to gain the final collagen count for each sample tested.

3.5.6 Northern blotting for procollagen α1(I)

To examine levels of procollagen α1(I) mRNA in response to RAD, RNA was extracted from cells treated with RAD (10, 100, 200nM) or from those maintained in control medium, and Northern blotting was then carried out according to section *2.7.2*. Procollagen mRNA was quantified using densitometry software.

Chapter 4

Development and Validation of an *Ex Vivo* Model of EMT

4.1 INTRODUCTION

It is now widely acknowledged that interstitial fibroblasts are an important cellular mediator of all progressive renal disease, regardless of etiology. Myofibroblasts in particular are key to the pathogenesis of scarring. However, much less is known about the derivation of these cells, and the regulation of their phenotype.

A growing body of evidence however highlights the interrelationship between tubular epithelial cells and interstitial fibroblasts. Fibroblasts, and by implication myofibroblasts, may be derived from a variety of sources including epithelial-mesenchymal transition (EMT). This chapter sets out to develop and validate an experimental model of EMT that can be used to examine the regulation of this important process. This is particularly relevant because EMT is a highly complex process that is inherently dependent on the surrounding microenvironment, numerous cellular programs and a plethora of cytokines including TGFβ1(Yang and Liu, 2001) and FGF-2 (Strutz et al., 2002), vasoactive peptides such as angiotensin II (Chen et al., 2006), and non-cytokine mediated pathways such as AGEs (Oldfield et al., 2001) and tPA (Yang et al., 2002b). Although pathways involved in EMT have been progressively identified, it is important to fully characterise the pathways involved in engaging the EMT proteome.

Initial studies on EMT concentrated on utilising nrk-52e cells, an immortalized line of rat tubular epithelial cells, thought to be of proximal tubule origin (Fan et al., 1999). As outlined in *section 4.2*, difficulties in its application led to the development and validation of a new model of EMT *(section 4.3),* based on the *ex vivo* culture of explanted renal tissue. Consequently, this model hopes to better represent the *in vivo* environment and over-comes some inherent difficulties with *in vivo* studies of EMT.

4.2 AN *IN VITRO* MODEL OF EMT USING TRANSFORMED EPITHELIAL CELLS

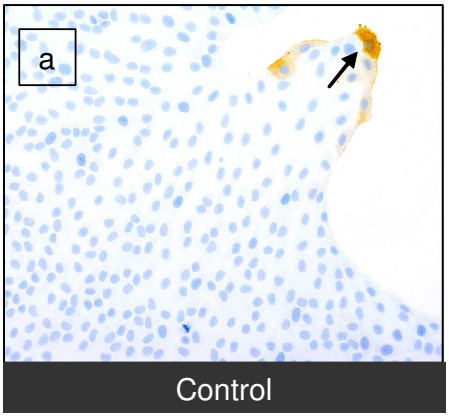
4.2.1 METHODS AND RESULTS

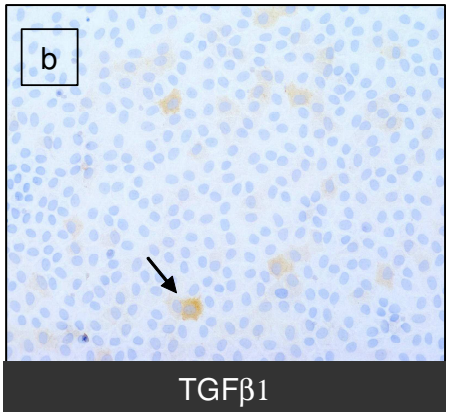
In pilot studies, $3x10^4$ trypsinised nrk-52e cells were seeded onto uncoated sterile 3cm diameter petri dishes, supplemented with DMEM + 10% FCS, and grown to confluence (approx 4-5 days). Cells were fixed in ice-cold methanol and stained with anti-αSMA using the techniques described in *2.6.1.* An unexpected and consistent finding was that approximately 60% of cells expressed αSMA, even when FCS was reduced to 1% (data not shown).

In an attempt to reduce basal αSMA expression, petri dishes were coated evenly with 800µl of cold Cellagen solution (bovine skin collagen I). Petris were then washed with 2-3ml of PBS. After incubating at 37°C for 30min, 3ml DMEM + 10% FCS medium was added to each petri to neutralise the pH, and they were incubated at 37°C for at least 15min. Coating with Cellagen consistently reduced expression of αSMA, although cells at the periphery were often αSMA-positive (*Fig. 4.1a*, arrow). This was presumably in response to lack of cell adhesion to collagen, compromising their integrity and stimulating the development of stress fibres.

Cells were then treated with 10ng/ml of TGFβ1, an established inducer of EMT in this cell line (Fan et al., 1999). TGFβ1 was replenished every second day, before cells were fixed and stained. Qualitative analysis of cells treated with TGFβ1 indicated an increase in αSMA staining, albeit of a highly focal nature (*Fig. 4.1b*, arrow). This was estimated to only comprise 1-2% of total cells. Western blotting with antisera to αSMA was also used, however changes were minimal when corrected for β-actin, and there were therefore no detectable differences in αSMA protein (data not shown).

Fig. 4.1





Once expression of αSMA expression was reduced in nrk-52e cells, cells were treated with 10ng/ml TGFβ1. Although an increase in αSMA expression could be detected (b), no significant changes could be detected by immunohistochemistry as compared to the control. Fig. 4.1a highlights the characteristic and focal expression of αSMA at the periphery of cells (arrow).

4.2.2 DISCUSSION

Initial experiments with the immortalised nrk-52e cell line indicated a consistent expression of αSMA when grown on plastic. In order to resolve this issue, cells were seeded onto a collagen matrix (Cellagen). Phenotyping of the cells confirmed that the basal expression of αSMA was reduced. Ironically, it has been shown that the α1NC1 domain of type I collagen induces EMT *in vitro* by inhibiting the assembly of type IV collagen (Zeisberg et al., 2001). In retrospect, it would therefore be expected that adherance to this type of collagen matrix, which is a type I collagen, would facilitate EMT and therefore increase the expression of the mesenchymal marker αSMA.

The ability of this cell line to undergo EMT was then examined with TGFβ1. Whilst Fan et al (Fan et al., 1999) found that nrk-52e cells stimulated with 10ng/ml showed profound changes in morphology and phenotype, chiefly through loss of the epithelial marker E-cadherin and *de novo* expression of αSMA, studies described within using 10ng/ml of TGFβ1 found that only 1-2% of cells underwent EMT. Consequently, changes in αSMA protein, as determined by western blotting, were minimal when compared to β-actin and no real changes were detected.

Given the issues that were raised using this cell line and the difficultly in establishing these cells as a reliable model of EMT *in vitro*, this model was felt to be of limited practical value in examining the regulation of EMT. It was therefore apparent that a different model of EMT was needed to study the mechanisms of this process.

4.3 AN *EX VIVO* RENAL EXPLANT CULTURE MODEL OF EMT

The next passages describe the development and validation of a novel *ex vivo* model of EMT based on explanting of the normal rat renal cortex.

4.3.1 DESIGN OF MODEL

Fortuitously, the observation of typical growth patterns that have been observed during explanting of cortical tissue derived from the normal kidneys of rats led to the hypothesis that EMT occurs during the explanting process. On many occasions, it has been observed that cells that grow out of tissue fragments of explant tissue seem to characteristically change their morphology as they travel further away from the deposited tissue. Given that EMT can occur in response to mechanical injury (Bascands and Schanstra, 2005), and that explanting also mimics a form of mechanical injury sustained by tubular epithelial cells, this underscores the potential for EMT to occur during this process. A number of key features observed during explanting (described below), along with the growing understanding of the process of EMT, indeed highlight the presence of EMT during the growth of cells from explants.

This chapter therefore examines the potential for epithelial cells growing out of explants to undergo EMT. This was specifically investigated by phenotyping cells for epithelial, endothelial and mesenchymal markers using immunoperoxidase techniques and double-labelling immunofluorescence. This model was then tested using established regulators of EMT.

4.3.2 EXPERIMENTAL DESIGN

4.3.2.1 Explants for the study of EMT

Cell explant outgrowths from normal rat kidneys were established using explanting methods described in *2.3.1*. Studies in this lab have repeatedly shown that the first cells to grow out of explants of normal kidney tissue are epithelial in nature. In each case, explants were grown in DMEM + 20% FCS, treated in DMEM + 10% FCS, and fixed in cold methanol after various intervals between 3 and 17 days post explanting. Morphology analysis and immunocytochemistry were used to identify, mesenchymal (FSP-1, αSMA+, desmin+), epithelial (cytokeratin+) and endothelial (RECA+) cells.

4.3.3 RESULTS

4.3.3.1 Primary cell outgrowth

After cortical renal tissue was minced into gelatin coated petri dishes, it took approximately 3 days before cell growth could be identified (*Fig. 4.2*). Those cells that grow out initially displayed a cuboidal phenotype, typical of epithelial cells. These cells grew out in sheets around the tissue presumably through the secretion of growth factors/cytokines and matrix interactions. Typical of epithelial cell growth, they grew out in a uniform manner representative of them being tightly bound by cell-cell junctions and adhesions (Lee et al., 2006).

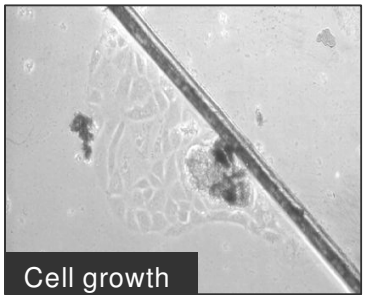
4.3.3.2 Two sub-populations of cells grow from explants of the renal cortex

Cells that surrounded explant tissue were characterised with phenotypic markers of mesenchymal cells, epithelial cells and endothelial cells. By day 7, cells were 21±18% αSMA+, 26±18% desmin+, and 79±12% were cytokeratin+ positive. At this stage it was evident that two cell predominant cell populations were present: epithlelial cells and

myofibroblasts. *Fig. 4.3a-b* illustrates that at day 7, explants typically contained clusters of E-cadherin and cytokeratin positive cells. These cells displayed a typical cobblestone morphology and were tightly bound to their neighbours. *Fig. 4.4a-b* illustrates the presence of myofibroblasts, as demonstrated by positive staining for the mesenchymal markers αSMA and FSP1. These cells displayed a spindle-shaped morphology and were dissociated from their neighbours. There were not however any RECA positive cells, confirming endothelial cells were not a feature of explanting *(Fig. 4.5).*

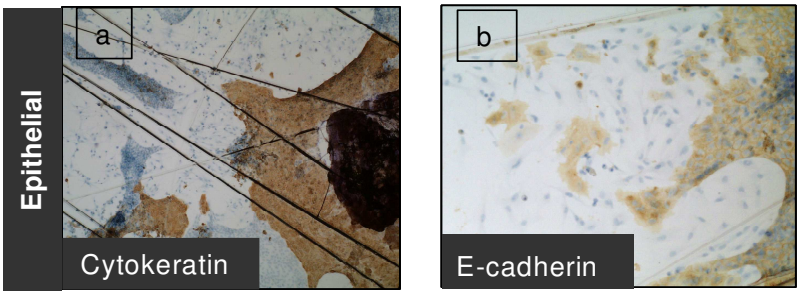
This process of phenotyping indicated that either epithelial cells or myofibroblasts grew out from tissue fragments. However, when examined further, it was revealed that these sub-populations may be related. Such speculation was based on (a) that there were sub-populations of cells that partially but consistently stained for αSMA *(Fig. 4.6a),* and although this may have represented different sub-populations of cells over-growing each other (fibroblasts over myofibroblasts or epithelial cells over myofibroblasts); (b) there were also cells that stained positive for cytokeratin but they clearly did not display an epithelial morphology *(Fig. 4.6b).* These cells looked larger, had started to dissociate from their neighbours and did not display the typical cobblestone morphology of epithelial cells. This strongly suggests that these cells were undergoing some type of transition process.

Fig. 4.2



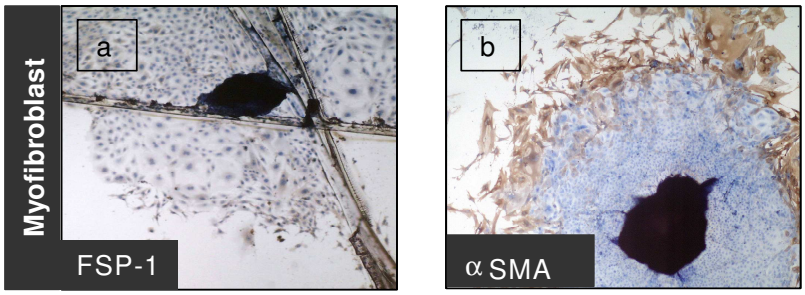
It took approximately 3 days before cell growth from explant tissue could be observed.

Fig. 4.3



Cells stain positive for cytokeratin (a) and E-cadherin (b), indicating that epithelial cells grow out from explants.

Fig. 4.4

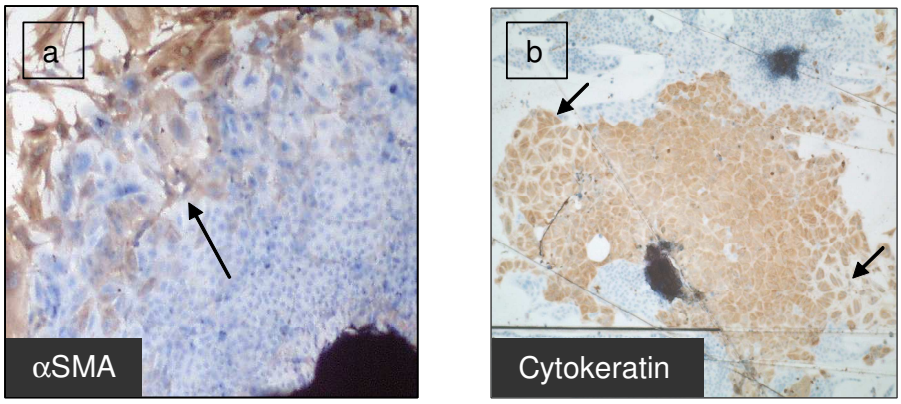


Sub-populations of myofibroblasts are also present, as demonstrated by positive staining for FSP-1 (a) and αSMA (b).

Fig. 4.5

Endothelial cells do not typically grow out of explant tissue as demonstrated by sub-populations of cells staining negatively for RECA.

Fig. 4.6

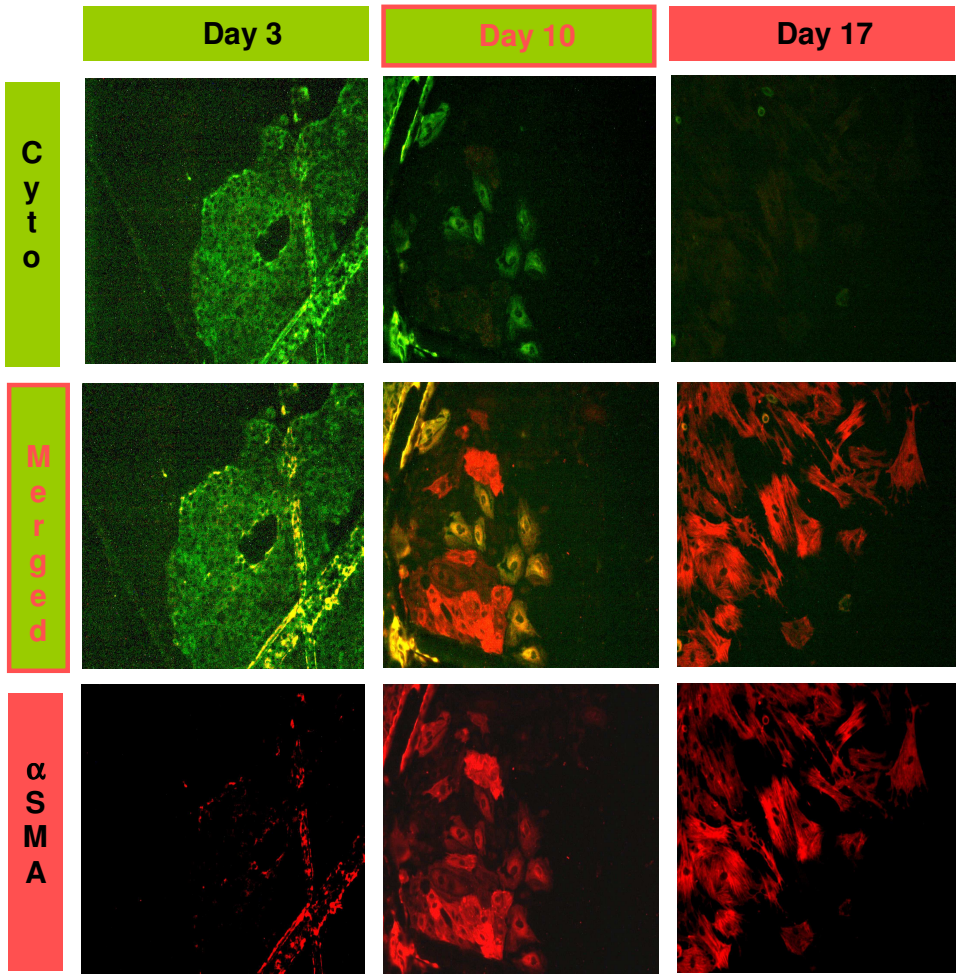


At day 7, cells typically stained for αSMA or cytokeratin, however there were also populations of cells present that stained partially for αSMA (a, arrow). Furthermore, morphological changes in cytokeratin positive cells could also be demonstrated (b). These cells displayed a morphology not typical to epithelial cells including hypertrophy, elongation and dissociation from neighbor cells (arrows).

4.3.3.3. Double labelling indicates cell progressive EMT in explants

To determine if the mesenchymal and epithelial cells shown in *Fig. 4.6* were indeed undergoing a phenotype change, double labelling immunofluorescence was performed at various time points post-explanting. At day 3, outgrowths from tissue labelled only for epithelial cell markers, with no cells expressing αSMA (day 3; *Fig. 4.7*). At day 10, myofibroblasts were also present, and by double-labelling, it was demonstrated that populations of cells co-expressed αSMA and cytokeratin (cytokeratin+/αSMA+) (day 10; *Fig. 4.7*). This indicated that a process of EMT was occurring and when quantified, it was revealed that 50±12% (n=5) of cytokeratin positive cells also co-expressed αSMA at day 10. By day 17, cultures were all myofibroblasts and it was apparent that those cells at day 10 that co-expressed cytokeratin had lost their staining for cytokeratin (cytokeratin-/αSMA+; day 17; *Fig. 4.7*). These changes were also accompanied by morphological changes. Cells lost their cobblestone appearance typical of epithelial cells, were dissociated from their neighbours, and became hypertrophic and elongated as they progressively changed into spindle-shaped myofibroblasts.

Fig. 4.7



Double-labelling immunofluorescence indicates that EMT occurs during growth of cells from tissue explants. Initially,cells can be characterised as cytokeratin positive however by the later stages of explanting, these cells acquire αSMA. 50% of these cells co-expressed αSMA and cytokeratin (n=5). By the later stages post-explanting, cells lose their epithelial markers and express αSMA only.

Chapter 5

The Role of Phosphatidylinositol 3-Kinase and mTOR in the Regulation of Myofibroblast Differentiation and EMT

5.1 INTRODUCTION

The process of myofibroblast differentiation is of pivotal importance to the progression of tubulointerstitial fibrosis as these are the cells that are responsible for the secretion of copious amounts of ECM proteins. We have come to understand that this form of differentiation is a highly complex process that depends not only on the differentiation of resident fibroblasts but also on the differentiation of a number of heterogenous cell types. These include blood-borne fibrocytes (Iwano et al., 2002), bone-marrow derived pluripotential cells (Iwano et al., 2002), mesangial cells (Sam et al., 2006), resident and perivascular fibroblasts (Hewitson et al., 1995), pericytes (Postlethwaite et al., 2004) and tubular epithelial cells (Roxburgh et al., 2006).

Of those cells in the tubulointerstitial compartment, fibroblasts and epithelial cells are known to be the most important sources of myofibroblasts. It is therefore important to characterise the signalling pathways involved in differentiation. This study therefore set out to elucidate the role of phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) in (a) the differentiation of fibroblasts and in (b) EMT.

Although fibroblasts represent a highly heterogenous population of cells, differentiation of these cells is based on a common mechanism. In response to mechanical or environmental stressors such as stretch or hypoxia respectively, these cells are thought to be able to transdifferentiate into proto-myofibroblasts *(Fig. 1.9)*. The proto-myofibroblast is an intermediate form of a fully differentiated myofibroblast that expresses stress fibres (α and β), however only in the presence of TGFβ1 can these cells then differentiate into myofibroblasts acquiring the ability to secrete large amounts of the ED-A splice variant of fibronectin and express αSMA (Tomasek et al., 2002). Given that TGFβ seems indispensable in mediating myofibroblast differentiation, many of the regulatory studies of myofibroblast differentiation have, perhaps not surprisingly, focused on the role of the TGFβ-Smad signalling axis.

The process of differentiation is likely however to involve more than one signalling axis. Whilst PI3K and mTOR are more well-known for their roles in proliferation and growth, a number of studies have identified this axis as a key mediator of cell differentiation. Recent work in VSMC's is of particular interest (Martin et al., 2004). In this study the investigators were able to establish that inhibition of mTOR was sufficient to induce VSMC differentiation, thereby implicating mTOR as a negative regulator of VSMC differentiation. The phenotypic similarity between vascular smooth muscle cells and fibroblasts suggests obvious parallels and underscores the potential for mTOR to be involved in mediating fibroblast differentiation.

Although epithelial cells display a higher level of homogeneity than fibroblasts, these cells can respond in a variety of different ways in the presence of an injurious stimulus. As outlined in *Chapter 4*, this seems to be inherently dependent of the state of the cell cycle (Bonneton et al., 1999; Yang et al., 2006c). Tubular epithelial cells can (a) proliferate, (b) degenerate and undergo apoptosis, (c) secrete a number of inflammatory cytokines including MCP-1 and RANTES, or (d) they can transition into myofibroblasts via EMT. Furthermore, epithelial cells may also de-differentiate in an attempt to regenerate the damaged tubular segments or repair the damaged area (Forino et al., 2006; Rastaldi et al., 2002). With the likelihood that EMT can act as a source for a substantial number of myofibroblasts and even replenish fibroblast pools late in fibrosis (Yang and Liu, 2001), it is important to identify the signalling axes through which EMT is mediated. Whilst members of the Smads (Valcourt et al., 2005; Zavadil and Bottinger, 2005), MAPK (Li et al., 2004b; Yu et al., 2002) and Rho (Kaartinen et al., 2002; Masszi et al., 2003) signalling pathways have notably gained the most attention, it is increasingly accepted that EMT is complex and likely to involve a number of signalling pathways. Furthermore, with the ongoing elucidation of signalling pathways in fibrosis, it is increasingly recognised that TGFβ, MAPK and PI3K can cross-talk in a number of different cells types. With this in mind, and also given that PI3K/Akt play roles in EMT in

various other contexts (Bakin et al., 2000; Grille et al., 2003; Irie et al., 2005), an investigation into the role of PI3K and mTOR in EMT is warranted. To carry out this investigation, the previously established model of EMT *(Chapter 4)* was utilised.

This study therefore set out to investigate the role of PI3K and mTOR in the regulation of rat renal fibroblast differentiation and renal EMT, which can both supply the myofibroblast population with significant numbers of myofibroblasts. Once again, using rapamycin derivative RAD and LY294002 (LY) acting as a inhibitor of the ATP binding domain of PI3K *(Fig. 3.1)*, a number of novel findings have been observed in this study.

5.2 EXPERIMENTAL DESIGN

This study utilised rat renal fibroblasts derived from 3 days post-UUO as outlined in *Chapter 3* to examine the differentiation of fibroblasts to myofibroblasts, and the model of EMT, established in *Chapter 4*, to study the transition of tubular epithelial cells into myofibroblasts. Unless specified, cells were treated for 48hr in medium alone, or medium supplemented with various doses of RAD (10, 100, 200nM), LY (10μM) and TGFβ1 (10ng/ml). These studies were based on the response of fibroblasts to serum-based growth factor/cytokine stimulation. In each case, medium consisted of 1xDMEM supplemented with 10%FCS, 1M HEPES, 2mM glutamine and 500Units/ml penicillin/streptomycin, unless specified.

5.3. RESULTS

5.3.1 PI3K and mTOR regulate fibroblast differentiation

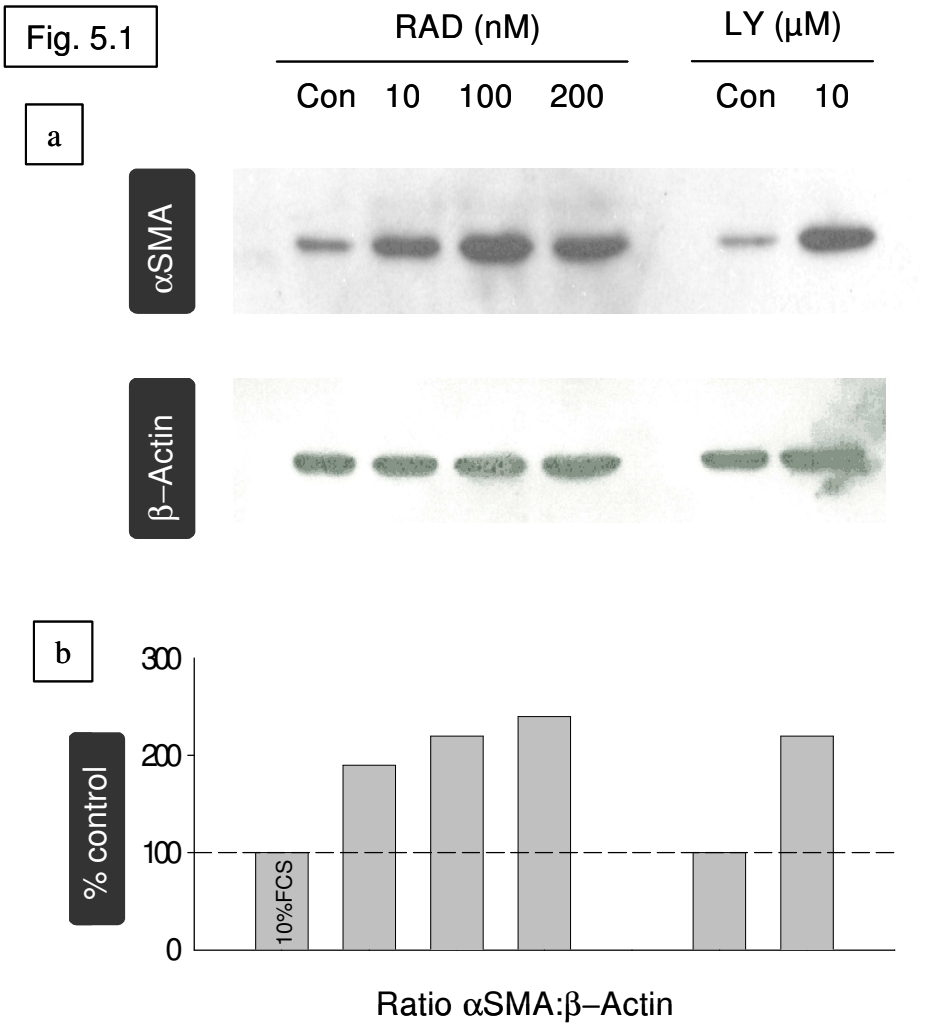
Martin et al (Martin et al., 2004) have recently demonstrated the ability of mTOR to regulate VSMC differentiation, as demonstrated by a rapamycin induced increase in αSMA, calponin, and SM myosin heavy chain. Western blotting and cytochemistry were therefore used to examine the role of the PI3K and mTOR in renal fibroblast differentiation.

5.3.1.1 Regulation of αSMA protein by PI3K and mTOR in renal fibroblasts

The functional significance of PI3K and mTOR in differentiation was confirmed by using western blotting to demonstrate that all doses of RAD (10, 100, 200nM) and LY (10μM) induced αSMA expression, an established protein marker of myofibroblast differentiation (*Fig. 5.1a*). Results of densitometry from a representative experiment indicated that RAD and LY increased αSMA expression relative to β–actin by 1.9, 2.2, 2.4 and 2.2 fold respectively (ratio αSMA: β-actin; *Fig. 5.1b*).

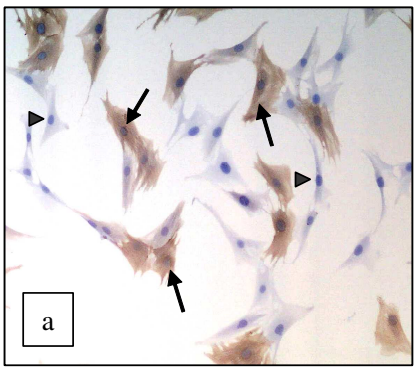
5.3.1.2 Cytochemistry for αSMA

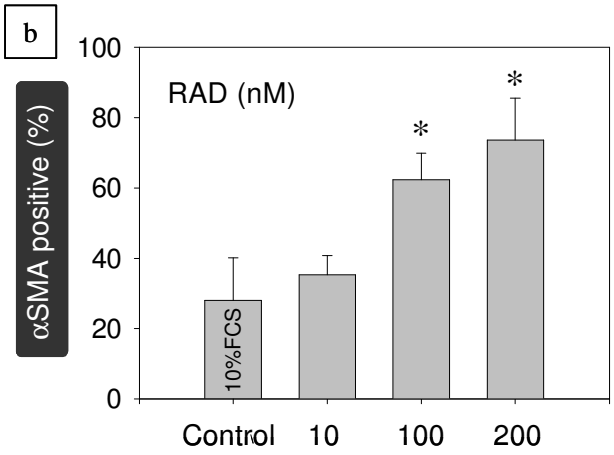
The change in total αSMA protein expression was reflected by an increase in the proportion of myofibroblasts, as determined by the number of cells staining for αSMA (*Fig. 5.2a*, arrows) which were visibly larger compared to negative staining cells (*Fig. 5.2a*, arrow heads). Treatment with 100, 200nM RAD and 10μM LY increased the percentage of cells staining for αSMA, and therefore myofibroblasts, by 34±8 %, 46±12% and 20±8% respectively (*Fig. 5.2b-c*; $p<0.05$ vs. control, n=3 each group), implicating both PI3K and mTOR in the regulation of fibroblast differentiation. No significant change in αSMA immunostaining in response to 10nM RAD was noted however.

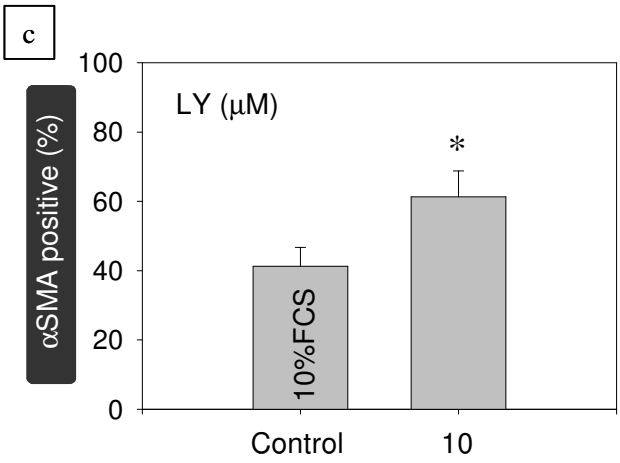


(a) Treatment with RAD and LY increased total αSMA protein expression. Membranes were probed with a monoclonal antibody against β-actin to correct for loading. (b) Compared to control groups, RAD increased αSMA by 1.9-fold (10 nM), 2.2-fold (100nM) and 2.4-fold (200nM) whilst LY (10μM) increased αSMA by 2.2-fold. The results illustrate a duplicate experiment.

Fig. 5.2







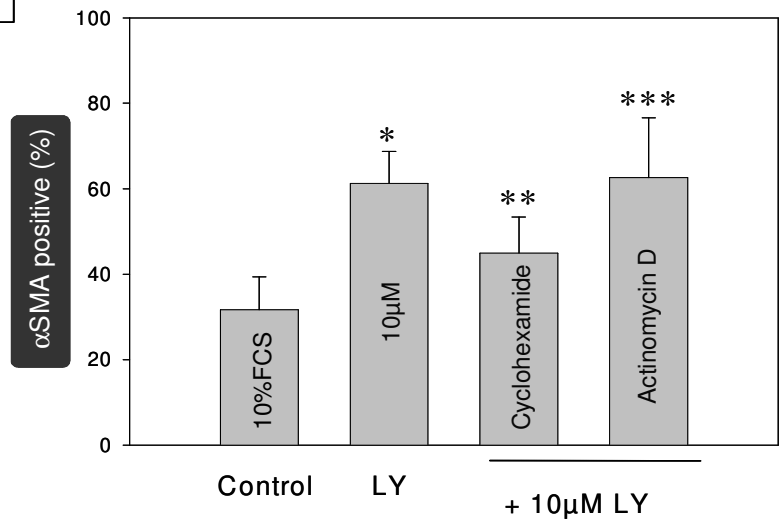
*The change in total αSMA expression was reflected by a corresponding increase in the proportion of myofibroblasts. After exposure to RAD or LY, cells were evaluated with immunocytochemistry to determine the effect on myofibroblast differentiation. (a) In each case, a minimum of 400 cells were evaluated for αSMA staining. αSMA positive cells (arrows) appeared larger than their αSMA negative counterparts (arrowheads). The proportion of myofibroblasts was increased by (b) 34±8% (100nM RAD) 46±12% (200nM RAD) and (c) 20±8% (10μM LY). Results are expressed as mean ± S.D.% and illustrate a duplicate independent experiment (*p < 0.05 vs. control).*

To confirm that these changes in differentiation were not simply a consequence of change in cell confluency, the proportion of myofibroblasts in cells seeded at varying densities was specifically examined. When seeded in control medium at $1x10^4$, $3x10^4$ and $6x10^4$cells/well, a similar proportion of cells stained for αSMA in each group after 72hr of treatment (data not shown).

5.3.2 Changes in αSMA expression may be independent of transcriptional activation

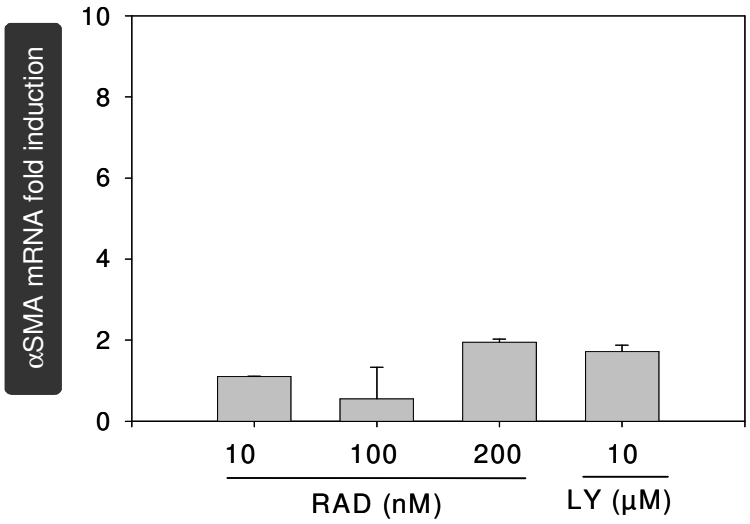
Steady state mRNA levels can be affected by the level of gene transcription as well as changes to mRNA stability (Jinnin et al., 2006). To determine if the increase in αSMA protein, regulated by PI3K and mTOR, was attributed to translational or transcriptional regulation, cells were treated with either actinomycin D or cycloheximide preceding treatment with 10μM LY. Based on the premise that LY should increase the proportion of αSMA cells (29.6±7.5% vs. control, $p<0.001$, n=7), cyclohexamide (translational inhibitor) was the only agent capable of inhibiting this response (p=not significant (ns) vs. control, n=7). Conversely, pre-treatment with Actinomycin D, a transcriptional inhibitor, did not inhibit αSMA expression as shown by *Fig. 5.3*, which illustrates a 31±14% increase compared to the control group ($p<0.001$ vs. control, n=7). These preliminary results suggest that PI3K regulates αSMA expression via translational activation rather than direct transcriptional activation of αSMA.

Fig. 5.3



*To determine if mTOR and PI3K regulate transcription or translation, cells were pre-treated with actinomycin D and cyclohexamide for 1hr prior to treatment with 10μM LY. Based on the premise that LY should increase the proportion of αSMA cells (29.6±7.5 vs. control, *p<0.001, n=7), cyclohexamide was the only agent capable of inhibiting this response (**p=ns vs. control, n=7). αSMA was still increased by 31±14% in the presence of actinomycin D (***p<0.001 vs. control, n=7). This suggests PI3K may not regulate αSMA at the transcriptional level. This data represents an average of three independent experiments.*

Fig. 5.4



Gene expression was then evaluated by real time PCR by probing for αSMA via Syber Green quantitative analysis. Pilot studies have utilised TGFβ1 as a positive control (data not shown). Changes in αSMA gene expression were measured by determining the fold change compared to the house keeping gene (Ribosomal protein L32) and to the control groups. αSMA gene expression did not significantly change in response to all doses of RAD (10, 100, 200nM) and LY (10μM LY).

5.3.3 Changes in αSMA expression independent of transcriptional activation can be further confirmed by real time PCR

Consistent with actinomycin D not being able to prevent the LY-mediated increase in αSMA, there was no demonstrable effect of LY on αSMA mRNA expression using real time PCR (*Fig. 5.4*). In both RAD (10, 100, 200nM) and LY (10μM) treated groups, αSMA gene expression was not meaningfully changed, as compared to the house-keeper gene, Ribosomal protein L32 (average of two experiments). These changes to αSMA gene expression were reasoned to be insignificant due to the sensitivity of this method as compared to western blotting where similar fold differences are likely to be more significant.

5.3.4 RAD and LY induce differentiation and decreases in proliferation in the same cell sub-populations.

Because mTOR and PI3K regulate proliferation and differentiation inversely in fibroblasts, it is possible that these kinases may be inducing mitogenesis and differentiation in different sub-populations of cells. To evaluate this, cells treated with LY or RAD were double-labelled with αSMA and proliferating nuclear cell antigen (PCNA). Consistent with studies that have evaluated fibroblast DNA synthesis (*Chapter 3*), qualitative evaluation indicated that treatment with either LY or RAD resulted in a reduction in PCNA positive nuclei (*Fig. 5.5a-c*). Double labelling for PCNA and αSMA indicated that 22±10% and 21±4% of fibroblasts expressed both markers in RAD (100nM) and LY (10μM) treated groups respectively, compared to 53±13% in the control group (*Fig. 5.6*, $p<0.05$ vs. control; n=3 each group). Consequently, of the RAD and LY treated groups undergoing proliferation, approximately 60% fewer cells (when the control is taken as 100%) are in a differentiated state as compared to the control group. Given that inhibition of PI3K and mTOR facilitates

Fig. 5.5

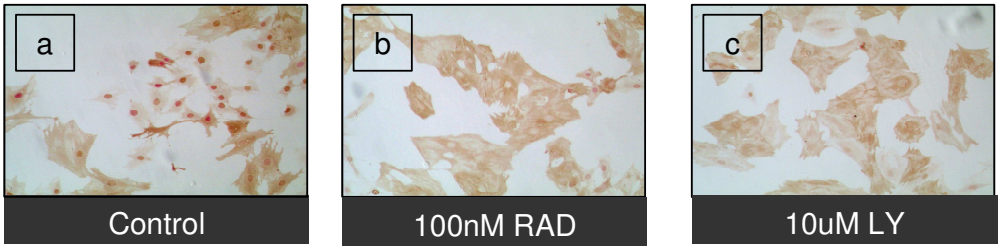
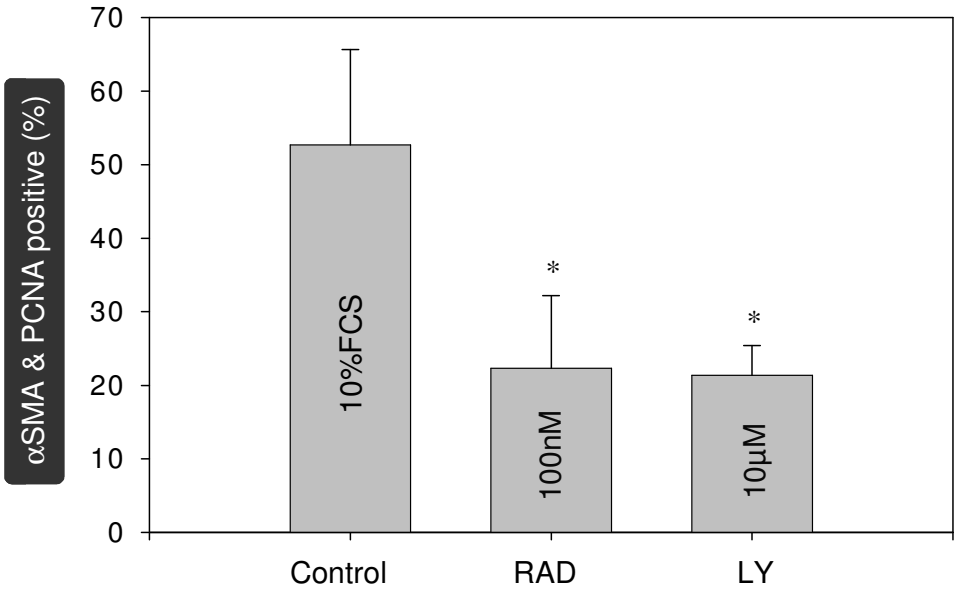


Fig. 5.6



Both RAD and LY affect proliferation and differentiation in the same population of cells. Combined immuno-cytochemical staining for myofibroblast differentiation (αSMA; cytoplasmic staining) and proliferation (PCNA; nuclear staining) illustrates that the increased myofibroblast differentiation was accompanied by a decrease in proliferation (Fig. 5.5 a-c). Quantification of these results indicated that the proportion of PCNA positive myofibroblasts decreased from 53±13% in the control group to 22±10 and 21±4% in the RAD and LY groups, respectively (Fig. 5.6; $^{*}p < 0.05$ vs. control; n=3 each group). This data represents an average of two experiments.

differentiation and inhibits proliferation, this suggests that the same sub-populations of cells are undergoing changes in proliferation and differentiation.

5.3.5 Role of PI3K and mTOR in the regulation of TGFβ1 synthesis

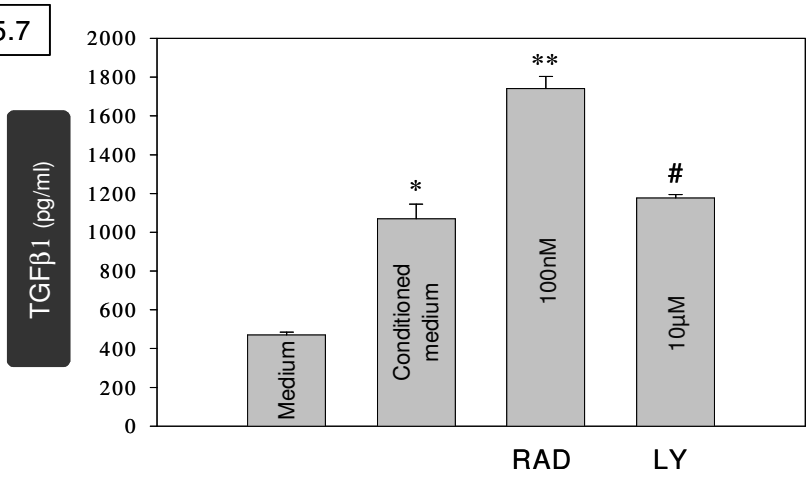
The recent literature suggests that mTOR and PI3K can regulate TGFβ1 synthesis (Runyan et al., 2004; Wu et al., 2006). Given the importance of TGFβ1 in renal fibrosis, this experiment was carried out to determine if mTOR and PI3K play roles in the regulation of TGFβ1 in renal fibroblasts. As indicated in *Fig. 5.7*, the concentration of TGFβ1 in the medium exposed to cells; *conditioned medium* (1069±76pg/ml) was markedly higher than that in the fresh unconditioned *medium* (not exposed to cells; 470±14pg/ml, $p<0.001$ vs. medium, n=3). Furthermore, RAD significantly increased TGFβ1 (1740±63pg/ml), more so than that in the *conditioned medium* ($p<0.001$ vs. conditioned medium, n=3). TGFβ1 levels in LY treated cells did not differ from that in conditioned medium (1176±17pg/ml; p=n.s. vs. control, $p<0.001$ vs. medium, n=3).

5.3.6 Role of PI3K and mTOR in the regulation of EMT

The heterogeneous derivation of myofibroblasts includes those that are derived via EMT. Apart from the role of Smads, the MAPK family and Rho, little is known about other regulatory pathways that govern EMT, although the potential for PI3K to cross-talk with Smads (Runyan et al., 2004) and Ras/MAPK (Pende et al., 2004; Shaw and Cantley, 2006) has previously been reported.

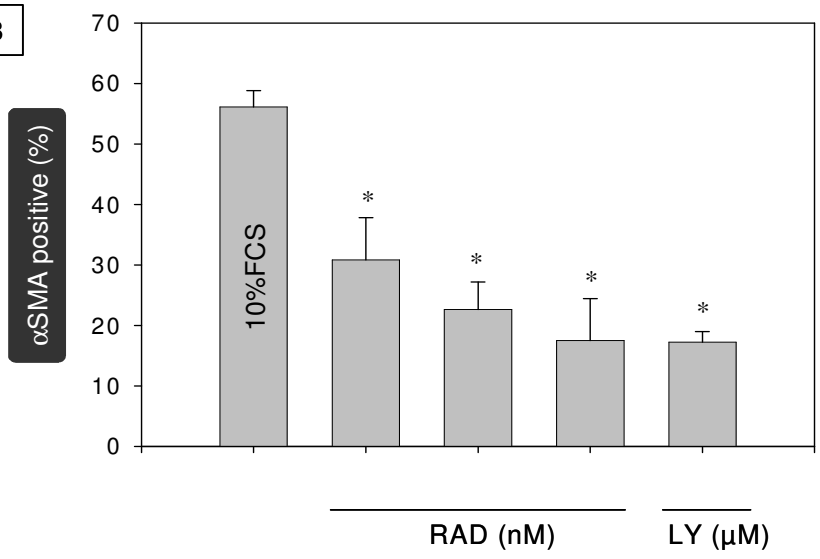
Renal explants *(Chapter 4)* were treated with either 10, 100, 200nM RAD, 10μM LY or maintained in control medium. At the end of this time period, explants were fixed in cold methanol and stained for αSMA. Addition of the mTOR and PI3K inhibitors decreased αSMA expression by 25.3±7% (10nM RAD), 33.5±4.6% (100nM RAD), 38.7±7% (200nM

Fig. 5.7



*TGFβ1 analysis was carried out to determine if RAD and LY affect differentiation through an up-regulation of TGFβ1 in renal fibroblasts. Cells were treated with either: (i) 100nM RAD in DMEM + 2.5% FCS, (ii) 10μM LY in DMEM + 2.5%FCS, or (iii) maintained in control medium (DMEM + 2.5% FCS; conditioned medium). For comparison, TGFβ1 concentration in medium not exposed to cells (Medium) was measured. Results suggest that a RAD-mediated increase in TGFβ1 synthesis facilitates differentiation, however LY had no effect on TGFβ1 secretion. TGFβ1 concentrations have been adjusted according to dilutions made throughout the detection process. (n=3 each group; *p<0.001 vs. medium;**p<0.001 vs. conditioned medium; #p=n.s. vs. conditioned medium; #p<0.001 vs. medium).*

Fig. 5.8



*Explants were treated with either RAD or LY for 48hr and stained for the myofibroblast marker αSMA. Cell enumeration indicated that all doses of RAD (10, 100, 200nM) and LY (10μM) decreased the acquisition of αSMA by by 25.3±7%, 33.5±4.6%, 38.7±7% and 38.9±1.8% respectively, indicating the potential for these kinases to regulate EMT in this model (*p<0.001 vs. control, n=5). The results illustrate a duplicate experiment.*

RAD) and 38.9±1.8% (10μM LY) (*Fig. 5.8*, all $p<0.001$ vs. control, n=5). This data provides novel insights into the mechanisms of EMT regulation.

5.4 DISCUSSION

Whilst the derivation of myofibroblasts is heterogenous, studies have indicated that two major sources are derived from the differentiation of local fibroblasts and from tubular epithelial cells via EMT. This study has therefore investigated the role of PI3K and mTOR in the regulation of fibroblast differentiation and EMT. Because the presence of αSMA-positive cells is often a key indicator of the severity of tubulointerstitial fibrosis, it is important to identify the mechanisms that govern this process. This study has shown that PI3K and mTOR play roles in regulating both fibroblast differentiation and EMT. Treatment with the respective inhibitors led to concurrent increases in αSMA protein, indicative of myofibroblast differentiation. TGFβ1 signalling, increasingly recognised for its ability to cross-talk with other pathways including PI3K and mTOR, was markedly up-regulated by the mTOR inhibitor RAD. Lastly, by using the *ex vivo* model of EMT established in *Chapter 4*, it was found that in contrast to their effect on fibroblasts, mTOR and PI3K inhibitors decreased the proportion of αSMA-positive epithelial cells, contributing to a down regulation of EMT in this model.

5.4.1 Role of PI3K and mTOR in myofibroblast differentiation

Recent evidence in a variety of cells suggests that PI3K and mTOR may play a role in fibroblast differentiation. PI3K has been shown to be essential for the terminal differentiation of skeletal muscle cells (Kaliman et al., 1996), as well as the differentiation of T cells (Okkenhaug et al., 2006), lens fibre cells (Weber and Menko, 2006) hepatic stellate cells (Reichard and Petersen, 2006) and osteoclasts (Lee et al., 2002). Furthermore, given the similarity between VSMC and myofibroblasts, the recent study on VSMC is perhaps the most compelling. In this work, Martin et al (Martin et al., 2004) found that RAD increased the expression of smooth muscle specific proteins including αSMA, calponin, and SM myosin heavy chain, suggesting that αSMA expression in VSMC is regulated by mTOR (Martin et

al., 2004). Like Martin's work and others (Yamamoto-Yamaguchi et al., 2001) this study has found that rapamycin and its derivatives increase αSMA protein expression in renal fibroblasts. The change in total αSMA protein expression was reflected by an increase in the proportion of myofibroblasts staining positive for αSMA. Of those cells that had reached a differentiated state, a contractile morphology was observed with αSMA filaments occupying a considerable proportion of the cytoplasm.

Whilst the studies in VSMC found no effect of LY on differentiation (Martin et al., 2004), the findings described herein suggest that LY also promotes differentiation, consistent with other studies that have implicated PI3K and Akt in PDGF-mediated regulation of αSMA in VSMC (Kaplan-Albuquerque et al., 2003). Furthermore, mTOR and PI3K function in a TGFβ1-dependent manner in the differentiation of hepatic stellate cells into a myofibroblast phenotype (Le Pabic et al., 2005). In the context of PI3K/mTOR's *in vivo* function in fibroblasts, these kinases may therefore negatively regulate αSMA expression. However, it is also worth noting that because cells were treated for an extended period of time (48hr), it may be possible that RAD and LY are inhibiting proteosomal degradation of αSMA through the inhibition of the ubiquitin-proteasome pathway, responsible for the degradation of intracellular proteins. Although this requires further studies, conceivably this would prevent the turnover of αSMA, leading to an accumulation of αSMA in the cell without requiring the need for new protein synthesis.

The effects of rapamycin on myofibroblast differentiation have also recently been examined *in vivo*. Interestingly, rapamycin has been shown to decrease protein expression and staining of peri-tubular and peri-glomerular αSMA in a model of UUO (Wu et al., 2006). Although the apparent reason for these discrepancies is unknown, it may be an indication of the importance of the *in vivo* environment in the study of fibrotic responses but it may also be indicative of the relevance of cell specificity to mTOR and PI3K signalling. Relevant to this, Sarbassov, who examined 33 transformed or cancer cells, found that mTORC2 assembly was

rapamycin sensitive in response to prolonged treatment in only one third of these cells (Sarbassov dos et al., 2006). Further studies are required to examine these mechanisms in a renal context.

5.4.2 Role of PI3K and mTOR in the regulation of αSMA gene expression

The results of the studies described herein suggest that the regulation of αSMA expression by PI3K and mTOR appears to be through regulation of protein synthesis, consistent with the role of mTOR in the positive and negative control of protein synthesis (Ruvinsky and Meyuhas, 2006). In order to examine whether these kinases regulate the gene expression of αSMA in renal fibroblasts, cyclohexamide and actinomycin D were used to study translational and transcriptional regulation respectively. These global inhibitors have been used by a number of other studies to examine the role of various substrates in gene expression (Jinnin et al., 2006; Martin et al., 2004; Price et al., 1989; Ricupero et al., 2001). Based on the premise that LY should increase αSMA expression, pre-treatment with these inhibitors established that only cyclohexamide prevented cells from undergoing differentiation. This observation is supported by the increase in total αSMA protein observed with western blotting, and the failure to detect any meaningful changes in αSMA mRNA expression by real time PCR.

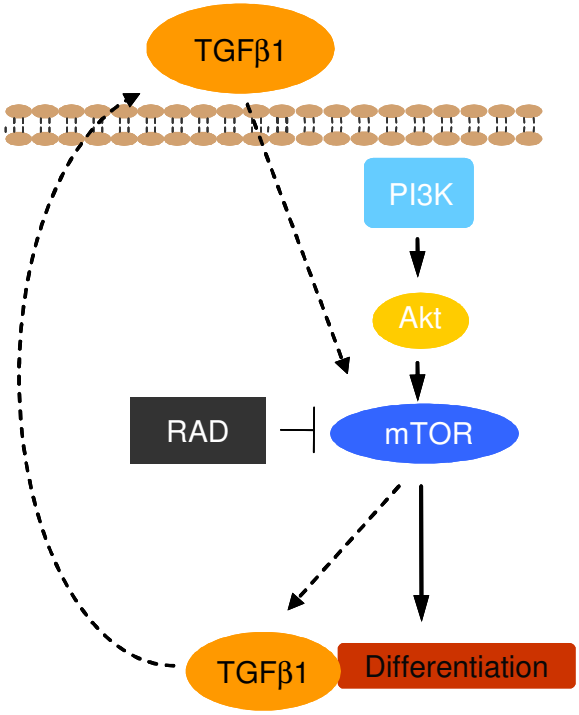
Post-transcriptional regulation plays an important downstream role in many signalling pathways in response to growth factors (Dibrov et al., 2006), and is a likely explanation for increases in protein synthesis that do not show comparative changes to transcription. Although αSMA expression has been shown previously to be controlled at the post-transcriptional level (Corjay et al., 1990), in support of the present findings, further studies will be required to confirm the preliminary data described within.

5.4.3 Role of TGFβ1 in PI3K and mTOR-mediated differentiation

TGFβ1 plays an important role in promoting fibroblast differentiation and an increasing appreciation of the complexity of signalling pathways has led to findings that the TGFβ signalling axis can cross-talk and integrate its responses with PI3K and mTOR (Runyan et al., 2004). In response to mTOR inhibition, it was found that total TGFβ1 levels were markedly increased. This suggests that the up-regulation of TGFβ1 by RAD treatment may mediate the differentiation of fibroblasts in an autocrine fashion, consistent with the present studies, which demonstrate a RAD-mediated up-regulation of αSMA expression *(Fig. 5.9)*. Other pathways are likely to be involved in the regulation of differentiation via PI3K as LY had no effect on TGFβ1 synthesis.

This study suggests that mTOR may play a role in negatively regulating TGFβ1 levels *in vivo*, and is consistent with studies showing a significant up-regulation of rapamycin-mediated TGFβ1 synthesis in human kidney transplant fine-needle aspiration biopsies (Oliveira et al., 2002), and in rapamycin-infused rats with chronic cyclosporine nephrotoxicity (Shihab et al., 2004a; Shihab et al., 2004b). However, other studies contradict these findings making the exact role of mTOR in TGFβ1 regulation *in vivo* ambiguous. Wu et al (Wu et al., 2006) has shown that TGFβ1 expression, induced by UUO, was significantly lowered in response to rapamycin whilst other studies (Jain et al., 2001) demonstrated a marked rapamycin-mediated decrease in TGFβ1 expression in an experimental model of renal ischaemia reperfusion injury. Similar to the regulation of collagen *in vivo* by PI3K/mTOR, it is apparent that the regulation of TGFβ1 levels *in vivo* by these kinases is also unclear.

Fig. 5.9



Potential signalling mechanisms involved in the regulation of differentiation by PI3K and mTOR.

5.4.4 Role of PI3K and mTOR in EMT

The process of EMT has been shown to contribute significant numbers of interstitial myofibroblasts *in vivo* (Iwano et al., 2002). Whilst TGFβ1 is a potent inducer of EMT, the intracellular signalling mechanisms governing EMT are far from being fully elucidated. TGFβ and the Smad2/3 axis are likely to play roles in most of the sequential events of EMT (Flanders, 2004; Roberts et al., 2006; Yang and Liu, 2001), whilst the MAPK cascade is thought to mediate αSMA expression and suppress E-cadherin (Li et al., 2004b; Rhyu et al., 2005; Xie et al., 2004). Furthermore, Rho has been implicated in the dissociation of epithelial cells from their neighbours (Bakin et al., 2000; Ozdamar et al., 2005) and in the formation of stress fibres (Bhowmick et al., 2001; Masszi et al., 2003), and ILK has been repeatedly shown to regulate E-cadherin expression through the regulation of *Snail* and β-catenin translocation to the nucleus (Li et al., 2003; Tan et al., 2001).

However, EMT is a highly complex process with the dependence on a number of cellular programs (Thiery and Sleeman, 2006) and it is unlikely that the plethora of EMT agonists including angiotensin II (Chen et al., 2006), AGEs (Oldfield et al., 2001), CTGF (Burns et al., 2006), TGFβ1 (Lan, 2003), IL-1 (Fan et al., 2001), collagen I (Zeisberg et al., 2001), FGF and EGF (Strutz et al., 2002), and tPA (Yang et al., 2002b) all mediate their responses through the currently identified pathways. The potential for cross-talk between the TGFβ axis and PI3K/mTOR in the mediation of EMT has become increasingly acknowledged in many cancer-related studies (Bakin et al., 2000; Irie et al., 2005; Larue and Bellacosa, 2005; Park et al., 2001) and a limited number of renal studies (Wu et al., 2006), suggesting that PI3K/mTOR may play roles in renal EMT.

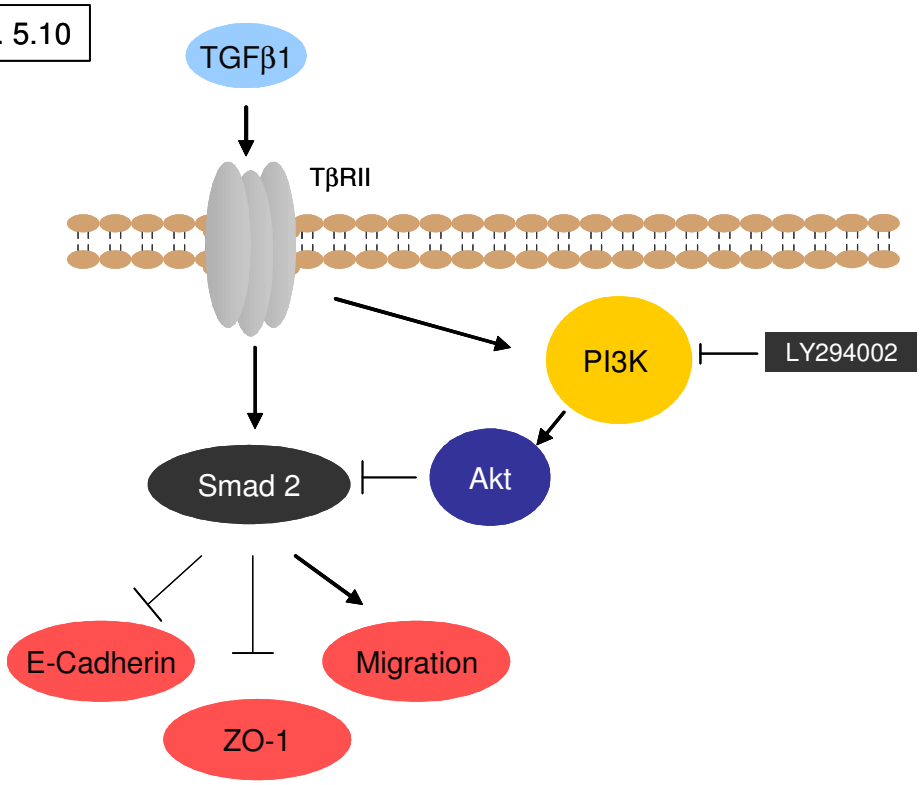
By using the previously established model of EMT *(Chapter 4)*, the role of mTOR and PI3K in renal EMT was determined. Using this model, these results indicate that whilst RAD has an agonistic effect on the differentiation of renal fibroblasts derived from UUO, cells that grow out of renal tissue explants, phenotyped as epithelial, demonstrate a RAD-

mediated inhibition in their capacity to undergo EMT. These cells down-regulated their expression of the myofibroblast marker αSMA in response to both RAD and LY. This provides an insight into the regulatory mechanisms of mTOR in accordance with *in vitro* studies by Wu et al (Wu et al., 2006), who demonstrated a rapamycin-mediated inhibition of TGFβ1-induced loss of E-cadherin expression and the *de novo* expression of αSMA, and Aguilera et al (Aguilera et al., 2005), who showed that TGFβ–mediated *Snail* expression, αSMA expression and loss of E-cadherin were prevented by rapamycin.

It is worthy to note that rapamycin has been shown to inhibit the proliferation of human renal epithelial cells *in vitro* (Pallet et al., 2005) underscoring the potential for rapamycin to actually be inhibiting proliferation rather than preventing expression of αSMA in this model. However, to overcome this problem, cells were only treated with rapamycin when a substantial number of epithelial cells were present and cell numbers around a selected number of tissue patches were counted to ensure there were not significant changes in cell proliferation in each of the treated groups compared to the control (data not shown). Furthermore, it has been shown that, as in the case of TGFβ1-mediated EMT, cell cycle arrest is necessary in the regulation of PI3K and mTOR-mediated EMT in epithelial cells (Shao et al., 2004). This once again suggests that epithelial cells do not undergo proliferation during EMT and the effect of RAD on EMT in this model is therefore unlikely to be a result of decreased proliferation rather than repression of αSMA.

The role of mTOR in EMT is further corroborated by its upstream liaison PI3K in this study. Inhibition of PI3K in epithelial cells that grow out of explants resulted in a decrease in αSMA expression. Evidence for a role of PI3K-mediated processes in EMT is also emerging in the literature. Bakin et al (Bakin et al., 2000) has demonstrated that TGFβ1–mediated EMT is dependent on PI3K where PI3K dominant-negative mutation or pharmacological inhibition (LY) leads to the inhibition of TGFβ1-mediated Smad2 phosphorylation and repression of the EMT proteome *(Fig. 5.10)*. It therefore seems likely

Fig. 5.10



Potential role of PI3K in TGFβ-mediated EMT

Adapted from Bakin et al (2000).

that PI3K may cross-talk with the TGFβ signalling axis during EMT (Bakin et al., 2000; Runyan et al., 2004). Furthermore, over-expressing mutated forms of Akt were shown to induce EMT by the up-regulation of *Snail* leading to repression of the E-cadherin gene (Batlle et al., 2000; Grille et al., 2003).

5.4.5 Summary

In conclusion, mTOR and PI3K play roles in the regulation of fibroblast differentiation and EMT. Inhibition of these respective pathways promotes the differentiation of fibroblasts. This process may result from autocrine TGFβ signalling via mTOR. Preliminary evidence also suggests that mTOR and PI3K may mediate changes to mRNA stability rather than direct changes to transcription. In contrast, the acquisition of αSMA filaments in epithelial cells during EMT is noticeably suppressed in response to inhibition of these kinases. The effect of rapamycin derivative RAD on tubular epithelial cells during EMT, as demonstrated *ex vivo*, implicates another important role for rapamycin in the context of renal fibrosis.

The differential regulation of αSMA in myofibroblast differentiation and EMT by PI3K and mTOR establishes an unusual finding and suggests that these kinases may act in a cell-dependent manner in fibroblasts and epithelial cells. This type of signalling mechanism has been established in response to TGFβ1, the key pro-fibrotic mediator in the kidney. TGFβ1 is well known for its ability to promote fibroblast proliferation however it can also inhibit epithelial cell proliferation. Whilst Smads have been identified as the generic signalling pathway in both epithelial cells and fibroblasts, intracellular cross-signalling mechanisms govern the response to TGFβ1 in these cells. Accordingly it has been shown that PI3K (Wilkes et al., 2005) and p21-activated kinase-2 (PAK-2) are activated in fibroblasts independently of the Smad axis however this inhibition of epithelial cell proliferation is mediated by the Smad signalling axis only (Wilkes et al., 2003). Given that both TGFβ1 and

PI3K are likely to be involved in fibroblast differentiation, it is possible that differential cross-talk mechanisms may dictate the response to cytokine or growth factor signals within different cells.

These studies provide novel insights into the regulation of cells that contribute to the derivation of myofibroblasts. Furthermore, this highlights the complexity of the cell signalling pathways involved in mediating fibroblast differentiation and EMT and provides a solid foundation for further elucidation of mTOR and PI3K in mediating pro-fibrotic events.

5.5 SPECIFIC METHOD PROTOCOLS

Method	Section
UUO	2.2.1
RNA and protein extraction	2.7.1
Western blotting	2.7.3
Cytochemistry	2.6.1
Cell enumeration	2.6.1.1
Real time PCR	2.7.4

Table 5.1: References for methods described in *Chapter 2* used in this chapter.

5.5.1 Western blotting for αSMA

In the case of Western blotting for αSMA protein, protein was extracted from cells treated with RAD (10, 100, 200nM) or LY (10μM). Western blotting was used to quantify total cellular αSMA where membranes were incubated with a 1:1000 dilution of anti-αSMA *(Table Apx. 3.3)*, followed by a 1:2000 incubation with HRP conjugated anti-mouse IgG *(Table Apx. 3.2).* Membranes probed for αSMA were stripped and re-probed with a 1:2000 dilution of mouse anti-β–actin O/N to confirm protein loading. Fold differences were quantified by densitometry.

5.5.2 Global inhibition of transcription and translation

To determine whether αSMA is regulated by PI3K and mTOR at the translational or transcriptional level, cells were pre-treated with actinomycin D (transcriptional inhibitor) or cycloheximide (translation inhibitor) and then treated with the PI3K inhibitor, LY (10μM). Cells were seeded in 36mm^2 multiwell plates at a density of 1X10^5 and grown to sub-confluence. 2.5ug/ml actinomycin D or 10ng/ml cycloheximide was then added for 1hr. Media was removed, and 10μM LY, or no additive (control medium), was then added for 24hr at 37°C. Cells were washed in PBS and fixed in cold methanol. Cells were then stained for αSMA and enumerated.

5.5.3 Real time PCR for αSMA

Real time PCR and was used to determine the role of PI3K and mTOR in αSMA gene expression. Trizol extraction was carried out on samples treated with RAD (10, 100, 200nM), LY (10μM), or from those maintained in control medium. RNA was analysed with 2% agarose gels and purified with an RNA clean up kit. Primers for αSMA (Accession number: NM_031004; 1361 bp), in comparison to the house-keeper gene, Ribosomal protein L32 (Accession number: NM_013226; 564bp) were used. Twenty μl (0.5μM) of each of the forward and reverse primers were added to dH_2O.

αSMA forward primer	αSMA reverse primer
5' ACT GGG ACG ACA TGG AAA AG 3'	5' CTA CTC CAG AGT CCA GCA CA 3'

Table 5.2: αSMA primer sequences

Previous experiments had established the reliability of this method by testing TGFβ1, an inducer of fibroblast differentiation, as a positive control (data not shown). Duplicates of each group were analysed and represented according to delta-delta fold differences.

5.5.4 Double labelling of fibroblasts for αSMA and PCNA

To determine if the same sub-populations of cells were responding by proliferation and differentiation, peroxidase double-labelling was carried out. An equal number of cells were seeded onto sterile coverslips and grown until subconfluent. Cells were then treated with RAD (100nM), LY (10μM), or maintained in control medium and left for 72hr. Cells were then fixed in cold methanol and washed with TBSx2. Cells were then blocked with normal dilute serum for 10min and incubated with PCNA O/N at 4°C *(Table Apx. 3.1)*. Cells were then washed in tris buffered saline (TBS) x2, Alk phos-anti-mouse conjugate added for 30min, washed in TBSx2 and incubated with Alk-phos anti-alk-phos for 40min *(Table Apx. 3.2)*. After washing with TBSx2 Texas fast red was added for 20min in darkness, cells were washed in TBSx2, and anti-αSMA (HRP-EPOS) was then added for 30min *(Table Apx. 3.1)*.

Cells were then washed in TBSx2, and developed with DAB until a colour change appeared. Finally, cells were mounted onto glass slides with aquamount. Approximately 600-800 cells were counted for positive staining for αSMA, PCNA or both markers.

5.5.5 Detection of total TGFβ1

To determine if mTOR and PI3K are involved in the regulation of TGFβ1 secretion, a TGFβ1 Quantikine® kit was used. Fibroblasts were grown to confluency in 6-well plates as described previously. In DMEM + 2.5%FCS, cells were then treated with 100nM RAD, 10μMLY or maintained in control medium (DMEM + 2.5%FCS), named *Conditioned medium* at 37ºC. Media (including fresh media not exposed to cells; named *Medium*) was then collected, centrifuged at 6000rpm for 10min, placed into sterile nunc tubes and snap frozen (into liquid nitrogen). Samples were activated to detect total TGFβ1 and the ELISA was carried out according to the manufacturer's instructions.

5.5.6 Pharmacological modification of EMT with RAD and LY

Explants were treated with TGFβ1 (10ng/ml), RAD (10, 100, 200nM), LY (10μM), or maintained in control medium (DMEM+ 10% FCS). After treatment for 48hr, cells were fixed in ice-cold methanol, stained for αSMA, and counted on the basis of positive staining.

Chapter 6

Constituents of the Coagulation Cascade are Spatially and Functionally Related to Experimental Tubulointerstitial Fibrosis

6.1 INTRODUCTION

Tubulointerstitial fibrosis and its damaging effects on the surrounding renal parenchymal tissue may be regulated by a cocktail of locally and systemically derived mediators, many of which are cytokines. Although these soluble factors are key mediators of fibrosis, they are not by any means the sole players and we increasingly acknowledge the role of non-cytokine mediated pathways in renal fibrosis. Those that have gained worthy attention over recent years include (a) tPA where fibrosis is significantly ameliorated in tPA gene knock-out mice (Yang et al., 2002b); (b) PAI-1 where over-expressing PAI-1 transgenic mice display severe fibrosis characterized by marked tubular injury and increased myofibroblast proliferation (Eddy, 2002; Matsuo et al., 2005); (c) angiotensin II which plays a significant role in fibrosis in animal models of UUO (Kellner et al., 2006), 5/6 nephrectomy (Kelly et al., 2006), anti-Thy1 glomerulonephritis (Yu et al., 2004), and diabetic nephropathy (Anjaneyulu and Chopra, 2004), and can also activate TGFβ signalling (Fukami et al., 2004; Kagami et al., 1994) and lastly; (d) AGEs which can induce cytokine release (Cooper, 2004) and EMT independently of TGFβ1 (Oldfield et al., 2001).

Similarly, although historically known for their role in minimising blood loss, the coagulation cascade can be directly activated during injury and inflammation (Grandaliano et al., 2001; Sekiya et al., 1994). Consistent with this, a number of studies have demonstrated that serine proteases have cell-mediated effects, in particular, influencing the key fibrogenic cells in progressive scarring (Enestrom et al., 1988; Grandaliano et al., 2000b; Hewitson et al., 2005; Howell et al., 2001; Liu et al., 2004; Monno et al., 2001; Tanaka et al., 2005; Vesey et al., 2005). Firstly, a number of studies on the role of the coagulation cascade in animal models of fibrotic lung disease have underscored the potential for the coagulation cascade pathway to mediate fibrosis. Howell et al (Howell et al., 2002) have shown that inhibition of thrombin in bleomycin-induced lung fibrosis attenuated ECM deposition and cytokine production, whilst Fujimoto et al (Fujimoto et al., 2006) demonstrated that knock-out of

thrombin-activatable fibrinolysis inhibitor (TAFI; a strong suppressor of fibrinolysis) markedly ameliorated lung fibrosis. Secondly, members of the coagulation cascade have been implicated in a diverse range of glomerular-based diseases including IgA nephropathy (Liu et al., 2000), Henoch-Schonlein purpura nephritis, lupus nephritis and hemolytic uremic syndrome (Takemura et al., 1987), mesangioproliferative glomerulonephritis (Liu et al., 2004), human (Cunningham et al., 1999; Grandaliano et al., 2000a) and murine (Cunningham et al., 2000) crescentic glomerulonephritis.

Thirdly and foremost, the presence of coagulation members have also specifically been described in various human and experimental models of tubulointerstitial renal diseases including ureteric obstruction, allograft rejection and acute ischaemic renal failure (Enestrom et al., 1988; Faulk et al., 1989; Grandaliano et al., 2000b; Wang et al., 1997; Wang et al., 1996; Wendt et al., 1995; Yamamoto and Loskutoff, 1997). However, unlike their role in the formation of glomerular crescents, the significance of such factors in mediating tubulointerstitial fibrosis is only beginning to be elucidated. Perhaps the greatest insight into this process can be drawn from studies on the serine protease thrombin. Thrombin has been implicated in facilitating fibroblast mitogenesis (Hewitson et al., 2005), the autocoid up-regulation of CTGF (Chambers and Laurent, 2002), TGFβ1 (Bachhuber et al., 1997; Grandaliano et al., 2001), and PDGF production (Ohba et al., 1994), and in the up-regulation of ECM synthesis (Bachhuber et al., 1997; Chambers et al., 1998; Dabbagh et al., 1998; Hewitson et al., 2005; Howell et al., 2001; Shirato et al., 2003; Vesey et al., 2005). Moreover, thrombin can stimulate fibroblast differentiation (Bogatkevich et al., 2001), and is present with these cells in the inflammatory and early fibrotic stages in a number of pathological conditions (Coughlin, 2000).

Thrombin carries out many of its fibrogenic cell-mediated effects through protease activated receptor-1 (PAR-1) (Bogatkevich et al., 2001; Grandaliano et al., 2001; Grandaliano et al., 2000b; Hewitson et al., 2005; Howell et al., 2001; Howell et al., 2002). This GPCR has

been identified on endothelial cells of glomerular and interstitial capillaries and arterioles, mesangial cells and podocytes, myofibroblasts and tubular epithelial. Although studies (Grandaliano et al., 2001; Grandaliano et al., 2000b) have localised PAR-1 to proximal tubular epithelial cells in chronic allograft nephropathy (CAN), the *in vivo* distribution of PAR-1 in a model of UUO has not yet been ascertained.

Given that the role of serine proteases in glomerular pathology has been well documented, this study has therefore examined the temporal and spatial distribution of coagulation factors after experimental UUO.

6.2 EXPERIMENTAL DESIGN

To assess the role of the coagulation cascade in tubulointerstitial fibrosis, this study has utilised tissue obtained from rats sacrificed at various time points post-UUO. This study is based on the examination of the *in vivo* temporal and spatial distribution of coagulation factors 0, 3, 14 and 21 days post-UUO which relies on immunohistochemistry and *in situ* hybridisation. Renal fibroblasts propagated from explants of rat renal cortex, 3-days post UUO were also used to for *in vitro* studies, whilst the model of EMT, established in C*hapter 4,* was also incorporated to study the role of non-cytokine-mediated pathways in EMT.

6.3 RESULTS

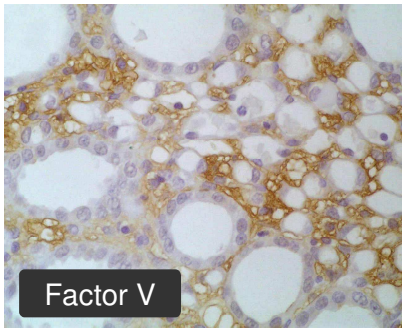
6.3.1 Factor V is present in the tubulointerstitium *in vivo*

Factor V acts as a membrane-bound co-factor and when present, has the ability to accelerate the generation of thrombin by 10^4 fold (Ono et al., 2001). Tissue sections derived from rat kidneys were stained with an antibody against factor V at 3 days post-UUO. This demonstrated that factor V staining was evident during renal injury as supported by Liu et al (Liu et al., 2000), and was predominantly localized to peri-tubular interstitial regions *(Fig. 6.1).*

6.3.2 Factor V is expressed by activated fibroblasts

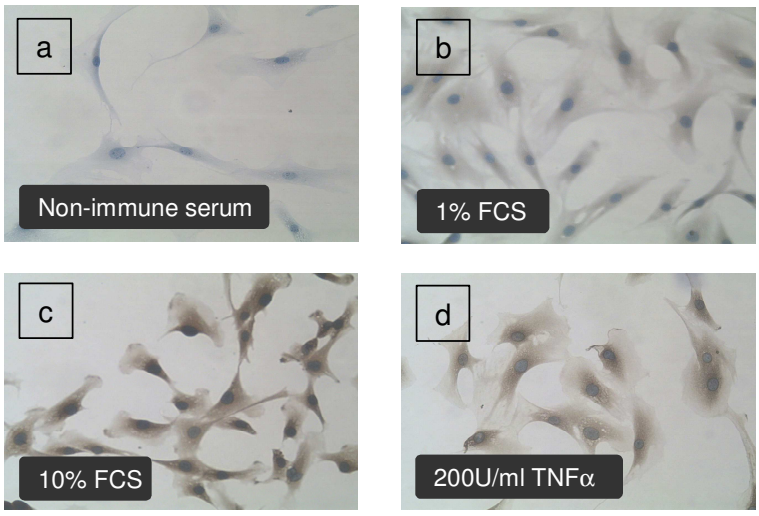
To determine if cells derived from UUO can actively express factor V in response to inflammation, cells derived from 3 days post-UUO were treated with 200U/ml of the inflammatory cytokine tumour necrosis factor-α (TNFα) for 24hr in 1%FCS+DMEM, and then stained for factor V. Obvious staining for factor V was demonstrated in cells grown in 1% FCS (*Fig. 6.2b*); this was markedly up-regulated in cells grown in 10% FCS (*Fig. 6.2c*) and when cells were treated with 200U/ml TNF-α (*Fig. 6.2d*). Specificity of staining was confirmed by replacing anti-factor V with species matched non-immune serum. No specific staining was noted in this group (*Fig. 6.2a*; rabbit immunoglobulins in 1% FCS). Inflammatory-mediated stimulation of factor V synthesis by renal fibroblasts and its ability to be modified *in vitro* by different culture media suggest that fibroblasts may be an alternate pathway for generation of this protease. Results are illustrative of a duplicate experiment.

Fig. 6.1



Tissue sections taken from day 3 post-UUO were incubated with primary antisera against the cell specific protein factor V. Increased Factor V staining can be found in the tubulointerstitium during experimental fibrogenesis. Factor V was localised mainly to peri-tubular regions (n=5).

Fig. 6.2

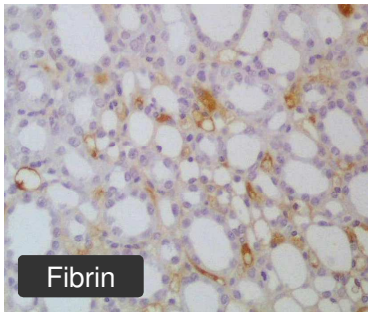


Factor V can be synthesized by fibroblasts derived from obstructed kidneys at 3 days post-UUO. Obvious staining for factor V was demonstrated in cells grown in 1% FCS (fig 6.2b); this was markedly up-regulated in cells grown in 10% FCS (fig 6.2c) and when cells were treated with 200U/ml TNF-α in 1%FCS (fig 6.2d). Specificity of staining was confirmed by replacing anti-factor V with species matched non-immune serum. No specific staining was noted in this group (fig 6.2a; rabbit immunoglobulins in 1% FCS). Photomicrographs are representative of a duplicate experiment, n=3 slides each group.

6.3.3 Fibrin is present in the tubulointerstitium

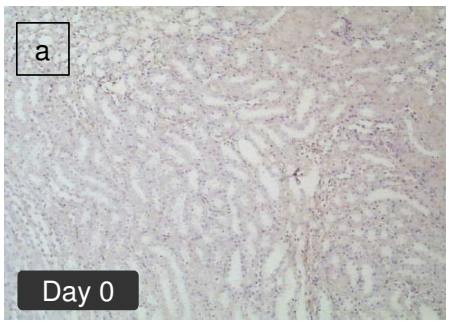
In light of thrombin's rapid turnover rate (Coughlin, 2000) and consequently the difficulty in identifying its presence in the interstitium, fibrin/fibrinogen (called fibrin from hereon) was used as a surrogate marker for thrombin. As shown in *Fig. 6.3*, fibrin has an interstitial distribution in a juxtaposition to factor V (*Fig. 6.1*), and was predominantly localized to interstitial peri-tubular regions. Sections taken from rat kidneys subjected to UUO were also stained at 0, 3, 14 and 21 days post-UUO (*Fig. 6.4a-d*). Cortical and medullary distribution of fibrin was enumerated and expressed as a percentage of the fractional area (FA). The distribution of fibrin was chiefly confined to the medullary and corticomedullary junction, with relatively small amounts in the cortex. Fibrin was distributed acutely around peri-tubular capillaries, and more diffusely in tubular casts at latter time points. The quantity of staining for fibrin was enumerated and expressed as a percentage of the FA over the same time points post-UUO (*Fig. 6.5*). Staining for fibrin was increased by 3-days post-UUO to 11%, further increasing by 14 days post-UUO, at which point it occupied 16.9% of the FA ($p<0.05$ vs. Day 0). By day 21, fibrin occupied 13.4% of the FA (n=3-6).

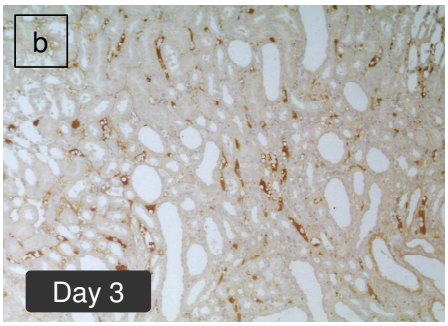
Fig. 6.3

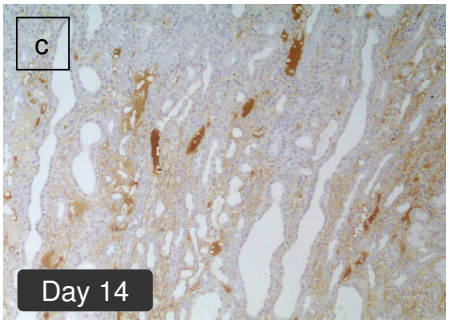


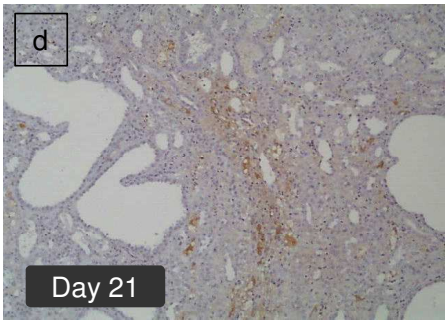
Using an antibody against fibrinogen/fibrin, fibrin deposits were localized to interstitial and peri-tubular regions in a juxtaposition to factor V. Because of the high turn over rate of thrombin, these mesh clots are representative of the location of the serine protease thrombin.

Fig. 6.4



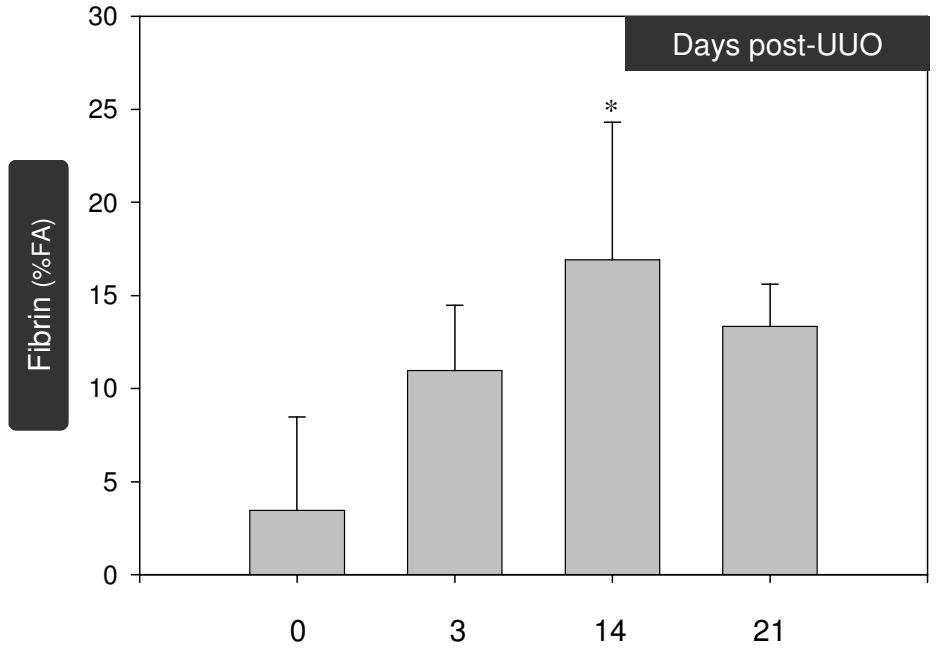






A temporal examination of fibrin distribution reveals a progressive increase in fibrin. The distribution of fibrin was chiefly confined to the medullary and corticomedullary junction, with relatively small amounts in the cortex. Fibrin was found acutely around peri-tubular capillaries, and more diffusely in tubular casts at latter time points. As renal injury progresses, the renal architecture becomes distended and tubular dilation becomes evident and marked by day 21.

Fig. 6.5



The quantity of staining for fibrin was enumerated and expressed as a percentage of the FA over fixed time points post-UUO. Staining for fibrin increased by 11% by 3 days post-UUO, further increasing by 14 days post-UUO, at which point it occupied 16.9% of the FA. At day 21 fibrin occupied 13.4% of the FA ($p<0.05$ vs. control, n=3-6).*

6.3.4 The distribution of fibrin and αSMA is spatially related

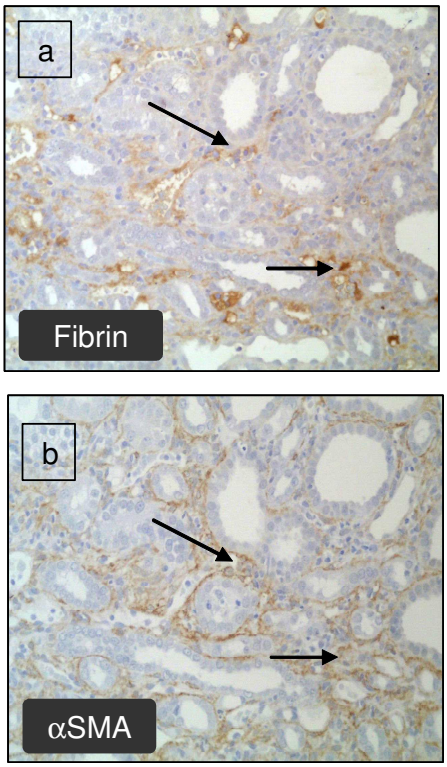
By staining serial sections of rat tissue derived from UUO at day 3 for fibrin and the myofibroblast marker αSMA, a spatial relationship can be established between a marker of the end point of the coagulation cascade (*Fig. 6.6a*, arrows), and a marker of tubulointerstitial pathology (*Fig. 6.6b*, arrows). This spatial relationship suggests that a cause and effect relationship may exist between serine proteases (thrombin) and fibrosis (myofibroblasts).

6.3.5 PAR-1 expression is confined mainly to renal tubule epithelial cells

To examine the functional significance of thrombin, immunohistochemistry was used to localise the distribution of the thrombin receptor PAR-1 in progressive fibrosis. PAR-1 expression was localised to the basolateral surface of renal tubular epithelial cells (*Fig. 6.7).*

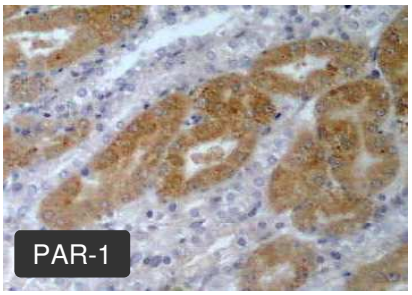
To determine whether there is a relationship between the localization of fibrin and the distribution of PAR-1, comparisons at different time-points were carried out. Staining for PAR-1 was maximal during the early time-points, (*Fig. 6.8a-b*, 0 and 3-days post-UUO), and decreased progressively with the progression of fibrosis (*Fig. 6.8 c-d*, 14 and 21 days post-UUO). At higher magnification it could be seen that loss of staining was focal, affecting occasional cells in tubular cross sections (*Fig. 6.9a-b,* arrow).

Fig. 6.6



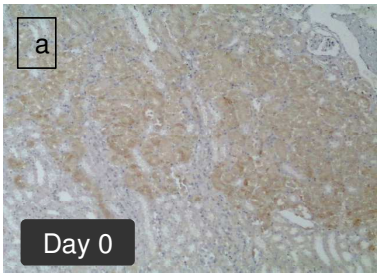
The presence of fibrin clots (a, arrows), indicative of thrombin, can be spatially related to the location of αSMA positive myofibroblasts (b, arrows) in the interstitium. Although it cannot be concluded that a cause and effect relationship does exist, it establishes a potential mechanism by which thrombin, present in fibrin clots may activate myofibroblasts

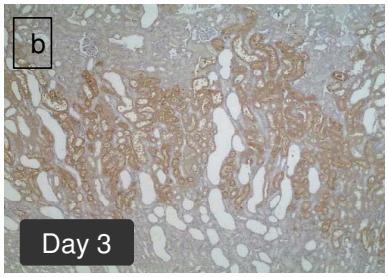
Fig. 6.7

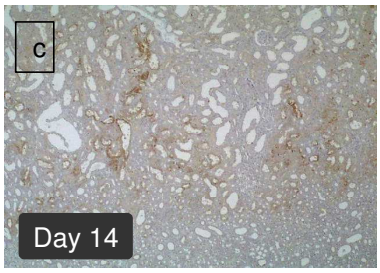


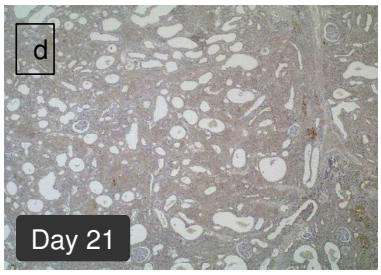
PAR-1 was detected at 3 days post-UUO by using a monoclonal PAR-1 antibody on paraffin embedded tissue. PAR-1 receptor staining was located to the basolateral surface of tubular epithelial cells.

Fig. 6.8



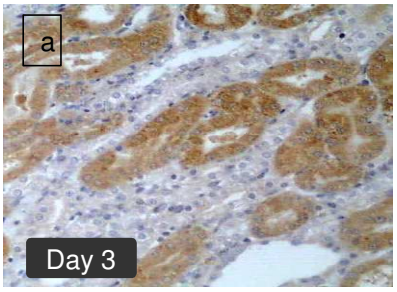


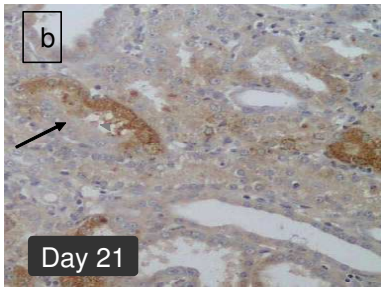




PAR-1 was found to be heavily expressed during experimental UUO. Staining for PAR-1 was maximal during the early time-points, (a-b, 0 and 3 days post-UUO), and decreased progressively with the progression of fibrosis (c-d, 14 and 21 days post-UUO).

Fig. 6.9





At higher magnification it could be seen that loss of staining was focal, affecting occasional cells in tubular cross sections (a-b, arrow).

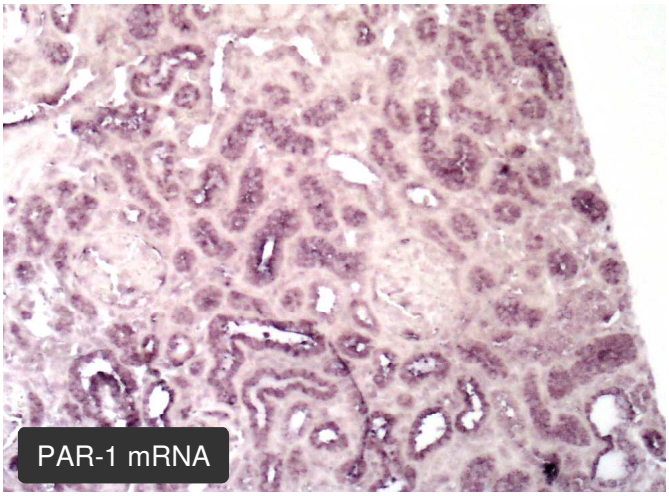
6.3.6 Expression of PAR-1 mRNA is maintained during progressive fibrosis

An investigation of PAR-1 receptor kinetics by non-radioactive *in situ* hybridisation illustrated that PAR-1 mRNA synthesis was present at 21 days post-UUO (*Fig. 6.10).* This suggests that PAR-1 synthesis is maintained in progressive fibrosis.

6.3.7 EMT can be demonstrated in UUO

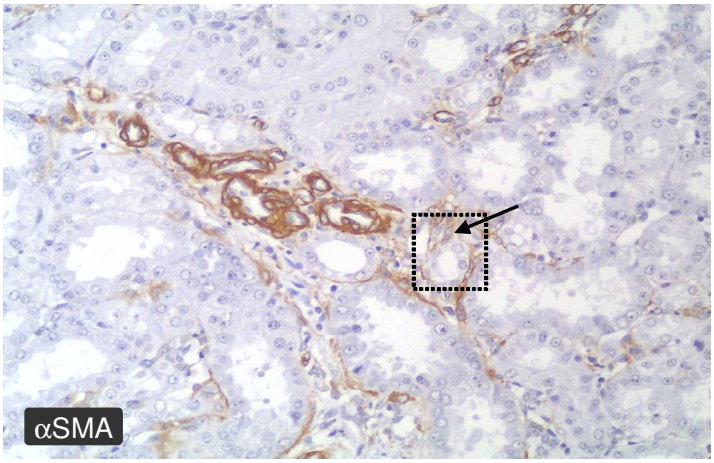
In addition to carrying out cell-mediated effects through PAR-1 to induce fibroblast differentiation (as shown by spatial positioning of fibrin with αSMA), thrombin may play a role in EMT, given the favourable position of PAR-1 on tubular epithelial cells. Given that EMT occurs in fibrosis induced by UUO (Rastaldi et al., 2002), sections were examined to localise this phenomenon, as illustrated in *Fig. 6.11* (day 3, arrow). The cross-section (within the demarcated dotted lines) demonstrates a typical distal convoluted tubule consisting of cuboidal epithelial cells surrounded by a basement membrane at its basolateral aspect and a luminal aspect devoid of a prominent brush-border. Whilst αSMA-positive cells can be seen surrounding the basement membrane in this region, astute observation indicates that an epithelial cell at the superior pole has changed its morphology, become αSMA-positive and has started to migrate into the interstitium. Because of the transient nature of this event, this was not a common occurrence. Given that key mediators fibrin/thrombin and PAR-1 are spatially co-localised in UUO, this suggests that they may play an inter-related functional role in EMT.

Fig. 6.10



In situ hybridization reveals that PAR-1 receptor mRNA in still present at 21 days post-UUO.

Fig. 6.11

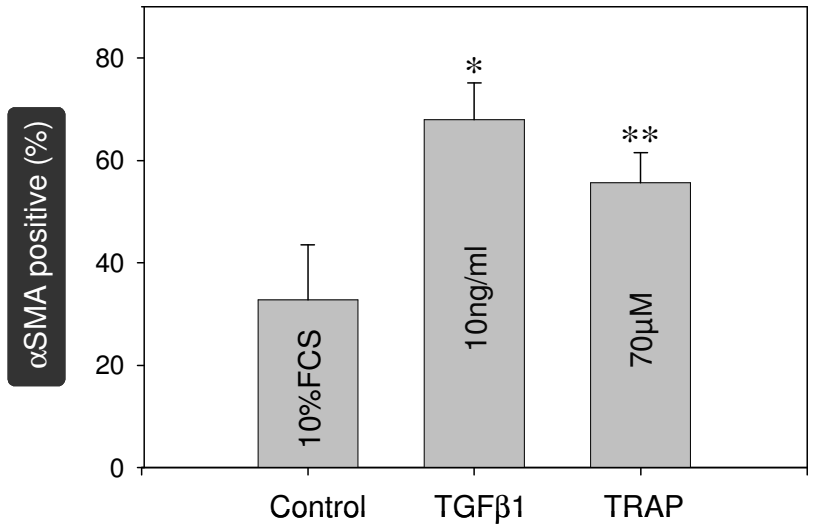


Demonstration of EMT in UUO at 3 days post-UUO. The distal tubular segment within the boxed outline and indicated by the arrow not only stains positive for αSMA but appears to be migrating into the surrounding interstitium. The morphology of this cell has also visibly changed and appears elongated and spindle shaped having lost any morphological similarity to tubular epithelial cells.

6.3.8 Activation of PAR-1 on tubular epithelial cells is a trigger for EMT

To determine if PAR-1 could induce EMT using the *ex vivo* model of explanting, TGFβ1 and thrombin receptor agonist peptide (TRAP; 70μM) were added to explant medium (10%FCS+DMEM). Treatment with 70μM of the synthetic peptide TRAP increased the transformation of tubule epithelial cells to myofibroblasts (αSMA +ve cells) by 63%, (*Fig. 6.12*; $p<0.001$ vs. control, $n = 3$). This compares to 110% seen with 10ng/ml TGFβ1 ($p<0.001$ vs. control, $p<0.05$ vs. TRAP). This confirms the potential for thrombin to induce EMT through PAR-1. The results illustrate a duplicate experiment.

Fig. 6.12



*Using the ex vivo model of EMT, explants were treated with either 10ng/ml TGFβ1 or 70µM TRAP. Staining for αSMA demonstrates that the synthetic agonist TRAP increased the transformation of tubule epithelia to myofibroblasts (αSMA +ve cells) by 63%, (**p<0.001 vs. control, n=3). This compares to 110% seen with 10ng/ml TGFβ1 (*p<0.001 vs. control, *p<0.05 vs. TRAP).*

6.4 DISCUSSION

It has become increasingly apparent that tubulointerstitial fibrosis, and its damaging effects on the surrounding renal parenchyma, does not exclusively dependent on cytokine mediators. Coagulation factors can be endogenously activated independently of the coagulation cascade during injury and inflammation (Chambers and Laurent, 2002; Sekiya et al., 1994), and as a result they have been implicated as having cell-mediated effects in addition to their traditional roles in coagulation (Enestrom et al., 1988; Grandaliano et al., 2000b; Howell et al., 2001). Consequently, a number of studies have demonstrated that members of the coagulation cascade may play a direct or indirect role in a number of pro-fibrotic processes highlighting their ability to contribute toward the pathogenesis of fibrosis (Hewitson et al., 2005; Liu et al., 2004; Monno et al., 2001; Tanaka et al., 2005; Vesey et al., 2005). To date, a more comprehensive account of the role of coagulation members localised to glomerular lesions has been provided, whilst little is known about the pathological significance of serine proteases in the tubulointerstitium.

This study has therefore examined the interstitial distribution of coagulation factors after the induction of UUO and has attempted to identify the significance of coagulation factor accumulation during fibrosis. Accordingly, the work described within has demonstrated the presence of coagulation factors in the tubulointerstitium *in vivo* and their temporal and spatial distribution in relation to the cell surface receptor PAR-1 and myofibroblasts. This study therefore establishes potential mechanisms whereby spatially related coagulation factors can induce cell-mediated effects through PAR-1. This was further supported by the temporal loss of PAR-1 receptor protein, likely to be indicative of receptor activation and degradation. Furthermore, in an attempt to understand the roles of these coagulation factors, this study has been able to identify an alternate pathway for factor V generation by renal fibroblasts, potentially facilitating the formation of thrombin. Lastly, EMT is an increasingly acknowledged phenomenon in fibrosis, and this study has identified

tubular epithelial cells that undergo EMT at the early stages of UUO. Using the *ex- vivo* model of EMT, the potential for thrombin to induce EMT through PAR-1 has been demonstrated, providing an insight into the significance of PAR-1 on tubular epithelial cells and confirming the likelihood for thrombin to carry out cell mediated effects through this GPCR.

6.4.1 Fibrin deposition in experimental tubulointerstitial fibrosis

Fibrin deposition has been infrequently recognised as a marker of renal tubulointerstitial damage and has been described in a number of human and experimental renal diseases including UUO, allograft rejection and acute ischaemic renal failure (Enestrom et al., 1988; Faulk et al., 1989; Grandaliano et al., 2000b; Wang et al., 1997; Wang et al., 1996; Wendt et al., 1995; Yamamoto and Loskutoff, 1997). These studies have documented fibrin deposits in the interstitium, peri-tubular capillaries and along the tubular basement membrane (Enestrom et al., 1988; Wang et al., 1997; Wendt et al., 1995). Consistent with this, the work described within has shown that interstitial fibrin deposition progressively accumulates after the induction of UUO, and it is still present at 21 days post-UUO. The accumulation of fibrin during progressive fibrosis is supported by numerous studies (Enestrom et al., 1988; Faulk et al., 1989; Grandaliano et al., 2000b; Smiley et al., 2001; Wang et al., 1997; Wang et al., 1996; Wendt et al., 1995; Yamamoto and Loskutoff, 1997) and as a result, fibrin deposition has become increasingly recognised as a hallmark of the inflammatory response (Smiley et al., 2001).

The significance of fibrin deposition can be ascertained from studies which show that TAFI knock-out mice display attenuated lung fibrosis suggesting that the anti-fibrinolytic activity of TAFI promotes lung fibrosis by hindering the rate at which fibrin is degraded (Fujimoto et al., 2006). Fibrin in its clotted form can promote ischaemia, influx of monocyte-macrophages and proliferation of epithelial cells in Bowman's space (Hertig and Rondeau,

2004) leading to the release of more cytokines which can facilitate inflammatory and fibrogenic processes.

The accumulation of fibrin is thought to be due to the up-regulation of PAI-1, primarily because of its ability to inhibit tPA and uPA which play the role of facilitating plasmin formation normally leading to the degradation of fibrin. The up-regulation of PAI-1, principally mediated by TGFβ1, has been noted in a number of animal disease models (Ishidoya et al., 2002; Kitching et al., 2003; Matsuo et al., 2005; Wang et al., 1997; Wang et al., 1996). Furthermore, increased levels of PAI-1 have been demonstrated in human CAN (Grandaliano et al., 2001) and thrombotic microangiopathy (Eddy, 2002) in concert with marked increases in fibrin deposition. This underscores the potential for PAI-1 to facilitate the accumulation of fibrin. However it has also been noted that activated fibroblasts may also invade fibrin deposits, and through the secretion of collagens, make the fibrin deposits more resistant to breakdown (Rerolle et al., 2000) underlining another mechanism which may lead to the accumulation of fibrin.

6.4.2 Spatial relationship between thrombin and myofibroblasts

This study has demonstrated a spatial relationship between fibrin, the end product of the coagulation cascade, and the myofibroblast, a key mediator of fibrosis at 3 days post-UUO. Because the serine protease thrombin has a rapid turn over rate (Coughlin, 2000), this study has utilised fibrin as a surrogate marker for thrombin. This study therefore supports other work showing that thrombin and the myofibroblast marker, αSMA co-exist in the inflammatory and early fibrotic stages in various pathological situations (Coughlin, 2000). Thrombin may be found in the interstitium as a consequence of increased capillary permeability (Plante et al., 1996) and misdirected filtration from the glomerulus to the interstitium (Kriz et al., 2001), features of interstitial pathology.

By establishing a spatial relationship between markers of fibrosis and thrombin, it can be hypothesised that accumulating fibrin clots in the tubulointerstitium act as thrombin reservoirs (Grandaliano et al., 2001) in interstitial spaces, thereby allowing thrombin to carry out its cell-mediated effects. Given that thrombin has a short half life, it is thought to act near the site where it is produced (Coughlin, 2000) and this therefore underscores the potential for thrombin to mediate local fibroblast mitogenesis (Hewitson et al., 2005), differentiation (Bogatkevich et al., 2001) or ECM synthesis (Bachhuber et al., 1997; Chambers et al., 1998; Dabbagh et al., 1998; Hewitson et al., 2005; Howell et al., 2001; Shirato et al., 2003; Vesey et al., 2005) on these αSMA-positive cells. However, because the data is correlative, further work needs to be performed to establish definitive functional relationships.

6.4.3 Factor V expression in experimental tubulointerstitial fibrosis *in vivo* and *in vitro*

The presence of the membrane bound co-factor, factor V has also been demonstrated in a juxtaposition to fibrin, and the work herein has been shown that like mesangial cells (Ono et al., 2001), renal fibroblasts can synthesise factor V *in vitro* in response to the inflammatory mediator TNFα. The generation of thrombin from prothrombin is potentiated by 10^4 fold in the presence of factor V (Ono et al., 2001). Accordingly, the presence of membrane-bound factor V in the tubulointerstitium, potentially secreted by fibroblasts, provides a mechanistic pathway by which the generation of thrombin can occur. This may then allow thrombin to act in an autocrine or paracrine fashion on resident interstitial fibroblasts.

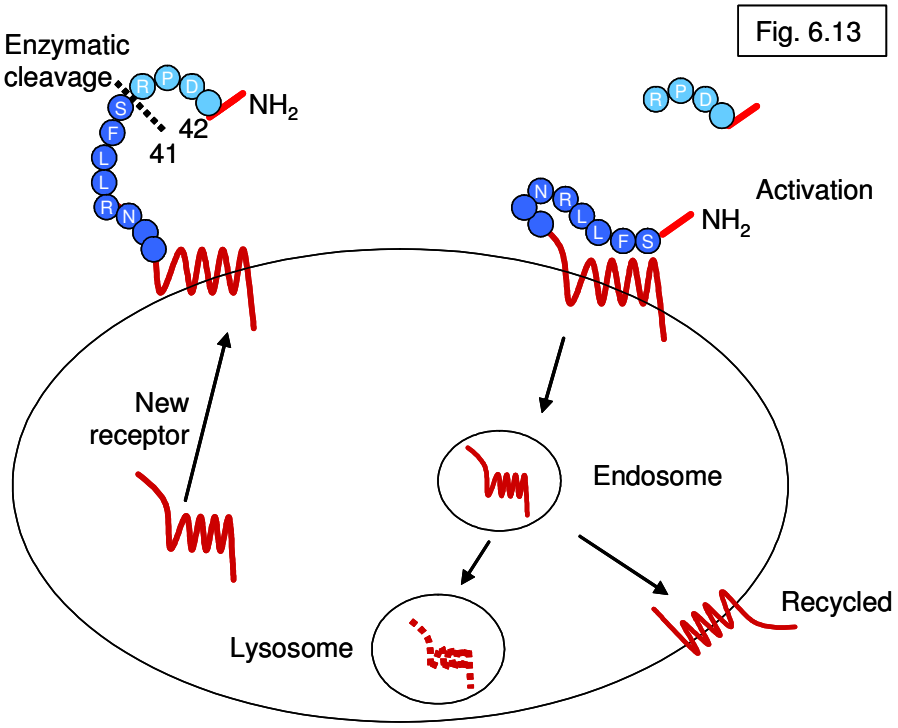
6.4.4 PAR-1 expression in experimental tubulointerstitial fibrosis

Thrombin may exacerbate fibrosis by inducing mitogenesis and collagen synthesis (Hewitson et al., 2005), potentially making it an important fibrogenic mediator. Cell-mediated effects of the serine protease thrombin occur via activation of the GPCR, PAR-1. When a protease binds to the PAR, it cleaves the amino terminal exo-domain exposing a new

amino acid sequence. This exposed N-terminus then acts as the tethered peptide ligand and binds to the heptahelical portion of the receptor to activate transmembrane signalling and G protein activation (Coughlin, 2000). Increasing evidence suggests that thrombin can act through this receptor to mediate a number of pro-inflammatory and fibrogenic events (Bogatkevich et al., 2001; Dabbagh et al., 1998; Grandaliano et al., 2001; Grandaliano et al., 2000a; Hewitson et al., 2005), while previous studies have shown that thrombin can induce increased expression of PAR-1 *in vitro* (Ellis et al., 1999). In human allograft nephropathy, the up-regulation of PAR-1 by tubular epithelial cells correlates with interstitial fibrin deposition and the severity of tubulointerstitial fibrosis. (Grandaliano et al., 2001). In a similar vein to Grandaliano et al [24], this study confirms that PAR-1 is expressed on the basolateral surface of tubular epithelial cells, and has established that PAR-1 is spatially related to fibrin and factor V deposits.

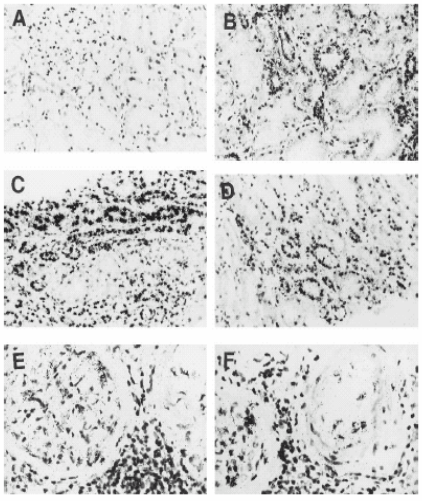
The results described within provide a further insight into the significance and kinetics of PAR-1 receptor activation during fibrosis. In contrast to previous findings (Xu, 1995), PAR-1 was detected at day 0 (before UUO was elicited). The reasons for this discrepancy are unknown. Whilst PAR-1 is prominent early during UUO, there is a gradual loss of protein staining with increasing fibrosis. This progressive loss of PAR-1 receptor staining was inversely related to the progressive accumulation of fibrin post-UUO. These fibrin mesh clots may house increasing concentrations of thrombin as they accumulate after UUO. The demonstrated loss of PAR-1 receptor staining is consistent with receptor activation and internal degradation, as demonstrated previously (Grandaliano et al., 2000a). Once extracellular thrombin activates PAR-1, PAR-1 is then uncoupled from the GPCR and internalised for lysosomal degradation [28]. A smaller proportion of these receptors may be recycled and returned to the cell membrane *(Fig. 6.13)* (Xu et al., 1995). Because this facilitates transient signalling, the magnitude of thrombin's signalling effect is dependent not

Fig. 6.13



Kinetics of PAR-1 receptor activation and degradation. After activation of PAR-1, it is rapidly phosphorylated and uncoupled from its G-protein. PAR-1 is then internalised into endosomes where approximately 75% of these receptors get degraded and a smaller proportion are recycled and returned to the cell membrane as naive receptors. Membrane expression of PAR-1 is replenished through an intracellular store that shuttles back and forth within the cytoplasm.

Fig. 6.14



PAR-1 mRNA in progressive renal disease. Grandaliano et al (2001) has shown that PAR-1 mRNA increases in CAN. Whilst no changes are detected in the control (a), marked up-regulation of PAR-1 is illustrated in the renal tubules (b-d), glomeruli (e) and vessels (f). Similarly, the study described within has demonstrated that whilst receptor staining for PAR-1 protein decreased with increasing fibrogenesis, PAR-1 mRNA was still evident at day 21.

CAN = chronic allograft nephropathy.

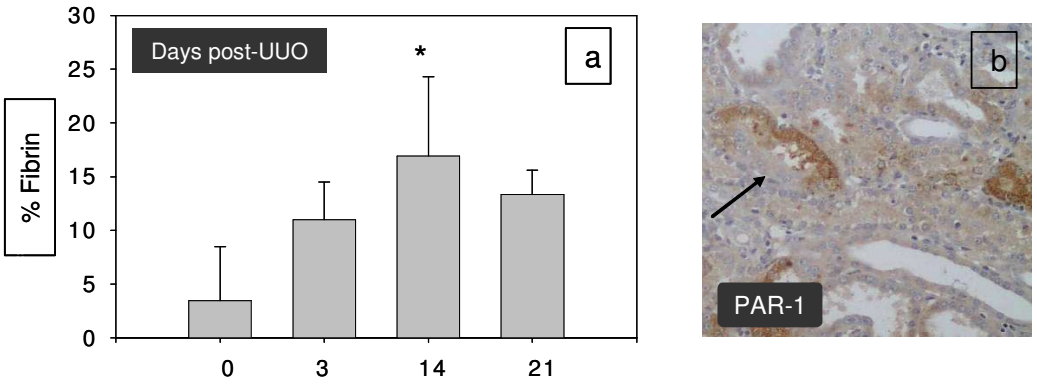
only on the rate of receptor cleavage and activation, but also on thrombin concentration (Bradshaw, 2004b). Furthermore, activation of PAR-1 by proteolytic cleavage means that one single thrombin molecule can activate multiple receptors. Consequently, in cases where high levels of thrombin are transiently present, a high rate of receptor cleavage and internalisation would be expected, in accordance with this study.

In situ hybridisation demonstrated that late in fibrosis (day 21), PAR-1 mRNA expression is still present. This finding is in the same theme as the work by Grandaliano et al (Grandaliano et al., 2001), who demonstrated an up-regulation of thrombin receptor mRNA levels in relation to increasing fibrin distribution in CAN *(Fig. 6.14)*. Furthermore, other groups support the likelihood that thrombin receptor protein does not always correlate with thrombin receptor mRNA level (Xu, 1995). Continued receptor activation, and an accumulating extracellular thrombin concentration residing in local fibrin clots is a likely explanation for new receptor synthesis late in fibrosis. Studies in human disease have shown that thrombin receptor protein expression is strikingly down-regulated (Grandaliano et al., 2000a). Similarly, in this study the loss of PAR-1 protein staining was also noted. These studies on PAR-1 receptor activation and degradation therefore describe a mechanism by which increasing fibrin deposition and thrombin concentrations throughout UUO lead to PAR-1 activation and degradation, as represented by a progressive loss of receptor staining *(Fig. 6.15a-b)*, and indicate that PAR-1 transcriptional activity occurs late in UUO.

6.4.5 Role of PAR-1 in EMT

Activation of PAR-1 in renal tubular epithelial cells may lead to pro-fibrogenic effects including up-regulation of MCP-1 and DNA synthesis (Grandaliano et al., 2000b). However, given that EMT was demonstrated in this model of UUO, and given the close proximity of PAR-1 and fibrin clots, the potential for PAR-1 to mediate EMT was investigated. This study was further warranted by a recent study which demonstrated that thrombin can induce PAR-1-

Fig. 6.15

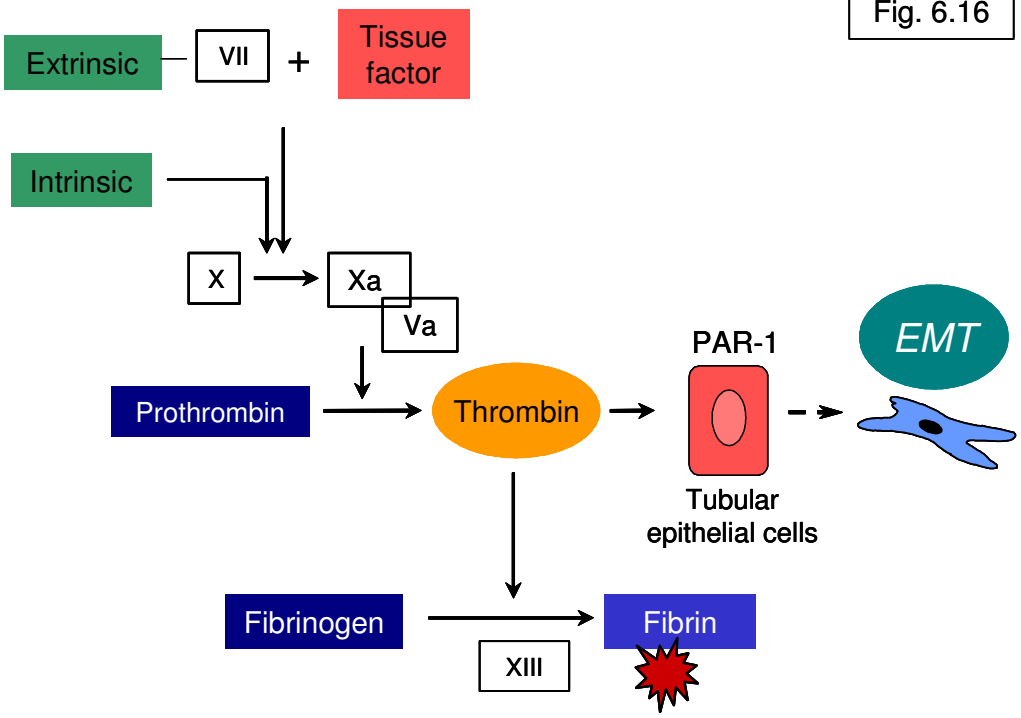


*Inverse relationship between accumulating fibrin clots and loss of PAR-1 with progressive UUO. Whilst fibrin accumulates progressively in the interstitial peri-tubular regions during UUO (a; *p<0.05 vs. control, n=3-6), PAR-1 staining is lost by the later stages of fibrogenesis (b; arrow). Indicative of receptor activation and degradation, this suggests that peri-tubular thrombin, housed in accumulating fibrin clots (potentially making it more resistant to degradation), may act through PAR-1 to carry out its cell-mediated effects.*

mediated endothelial-mesenchymal transition by facilitating the spread and migration of endothelial cells (Archiniegas et al., 2004). To assess the significance of PAR-1 in renal EMT, renal explants were treated with TRAP. TRAP contains the amino-acid sequence (SFLLRN) of the tethered ligand and effectively mimics thrombin's actions *in vivo*. It is commonly used in thrombin-related studies (Cunningham et al., 2000; Dabbagh et al., 1998; Grandaliano et al., 1994) because its longer half-life offers improved efficacy.

Exogenous TRAP facilitated EMT in the *ex vivo* model of EMT in this study highlighting the potential for thrombin to act as a novel mediator of EMT via PAR-1 *(Fig. 6.16),* in addition to the multiplicity of agonists already capable of carrying out this phenomenon. Whilst thrombin has been shown to act through PAR-1 to induce ECM synthesis (Hewitson et al., 2005; Howell et al., 2001; Shirato et al., 2003), αSMA expression (Bogatkevich et al., 2001) and mitogenesis (Hewitson et al., 2005), this study therefore highlights another pro-fibrotic role for thrombin, mediated through PAR-1. However, given that factor Xa is capable of activating PAR-1 and PAR-2 (Chambers and Laurent, 2002), the possibility that factor Xa is also involved in stimulating PAR-1-mediated EMT cannot be excluded. Furthermore, because thrombin also stimulates TGFβ1 release from proximal tubular epithelial cells (Vesey et al., 2005), it should be noted that TGFβ1 may act in an autocrine fashion to induce EMT in these cells. In line with the predominance of TGFβ1 in EMT, thrombin may therefore act in a TGFβ-dependent manner to induce EMT. Further studies are required to clarify the mechanism of thrombin's action.

Fig. 6.16



Schematic demonstrating the involvement of PAR-1 in EMT. This study describes the potential for PAR-1 to mediate EMT by demonstrating the ability of TRAP to up-regulate the expression of αSMA in the ex vivo model of explanting.

6.4.6 Summary

In conclusion, these investigations continue to re-define the theory that cytokines are the principle mediators of inflammation and highlights the potential for coagulation factors, through cell- and receptor-mediated mechanisms, to be involved in non-cytokine mediated pathways during the pathogenesis of tubulointerstitial fibrosis. This study provides insight into the distribution of serine proteases during experimental fibrosis entailing a possible mechanism whereby thrombin, present in fibrin clots, may be potentially generated through factor V expression by fibroblasts, and in turn may act in an autocrine or paracrine manner on local interstitial fibroblasts to induce a cascade of fibrotic events. It also provides an insight into the *in vivo* distribution of PAR-1 in a model of UUO and establishes possible mechanisms for the kinetics of PAR-1 receptor activation and degradation. Furthermore, through utilisation of the model of EMT, it has been shown that TRAP can induce PAR-1 mediated EMT in renal explants and provides a novel insight into the mechanisms of EMT.

6.5 SPECIFIC METHOD PROTOCOLS

Method	Section
UUO	2.2.1
Immunohistochemistry	2.6.2
Cytochemistry	2.6.1

Table 6.1: References for methods described in *Chapter 2* used in this chapter.

6.5.1 Point counting of fibrin

Point counting was carried out to determine the temporal distribution of fibrin at 0, 3, 14 and 21 days post-UUO. Slides were placed under an 11×11 grid and counted according to the intersection of the cell with a grid line. Only regions with a viable and real representation of the staining characteristics were counted.

The percentage of fractional area represented by the item of interest was found by:

$$\%FA = \frac{\text{Staining under grid intersections} \times 100}{\text{Total grid intersections}}$$

Results are presented as a percentage of total fractional area.

6.5.2 Factor V expression in activated fibroblasts

Fibroblasts derived from UUO were characterised for their ability to express factor V in response to the inflammatory cytokine TNF-α (200U/ml). Cells were grown on coverslips in DMEM medium supplemented with DMEM + 1% FCS, DMEM + 10% FCS, or treated with 200U/ml TNF-α in DMEM medium supplemented with 1% FCS for 24hr. Cells were then fixed in cold methanol, washed in PBS and stained for factor V. Non-immune serum was added for 10min at room temperature, acted as a negative control. Groups were qualitatively compared.

6.5.3 *In Situ* hybridisation

In situ hybridisation was carried out to determine PAR-1 mRNA expression during progressive UUO according to established protocols (Darby, 2000). Tissue sections from kidneys taken at 21 days post-UUO were fixed in 4% paraformaldehyde, dehydrated, placed into a humid chamber and digested with proteinase for 25min at 37°C. Sections were washed in water, dehydrated and air-dried through 95% ethanol. The Digoxigenin (DIG) PAR-1 riboprobe was then added and sections were cover-slipped and incubated for 1.5hr at 55°C. Slides were then immersed in pre-heated stringent washing buffer and incubated for 25min at 55°C with shaking, immersed in TBS and placed in a humidity chamber with 2-5 drops anti-DIG alkaline phosphatase to each section. After 30min of incubation and washing in TBS and water, 2-3 drops of substrate was added to each solution and incubated for 30-60min. Substrate was then washed off and slides were immersed in tap water. Finally, tissue sections were counterstained in haematoxylin and mounted in aqueous mountant.

6.5.4 Pharmacological modification of EMT with TRAP

Explants were treated with DMEM + 10% FCS supplemented with either 10ng/ml TGFβ1, 70μM TRAP or maintained in control medium (DMEM+ 10% FCS) for 72hr. Drugs were replenished for another 72hr period, and explants were then fixed in cold methanol. Cells were stained for αSMA and quantified on the basis of positive staining for αSMA as a percentage of total cell number.

Chapter 7

Summary of Findings and General Discussion

7.1 SUMMARY OF EXPERIMENTAL FINDINGS

Tubulointerstitial fibrosis is dictated by a complex interplay between a cocktail of interstitial inflammatory and non-inflammatory mediators, and their effect predominantly on renal fibroblasts. Integral to these processes are the cell signalling pathways that govern the responses of these cells. Although the TGFβ signalling axis has been identified as the chief mediator of tubulointerstitial fibrosis, this thesis has identified additional molecular mechanisms involved in the regulation of tubulointerstitial fibrosis by examining the regulation of the renal interstitial fibroblast, a key mediator in tubulointerstitial fibrosis. It has primarily examined those features of fibroblast function which are most devastating to renal function: (a) fibroblast proliferation (b) myofibroblast differentiation, (c) ECM synthesis and (d) EMT. By using fibroblasts and tissue derived from a model of UUO, or tissue exposed to mechanical stress (to study EMT), this thesis has ascertained the following findings:

7.1.1 Chapter 3: Role of phosphatidylinositol 3-kinase and mTOR in the regulation of renal fibroblast proliferation and collagen synthesis

This study was aimed at identifying additional novel signalling pathways involved in regulating fibroblast proliferation and collagen synthesis by using pharmacological inhibitors of PI3K and mTOR. The signalling circuitry encompassing PI3K and mTOR in renal fibroblasts was initially examined by determining Akt S-473 and S6RP S-235/235 phosphorylation status in response to serum-based mitogen stimulation. This established (a) Akt S-473 as a downstream mediator of PI3K and (b) S6RP S-235/235 as a downstream target of mTOR. In response to RAD treatment for the standard experimental treatment period (48hr), Akt S-473 phosphorylation was also down-regulated. Functional studies of these pathways have confirmed the involvement of PI3K and mTOR in the regulation of fibroblast proliferation, total collagen synthesis and gene expression of α1(I) procollagen

mRNA (mTOR). Results also suggest that the functional studies were independent of increases in apoptosis or compromised cell viability.

7.1.2 Chapter 4: Development and validation of an *ex vivo* model of EMT

This chapter has described the development of an *ex vivo* model of EMT that allows for easy tracking of transitioning epithelial cells in an environment that is physiologically relevant to its *in vivo* counterpart, but is displaced from the organism. By explanting the cortex derived from normal kidneys, it has been established that these cells (derived from proximal tubules; shown by lectin staining), initially phenotyped as cytokeratin positive, progressively acquire a mesenchymal phenotype and gradually lose their epithelial markers. These cells became αSMA-positive myofibroblasts and lost morphological characteristics of epithelial cells. Approximately half of the cell population underwent EMT at a given time point consistent with studies indicating that a large proportion of renal myofibroblasts may be derived from EMT. Testing this model with the most well known inducer of EMT (TGFβ1) and a newly established negative regulator of EMT (lovastatin) has effectively validated this model.

7.1.3 Chapter 5: The role of phosphatidylinositol 3-kinase and mTOR in the regulation of myofibroblast differentiation and EMT

Recent studies have established the role of mTOR in VSCM cell differentiation providing a basis to investigate the role in PI3K and mTOR in renal fibroblasts. In response to inhibition of PI3K and mTOR, the expression of αSMA protein was facilitated (when exposed to serum-based mitogen stimulation). Preliminary studies described within this chapter have indicated that up-regulation of αSMA may not be due to changes in gene transcription. Further investigation into the regulation of differentiation by these pathways by examining total TGFβ1 secretion indicated that mTOR, but not PI3K, may regulate TGFβ1 by controlling a TGFβ1 autocrine loop.

In addition to the well known TGFβ/Smad signalling axis, MAPK and Rho signalling, this study has also established a role for PI3K and mTOR in the regulation of EMT by using the *ex vivo* model of EMT. Consistent with *in vitro* studies in mammary epithelial cells (Bakin et al., 2000) and in nrk-52e cells (Wu et al., 2006), PI3K and mTOR down-regulate EMT by inhibiting the expression of αSMA. This chapter suggests a possible paradox in the ability of PI3K and mTOR to negatively regulate fibroblast differentiation whilst facilitating EMT *ex vivo*.

7.1.4 Chapter 6: Constituents of the coagulation cascade are spatially and functionally related to experimental tubulointerstitial fibrosis

It is apparent that tubulointerstitial fibrosis is highly complex and the interstitial milieu involves copious amounts of inflammatory cytokines, vasoactive peptides and an abundance of activated cell types. These factors drive the relentless inflammatory cycle, which can propagate a marked decline in renal function over time. As demonstrated in a number of experimental renal models and human renal disease, non-cytokine mediated pathways can also mediate fibrosis and in addition to AGEs, tPA and PAI-1, this study has highlighted the likely involvement of the coagulation cascade during tubulointerstitial fibrosis. It has primarily localised the interstitial distribution of factor V (and its potential role in fibrosis) and fibrin, the latter of which has become a hallmark of the inflammatory response. This study has also co-localised fibrin (acting as a surrogate marker for thrombin) to αSMA myofibroblasts providing the framework for thrombin to carry out a number of cell-mediated effects on these cells. Furthermore, an evaluation of the specific thrombin receptor PAR-1 at time points post-UUO has provided a likely mechanism whereby increasing fibrin clots, likely to house substantial concentrations of thrombin may lead to PAR-1 activation and degradation. Lastly, this study has underscored the likelihood of PAR-1 to mediate EMT by using the *ex vivo* model of EMT.

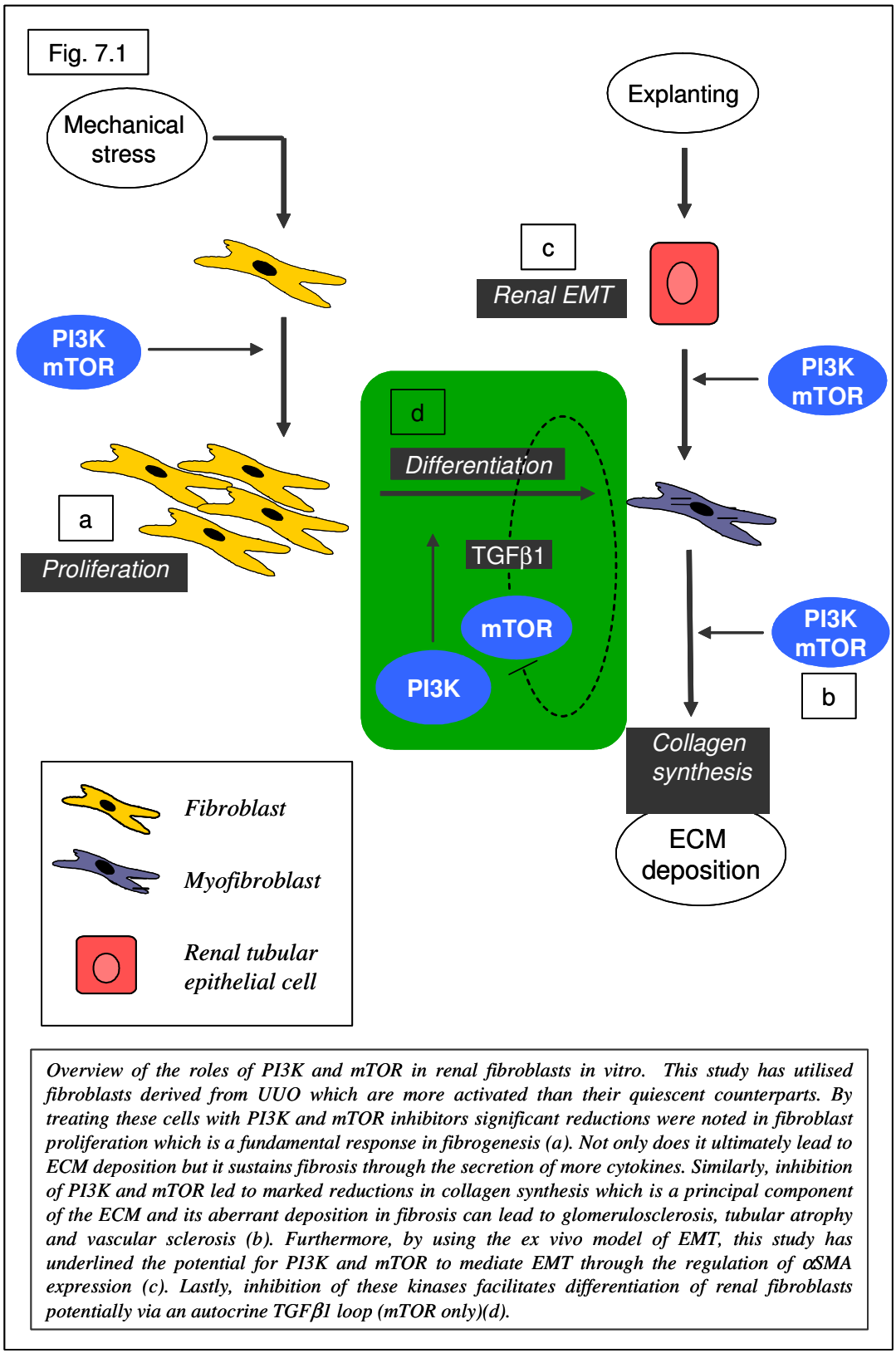
7.2 GENERAL DISCUSSION

When taken together, a number of interesting and paradoxical findings emerge from the work described within this thesis which deserve further discussion.

7.2.1 The paradox of PI3K and mTOR in the regulation of proliferation, ECM synthesis and differentiation

Whilst inhibition of PI3K and mTOR resulted in inhibition of key events during fibrosis including fibroblast proliferation *(Fig. 7.1a)* ECM synthesis *(Fig. 7.1b)* and EMT *(Fig. 7.1c)*, the differentiation of fibroblasts to myofibroblasts was clearly facilitated, as outlined in *Chapter 5 (Fig. 7.1d).* Whilst this finding may immediately seem paradoxical given that it is thought that inhibition of the cell cycle generally parallels an inhibition of differentiation (Wainwright et al., 2001), a number of studies suggest that these processes may not be proportionally related.

Whilst the differentiation and proliferation of both neutrophils (Kanayasu-Toyoda et al., 2002) and epithelial cells (Shao et al., 2004) is differentially regulated by PI3K and mTOR, more compelling is the parallel role that mTOR plays in regulating functionally distinct VSMC phenotypes (Martin et al., 2004; Marx et al., 1995). VSMC have an inherent functional plasticity whereby they can vary between a mature phenotype in which the primary function is contraction, to a less differentiated (synthetic) state where the predominant functions are protein synthesis and proliferation (Worth et al., 2001). Similarly to the effect of RAD in fibroblasts, rapamycin has also been shown to inhibit proliferation and ECM synthesis in VSMC whilst facilitating differentiation in these cells (Martin et al., 2004; Marx et al., 1995).



Overview of the roles of PI3K and mTOR in renal fibroblasts in vitro. This study has utilised fibroblasts derived from UUO which are more activated than their quiescent counterparts. By treating these cells with PI3K and mTOR inhibitors significant reductions were noted in fibroblast proliferation which is a fundamental response in fibrogenesis (a). Not only does it ultimately lead to ECM deposition but it sustains fibrosis through the secretion of more cytokines. Similarly, inhibition of PI3K and mTOR led to marked reductions in collagen synthesis which is a principal component of the ECM and its aberrant deposition in fibrosis can lead to glomerulosclerosis, tubular atrophy and vascular sclerosis (b). Furthermore, by using the ex vivo model of EMT, this study has underlined the potential for PI3K and mTOR to mediate EMT through the regulation of αSMA expression (c). Lastly, inhibition of these kinases facilitates differentiation of renal fibroblasts potentially via an autocrine TGFβ1 loop (mTOR only)(d).

Given the similarities between VSMC and fibroblasts, it is interesting to speculate whether the parallels in the differential regulation by PI3K and mTOR in VSMC and fibroblasts (as described within this thesis) can be drawn further to underscore the potential for fibroblasts to also exhibit a functional plasticity, similar to VSMC. In tubulointerstitial fibrosis, it could be possible that this response may be dictated by the interstitial milieu (ie. mitogen specific), which may differentially regulate fibroblast proliferation and differentiation by activating different pathways. This type of response is likely to depend on cross-talk signalling mechanisms and is also consistent with findings that (a) only a subset of myofibroblasts express αSMA (Strutz and Zeisberg, 2006), (b) myofibroblasts only transiently express αSMA during tubulointerstitial fibrosis (Hewitson et al., 1995), and that (c) results described within indicated that the changes to proliferation and differentiation occurred within the same cells, not within different cell populations (which may suggest sub-populations of cells respond differently). Future studies that are suggested by these outcomes should examine the hypothesis that the expression of αSMA in myofibroblasts consistently coincides with the secretion of increased ECM proteins (Hinz and Gabbiani, 2003).

7.2.2 The controversy over the effect of rapamycin *in vivo*

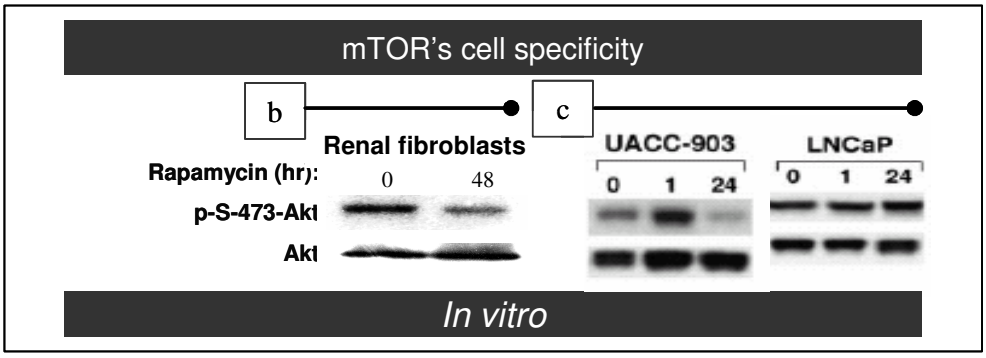
Whilst an increasing number of studies highlight the potential for rapamycin to ameliorate fibrosis *in vivo* (Bonegio et al., 2005; Jain et al., 2001; Lloberas et al., 2006; Stallone et al., 2005; Wu et al., 2006) other studies have also indicated that rapamycin can in some cases exacerbate fibrosis by activating the synthesis of TGFβ protein and contributing to the deposition of ECM proteins (Shihab et al., 2004a; Shihab et al., 2004b). Although the reasons for these discrepancies are unclear, it is apparent that mTOR signalling is highly dependent on the cell context. Whilst shown in these studies in renal fibroblasts *(Fig. 7.2b)* and by Sarbassov et al (Sarbassov dos et al., 2006) that prolonged RAD/rapamycin treatment can inhibit Akt phosphorylation, this effect is highly cell-specific, only being observed in a

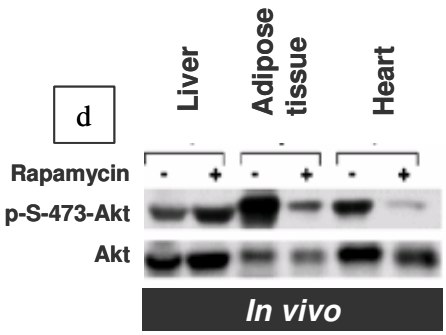
third of the 33 cell lines tested under identical experimental conditions *(Fig. 7.2c)*. This type of differential response has been shown to occur *in vivo (Fig. 7.2d)* (Sarbassov dos et al., 2006). Whether this reflects the ability of rapamycin to have variable effects *in vivo* is currently unknown.

Moreover, because rapamycin also inhibits the downstream S6K which normally forms a negative feedback loop with PI3K and Akt (therefore inhibiting Akt) (Harrington et al., 2004; Sarbassov et al., 2005; Shah and Hunter, 2006; Um et al., 2004), prolonged rapamycin treatment can actually activate Akt signalling (Sarbassov dos et al., 2006). It is therefore evident that rapamycin can (a) inhibit Akt (b) activate Akt or (c) have no effect (as demonstrated previously (Sarbassov dos et al., 2006). Although shown to be clinically relevant in the treatment of cancer with rapamycin (Eng et al., 1984; Sawyers, 2003; Sun et al., 2005), the implication of this circuitry in fibrosis *in vivo* is unknown, however potentially it suggests that in cases where rapamycin activates Akt, cell proliferation would be facilitated (given the predominant role of Akt in proliferation).

It therefore seems evident that whilst rapamycin has the potential to act as an anti-fibrotic agent *in vivo*, more experimental models are needed to confirm these findings in concert with further elucidation of mTOR signalling, which is developing at a fast pace.

Fig. 7.2





The differential effects of rapamycin in vitro and in vivo. mTORC2 assembly can be inhibited in response to prolonged rapamycin treatment (a), however this is highly cell-specific. Whilst rapamycin has been shown to inhibit Akt S-473 after prolonged treatment in my results (b) and others; UACC-903 (melanoma cell line) (c), rapamycin can also have no effect; LNCapP (prostate cancer cell line). This has also been demonstrated in vivo (d) where rapamycin can markedly inhibit Akt (eg. heart) or have no effect (eg. liver).

(c)-(d); Sarbassov et al, 2006.

7.2.3 The reality of EMT in renal tubulointerstitial fibrosis and the fate of EMT-derived cells

7.2.3.1 EMT in renal tubulointerstitial fibrosis

Although there has been a growing enthusiasm and acceptance of the significance of EMT in renal fibrosis, the identification of this process *in vivo* has to date proved difficult (due to the kinetics of EMT and difficultly in tracing these cells). It has therefore been noted that renal EMT is the least well-proven conceptually (Zavadil and Bottinger, 2005) suggesting that more *in vivo* models are needed to substantiate the contribution of this process during renal tubulointerstitial fibrosis.

Moreover, it has been noted that the different types of EMT (renal, cancer and embryonic) represent different physiological/pathological contexts and should not be interpreted as a universally regulated process (Zavadil and Bottinger, 2005). A glance at the literature confirms this and indicates that there are many notable differences between cancer and renal-related EMT including the ability of HGF (Li et al., 1994; Muller et al., 2002) and PDGF (Jechlinger et al., 2006; Yang et al., 2006a) to induce EMT in cancer, the former of which is a major negative regulator of renal EMT (Zeisberg et al., 2003). This raises concerns over the use of nrk-52e cells, which is indeed a transformed immortalised cell line, and the most well-known cell line used to study renal EMT *in vitro*. When studies use nrk-52e cells, it could be questioned whether we are really examining the proteome involved in producing populations of myofibroblasts during renal EMT.

7.2.3.2 Fate of EMT-derived cells

Whilst most studies indicate that cells that undergo EMT are bound to become myofibroblasts, other studies indicate that EMT serves different purposes. Whilst this study and other studies (Fan et al., 1999; Masszi et al., 2003; Shimizu et al., 2006; Strutz et al., 2002) have shown that (tubular) epithelial cells can transition into myofibroblasts, other studies have highlighted the ability of epithelial cells to undergo de-differentiation (Bonventre, 2003; Forino et al., 2006) in order to potentially repair damaged tubular segments (Rastaldi et al., 2002). Furthermore, it has been noted *in vivo* that EMT can be mistaken for encroachment into the renal tubule by αSMA-positive cells during fibrosis (Faulkner et al., 2005). Current studies are hindered by the lack of specific markers to detect EMT making it difficult to distinguish these events in some cases, however consistent with the complexity of the fibrotic process, it is likely that of the above phenomena can occur. It remains to be shown *in vivo* that cells actually migrate away from the tubule. Lastly, if one is to accept the growing school of thought that EMT is a key pathological process in renal fibrosis, then what is the terminal fate of these cells? Whilst Zeisberg et al has shown that EMT can be reversed by exogenous BMP-7 *in vivo* (Zeisberg et al., 2003), whether these cells can also revert to their epithelial phenotype after tissue damage is currently unknown.

7.3 FUTURE DIRECTIONS

This study has established a number of novel findings that not only provide a solid basis to assess the *in vivo* role of PI3K and mTOR, but also provide a foundation for the further elucidation of the mechanisms involved in various experimental aspects of this work. These include the following:

7.3.1 Growing roles for mTOR

As the identification of mTOR as a central kinase involved in the regulation of proliferation, growth and hypertrophy (Chen et al., 2005; Sakaguchi et al., 2006), cancer (Hidalgo and Rowinsky, 2000; Manning, 2004; Sabatini, 2006) type II diabetes (Lloberas et al., 2006; Um et al., 2004) cell survival and glucose metabolism (Cheatham et al., 1994; Ruvinsky et al., 2005), inflammation (Bonegio et al., 2005; Loverre et al., 2004) and fibrosis has unravelled over recent years, it is likely that the roles of the downstream substrates and furthermore, their upstream regulators will become clearer over the coming years. Although the effectors involved in the studies within have not been identified, elucidation of effectors would be valuable in defining potential therapeutic intervention strategies.

Specific to this study, it would also be valuable to further investigate the following:

(a) The reproducibility of these results with transfection, gene mutation knock-in or small interfering RNA (siRNA) of mTOR and PI3K (and the specific isoforms of PI3K). The recent development of specific PI3K subunit inhibitors also holds promise to elucidate the roles of PI3K subunits (Jackson et al., 2005). Furthermore, it would be useful to determine which Akt isoform is involved in fibrosis in order to identify the isoforms involved in metabolism and growth (Cully et al., 2006). The current literature suggests that p110α and Akt 1 are the predominant isoforms that control proliferation (Easton et al., 2005; Engelman et al., 2006).

(b) The *in vivo* effect of rapamycin on EMT given that growing evidence suggests a role for this kinase in EMT *in vitro*. Furthermore, given that EMT signalling involves a number of pathways, elucidation of the role of PI3K and mTOR in this signalling cascade as well as its roles (other than induction of αSMA) in the engagement of the EMT proteome would enhance our understanding of this complex event. Since both PI3K (Grille et al., 2003) and mTOR (Aguilera et al., 2005) have been shown to up-regulate *Snail*, it would be useful in future studies to examine the roles of these kinases in the suppression of E-cadherin.

(c) Whilst preliminary data indicates αSMA gene expression is not regulated by PI3K and mTOR at the transcriptional level, further studies are required to fully elucidate this signalling mechanism.

(d) The role of mTOR and PI3K in the regulation of proliferation, differentiation and ECM synthesis *in vivo*.

7.3.2 The *ex vivo* model of EMT

It would be valuable to further delineate whether the reversal of EMT in this model is possible. This would provide further insight into the cellular fate of EMT-derived cells, and allow us to understand the extent of epithelial plasticity. Similarly, an analysis of loss or gain of other epithelial and mesenchymal markers would provide further insight into the mechanisms of EMT in this model.

Whilst it is currently difficult to study cellular mechanisms of EMT with the *ex vivo* model of EMT, the potential to improve or improvise using this model can be drawn from the advancements made in models used to study cancer-related EMT. Acknowledgement of the inherent problems with structural organization and functional differentiation in *in vitro* epithelial cancer studies (Debnath and Brugge, 2005) led to the development of three-dimensional culture models that involve the generation of physiologically equivalent epithelia

structures which are completely embedded in an ECM (Debnath and Brugge, 2005). Consequently, models such as these may be promising for the study of renal EMT as they overcome micro-environmental isolation and allow for genetic as well as cell biological studies with a high throughput (Schmeichel and Bissell, 2003).

7.3.3 Further elucidation of coagulation in fibrosis

Whilst PAR-1 knock-out studies have been successfully utilized in crescentic glomerulonephritis (Cunningham et al., 2000), the effect of PAR-1 knock-out in experimental models of fibrosis remains largely unexplored. It would be particularly interesting to see what effect this has on EMT and also on myofibroblast recruitment, given that (a) thrombin can facilitate fibroblast differentiation (Bogatkevich et al., 2001) and (b) that PAR-1 has been shown in this study to mediate EMT. Furthermore, because there is some ambiguity over the specificity of proteases for PARs (TRAP has been shown to cross-react with PAR-2 whilst factor Xa has been shown to activate PAR-1 (Blanc-Brude et al., 2005) and PAR-2 (Grandaliano et al., 2003) it would be useful to examine the effect of specific inhibitors of thrombin. The examination of whether coagulation factors can independently mediate pro-fibrotic events would also be useful as it may be possible that these pathways depend on the TGFβ signalling axis (Grandaliano et al., 2003; Vesey et al., 2005). Because of the highly correlative nature of this chapter, an obvious future direction would be to explore and establish causal relationships. In this sense, it may be useful to include the use of pharmacological agonists or antagonists for *in vitro* studies whilst double labelling would also provide a more definitive insight.

7.4 CONCLUSION

Tubulointerstitial fibrosis can be induced through the activation of tubular epithelial cells leading to interstitial inflammation and activation and recruitment of interstitial fibroblasts. Beyond this point tubulointerstitial fibrosis may ensue independently of the initial stimulus and consequently this can lead to a progressive loss of renal function. The processes involved in these events depend inextricably on cell signalling cascades. Consistent with the complexity of tubulointerstitial fibrosis, studies are beginning to identify the complexity of cell signalling events which can act in a cell-specific, stimulus specific or context specific manner. The identification and characterisation of signalling pathways involved particularly in propagating a decline in renal function is therefore essential to be able to design effective and rational therapeutic interventions to ameliorate fibrosis. This study has contributed towards our understanding of renal fibroblast signalling and myofibroblast derivation and highlights the role of non-cytokine mediated pathways in tubulointerstitial fibrosis, providing a solid foundation for further studies in these areas.

APPENDIX 1

LIST of MATERIALS and SUPPLIERS

Unless indicated, all materials have been obtained from Sigma, St Louis, Mi, USA.

MATERIAL	SUPPLIER
Apx. 1.1 Immunohistochemistry	
Avidin-Biotin Complex (Elite)	DAKO, CA USA
Alcohols (70%-100%)	BDH, Kilsyth, Vic, Australia
Antibody diluent	DAKO
Aquamount Gurr®	BDH
Aqueous mounting medium	DAKO
Digital microscope camera	DP10, Olympus, Tokyo, Japan
Disodium hydrogen orthophosphate	BDH
Fluorescent microscope	Leika Microsystems, Wetzlar, Germany
Harris' haematoxylin	BDH
Histoclear	National Diagnostics, Adelaide, Australia
Hydrogen peroxide	BDH
Methanol	BDH
Neutral Buffered Formalin	Histolabs (Fronine) Riverstone, NSW, Australia
Non-immune serum	Vector laboratories Burlingame, CA USA
Normal horse serum	Vector laboratories,
Normal goat serum	Vector laboratories
PaintShop Pro	Jasc Sofware, Minnetonka, MI, USA.
Paraformaldehyde	BDH
Potassium chloride	BDH
Potassium dihydrogen phosphate	BDH
Sodium chloride	BDH
Sodium thiosulphate	BDH
Glass Slides	HD Scientific, Sunshine, Vic, Australia
Streptavidin Texas red conjugate	Molecular Probes, Inc, Eugene, Oregon
Vectastain kits	Vector
Xylene	BDH
Wax pen	DAKO
Whatman No. 1 filter paper	DAKO
Apx. 1.2 Cell culture	
Cellagen®	ICN Pharmaceuticals, Costa Mesa, CA, USA
DMEM	CSL, Vic, Australia
EDTA	Boehringer Mannheim, Mannheim, Germany
Falcon tubes	BDH
Flasks	Nunc, Roskilde, Denmark

Fetal calf serum	CSL
Hanks' salt solution	MP Biomedicals, Costa Mesa, Ca, USA
HEPES	JRH Biosciences Brooklyn Vic Australia
Gentamycin	ICN Pharmaceuticals
Nunc tubes	Nunc
OptiMEM®	Invitrogen, Mount Waverley, VIC Australia
Penicillin/Streptomycin antibiotics	MP Biomedicals
Petri dishes	Nunc
Scintillation counter	Beckman Coulter, Fullerton, CA, USA
Scintillation fluid	Perkin-Elmer Life and Analytical Sciences, Wellesley, MA USA
TCA	BDH
[^{3}H] Thymidine	Amersham Pharmacia Biotech, Rydalmere NSW, Australia

Apx. 1.3 Extraction of cellular components

Trizol®	Invitrogen
Chloroform	BDH
Guanidine hydrochloride	Boehringer Mannheim
Isopropanol	BDH
Molecular grade alcohols	BDH
SDS	BDH
Sodium citrate	BDH
Sodium hydroxide	BDH

Apx. 1.4 Real time PCR

αSMA forward and reverse primers	Sigma Genosys
Ribosomal protein L32 primers	Sigma Genosys
Dnase	Promega, Madison, WI, USA
DNase buffer	Promega
$MgCl_2$	Promega
DNTP	Promega
Random hexamers	Promega
RNAse out	Promega
RNA clean up kit (RNeasy kit)	Biorad, San Francisco, Ca, USA
Superscript III enzyme	Invitrogen
SYBR Green RNA kit	Invitrogen

Apx. 1.5 Northern blotting

^{32}P-nucleotide	Amersham Pharmacia Biotech
Ethidium bromide	Invitrogen
Formamide	BDH
Hybond®-N membrane	Amersham Pharmacia Biotech
Formaldehyde	BDH
Glycerol	BDH

Klenow enzyme	Amersham Pharmacia Biotech
Megaprime DNA labelling system kit	Amersham Biosciences, Piscataway, NJ, USA
Mixed bed resin	Promega
Sephadex-G50 column	Amersham Pharmacia Biotech
Sodium chloride	BDH
ssDNA	Promega

Apx. 1.6 Western blotting

Acetic Acid	BDH
Biotinylated protein ladder/HRP-conjugated	Cell Signaling
Agarose (Na)	Amersham Pharmacia Biotech
Ammonium persulphate	Biorad
Coomassie blue	Biorad
Film developer	Kodak, Rochester, NY, USA
Densitometry software	Kodak
ECL® detection kit	Amersham Biosciences
Film Fixative	Kodak
Glacial acetic acid	BDH
Hybond® nitrocellulose PVDF membrane	Amersham Biosciences
Rainbow standard	Biorad
SDS	BDH
Solution N (Acrylamide)	Biorad
TEMED	Biorad
Tris Base	BDH
Tween 20	Biorad
X-ray film	Kodak

Apx. 1.7 Hydroxyproline incorporation

[2,3-^{3}H Pro]-Pro	Amersham Biosciences
Collagenase	Worthington Biochemical Corporation, NJ, USA

Apx. 1.8 *In situ* hybridisation

Probe labelling kit and detection system	Roche, Castle Hill, N.S.W., Australia

Apx. 1.9 Apoptosis

Apx. 1.9.1 TUNEL

DeadEnd kit	Promega
DNase	Promega
Fluorescent microscope	Leika Microsystems

Apx. 1.9.2 Propidium Iodide

Propidium iodide	Invitrogen
Triton X-100	BDH

Apx. 1.10 Drugs, growth factors and peptides

Lovastain	Merck, Whitehouse Station, NJ, USA
LY294002	Cell Signaling Technology
PD98059	Cell Signaling Technology
RAD	Novartis, Basel, Switzerland
TGFβ1	Peprotech Inc., Rocky Hill, NJ, USA
TRAP	Auspep, Parkville, Australia
TNFα	Cytolab, Rehovot, Israel

Apx. 1.11 ELISAs

Apx. 1.11.1 MTT

DMSO	BDH

Apx. 1.11.2 S6RP ELISA (S-235/236)

S6RP ELISA	Cell Signaling Technology
Sunrise ELISA Plate Reader	Tecan, Salzberg, Austria

Apx. 1.11.3 TGFβ1 ELISA

TGFβ1 Quantikine® kit	R&D Systems, Gymea, N.S.W. Australia

APPENDIX 2

LIST of REAGENTS, BUFFERS and STOCK SOLUTIONS*

Apx. 2.1 Immunohistochemistry

PBS (10x)

Sodium chloride	80g
Potassium chloride	2g
Disodium hydrogen orthophosphate	11.5g
Potassium dihydrogen phosphate	2g
dH_2O	900ml

The pH was adjusted to 7.2 before final volume was made up to 1L using dH_2O. 1x PBS was made up at a dilution of 1:10.

Harris' Haematoxylin

Haematoxylin	5g
Ethanol (100%)	50ml
Ammonium alum	100g
dH_2O	1000ml

Haematoxylin was dissolved in ethanol. Ammonium alum was dissolved in distilled water. The 2 solutions were added together and heated until boiling. The solution was removed from heat and 1.5g mercuric oxide was added slowly. The solution was cooled before use.

2.1.1 Fixatives

Mercuric Formalin

37-40% formaldehyde solution	100ml
Distilled water	900ml
Sodium chloride	9g

Ingredients were stirred and mercuric chloride was added until solution was saturated.

Neutral Buffered Formalin

Formulation according to manufacturer's specifications

Formaldehyde	10%w/v

Disodium phosphate, sodium dihydrogen phosphate were used to buffer the solution to pH 6.95-7.05.

4% Paraformaldehyde

Paraformaldehyde	4g
0.1M PBS pH 7.2	100ml

Paraformaldehyde powder was dissolved in 65°C water and cleared by gradually adding 1M NaOH. Solution was cooled before use.

* Unless specified, all agents were stored at room temperature

TBS-Tween

1M Tris-HCl (pH 7.6)	2ml
1M NaCl	15ml
dH_2O	83ml
Tween-20	5µl

Stored at 4ºC

Apx. 2.2 Cell culture

10% BSA

1g BSA was dissolved in 10ml dH_2O.
Stored at 4°C.

Dialyzed 50% FCS

One end of dialysis membrane tubing was tied with silk to make a bag enclosed at one end. The membrane was filled with 10ml of FCS and top end tied with silk. Membranes containing FCS were soaked in 1L of dH_2O in a sterile glass beaker O/N at 4°C to allow the FCS to dialyze. The following day the tubing was untied and dialyzed FCS poured into a sterile cylinder. Dialyzed FCS was diluted to 50% with dH_2O and filter using a 0.2µm acetate membrane with a pre-filter membrane. The serum was then aliquoted and stored at -20°C.

DMEM + 20% FCS

1xDMEM	400ml
*FCS	80ml
HEPES	10ml
Penicillin (5000U/ml) & streptomycin (5000µg/ml)	8ml
Glutamine	4ml

Using sterile forceps, a filter unit and a disposable 0.2µM filter, the solution was poured into apparatus and filtered through a vacuum pump, and once completed, transferred into a Schott bottle and stored at 4°C.

*DMEM + 10, 5 and 1% FCS contained 40ml, 20ml and 4ml of FCS respectively, all other constituents remained the same.

Hanks buffer solution

Gentamycin	2.5µl
HEPES	200µl
Sodium Bicarbonate (7.5%)	47µl
Hanks solution (x1)*	100ml

Using a 60ml syringe and a disposable 0.2µM syringe filter, 60ml of the Hanks solution was filtered and the remaining 40ml of Hanks, gentamycin, HEPES and sodium bicarbonate were then added as outlined above. The 1xHanks and additives were stored in an autoclaved bottle at 4°C for up to 4 days.

*Hanks solution may exist as a 1x solution or a 10x solution. If using the 10x solution, Hank's buffer was diluted 1:10 with dH_2O before beginning the procedure.

Incubation Medium (Collagen Assay)

DMEM with additives*	80ml
50% dialyzed FCS	20ml

100mM Ascorbic acid	250ml

Media was prepared and filtered in a 60ml sterile syringe with a 0.2µM filter.
*Additives = pen/strep, glutamine and HEPES

Labelling Isotope (Collagen Assay)

[2,3-^{3}H Proline]	250µl
DMEM	245µl
0.5M Proline	5µl

These components were filtered through a 0.2µM filter.

Serum-Free Media (OptiMEM®)

A sachet of OptiMEM powder was dissolved in 900ml sterile water at room temperature and 2.4g of sodium bicarbonate was added. The pH was then adjusted to 7.3 with 1M sodium hydroxide or 1M HCl and make up the final volume to 1L with sterile water. The required volume was filtered and penicillin 5000U/ml / streptomycin 5000µg/ml was added. The solution was then stored at 4°C.

Apx. 2.3 Real time PCR

TE buffer

10mM Tris	0.1ml
1mM EDTA	20ml

Adjust pH to 8

Apx. 2.4 Northern blotting

Agarose-formaldehyde Gel (1%)

NA agarose	0.5g
MOPS (x10)	5ml
Formaldehyde (40%w/v)	8.93g
dH_2O	36.07ml

The agarose, MOPS and water were heated until the agarose had melted. The solution was cooled to approximately 50°C and the formaldehyde was added.

Denhardt's Solution

PVP	10g
BSA	10g
Ficoll 100	10g
dH_2O	230ml

The solution was passed through a sterile 0.2µm filter, aliquoted into 10-20ml batches and stored at -20ºC.

50% Dextran Sulphate

30g dextran sulphate was added to 60ml dH_2O in a sterile bottle, stirred and heated until it dissolved, and stored at 4°C.

Formamide (deionised)

Formamide	50ml
Mixed bed resin	5g

Components were stirred at room temperature until resin was exhausted, filtered through Whatman No. 1 filter paper and stored at 4°C.

Hybridisation Buffer

10x salts	50µl
Formamide (deionised)	250µl
Dextran sulphate (50%, heated to 80°C)	100µl
tRNA	18µl
Probe in dH_2O	82µl

10x MOPS, pH 7.0

MOPs powder	41.86g
EDTA (Na2)	3.7g
Sodium acetate (3M, pH 5.5)	16.66ml
NaOH pellets	5.0g
dH_2O	932.78ml

pH was be adjusted to 7.0 and the solution was autoclaved.

Pre-Hybridisation Solution

Deionised formamide	26ml
20xSSPE, pH 7-7.5	12.5ml
Denhardt's solution	2.5ml
50% Dextran sulphate	10ml
20% SDS	2.5ml

The solution was stored at 4^oC for up to 2 weeks.

RNA Gel Buffer

MOPS (10x)	25ml
Formaldehyde (40%w/v)	45ml

dH_2O was added to 250ml

RNA Sample Load Buffer

Formamide	0.72ml
MOPS (10x)	0.16ml
Formaldehyde (40%w/v)	0.26ml
dH_2O	0.18ml
Glycerol (80%)	0.1ml
Bromophenol blue	0.08ml

The solution was aliquoted and stored at -20°C

2 x SDS

4 x Tris-Cl pH 6.8	25ml
Glycerol	20ml
SDS	4g
BME	2ml
Bromophenol blue	1mg
dH_20	53ml

This was then aliquoted and stored at -20ºC.

20x SSC, pH 7.0-7.5

3M Sodium chloride	350.6g
Sodium citrate	176.49g
dH_2O	1472.91ml

pH was adjusted to 7.0-7.5 and the solution was then autoclaved.

2xSSPE, 0.1%SDS

*20xSSPE	10ml
SDS	1ml

Made up to 100ml with dH_2O.

*For 5xSSPE and 10xSSPE respective amounts of 25 and 50ml of SSPE were added.

20×SSPE buffer

Sodium chloride	87.65g
Sodium dihydrogen orthophosphate (NaH2PO4)	13.8g
EDTA	3.7g
dH_2O	394.85ml

pH was adjusted to 7.4 and the solution was autoclaved.

TEN (tris-EDTA-NaCl-) buffer

Tris	3.03g
EDTA	0.29g
NaCl	8.77g

Components were dissolved in dH_2O and the solution adjusted to a pH of 7.6 using HCl. The buffer was then made up to a volume of 1L with dH2O.

Tris Buffer (10x)

Sodium chloride	87.7g
Tris	60.6g
dH_2O	900ml

Salts were dissolved in distilled water and pH adjusted to 7.4. The final volume was then adjusted to 1L using dH_2O. The solution was diluted 1:10 in dH_2O before use. Stored at 4ºC

Apx. 2.5 Western blotting

10% Ammonium persulphate

1g of ammonium persulphate was dissolved in 10ml dH_2O and aliquots were stored at -20°C.

Coomassie Blue

Coomassie Blue R250	2g
Methanol	500ml
Glacial acetic acid	100ml

Made up to 1L with dH_2O and filtered before use.

Destain

Destainer was made up at a ratio of 50% Methanol:10% Acetic Acid.

1xPBS-Tween

10xPBS	100ml
dH_2O	900ml
Tween-20	1ml

The solution was stored at 4°C.

Polyacrylamide Gel (7.5%)

Solution N (acrylamide)	2.5ml
dH_2O	4.85ml
Solution L pH 8.8	2.6ml
Ammonium persulphate (10%)	50µl
TEMED	5.0µl

Polyacrylamide Gel (12.5%)

Solution N (Acrylamide)	4.67ml
dH_2O	3.33ml
Solution L pH 8.8	2.5ml
Ammonium persulphate (10%)	20µl
TEMED	8.3µl

S20 Load Buffer

Sucrose	20g
Solution M pH6.8	10ml
Urea (4.8M)	41.66ml
SDS (10%w/v)	1ml
Bromophenol blue	0.1g

The solution was aliquoted and stored at -20°C

5% skim milk / PBS-Tween

Skim milk	10g
1xPBS-Tween	200ml

The solution was mixed until dissolved and stored at 4ºC.

Solution L (Lower gel buffer)

Tris base	18.16g
SDS	0.4g
dH_2O	100ml

pH was adjusted to 6.8 with concentrated HCl and stored at 4°C.

Solution M (Upper gel buffer)

Tris-Cl	6.06g
SDS powder	0.4g
dH_2O	100ml

pH was adjusted to 6.8 with concentrated HCl and the solution stored at 4ºC

Solution N (Acrylamide)

Acrylamide	29.2g
Bisacrylamide	0.8g
dH_2O	100ml

Stored at 4°C.

Stacking gel (4.5%)

Solution N (acrylamide)	1.5ml
dH_2O	6.0ml
Solution M	2.5ml
Ammonium persulphate (10%)	30µl
TEMED	15µl

Transfer buffer

Tris base	3.03g
Glycine	14.4g
Methanol	200ml
dH_2O	800ml

Contents were stirred until dissolved and the pH adjusted to between 8.3-8.6, and stored at 4ºC.

Apx. 2.6 Hydroxyproline incorporation

100mM Ascorbic Acid

1.761g of ascorbic acid was dissolved in 100ml dH_2O and stored at 4°C.

10% BSA

Dissolve 1g BSA in 10ml dH_2O.
Stored at 4°C.

1M $CaCl_2$

147.02g $CaCl_2$ in 1L dH_2O
(25mM = 3.675g in 1L dH_2O)
Stored at 4°C

Collagenase

Made up as 1mg/ml in dH_2O (1054 U/mg)
Stored at -20°C.

Collagenase Negative Solution

25mM $CaCl_2$	10µl
62.5mM N-ethylmalemide	20µl
0.05M Tris 5M $CaCl_2$ pH 7.6	10µl

A master mix was made up and 40µl was pipetted into each test sample from this mix.

Collagenase Positive Solution

25mM $CaCl_2$	10µl
62.5mM N-ethylmalemide	20µl

Collagenase (Worthington)	10µl

A master mix was made up and 40 µl was pipetted into each test sample from this mix.

Apx. 2.7 *In situ* hybridisation

Salts (10x)

3M Sodium Chloride	17.35g
0.1M Disodium hydrogen orthophosphate (NaH2PO4)*	1.56g
0.1M Tris-HCl (pH 7.5)	10ml
0.05M EDTA (pH8.0)	10ml

*1.56g Disodium hydrogen orthophosphate was dissolved in 100ml dH_2O (pH6.8) and autoclaved. 0.2g each of bovine serum albumin, Ficoll and PVP was then added (final concentration was 0.2%). The solution was filtered using a 0.2µm filter before use.

Apx. 2.8 ELISAs

Apx. 2.8.1 MTT

Sorensen's glycine buffer

0.1M Glycine
0.1M NaCl
pH 10.5
Stored at 4ºC

MTT

0.0015gm MTT was diluted in 3ml PBS.

Apx. 2.8.2 S6RP ELISA (S-235/236)

1mM PMSF

1ml 20mM PMSF was diluted into 20ml dH_2O and mix until dissolved. Stored at 4°C.

APPENDIX 3

PRIMARY AND SECONDARY ANTISERA

Primary Antigen	Supplier	CLone	Raised	Specificity	Dilution
*Vimentin	DAKO	V9	Mouse	Mesenchymal cells	1:50
*#αSMA	DAKO	1A4	Mouse	Smooth muscle cells, myofibroblasts, mesangial cells	1:50
*Anti-αSMA Cy3 conjugate	Sigma	1A4	Mouse	αSMA isoform (see αSMA)	1:50
*EPOS Anti-human αSMA	DAKO	1A4	Mouse	αSMA isoform (see αSMA)	-
*Desmin	DAKO	D33	Mouse	Myofibroblasts, smooth muscle cells, glomerular podocytes	1:50
*Collagen III	Southern Biotech, Birmingham, Alabama, USA	-	Goat	Collagen III	1:300
*Cytokeratin	DAKO	LP34	Mouse	Epithelial cells	1:10/1:25
*S100-A4 (FSP1)	DAKO	-	Mouse	Various	1:50
*RECA	Serotec, Oxford, UK	HIS 52	Mouse	Endothelial cells	1:100
*E-Cadherin	BD Biosciences Pharmingen, San Jose, CA, USA	36	Mouse	Epithelial cells	1:100
#Fibrin / fibrinogen	DAKO	-	Rabbit	Fibrinogen, fibrinogen fragments D, E.	1:400
#PAR-1	Santa Cruz Biotechnology, CA, USA	-	Mouse	PAR-1	1:100
#*Factor V	Nordic Immunological s, Netherlands	-	Rabbit	Activated and degraded Factor V	1:400
*PCNA	DAKO	PC10	Rat	Linear epitope in PCNA, maximal during S phase	1:50
*βigH3	Gift of Professor P.Gibson, University of Adelaide	-	Rabbit	β-inducible gene-H3	1:100

Table Apx. 3.1: Antibodies used for cytochemistry (*) and immunohistochemistry (#)

Antisera	Supplier	Raised	Dilution
Anti-mouse FITC	DAKO	Mouse	1:50
Alk phos-anti mouse IgG	Sigma	Goat	-
Alk phos anti-alk phos	DAKO	Mouse	1:50
Biotinylated anti-mouse IgG	DAKO	Mouse	-
Biotinylated anti-rabbit IgG	DAKO	Rabbit	-

Table Apx. 3.2: Secondary antibodies

Antibody/conjugate	Supplier	Raised	Specificity	Dilution
αSMA	DAKO	Mouse	Smooth muscle, myofibroblast and mesangial cell protein	1:1000
Total Akt	Cell Signaling Technology	Rabbit	Akt 1, Akt 2 and Akt 3	1:1000
p-Akt (S-473)	Cell Signaling Technology	Rabbit	S-473 residue of Akt	1:500
β–actin	Sigma	Mouse	β–actin	1:2000
HRP anti-rabbit IgG	DAKO	Rabbit	Rabbit immunoglobulins	1:2000
HRP anti-mouse IgG	DAKO	Mouse	Mouse immunoglobulins	1:2000

Table Apx. 3.3: Western blotting antisera

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