Regulation of Transforming Growth Factor Beta-1 Signalling in the Renal Proximal Tubular Epithelial Cells

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Dedication

For Nan-Nan

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Summary

Transforming growth factor beta 1 (TGF-β1) is an important mediator of progressive renal tubulointerstitial fibrosis, and therefore the TGF-β1 signalling pathway plays an important role in progressive renal disease. Renal inflammation is also thought to be an important determinant of progression, involving infiltration of the tubulo-interstitum by microphages, and consequent release of cytokines including interleukin-1 beta (IL-1β). Bone Morphogenetic Protein-7 (BMP-7) is a powerful inhibitor of TGF-β dependent fibrosis in animal models of renal disease and has attracted substantial interest as a potential therapeutic target. The aim of my thesis is to examine the regulation of TGF-β1 signalling pathway by IL-1β and BMP-7 in proximal tubular epithelial cells (PTC) and to investigate the mechanisms underlying them.

The data presented show that IL-1β has a biphasic effect on PTC TGF-β1 signalling, with early NF-κB-mediated inhibition and delayed sensitization via an autocrine IL-6 loop, and possibly also via a switch from NF Kappa B p65/p50 heterodimer to p50/p50 homodimer formation. Secondly, the data indicate that BMP-7 prevents TGF-β1-mediated loss of the transcriptional repressor SnoN and hence specifically limits Smad3 DNA binding, altering the balance of transcriptional responses to TGF-β1 in PTC.

Publications and Presentations arising from this thesis

Publications

1. Bone morphogenetic protein-7 inhibits proximal tubular epithelial cell Smad3 signalling via increased SnoN expression. Luo DD, Phillips A, Fraser D.Am J Pathol. 2010 Mar; 176(3):1139-47.

2. Interleukin-1 beta regulates proximal tubular cell transforming growth factor beta-1 signalling. Luo DD, Fielding C, Phillips A, Fraser D. Nephrol Dial Transplant. 2009 Sep; 24(9):2655-65.

Presentations

1. Dong-Dong Luo, Aled Phillips, and Donald Fraser: Modulation of TGF beta signalling by BMP-7 in Proximal Tubular Epithelial Cells. American Society of Nephrology Annual Meeting, 2009, Oral.

2. Dong-Dong Luo, Aled Phillips, and Donald Fraser: Modulation of TGF beta signalling by BMP-7 in Proximal Tubular Epithelial Cells. UK Renal Association, 2008, Oral. The best abstract winner.

3. Dong-Dong Luo, Aled Phillips, and Donald Fraser: BMP-7 Opposes TGF Beta Signalling Via Altered DNA Binding and Id Expression. MR2 and IRG Annual Meeting, 2008, Oral.

4. Dong-Dong Luo, Aled Phillips, and Donald Fraser: Modulation of TGF beta signalling by BMP-7 in Proximal Tubular Epithelial Cells. CITER, 2008, Oral.

5. Dong-Dong Luo, Aled Phillips, and Donald Fraser: Modulation of TGF beta signalling by BMP-7 in Proximal Tubular Epithelial Cells. European Renal Cell Study Group 20th Annual Meeting, 2008, Oral.

6. Dong-Dong Luo, Aled Phillips, and Donald Fraser: IL-1β Sensitises Proximal Tubular Epithelial Cells to TGF beta. The 21st Annual Postgraduate Research Day, Cardiff University, 2006, Poster.

Abbreviations

BMPs	Bone morphogenetic proteins
BMP-7	Bone morphogenic protein-7
BSA	Bovine serum albumin
CBP	CREB binding protein
ChIP	Chromatin immunoprecipitation
CKD	Chronic kidney disease
Co-Smad	Common-mediator Smad
C-terminal	Carboxyl-terminal
CTGF	Connective tissue growth factor
E3	Ligase enzyme
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EEA1	Early endosome antigen-1
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
EMSA	Electrophoretic mobility shift assay
EndMT	Endothelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
ESRD	End stage renal disease
ET-1	Endothelin-1
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FSP-1	Fibroblast-specific protein-1
GFR	Glomerular filtration rate

GSK-3	Glycogen synthase kinase-3
HA	Hyaluronic acid (Hyaluronan)
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HECT	Homologous to the E6–AP Carboxyl Terminus
HGF	Hepatocyte growth factor
Hyal	Hyaluronidases
ID	Inhibitor of DNA binding/differentiation
IFN-γ	Interferon gamma
IKK	IκB kinase
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
I-Smad	Inhibitory Smad
JNK	c-Jun N-terminal kinase
LAP	Latency-associated protein
LB	Luria-Bertani
LTBP	Latent TGF-β binding protein
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic peptide-1
MET	Mesenchymal to epithelial transition
MH domain	Mad-homology domain
N-terminal	Amino-terminal
NF-κB	Nuclear Factor-kappaB
PAI-1	Plasminogen activator inhibitor-1
PI3K	Phosphatidylinositol 3-kinase

PTCs	Proximal tubular cells
RING	Really Interesting New Gene
QPCR	Quantitative polymerase chain reaction
R-Smad	Receptor-activated Smad
SARA	Smad anchor for receptor activation
SBE	Smad-binding element
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
Ski	Sloan-Kettering Institute proto-oncogene
Smurf	Smad-ubiquitination related factors
SnoN	Ski-related novel gene, non Alu-containing
Sp1	Signal protein-1
SUMO	Small ubiquitin-related modifier
TBM	Tubular basement membrane
TGIF	TG-interacting factor
TβRI	TGF-β receptor type I
TGIF	TG-interacting factor
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
TSP-1	Thrombospondin-1
UPP	Ubiquitin–proteasome pathway
UUO	Unilateral ureteral obstruction

Chapter One:

Introduction

1.1 *End stage renal disease and Chronic Kidney Disease*

End stage renal disease (ESRD), or stage V of chronic kidney disease (CKD) is defined as GFR (Glomerular Filtration Rate) less than 15 mL/min/1.73 m^2, according to the classification system established by the National Kidney Foundation Kidney Disease Outcomes Quality Initiatives. This is taken to be the point at which an irreversible deterioration of renal function has occurred, and where renal replacement therapy is typically required. The majority of patients with ESRD present initially with CKD, whereas acute renal failure with no recovery is a relatively uncommon cause of ESRD. Beyond a certain degree of CKD, progression to ESRD is likely therefore predictable but not avoidable [1, 2].

1.2 *Tubulointerstitial fibrosis and ESRD*

1.2.1 Tubulointerstitial components of normal kidney

Macroscopically, the kidney comprises two layers: an external layer, the cortex, displaying an intense and red coloration; and an inner pale layer, the medulla. The components of the kidney are nephrons, the collecting duct system, a unique vasculature, and the interstitium.

The nephrons are renal functional units, consisting of the glomerulus and renal tubules. Regarding physiological function of the nephron, the glomerulus is responsible for the production of an ultrafiltrate of plasma. The renal tubules play a vital role in regulation of fluid, electrolyte and acid-base homeostasis. The renal

interstitium is composed of cells, such as fibroblasts, perivascular cells, and other non-resident cells, and extracellular components including fibrillar structures, and ground substance such as proteoglycans, glycoproteins and interstitial fluid [3]. The extracellular components of the interstitium comprise a matrix, containing type I and III collagens in addition to fibronectin, non-collagenous glycoproteins and proteoglycans [4]. There are very few resident cells in the interstitium of normal kidney. Among them, interstitial fibroblasts constitute the major cell type and are assumed to be the major source of matrix production in the normal kidney [5].

Due to the close relationship between tubules and interstitium, they are often regarded as one term 'tubulointerstitium', or 'tubulointerstitial fibrosis'. The tubulointerstitium comprises the tubules, vascular structures, and interstitium, together occupying more than 90% of the kidney volume [6].

1.2.2 Tubulointerstitial fibrosis

1.2.2.1 The importance of tubulointerstitial fibrosis in progressive renal disease

Progressive renal failure results from the loss of nephrons and is associated with glomerulosclerosis, tubulointerstitial fibrosis and vascular sclerosis pathologically. Risdon *et al.* and Schainuck *et al* showed that in patients with glomerulonephritis, the decline of renal function such as GFR correlated well with structural abnormalities of the tubules and interstitium, but less with histologic glomerular injury [7, 8]. Studies by Bohle *et al* also revealed that tubulointerstitial fibrosis grading was more closely correlated with functional impairment than glomerular injury in a wide range of renal diseases including chronic sclerosing interstitial

nephritis, mesangioproliferative glomerulonephritis (GN), membranoproliferative GN, diabetic glomerulosclerosis and glomerular amyloidosis using renal biopsies [9-12]. Furthermore, in animal models, work has demonstrated that interstitial leucocyte accumulation has an important role in improving renal function [13]. In addition, cellular immunity has been proposed as a principal effector of interstitial injury leading to renal failure [14].

1.2.2.2 The histological changes of interstitium in tubulointerstitial fibrosis

The normal biological function of the renal interstitium depends on an integrated network of cellular and extracellular matrix (ECM) interactions [15]. Any alteration in the elements and structure of the resident cells and ECM is likely to have important effects for the function of the kidney [16].

Under physiological conditions, only a few renal fibroblasts can be found in the interstitium. During renal fibrogenesis, fibroblast accumalation occurs in the tubulointerstitium. These pathologic, activated fibroblasts directly mediate fibrosis by leading to excessive deposition of ECM, and also by secretion of many pro-fibrotic factors such as transforming growth factor (TGF-β), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) [17].

There are several potential origins for the fibroblasts that drive tubulointerstitial fibrosis, including local proliferation of resident fibroblasts, migration from the perivascular region, and recruitment of bone marrow derived precursors (reviewed by Hewitson) [18, 19]. Fibroblasts can also originate from acquisition of a fibroblast phenotype by other highly differentiated cells. This can apply to epithelial and

endothelial cells, through the processes of epithelial-mesenchymal transition (EMT) [20, 21] and endothelial-mesenchymal transition (EndMT) [22] respectively. Data from animal models and clinical biopsy samples suggests an important role for proximal tubular cells, from which a significant proportion of tubulointerstial fibroblasts may develop via EMT [23-25]. The compelling study by Iwano *et al* has indicated in mice with genetically tagged proximal tubular cells and bone marrow chimeras during EMT in fibrosis, that 36% of renal fibroblasts originated from the epithelium whereas 15% came from the bone marrow [20].

1.2.2.3 The role of proximal tubular epithelial cell in tubulointerstitial fibrosis

Renal proximal tubular cells (PTC) represent the most abundant cell type in the cortex. *In vivo* the proximal tubule is formed as an intact single layer of polarized columnar epithelial cells integrating with tight junctions and adherens junctions. The apical side of PTC faces the tubular lumen with a well developed brush border, which results in an increase of the luminal surface area of the cell. The basal side of the PTC contacts the tubular basement membrane separating PTC from the interstitium. This polarised apical and basolateral surface membrane structure of PTC is important to maintain their unique function for electrolyte, acid-base or solute balance. Also, the PTC reabsorbs macromolecules such as polypeptides and proteins that have passed through the glomerular filter with a prominent lysosomal system. Furthermore, PTCs is required for tubular basement membrane (TBM) formation by the secretion of type IV collagen and laminin [3].

It has become apparent that tubular epithelial cells actively participate in the interstitial fibrosis in both immunologically and non-immunologically-mediated

renal diseases [26-28]. PTC can act on T-cell activation and produce a variety of chemokines, cytokines and growth factors such as TGF-β1, endothelin-1(ET-1), tumour necrosis factor α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin 8 (IL-8) and interleukin 6 (IL-6), thus contributing to interstitial inflammatory and reparative events [29-37]. Also, in response to the above secreted profibrotic cytokines, they can produce matrix proteins, leading to accumulation of ECM.

In addition to being a source of profibrotic cytokines, PTCs may directly mediate fibrosis by acquiring a myofibroblast phenotype during renal injury, a process called EMT. EMT has been indicated to be particularly significant in the pathogenesis of tubulointerstitial fibrosis that accompanies all progressive kidney disease [18]. During EMT, tubular epithelial cells acquire mesenchymal gene expression and a migratory phenotype, accumulate excess ECM and secret of pro-fibrotic factors. In renal tubular cells, fibroblast-specific protein 1 (FSP1, also called S1004A), a member of the S100 family of calcium-binding proteins exclusively expressed in fibroblasts, has been detected in numerous independent studies, in the process of EMT in different animal models of chronic renal disease and also in human kidney biopsies [21, 38-41]. Blocking EMT such as by BMP-7 attenuates renal fibrosis [42, 43]. Tubular EMT is proposed as a highly regulated process involving four crucial events. These include: (1) loss of epithelial cell adhesion, (2) *de novo* α-smooth muscle actin expression and remodelling, (3) disruption of tubular basement membrane, and (4) enhanced cell migration and invasion [43].

A series of reports have shown TGF-β1 is a vital pro-fibrotic factor in renal fibrosis, and is the principal stimulus of related processes including EMT [40]. Recent studies by Zeisberg *et al* show that inhibiting TGF-β1 function can reverse EMT [44].

1.3 Transforming growth factor-β1 (TGF-β1)

1.3.1 Superfamily and isoforms

TGF-β1 belongs to a group of polypeptide growth/differentiation factors called the TGF-β superfamily, which comprises five distinct TGF-β isoforms, inhibins/activins, bone morphogenetic proteins (BMPs), and other structurally related polypeptide growth factors in vertebrates and invertebrates. These cytokines play very vital roles during organ development, as well as in physiological and pathophysiological processes, by regulating a broad range of cellular processes, such as cell growth, differentiation, migration, apoptosis, and extracellular matrix production in a cell and context specific manner [2, 45, 46]. They can be expressed by most cell types and are abundant both in circulating forms and bound to the extracellular matrix [47], exerting a wide range of effects in a context-dependent autocrine, paracrine, or endocrine fashion [2].

In mammals, the TGF-β branch exists in 3 highly homologous isoforms [48], TGF-β 1, 2 and 3 [49] arising from different genes and chromosomes, 19q13, 1q41, and 14q24, respectively [50]. The three TGF-β isoforms in mammalian have a molecular weight of approximately 25,000 and share a high degree of sequence homology (70–80%) in the carboxy-terminal mature region across species [2]. The deletion of the

three distinct TGF-βs isoforms in mice provides evidence that these TGF-β isoforms play entirely different roles in embryonic growth and development. Knock-out of TGF-β1 gene does not display any gross embryonic abnormality, but the newborn mice exhibit lethal multifocal inflammation, starting three weeks after birth [51]. Therefore, TGF-β1 appears as an essential regulator of immune system. The TGF-β3 null mice born with cleft palate die within a few hours due to a concomitant lack of pulmonary development, indicating the responsibility of TGF-β3 for normal embryonic growth [52]. Also, TGF-β2 knockout mice represent perinatal mortality and a wide range of organ defects including the heart, lung, , kidney, eye, and inner ear [53, 54].

1.3.2 Structure and synthesis of TGF-β1

Of the TGF-βs isoforms, the effects of TGF-β1 on tissue homeostasis and response to injury have been the most fully characterized [55]. *In vitro* the three isoforms have similar functions, but *in vivo* TGF-β1 is most strongly implicated in renal fibrosis [56].

The TGF-β1 gene encodes a 390-amino acid precursor molecule that contains a signal peptide, the active TGF-β1 molecule, and a latency-associated peptide (LAP) [57]. After removal of the signal peptide, the TGF-β1 gene product is proteolytically cleaved within the endoplasmic reticulum to form mature TGF-β1 and the LAP [58]. Before secretion, TGF-β1 noncovalently associates with the LAP to produce an inactive latent TGF-β1 complex [59, 60]. TGF-β1 is activated when released from

LAP, therefore conditions of extremes pH, heat and radiation, proteases such as plasmin and cathepsin D, detergents, and thrombospondin all result in TGF-β1 activation [2, 56, 61-63]. Once activated, TGF-β1 is capable of binding its cell surface receptors, thereby initiating intracellular signalling pathway activation.

1.3.3 Biological functions of TGF-β1

Abundant studies have shown that all TGF-β isoforms including TGF-β1 are potent endogenous mediators of tissue repair via stimulating chemotaxis, angiogenesis, and ECM deposition following wounding [2]. It is also known to suppress immunological performance by inhibiting B- and T-cell activities via other cytokines directly or indirectly. The disorder of TGF-β1 expression or activity has been implicated in a wide ranges of disease including autoimmune disorders, fibrotic disease and chronic inflammation, neurodegenerative disease, and carcinogenesis [64, 65].

Pathologic autoinduction of TGF-β1 may be characteristically involved in the progress of fibrosis in the kidney and other organs, such as lung, liver and related systemic sclerosis [55, 56]. Also, TGF-β1 is regulated by YB-1 and itself [66, 67]. TGF-β1 exerts a biphasic role during inflammation. At the early stage it promotes the inflammatory process. Serving as a powerful chemoattractant for leukocytes, TGF-β1 assembles monocytes with enhanced mRNA levels for various cytokines. At the late stage, however, TGF-β1 has immunosuppressive effects. It decreases T cell mediated immune response [68], cytokines production by macrophages [69], and the severity of inflammation [70]. In cancer, it has been considered that TGF-β1 acts

as both a tumour suppressor and tumour promoter [71]. Overall, these pleiotropic and sometimes apparently contradictory actions of TGF-β1 are regulated in a cell-type and –context dependent fashion [72].

1.3.4 The role of TGF-β1 in fibrotic renal disease

There are several lines of evidence supporting the role of TGF-β1 as a key regulator of fibrosis in kidney disease. The first milestone observation of the correlation between TGF-β1 and renal disease was reported by Border et al., who discovered a causal role of TGF-β1 in ECM accumulation in an experimental rat model of acute mesangial proliferative glomerulonephritis [73]. *In vivo* and *in vitro*, many studies have shown that increased expression or increased activity of TGF-β1 is seen in various chronic kidney diseases. In addition, elevated plasma and urinary TGF-β1 protein was detected in patients with renal fibrotic diseases including chronic allograft nephropathy, focal segmental glomerulosclerosis, and diabetic nephropathy [74-76]. In cultured rat mesangial cells, renal fibroblasts, and tubular epithelial cells, all three TGF-β isoforms have fibrogenic effects, notably TGF-β2 and TGF-β3 effects may be partially mediated by TGF-β1 [77]. In cultured kidney tubular epithelial cells, TGF-β1 can induce IL-8, MCP-1, and fibronectin production [78].

On the other hand, several therapeutic strategies have been applied to inhibit profibrotic TGF-β1 activity in experimental models of renal fibrosis. Treatment with TGF-β1 antibodies and with TGF-β1 antisense oligodeoxynucleotides has been associated with reduced histological evidence of glomerular injury and reduced ECM deposition in various animal models of renal disease (reviewed by Gagliardini E)

[79].

In normal cells, TGF-β1 has various cellular functions such as blocking the cell cycle at the G1 stage to inhibit proliferation as a tumor suppressor, inducing differentiation, and boosting apoptosis. It is also an immune suppressor, and a promoter of extracellular-matrix components, which are essential for wound healing and tissue repair progress [80]. Overproduction or overactivity of TGF-β1 can result in excessive ECM deposition leading to scar tissue and fibrosis [56]. But in cancer cells, TGF-β1 causes immunosuppression and angiogenesis thus increasing the invasiveness of the tumor [65].

But, TGF-β1 blockade has not yet translated into an effective and safe therapeutic approach in clinical patients [81]. This probably stems from the multifunctional biological activities of TGF-β1, such as anti-proliferation, differentiation, anti-tumorigenesis, anti-migration and apoptosis. These additional actions of TGF-β1 mean that complete blockade is hazardous, and that understanding the fundamental signalling events underlying TGF-β1-dependant process is important. It is also notable that, in the recovery phase of Acute Renal Failure (ARF), the expression of TGF-β1 is enhanced [82]. This indicates that increased expression of TGF-β1 alone is not the whole cause of progressive CKD, and suggests that the modulation of TGF-β1 signalling may be a key step in progressive CKD.

1.4 TGF-β signalling pathway

As with other signalling systems, key steps of TGF-β signalling include TGF-β ligand binding to the cell surface receptors, intracellular transduction of signal from the activated receptor to the nucleus, integration with other signalling pathways, and together with other co-factors, changes in target gene expression. Recent developments in these aspects of TGF-β signalling are now summarized below.

1.4.1 Smad signalling pathway

1.4.1.1 TGF-β1 receptors

TGF-β signalling is initiated through the formation of a heteromeric transmembrane receptor complex consisting of activated ligand, two TGF-β type I and two type II receptors. Type I and II receptors are glycoproteins of approximately 55 kDa and 70 kDa, respectively, with core polypeptides of 500 to 570 amino acids including the signal sequence [45]. After activated TGF-β binds to the type II receptor, the type II receptor recruits and phosphorylates the type I receptor within its GS domain because of a characteristic SGSGSG sequence it contains in its juxtamembrane region. Then the activated type I receptor phosphorylates cytoplasmic substrates (the receptor-regulated Smad proteins), which subsequently form R-Smad-Smad4 complexes that translocate to the nucleus, thereby regulating transcription of target genes with the assistance of other transcriptional factors.

As well as type I and II receptors, the original search for cell surface TGF-β binding proteins using ligand crosslinking methods also revealed type III receptors [83].Type III receptors were discovered to correspond to two related proteins, betaglycan and endoglin [84-86]. The evidence suggested that type III receptors do not have an

intrinsic signalling function but only regulate TGF-β access to the receptors. Their functions are varied in line with TGF-β isoforms, and cellular phenotype. There is no concrete evidence for type III receptors for other TGF-β family members [45].

Betaglycan is a membrane-anchored proteoglycan with an 853–amino acid core protein [84, 85, 87]. Betaglycan binds all three TGF-β forms with high affinity and facilitates TGF-β access to the type II receptor forming a betaglycan/TGF-β/TβR-II complex in the process [84, 85, 88, 89].

Endoglin, known as CD105, is a cell surface molecule mainly expressed in vascular endothelial cells, and at lower levels in vascular smooth muscle cells, leukemic cells of pre-B and myelomonocytic origin, fibroblasts, macrophages, and erythroid precursors [90, 91]. Endoglin binds TGF-β1 and -β3 in the presence of TGF-β type I and II receptors, but unlike betaglycan, it does not bind TGF-β2 [91, 92]. Furthermore, endoglin can bind to type I or II receptors even in the absence of ligand [93]. But endoglin alone may not bind TGF-β in endothelial and leukemic cells [92]. Some work show that endoglin counteracts TGF-β1-induced extracellular matrix genes (*i.e.* collagen, fibronectin), PAI-1, and connective tissue growth factor via mediating inhibition of TGF-β1 signalling [93-95].

1.4.1.2 Smads family and nuclear accumulation

Based on structural and functional features, Smads fall into three subfamilies: receptor-regulated Smads (R-Smads), a common pathway Smad (co-Smad), and inhibitory Smads (I-Smads). R-Smads include Smad2 and Smad3, which are recognized by TGF-β and activin receptors, and Smads 1, 5, and 8, the substrates of

BMP receptors. TGF-β-Smads are phosphorylated directly by TGF-β type I receptor. Smad4 is a common pathway Smad (also called cooperating Smad or co-Smad), which is not phosphorylated by the TGF-β type I receptor [45]. I-Smads include Smad6 and Smad7, which regulate TGF-β signalling in a negative feedback pathway.

TGF-β type I receptor phosphorylates the C-terminal sequence SSXS of R–Smads to free these two domains from a basal mutually inhibitory interaction, leading to R-Smad activation and combination with Smad4. Co-Smads and I-Smads lack the SSXS sequence and therefore can not be phosphorylated by the activated type I receptor.

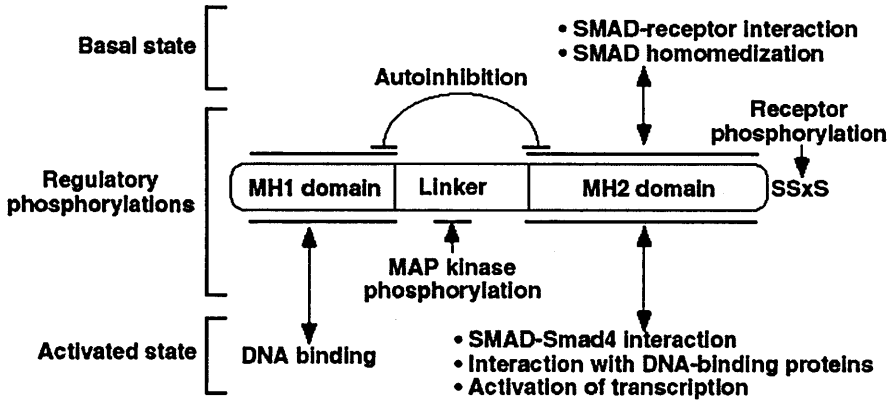


Fig 1 adapted from [45]

Smads contain an N-terminal mad homology 1 domain (MH1), and a C-terminal MH2 domain. MH1 domain has DNA-binding activity, whereas MH2 triggers Smads complex accumulation into the nucleus and regulates target genes transcription by combing with co-activators or co-repressors. The MH1 domain, is highly conserved among R-Smads and Co-Smads, regulating nuclear import and transcription by binding to target DNA promoter elements and interacting with nuclear transcriptional factors [94, 95]. The MH1 domain of the R-Smads and Smad4 also contains a nuclear localization signal (NLS) and in the R-Smads the

MH1 domain appears to inhibit the functional activity of the MH2 domain prior to receptor phosphorylation [96].The MH2 domain is highly conserved among all Smads. The MH2 domain regulates Smad oligomerisation and recognition by type I receptors and interacts with cytoplasmic adaptors and several transcription factors [94]. The linker region is highly variable in length and sequence. In R-Smads, the linker region contains Mitogen-activated protein kinase (MAPK) phosphorylation sites [45, 97]. MAPK activated phosphorylation of these sites inhibits nuclear translocation of Smads. Also, the linker region of I-Smads contains a proline-tyrosine (PY) motif, which specifically interacts with an E3 ubiquitin ligase implicated in the regulation of the TGF-β and BMP pathways [98-100]. The I-Smads are structurally related to the R-Smads and Smad4 in their MH2 domain, but have a more divergent N-terminus [94] (see Fig.1).

R-Smads and Smad4 are continuously shuttling between the cytoplasm and nucleus in the absence or presence of signal [101-103]. The Smad shuttling in the presence of TGF-β allows the Smads to continuously monitor membrane receptor activity. Particularly with respect to Smad2, additional proteins are required to facilitate the phosphorylation of R-Smads by the activated receptor complex. The MH2 domain of R-Smads interacts with a Smad-binding domain in Smad Anchor for Receptor Activation (SARA) [95], an accessory protein that forms a bridge between the type I receptor and R-Smads [97]. Upon phosphorylation R-Smads are released from SARA. In addition, evidence in both early vertebrate embryos and mammalian cells showed that the cellular microtubule network and the kinesin-1 motor are necessary for Smad2 phosphorylation, by assisting Smad2/receptor interaction [104]. Following receptor-mediated phosphorylation, R-Smads oligomerise with the co-

Smad, Smad4, and accumulate in the nucleus. Although Smad4 is not required for nuclear accumulation of R-Smad complexes, it is necessary for the formation of transcriptional R-Smad-DNA-binding complexes. The precise stoichiometry of R-Smad/co-Smad interaction has been a subject of debate, however, Isothermal titration calorimetry and mutational studies have revealed the crystal structure of heterotrimers recruited by two phosphorylated R-Smad subunits and one Smad4 via the MH2 domains of phospho-Smad2–Smad4 and phospho-Smad3–Smad4 complexes [105-107].

1.4.1.3 DNA-binding and transcriptional factor interaction

After R-Smad phosphorylation and association with Smad4, the complex translocates into the nucleus, participating in DNA binding via a β-hairpin structure protruding from the surface of the MH1 domain and recruitment of transcriptional cofactors to initiate the transcription of target genes, both positively and negatively.

Utilizing TGF-β-activated plasminogen activator inhibitor-1 (PAI-1) promoter, DNA-binding site selection experiments and mutation analysis identified the Smad3-Smad4 recognized DNA binding element. It contains a repeated AGAC sequence or its reverse complement GTCT in the opposite strand, [38, 108], and is known as a Smad-binding element (SBE). The most common splice form of Smad2 cannot bind DNA directly because of a small extra exon-3-encoded insert [46].

R-Smad-containing complexes have been shown to recruit the ATPase subunit of the SWI–SNF chromatin remodelling complex, Brg1 [102, 109], Fast1, 3 and also histone modifying enzymes such as the histone acetyltransferases p300 and CREB

(cAMP response element binding protein) binding protein (CBP) to participate in TGF-β-induced transcription responses [110, 111]. Smad-dependent chromatin remodeling and histone modifications are thought to facilitate the subsequent recruitment of the general transcription machinery to target gene promoters. Once within the nucleus, the R-Smads may associate with specific nuclear transcription coactivators such as activating protein-1 (Ap-1) family of transcription factors, P300/CBP [112-115], nuclear factor-kappaB (NF-kB) [116-118], runt-related transcription factor-2 (Runx2) [119, 120], signal protein-1 (Sp1) [121] and transcriptional corepressors such as Sloan-Kettering Institute proto-oncogene (Ski), TG-interacting factor (TGIF) and Ski-related novel gene (SnoN, non Alu-containing) by MH2 domain [122-129].

1.4.2 Crosstalk with Non-Smad Signalling Pathways

It has been firmly established that Smad pathways are central mediators of TGF-β signalling. However, there are several non-Smad signalling pathways that are involved in the TGF-β1 signal pathway including MAPK, phosphatidylinositol 3-kinase (PI3K), RhoA and Rac1 small GTPases. These non-Smad pathways are implicated as mediators of TGF-β-induced outcomes such as EMT, apoptosis, cell proliferation and differentiation [130-132]. There are three fashions of cross-talks contributing to responses to TGF-β: (1) non-Smad signalling pathways directly modify the Smads activity and thus regulate the TGF-β signalling pathway, such as MAP-kinase activated phosphorylation of Smads linker region; (2) Smads directly interact and modulate the other signalling mediators, thus transmitting Smads signals to other pathways. For instance, TGF-β1 can activate ERK (extracellular signal-

regulated kinase), c-Jun N-terminal kinase (JNK) and P38 MAPK kinase pathway; and (3) at the TGF-β receptors level, the non-Smad proteins directly can be activated and initiate parallel signalling collaborating with the Smad pathway[131]. An example for this is the link between TGF-β1 receptors and RhoGTPase to regulate EMT. TGF-β1 type II receptor phosphorylats type I receptor as well as type I receptor-tethered Par6, leading to the recruitment of Smad ubiquitination related factor 1 (Smurf1) and then final degradation of RhoA [133].

The MAPK is a major signalling system involved in TGF-β1 classical signalling pathway [134]. Three major subgroups of the MAPK superfamily members have been identified: the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2); JNK; and the p38 MAPK. Besides MAPK pathway, RhoGTPases, PI3K/Akt and protein kinase c (PKC) signalling pathway are also key participants in TGF-β1-Smad signalling pathway [131].

These non-Smad pathways are initiated by activated TGF-β1 receptors through either phosphorylation or direct interaction independently of Smads, then the activated non-Smad pathways interact with R-Smads such as phosphorylating linker region of R-Smads or recruiting other co-factors to take part in the Smads-regulated target genes ,such as matrix metalloproteinase-1 (MMP-1) and tissue inhibitors of MMP-1 (TIMP-1) [132].

1.5 Regulation of TGF-β1 signalling

1.5.1 Regulation of TGF-β1 activation and interaction with TGF-β receptors

The production of TGF-β1 in a latent form is likely to be an important determinant for the strength of TGF-β1 activity, ensuring that TGF-β1 activity is only generated in a specified situation. Latent TGF-β1 can be activated by denaturing and by heat, detergents, extremes of pH, and radiation [135, 136].

Thrombospondin-1 (TSP-1) may also serve to activate TGF-β1 in the kidney. TSP-1 is expressed in platelets, platelet alpha-granules, vascular smooth muscle cells and mesangial cells during inflammation and wound healing. It is a trimer of disulfide-linked 180 kDa subunits, consisting of several domains that bind to matrix and cell surface proteins [137-140]. In human renal disease and experimental renal disease models, there is increased TSP-1 expression in human focal sclerosis, vasculitis, membranous glomerulonephritis (GN), and diabetic GN as well as in several animal models of glomerulonephritis (such as anti-Thy1 model, aminonucleoside nephrosis, passive Heymann nephritis) [141, 142]. With specific strategies to block TSP1 activity, such as synthetic peptides (GGWSHW or LSKL) or TSP-1 antisense oligonucleotide, the latent activation of TGF-β1 to bioactive growth factor was inhibited, especially in glomerular mesangial cells exposed to high glucose *in vitro* or *in vivo* [139, 140, 143]. Hereby, TSP-1 might trigger latent TGF-β1 activation via binding to the LAP, as a non-protease mediated release of active TGF-β1.

Plasmin and the lysomal serine protease cathepsin D, both of which are proteases, can activate latent TGF-β1 in fibroblasts under cell-conditioned medium *in vitro*. This result suggested that activation of latent TGF-β1 may occur by proteolytic nicking within the LAP, thereby resulting in the release of active, mature TGF-β1 [63, 144]. In terms of another latent TGF-β binding protein (LTBP), evidence

showed that TGF-β1 activation can be achieved via proteolytic release of LTBP from ECM by cleavage at a specific protease-sensitive site in LTBP [145, 146] .

Activation of TGF-β1 may also be mediated by integrins. Induction of integrin alphavbeta6 led to activation of TGF-β1, whereas inhibition of integrin with its specific antagonist EMD527040 blocked TGF-β1 activation, suggesting integrin was required for this process [147, 148]. There are two possible models to explain how integrins is involved in the latent TGF-β1 activation. Firstly, in a protease-dependent mechanism, integrins are proposed to simultaneously bind the latent TGF-β1 complex together with proteinases and the adjacent proteinases improve enzymatic cleavage of the latent complex to release active TGF-β1. Secondly, Integrin binds LAP and changes the conformation of the latent TGF-β1 complex by transmitting cell traction forces with the ECM support of mechanically resistant ECM in a non-protease method [124, 149].

As discussed before, betaglycan can facilitate TGF-β1 binding to the type II receptor and promote TGF-β1 responses. Wang *et al* also found in L6 myoblasts lacking the endogenous type III receptor, the recombinant receptor increases ligand-receptor bound and integrates with type II TGF-β receptors. This indicates that the type III receptor may regulate the ligand-binding ability or surface expression of the type II receptor [85, 150]. Additionally, CTGF (connective tissue growth factor) can increase the crosslinking between TGF-β1 and its three receptors via its carboxy-terminal cystine knot (CT) domain *in vitro* to facilitate TGF-β signalling pathway [151] .

1.5.2 Regulation of TGF-β receptor turnover and degradation

Cell surface receptor internalization is a primary step to initiate signal transduction. In higher eukaryotic cells, internalization of cell surface proteins occurs through both clathrin-dependent and -independent pathways [152], Studies have demonstrated that TGF-β receptor internalization is through the classic clathrin-dependent pathway as well as the raft-caveolin non-clathrin pathway [153]. The clathrin-dependent internalisation into the EEA1 (early endosome antigen-1) positive vesicles, promotes TGF-β1-activated Smads signalling [154]. In contrast, the Smad7-Smurf2 – dependent receptor degradation is dependent on the caveolar pathway [153]. Thus, segregation of TGF-β receptors into the two distinct endocytic membrane pools regulates receptor turnover and subsequently Smad activation. In addition, the E3 ligase Smurf1 is also targeted to the activated TGF-β type I receptor by Smad7 and promotes receptor degradation [99, 155].

In our lab, previous work has shown that the interaction of hyaluronan (HA) with its receptor CD44 leads to the majority of TGF-β receptors trafficking to caveolin-1 lipid raft-associated membrane pools in PTC. In the presence of HA, concomitant with TGF-β receptor partitioning into the lipid raft compartment, there was an increase in the association of the TGF-β receptor with Smad7, and a decrease in the association of phosphorylated Smad proteins. This implies that HA-mediated downregulation of TGF-β signalling is related to increased TGF-β receptor trafficking to lipid raft compartment away from the endosomal signalling compartment [156].

1.5.3 I-Smads: Negative feedback regulators of TGF-β signalling

I-Smads negatively regulate the TGF-β signalling pathway in a feedback loop primarily. I-Smads (Smad6 and Smad7) bind directly to type I receptors and compete with R-Smads phosphorylation by the receptors and then interfere with their association with Smad4, resulting in inhibition of TGF-β signalling [72, 157-159]. I-Smads also bind to the Smurf family of E3 ligase as adaptors to recruit Smurfs to the receptor complex and thereby mediate receptor degradation and downregulation of TGF-β signalling [99, 100]. I-Smads inhibit transcriptional factors recruitment by the R-Smads [160] or disrupt the formation of the TGF-β-induced functional Smad-DNA complex in nucleus [161].

1.5.4 Regulations of Smads

1.5.4.1 Ubiquitin proteasome system related Smads degradation

The ubiquitin–proteasome pathway (UPP), a main path to degrade protein, regulates the stability of proteins. It consists of ubiquitin, ubiquitin ligases and 26S proteasomes. Degradation of target protein by UPP system involves two steps: target proteins are poly-ubiquitinated by ubiquitin ligases (ubiquitination), and 26S proteasomes subsequently recognize and degrade poly-ubiquitinated target proteins [162, 163]. Ubiquitin ligases which link ubiquitin to target proteins include three distinct classes of enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. E1 and E2 enzymes are not highly substrate specific, whereas E3 ubiquitin ligases play key roles in the recognition of

target proteins and subsequent degradation by 26S proteasomes specifically [164]. The E3s can be divided into two main functional classes, the Really Interesting New Gene (RING) domain E3s that function primarily as adapter proteins and the Homologous to the E6–AP Carboxyl Terminus (HECT) domain E3s that catalyse the transfer of the ubiquitin from the E2 to the substrate [165].

The degradation of R-Smads, including Smad2 and Smad3 by ubiquitin-proteasome pathways in a ligand-dependent manner results in TGF-β signalling termination [166]. Smad3 activated by TGF-β is degraded by ROC1-SCF complexes in the nucleus [167].

Smurf1 (Smad ubiquitination related factor) and Smurf2 are HECT type E3 ligases. In *in vitro* studies, it was shown that Smurf1/2 can interact with R-Smads including Smad1, -2, and -3, and degrade them [168, 169]. Similar to Smad2, once bound to and ubiquitinated by the E3 ligase complex, nuclear Smad3 is exported from the nucleus and proteasomally degraded in the cytoplasm. Moreover, recruitment of the transcriptional coactivator p300 to activated Smad3 facilitates the interaction with the E3 ligase complex, and triggers the process of Smad3 degradation [166]. In addition, Smurfs stimulate I-Smads to export from the nucleus to the cytoplasm after forming Smurfs-I-Smads complex. Then the I-Smads are recruited to TGF-β and BMP receptors as an adaptor, initiating the degradation of these receptors via UPP pathway [99, 100, 170]. Thus, Smurfs support inhibitory activities of I-Smads in TGF-β superfamily signalling pathways.

Arkadia was originally isolated through gene-trap insertion mutagenesis in mice [99].

Arkadia contains a RING finger domain in its C-terminal region, suggesting activity as an E3 ubiquitin ligase. Arkadia is expressed ubiquitously in embryonic and adult tissues [171, 172]. In normal renal tissue, Arkadia protein was expressed weakly in medullary renal tubular, abundantly in cortical renal tubular, glomeruli and renal interstitium. Arkadia only interacts strongly with I-Smads, and associates with Smad3 weakly, but not with other Smads (Smad1, Smad2, Smad4, Smad5, Smad8) [173]. Immunostaining of Arkadia in unilateral ureteral obstruction (UUO) kidneys in mice showed it is gradually enhanced, but that of Smad7 is decreased [173]. In contrast, Koinuma D et al have shown that Arkadia expression is transiently repressed but Smad7 induced by TGF-β in HaCaT cells and that it enhances TGF-β signalling by interacting with Smad7 and then inducing degradation of Smad7 [172]. During renal tubular EMT in HKC cells, Arkadia expression was increased by TGF-β treatment, and Arkadia can positively contribute to EMT through degradation of Smad7 [174]. Overall, Arkadia regulates the transcriptional activity of TGF-β through degradation of Smad7. In contrast to Smurfs, Arkadia is not recruited to TGF-β receptors [173].

1.5.4.2 R-Smads dephosphorylation at C-terminals

Clearly, regulation of R-Smad degradation is an important potential mechanism of regulation of TGF-β signalling. However, more recent findings suggest that the majority of activated R-Smads are not degraded but recycled [101] and that dephosphorylation is a prerequisite for the recycling of R-Smads [102]. It was demonstrated that the TGF-β-regulated R-Smads, Smad2 and Smad3, constantly shuttle between the nucleus and the cytoplasm both in basal context [175] and during TGF-β signalling. Smad nucleocytoplasmic shuttling was suggested as a mechanism

whereby the Smads monitored receptor activity [102]. Most importantly, cycles of Smad phosphorylation and dephosphorylation are required to maintain this nucleocytoplasmic Smad shuttling in the presence of a TGF-β signal. Thus phosphatases have been proposed to be key regulatory molecules in these important signal pathways. Xin-Hua Feng and colleagues [176] have identified PPM1A to dephosphorylate TGF-β-activated Smad2/3. Removal of C-terminal phosphates from R-Smads terminates TGF-β signalling without proteasome-mediated R-Smad degradation. This indicates another crucial regulation partner of R-Smads phosphorylation in TGF-β signalling pathway.

1.5.4.3 Acetylation of Smads

Acetylation is a lysine-specific modification that regulates most histones, but also non-histone proteins such as transcriptional regulators [177]. Smad7 was shown to be a substrate of acetylation by the histone acetyltransferase (HAT) activity of the co-activator p300, preventing Smad7 ubiquitination and degradation in the proteasome [178]. Also, decreased acetylation of Smad7 is correlated with upregulation of Smad7 ubiquitination and decrease of Smad7 stability [178]. In keeping with this, histone deacetylase (HDAC) can mediate Smad7 deacetylation thus leading to an acceleration in Smad7 degradation [179]. Therefore it appears that there is a balance between acetylation and HDAC-medicated deacetylation regulating Smad7 stability [178, 180].

The TGF-β-R-Smads have also been shown to be acetylated by p300 and CBP. Acetylation of Smad2 and Smad3 enhances transcriptional activity, however unlike Smad7, these modifications do not affect the stability of the R-Smads [111, 180,

181].

1.5.4.4 Sumoylation of Smad4

Small ubiquitin-like modifier (SUMO), an ubiquitin-related polypeptide, has also been shown to be important in altering target protein functions. Some studies demonstrated that Smad4 is modified by sumoylation localizing in its linker segment or MH1 domain [182, 183]. Sumoylation of Smad4 regulates its stability, represses Smad4 transcriptional activity, and increases its nuclear accumulation at basal level and in the presence of TGF-β [183, 184]. However, some tumour-associated mutations allow ubiquitination and/or decrease the stability of Smad4[185]. The Smad activation domain (SAD) in the linker region of Smad4 has been showed to play an essential role in Smad4 transcriptional activity [186]. In addition, SUMO modification of Smad4 may change the complex conformation, thus inhibiting the assembly of active transcriptional complexes or promoting the assembly of transcriptional repressor complexes [183].

1.5.5 Transcription modulation

In the nucleus, R-Smad-Co-Smad complexes are involved in transcriptional regulation of target genes, but Smads do not act as transcription factors alone. The complex of Smads with other appropriate transcription factors confers DNA binding specificity to the Smad-containing transcription complex [187]. The Smads transcriptional regulation on DNA-binding is mainly through the modulation of chromatin structure. There are two essential ways to modulate transcription via chromatin structure: either post-translationally modify histones or move the position

of nucleosomes on DNA. The Smads have been shown to interact with a series of co-activators and co-repressors to modify chromatin activities or a chromatin-remodelling complex.

1.5.5.1 HATs

Histone acetylation enables transcription factors and the transcription machinery to access DNA, and many transcription co-activators have histone-acetyltransferase activity. Co-activators, p300/CBP are identified as histone acetyltransferases that potentiate transcription by loosing the acetylation-dependent chromatin structure, and then enhancing the DNA-binding activity. There are several mechanisms to undergo the chromatin modulation structurally and functionally. They act as a bridging factor between transcription factors and the basal transcription machinery [188]. TGF-β1-mediated phosphorylation of Smad3 promotes the conformational assembly between Smad3 and p300, leading to transcriptional regulation [109, 189, 190].

In addition p300/CBP alone, several other factors modulate TGF-β-dependent gene expression related to p300/CBP. One of the ZEB family, ZEB-1/deltaEF1 binds to p300 and promotes the p300-Smad transcriptional complex formation [191, 192]. The nuclear factor MSG1 and the steroid receptor co-activator 1 (SRC1) both enhance TGF-β-regulated transcription in a p300/CBP dependent manner [193, 194]. Whereas transcriptional co-repressors such as Smad nuclear interacting protein 1 (SNIP1) and TGIF suppress TGF-β-regulated gene expression through interfering with the p300/CBP–Smad complex partly [125, 195]. In addition, Lithium and BCL6 disrupts Smad3/4-p300/CBP complex and suppresses transcriptional Smad3/4-dependent genes transcription [196, 197].

1.5.5.2 HDACs

HDACs remove acetylation groups from histones to prevent the transcription complex from binding to the promoter DNA-binding sites, thus leading to transcription inhibition. HDACs are recruited directly by Smads or by associated co-repressors [109].

Antagonizing Smad-mediated gene transcription by co-repressors, Ski, SnoN and TGIF, is also an important regulatory component [126]. In response to TGF-β, SnoN, and to a certain extent Ski, is rapidly degraded, therefore allowing the the activation of TGF-β1 target genes. However, SnoN expression is also induced in response to TGF-β acting as a negative feedback loop to terminate target gene transcription [198, 199]. Ski and SnoN repress TGF-β-induced transcription by disrupting the interaction of the Smads with co-activtor p300 and on the other hand actively recruit a co-repressor complex consisting of HDAC1 [122, 200]. Some co-repressors including Smad-interacting protein 1 (SIP1), TGIF and ecotropic virus integration site 1 protein homolog (Evi-1) can inhibit Smad-mediated transcription, possibly through the recruitment of HDAC activity [201-204]. Overall, the final transcriptional outcome by TGF-β1 may depend on the balance between HATs and HDACs under specific cellular conditions.

1.5.5.3 Chromatin remodelling by the Smads

ATP-dependant chromatin remodelling complexes such as switching of mating type/sucrose nonfermenting (SWI/SNF), imitation switch (ISWI), nucleosomes remodelling and deacetylase (NuRD) are involved in the regulation of gene expression, both activating transcription by moving nucleosomes and repressing

transcription by altering the chromatin structure of promoters. For example, *in vitro* experiments with assembled chromatin templates have shown that Brg1 is essential for phosphorylated Smad2 complexes to activate transcription [109].

1.5.6 Regulation of Non-Smad pathway on TGF-β signalling

TGF-β1 stimulates ERK, JNK, and p38 via TGF-β1 receptors independently of Smads [205, 206]. p38, ERK, and JNK MAP kinase inhibition blocks the expression of TGF-β-induced CCN2/CTGF in lung or with gingival fibroblasts [207], suggesting the involvement of MAPK pathways in TGF-β1 signalling cascade.

On the other hand, ERK phosphorylation in the linker region of Smad proteins associates with decrease of Smads nuclear translocation, suggesting that ERK may also act as a negative regulator of TGF-β1 signalling pathway [45]. ERK, JNK, and p38 have all been implicated in the transcriptional regulation of Smad7, therefore indirectly regulating TGF-β signalling [222].

TGF-β1 can up or down-regulate Rho-like GTPases including RhoA and P13K/Akt pathways to contribute to TGF-β1-induced functions independent of R-Smads activation [208-212].

The linker-region of Smads, which contains several serine and threonine residues, allows for regulation of R-Smads by multiple signalling participants including MAPK. The linker region of Smad1 contains four MAPK phosphorylation sites (Ser-187, Ser-195, Ser-206, and Ser-214), whereas Smad2/3 linker regions contain four

SP/TP sites for proline-directed kinases. In response to mitogens, ERK MAPK mediates the phosphorylation of these sites *in vivo* [213]. Cyclin-dependant kinases (CDK) CDK-2 and -4 have also been reported to mediate the phosphorylation of some of the linker residues in Smad2/3 in addition to residues at the N-terminus of Smad2/3. p38 MAPK and JNK also phosphorylate the linker region of Smad2/3 and regulate their transcriptional activity. MAPK mediated attenuation of Smad2 activity has been attributed to Smad2 linker phosphorylation [213]. The phosphorylation of MAP-kinase sites in the Smads linker region can inhibit the accumulation of Smads in the nucleus [214]. As for the importance of Smad2 and Smad3 in fibrosis, some work has implied that Smad3 probably is the more vital actor [215].

In summary, non-Smad signalling in response to TGF-β1 plays an important role in determining the response to TGF-β1, but in some cases this is via modulation of Smad responses. Most of the evidence is consistent with Smad signalling being the key determinant of TGF-β1 responses.

Even though massive amounts of work have been done to reveal the mechanisms of TGF-β1 signalling regulation, a lot of the detail of how cellular response to TGF-β1 is altered by other signalling pathways remains unclear. The aim of my work was to explore regulation of TGF-β1 signalling in PTC in renal fibrosis. Two stimuli were chosen, namely IL-1β and BMP-7.

1.6 Interleukin-1 beta *(IL-1β)*

Inflammation is a key contributor to kidney diseases. Acute inflammation is associated with repair following acute injury [216], but more prolonged inflammation is linked to fibrosis, in which inflammation, tissue remodeling and repair processes occur simultaneously [217, 218]. Most chronic fibrotic disorders have a long-term stimulation that sustains the production of growth factors, proteolytic enzymes, and fibrogenic cytokines, causing the excessive deposition of extra cellular connective tissue elements that progressively destroy and remodel the normal tissue architecture [218]. TGF-β1 is a key profibrotic cytokine but also plays an important role in repair following acute injury. Pro-inflammatory cytokines are important negative regulators of TGF-β signalling in acute inflammation [219]. However, recent work suggests that chronic inflammation may promote fibrosis by enhancing epithelial cell signalling responses to TGF-β1 [220, 221]. TGF-β1, with its broad and crucial functions in the immune system, inevitably also alters signalling responses to ILs, TNFα, and Interferon gamma (IFN-γ) [222]. Taken together, there is a complex interplay between TGF-β and pro-inflammatory cytokine signalling, with the final transcriptional and other cellular responses determined by the integration of these hypothetically distinct signalling pathways in a manner that is dependent on on stimulus, cellular context, and time.

Interleukin-1 beta (IL-1β) is a key regulator of inflammatory responses, and many other physiological/pathological functions. IL-1β induces local Smad3-dependent tissue fibrosis when over-expressed in the murine lung [223] or peritoneum[224], and chronic IL-1β administration induces EMT [225], a key step in fibrogenesis [226]. Conversely, IL-1β inhibits renal mesangial cell TGF-β1 generation [227], impairs TGF-β1 response in chondrocytes via decreased type II receptor expression

[228, 229], and delays TGF-β1-induced fibroblast to myofibroblast differentiation [230]. Furthermore, Interleukin Receptor Antagonist deficient mice, in whom IL-1β signalling is enhanced, exhibit suppressed TGF-β1 signalling [231].

Therefore, IL-1β was studied as a prototypic proinflammatory stimulus, because the above apparently contradictory outcomes in respect of both stimulatory and inhibitory effects of IL-1β in different contexts. Furthermore, our lab has previously delineated altered PTC HA synthesis in response to IL-1β, and altered TGF-β1 signalling via HA, a possible mechanism link shared by IL-1β and the second stimulus chosen for study, BMP-7.

1.7 Bone Morphogenetic protein 7 (BMP-7)

1.7.1 General introduction of BMP-7

BMPs are a group of proteins that were originally identified based on their unique ability to stimulate cartilage and bone formation *in vivo* [232]. More than 20 BMP-like proteins have been cloned in vertebrates. Except for BMP-1, all share sequence homology with transforming growth factor-beta superfamily members [233]. BMPs also function in a number of non-osteogenic development processes. Bone morphogenic protein-7 (BMP-7), also referred to as osteogenic protein-1 (OP-1) or DVR-7, is a 35-Kd homodimeric protein and was originally identified as a potent osteogenic factor purified from bone [234]; BMP-7 is a member of the TGF-β superfamily that is required during embryogenesis for normal skeletal, kidney, and eye development [235]. BMP-7 deficient mice die shortly after birth because of poor

kidney development [236, 237]. In the adult murine organism, BMP-7 is expressed in many tissues but kidney is the organ with the highest level of expression [238]. BMP-7 is detected in circulating blood and *in vivo* studies demonstrated BMP-7 acts as an endocrine modes at distant targets to promote liver regeneration [239]. Moreover, BMP-7, similar to other peptides within the TGF-β superfamily, binds to extracellular matrix proteins by paracrine mode [240].

1.7.2 BMP-7 and receptor distribution in developing and normal adult kidney

In adult mouse kidney, BMP-7 is abundantly expressed in the medullary region and ureter, and to a lesser extent in the cortical region. BMP-7 is expressed in podocytes, collecting ducts, thick ascending limb, distal convoluted tubule, but not in proximal tubules, thin descending limb, loop of Henle, or in the peritubular vasculature [241]. In adult rat kidney, BMP-7 is expressed immunohistologically lightly in some cortical proximal tubular cross-sections, but strongly in distal convoluted tubules and collecting ducts. There is evidence to show BMP-7 expression in glomerular podocytes *in vivo* specifically and exclusively in rats and in mice. But BMP-7 expression from normal human kidney is rather scarce [242-244]. In situ hybridization and immunostaining have shown localization of mRNA transcripts and the protein for BMP type II receptor in convoluted tubule epithelium and the glomeruli in the cortex, and the collecting ducts in the medulla region in rat kidney, suggesting that the cellular targets for BMP-7 in the kidney are in similar areas of the cortex and medulla [245]. *In vitro*, BMP receptors are abundant in human proximal tubule HK-2 cells but also are present in mesangial cells [241]. In human primary

proximal tubular epithelial cells (PTEC), all known BMP type I and II receptors excluding the type I receptor, BMPR-IB were defined by RT-PCR [246].

1.7.3 Evidence of BMP-7 protective effects on renal acute and chronic renal injury

BMP-7 mRNA expression is decreased in the outer medulla and glomeruli in the acute ischemic rat kidneys after 16 h of reperfusion [244]. Expression of BMP-7 is reduced in obstructed kidney UUO animal model, pyelonephritis, chronic allograft nephropathy [247-250]. Decrease of BMP-7 expression was also detected in mouse podocytes under high glucose culture *in vitro*, and in renal biopsies of patients with diabetic nephropathy [240, 251]. In diabetic rats, the expression of BMP-7 in renal tubules is decreased gradually, along with BMP-7 receptors. In contrast, more and more TGF-β1 is detected with the development of diabetes. Gremlin is an antagonist of BMP-7. Gremlin mRNA levels were significantly increased [252], suggesting overall that there is a balance between BMP7 and TGF-β, as has been suggested by some investigators.

Increasing evidence showed BMP-7 plays a key role in kidney development and postnatal function [253]. In addition, loss of BMP-7 signalling pathway activity, such as less phosphorylation of Smad1/5 protein, was also observed in experimental nephrotoxic serum nephritis and diabetic nephropathy [44, 254].

Several *in vivo* studies using animal models of acute and chronic renal failure demonstrated that BMP-7 not only improves renal function and maintains, to a

variable degree, renal epithelial cell morphology [244], but also prevents complications of chronic kidney diseases, like renal osteodystrophy and vascular calcification [255, 256].

BMP-7 induces differentiation of metanephric mesenchymal cells into epithelial cells [244]. Blocking BMP-7 activity increased expression of fibronectin significantly. Also, exogenous BMP-7 blunted the epithelial cell apoptosis as well as increased interstitial volume based on excessive collagen IV matrix production and tubular atrophy, which is beneficial for the maintenance of tubular epithelial integrity [247, 257, 258]. In addition, transgenically expressed BMP-7 or exogenously rhBMP-7 adminstration reduces the diabetic podocyte injury and postpones initiation and progression of experimental diabetic nephropathy [240]. BMP-7 inhibits tubular epithelial cell damage in UUO model and maintains renal function by preventing tubular atrophy and attenuating tubulointerstitial inflammation and fibrosis activation [247, 258, 259] .

BMP-7 diminished the activation of tubulointerstitial inflammational monocyte/macrophage interstitial infiltration [247] and CTGF [260]. In our lab, previous work has shown that BMP-7 inhibits monocyte-stimulated TGF-β1 generation in human renal proximal tubular epithelial cells (HK-2) [261]. Gould et al have found that BMP-7 significantly reduces the expression of pro-inflammatory cytokines including IL-6 and IL-1β, the chemokines IL-8 and MCP-1, the vasoactive peptide endothelin-2 (ET-2) in primary human proximal tubule cells [246].

Michael Zeisberg et al showed that administration of BMP-7 leads to reversal of

TGF-β1-induced EMT by reduction of E-cadherin *in vivo* and *in vitro*, in association with reversal of chronic renal injury in chronic renal injury mouse model [262]. Furthermore, BMP-7 might induce mesenchymal to epithelial transition (MET) involving adult renal fibroblasts in the injured kidney, generating functional epithelial cells [263]. On human peritoneal mesothelial cells (HPMcs), decreased expression of BMP-7 was observed in high glucose induced EMT and treatment with BMP-7 blocked this transition. *In vivo*, further evidence showed BMP-7 ameliorated peritoneal fibrosis in an animal model of peritoneal dialysis [264]. Controversially, Paul L. Dudas et al found BMP-7 was unable to inhibit EMT in either primary or immortalized human proximal tubule cells, however a protective effect observed at an elevated BMP-7 concentration in mouse renal tubular epithelial cells [265].

1.8 HA-CD44: A possible common mechanism for TGF-β signalling regulation?

HA is a ubiquitous connective tissue polysaccharide as a high molecular mass component of ECM *in vivo*. HA is a nonsulphated, linear glycosaminoglycan (GAG) consisting of the repeating disaccharide units, d-glucuronic acid and N-acetyl-dglucosamine. It exists as a polymer of approximately 104–107 kDa molecular weight [266]. The synthesis of HA is regulated by three mammalian HA synthase isozymes: HAS1, HAS2 and HAS3 [267, 268]. HA degradation is carried out by a family of six endo-N-acetylhexosaminidases, the hyaluronidases (Hyal) identified in human [269].

In addition to provide cellular support, it is now known that under normal

circumstances, hyaluronan regulates cell-cell adhesion, migration, proliferation, differentiation, and the movement of interstitial fluid and macromolecules [266]. HA is only expressed in the interstitium of the normal renal papilla, and its alteration has been invovled in renal water handling regulation by affecting physiochemical characteristics of the papillary interstitial matrix and the interstitial hydrostatic pressure[270].

CD44 is a principal signal-transducing cell-surface glycoprotein receptor of HA that influences cell proliferation, survival and motility relevant to cancer. It contains an ectodomain, a transmembrane domain and a cytoplasmic domain [271, 272]. CD44 also mediates the cellular uptake and degradation of hyaluronan, which affects growth regulation and tissue integrity in tumor growth and progression [273-276] . CD44 is expressed by tubular epithelial cells in areas of tubular injury, but hardly detected in normal kidneys [277, 278]. In ischemic DM kidneys, HA and CD44 staining started to increase in the cortex and outer medulla [279]. The CD44 expression is correlated closely with the degree of glomerular and interstitial damage histopathologically. There is also a positive correlation between proteinuria and the expression of CD44 in the tubulointerstitial compartment [280]. The work in our lab has shown in PTC, standard CD44 and CD44 variants were detected [281].

HA is expressed in the interstitium following renal injury caused by diverse diseases, such as interstitial nephritis, ischemia/refusion injury and IgA nephrology, lupus nephritis, various animal models [282-287] . In progressive renal disease associated with IgA nephropathy, the correlation between the excess deposition of interstitial

HA and renal function has been investigated [284], but the role in the pathogenesis of these diseases is not clear.

Previous work in our lab has investigated the relationship between HA-CD44 and TGF-β1. Addition of HA antagonized TGF-β1-mediated functional increase in type III and type IV collagen and anti-migration in a CD44-dependent manner in HK-2 cells. Furthermore in TGF-β1 signalling pathway, Ito *et al* have shown that HA decreased TGF-β1 activation of a luciferase-Smad responsive construct, and translocation of Smad4 into the cell nucleus [156], and HA increased trafficking of TGF-β1 receptors to lipid raft-associated pools, which facilitates increased receptor turnover and attenuation of TGF-β1-dependent alteration in proximal tubular cell function. This suggests that HA attenuates TGF-β1-mediated function in PTC at least partially via altering TGF-β1 receptor compartmentalization [288].

The above data shows that binding of HA to CD44 can inhibit TGF-β signalling in PTC. However, there is also good evidence for enhancement of TGF-β signalling activity by HA/CD44 interactions in some contexts. In a malignant breast cell line, HA binding to CD44v3 induces Smad signalling via direct Smad phosphorylation, dependent on an interaction between variable stalk region of CD44 coded for by the v3 exon [289]. Interestingly, mice lacking CD44, show decreased proliferation and increased apoptosis of tubular epithelial cells following acute unilateral uretic obstruction, but subsequently also show a reduced fibrotic response. These effects are associated with reduced TGF-β1 signalling, characterized by the phosphorylation and nuclear translocation of Smad-2 and Smad-3, and suggest that CD44 exerts renal protective effects on tubuli but contributes to renal fibrogenesis related to

enhancement of TGF-β1 signalling pathway in obstructive nephropathy [290].

There are also data showing that both IL-1β and BMP-7 interact with HA/CD44. In PTC, previous work in our lab has shown IL-1β stimulated HA synthesis and promoted HA binding to CD44. Moreover, Smad1, a key mediator of BMP-7 signalling, is known to interact with the cytoplasmic domain of CD44. Further, interrupting extracellular hyaluronan-cell interactions with hyaluronidase inhibited BMP-7-mediated Smad1 phosphorylation, nuclear translocation of Smad1 or Smad4, and SBE4-luciferase reporter activation [291]. Collectively, the above data led us to wonder if HA/CD44 might be a "master" regulator of TGF-β1 signalling in PTC, potentially responsible for alterations in PTC TGF-β responsiveness secondary both to pro-inflammatory stimuli such as IL-1β, and to reparative cytokines such as BMP-7.

1.9 Aim of this thesis

The aim of this thesis was to characterize changes in TGF-β1 signalling caused by IL-1β and BMP7 in proximal tubular epithelial cells, and to investigate the mechanisms underlying them.

In summary, my objectives were:

1 To determine the effects of IL-1β and BMP-7 in response to TGF-β1 in PTC

2 To characterise the mechanisms of effects on TGF-β1 signalling by IL-1β and BMP-7 in PTC

3 To assess the role of HA-CD44 in the TGF-β1-induced signalling regulation by IL-1β and BMP-7

Chapter Two:

Methods

2.1 Cell Culture

2.1.1 Selection of HK-2 cell line

Human Kidney-2 (HK-2) is a clonal cell line derived from transformation of human proximal tubular epithelial cells with Human papilloma Virus 16 E6/E7 genes [292]. They retain the functional characteristics of fully differentiated PTCs, showing sodium dependent and phlorizin sensitive sugar transport and adenylate cyclase responsiveness to parathyroid hormone but are not responsive to anti-diuretic hormone. They are positive for proximal tubular markers including alkaline phosphatase, gamma glutamyltranspeptidase, leucine aminopeptidase, acid phosphatase, cytokeratin, alpha 3 beta 1 integrin, fibronectin. They are negative for distal tubular cell markers, including factor VIII-related antigen, 6.19 antigen and CALLA endopeptidase.

There are some concerns with extrapolating the results obtained with transformed cells to those predicted with primary cell cultures, and it is worth noting that over-expression of the E7 gene (used in transformation of HK-2 cells) in an already malignantly transformed cell line caused a downregulation of Smad signalling [293]. However, extensive comparison of HK-2 cells and primary proximal tubular epithelial cells by our laboratory has not yielded any difference in phenotype as assessed by light microscopic appearance, expression and distribution of epithelial cell markers such as E-cadherin, Smad expression and activity, and alteration in

proliferation, migration and cytoskeletal reorganisation in response to TGF-β1 [281, 294-298].

2.1.2 Culture of HK-2 cells

A single frozen aliquot of HK-2 cells were purchased (American Type Culture Collection, Manassas, VA, USA). Stock cells were frozen in liquid nitrogen after three passages. Cells for freezing were removed from their flask with trypsin and pelleted by centrifugation for cell passage, before resuspension in complete HK-2 cell culture medium supplemented with 10% v/v DMSO and 20% v/v bovine calf serum.

HK-2 cells were cultured in 1:1 mixture of DMEM/Ham's F12 (Gibco, Sigma, UK) supplemented with 10% fetal calf serum (FCS) (Biological Industries Ltd, Cumbernauld, UK), 20 mM HEPES buffer (Gibco BRL, Paisley, UK), 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Sigma Chemical Company Ltd, Poole, UK). Cells were grown in a humidified incubator (Cell house 170, Heto Holten, Derby, UK) at 37°C in an atmosphere of 5% CO2. Fresh growth medium was replaced every 2-3 days until cells reached confluence.

To achieve cell cycle synchronisation, cells were serum-deprived for 2 days prior to further manipulation, except as indicated in transfection experiments. All experiments were performed under serum free conditions to avoid the confounding influence of serum on cell function.

2.1.3 Subculture of HK-2 cells

After achieving confluency, cell monolayers were treated with Trypsin/EDTA (0.05w/v / 0.02%v/v, Gibco/BRL, UK) diluted 1:1 in PBS for no more than 10 minutes in the incubator. Cell detachment was monitored by light microscopy and accelerated by agitation of the flask. After detachment, an equal volume of FCS was added to terminate the activity of Trypsin and the solution was collected for centrifugation at 2000rpm for 6 minutes at 4°C. The cell pellet was then resuspended in culture medium containing 10% FCS, and seeded on to culture flasks for experimental manipulation. All experiments were performed using cells below passage 30. Except the cells used for transfection and reporter gene assay, cells were growth arrest in serum-free and insulin-free medium for 48 hours before use in experiments unless otherwise noted. All experiments were performed in serum-free and insulin-free conditions.

2.2 Transfection

2.2.1 Transient transfection

Plasmids preparation

A range of plasmids were acquired for experiments listed below. Plasmids from other investigators were received dried into a filter paper. The spot containing the plasmid was rehydrated in 20ul 50mM Tris.-HCl pH 8.0. Approximately 1ng of the plasmid to be amplified was introduced into competent E.coli using heat shock at 42°C for 45 seconds before culture in Luria-Bertani (LB) medium supplemented with 100ug/ml ampicilin for 1 hour. Bacteria were smeared on the LB medium/ampicilin for culture

overnight. Only the bacteria transformed by the plasmids, which contained the ampicillin resistant gene, were able to grow on the plates.

The following day a single colony of transformed ampicillin resistant *e.coli* was inoculated and grown in LB medium containing ampicillin (LB/amp+medium) at 37°C overnight. Plasmid DNA was extracted and purificated using Maxi Kit (QIAGEN). The remaining culture was stored in LB medium containing 20% glycerol (v/v) at -70°C until further plasmid production.

The pGL3-basic (SBE) 4-Lux, CAGA (4) Smad3 responsive reporter construct was a gift from Aristidis Moustakas [299]. It is Smad 3/4-specific. The ARE and MF1 Smad2 responsive reporter constructs were a gift from Lalage Wakefield [300]. It is Smad 2/4-specific. p65, p50 overexpression vectors are pcDNA3-based expression vectors [301].

Reporter gene transfection

Transient transfection was achieved using the mixed lipofection agent Fugene 6 (Roche, UK). Briefly, cells were seeded onto 6-well plates and grown in serum-containing medium until 50~80% confluent. The culture medium was then replaced by serum-free medium prior to transfection. To introduce plasmids into cells, each plasmid was mixed with Fugene 6 in a ratio of 1:3 (μg : μl) and serum-free medium to make a final volume 100 μl, followed by incubation at room temperature for 15 minutes before adding to each well. Following addition of plasmid-Fugene 6 mixture, cells were incubated overnight prior to further manipulation according to experimental design. At the end of the experiments, the medium was removed and

the remaining cells were washed with PBS and subsequently subjected to cell lysis for measurement of luciferase activity.

Measurement of luciferase activity

To assess the luciferase activity of the reporter, 0.9 μg of each plasmid including the reporter gene and 0.1 μg of the Renilla, were co-transfected in 6-well plates using a transient transfection as described above. The purpose of co-transfection with the Renilla was to normalise data to take account of transfection efficiency.

Following the transient transfection and at the end of the experiments, the medium was removed and cells washed with ice-cold PBS. The cells in each well of 6-well plates were lysed by adding 400 μl of 1x Passive Lysis Buffer (Promega), followed by incubation at –20°C overnight. The following day, the cell lysate were thawed at room temperature for at least 30 minutes to ensure complete lysis of the cells. After pipetting the mixtures, 30μl of cell lysate in each sample was gently mixed with 100μl of Luciferase Assay Reagent II (Promega) to generate a stabilized luminescent signal. Because the total amount of light measured during a given time interval is proportional to the amount of luciferase reporter activity in the sample, firefly luciferase measured using a luminometer (MLX micro titer plate luminometer, Dynex Ltd) represents the reporter activity.

Firefly and Renilla luciferase have distinct evolutionary origins, so they have dissimilar enzyme structures and substrate requirements. Firefly luciferase catalyses a reaction using D-luciferin and ATP in the presence of oxygen and Mg^{2+}, resulting in light emission, whereas Renilla luciferase utilizes oxygen and coelenterate

luciferin to generate light emission. These differences make it possible to selectively discriminate between their respective bioluminescent reactions. Thus, the luminescence from the Firefly luciferase reaction may be quenched while simultaneously activating the luminescent reaction of Renilla luciferase.

To evaluate transfection efficacy, Renilla luciferase activity was measured in the same cell lysates using Dual-Luciferase Reporter Assay System (Promega Ltd.). After quantifying the Firefly luciferase luminescence using a luminometer (Fluostar Optima, BMG), 100μl of Stop & Glo Reagent (Promega Ltd.) was added to the same sample. That means firefly luciferase reaction was quenched and then Renilla luciferase luminescence reaction was activated and measured using the same luminometer again.

Presentation of luciferase activity

The luciferase activity was calculated as the amount of Firefly luciferase luminescence divided by the amount of Renilla luciferase luminescence in each sample, and the data was presented as the relative change in luciferase activity compared to the control for each experiment.

2.2.2 Small interfering RNA (siRNA) transfection

RNA interference (RNAi), or gene silencing, is a technique for down-regulating the expression of a specific gene in living cells by introducing a double-stranded RNA (dsRNA) with homology to the gene of interest. In my work, siRNA transfection was used to study the role of ski, SnoN in BMP-7 effect on TGF-β1 mediated activation

of Smad3, by introduction of a gene silencing using CD44, ski, SnoN, Id (inhibitor of DNA binding/differentiation)1-3 siRNA to inhibit mRNA and protein expression.

siRNA/ transfection agent complexes preparation

All siRNA were purchased from Ambion. The sense and antisense siRNA strands are chemically synthesized, column purified, and annealed.

Delivery of siRNA into cultured cells

Delivery of small interfering RNA (siRNA) into HK-2 cells was optimised using the Silencer siRNA Transfection Kit II (Ambion) according to the manufacturer's instruction. Briefly, $9x10^4$ cells per 12-well plate well were transfected in suspension with 30nM siRNAs and 5μl siPORT Amine (Ambion) in a final volume of 1000μl per well. Subsequently, the transfected HK-2 cells were incubated at 37°C prior to further manipulation according to experimental design. At the end of the experimental time point, the medium was removed and the remaining cells were treated to assess gene expression using QPCR (Quantitative polymerase chain reaction) or Westren Blot to detect protein expression or to compare luciferase activity using reporter gene transfection. Mock-transfected and scrambled siRNA transfected controls were included parallell in these experiments. To evaluate the target gene knockdown efficacy, mRNA levels were quantitated by RT-qPCR, protein levels were detected by Western blot (See Table 2.1).

2.3 Protein extraction and analysis

2.3.1 Protein extraction

Protein extraction was performed as follows: cell monolayers were washed three times with ice cold PBS and were subsequently put in complete lysis buffer on ice for 10 minutes. Then the lysate was collected by scraping the cells into the tube.

Table 2.1 The knockdown efficiency of siRNAs

	Knockdown efficiency	Method
CD44	92%	QPCR(48h)
Id1	60%	QPCR(48h)
Id2	88%	QPCR(48h)
Id3	68%	QPCR(48h)
SnoN	71%	QPCR(48h)
Ski	67%	Immunoblot(72h)

Whole cell protein extraction

To obtain the whole cell lysates, RIPA lysis buffer was used (1x TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% Sodium dodecyl sulphate-polyacrylamide (SDS), 0.04% sodium azide), with added 2mM PMSF, 1mM sodium orthovanadate, protease inhibitor cocktail freshly each time. Samples were centrifuged at 13,000 rpm at 4°C for 30 minutes and then the supernatant was transferred to a separate tube and kept at –70°C until use.

Nuclear extraction

To obtain nuclear fractions, the confluent monolayers were washed three times with cold PBS, scraped and rinsed into 2ml cold PBS. After centrifugation at 3,000 rpm at room temperature for 10 minutes, cell pellets were extracted in buffer A (10mM Hepes, pH7.9, 1.5mM MgCl2, 10mM KCl, 50μM DTT, 100μM sodium orthovanadate, 0.2mM PMSF, 50mM NaF), and then put on ice for 10 minutes. Following centrifugation at 13,000rpm for 1 minutes at 4°C, the supernatant was collected as cytoplasmic protein, and the remaining pellets were extracted in buffer C (20mM Hepes pH 7.9, 25% v/v Glycerol, 420mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 50μM DTT, 100μM sodium orthovanadate, 0.2mM PMSF, 50mM NaF), and put on ice for 20 minutes. Following centrifugation at 13,000rpm for 1 minute at 4°C, the supernatant containing nuclear proteins was collected and stored at -70°C until use.

2.3.2 Quantification of proteins

To ensure equal protein loading of each sample subjected to Western blot analysis, the amount of protein in cell lysate was measured by a dye-binding method (Bradford, Bio-Rad protein assay). Briefly, each sample was diluted with distilled water (typically 1/4 v/v) and 5ul added in triplicate to a flat-bottomed 96-well microtitre plate. Following gentle agitation, the sample was mixed with 250μl of 20% Bio-Rad dye binding reagent per well to give a colour reaction. Concentration of sample proteins could be calculated by comparing with known concentrations of bovine serum albumin (BSA, Sigma-Aldrich Ltd), which are available as standard proteins, ranging from 0~2000 μg/ml. Where necessary, the assay was repeated with altered dilution of samples to achieve readings in the linear range of the assay.

2.3.3 Western blot

Western blot is a technique for separation, transfer, and detection of a particular protein using specific antibodies. Briefly, using gel electrophoresis denatured proteins are separated, and then transferred onto a nitrocellulose membrane by transblot. Subsequently, membranes are probed using specific antibodies to detect the proteins.

Protein separation

Equal amounts of protein (30μg) were mixed with reducing SDS sample buffer (2% v/v SDS, 10% v/v glycerol, 60mM Tris and 0.05% v/v mercaptoethanol) boiled for 5~7 minutes at 95°C. After cooling on ice for 1 minute, the samples and the molecular weight marker (SeeBlue Plus2 Pre-Stained Standard, Invitrogen) were loaded onto SDS-PAGE gels.

Subsequently, electrophoresis was carried out using discontinuous vertical minigel apparatus (Becton Dickinson) in running buffer (25mM Tris-HCl, pH 8.3, 192mM glycine, 1% SDS, Sigma) at 150V until the 3kDa standard had just run off the bottom of the gel. Following electrophoresis, the separated proteins in the gel were transblotted onto a nitrocellulose membrane (Amersham, Little Chalfont, UK) using a Biorad miniblot apparatus in transfer buffer containing 25mM Tris-HCl, pH8.3, 192mM glycine, 20% (v/v) methanol for 90 minutes at 150 V.

Protein detection

After transblotting, the membrane was incubated with Tris-buffered saline-Tween (TBS-Tween, 50mM Tris-HCl, 200mM NaCl, and 0.1% (v/v) Tween-20, pH 7.6) containing 5% non-fat powdered milk for 1 hour at room temperature to block non-specific binding. After washing 3 times with TBS-Tween, the membrane was incubated with primary antibody in Tris-buffered saline-Tween containing 5% non-fat powdered milk or BSA overnight at 4°C. The following day, the blots were washed 3 times with TBS-Tween, and subsequently incubated with an appropriate horseradish peroxidase (HRP) conjugated secondary antibody in Tris-buffered saline-Tween containing 5% non-fat powdered milk for 1 hour at room temperature (Goat anti-rabbit HRP conjugated IgG or goat anti-mouse HRP conjugated IgG, Santa Cruz, USA, 1:10000 diluted concentration). After three times further washes with TBS-Tween, proteins were detected using enhanced chemiluminescence (ECL plus Western Blotting Detecting Reagents, Amersham, UK) according to the manufacturer's instruction.

Briefly, the blot was dabbed dry on filter paper and its protein side was placed face up on a sheet of SaranWrap. Followed by mixing detection solution A and B (Amersham) in a ratio of 40: 1, the mixed detection reagent is pipetted onto the membrane. After incubation for 5 minutes at room temperature, excess detection reagent was drained with filter paper, and the blot was put down on to a fresh piece of SaranWrap. The blot was gently wrapped up and smoothed out any air bubbles before its being placed protein side up in an x-ray film cassette. In a dark room, a sheet of Hyperfilm ECL (Amersham) was put on the top of the membrane and exposed for 15-60 seconds. Then the film was removed from the x-ray cassette and developed immediately.

Stripping and reprobing

To detect the other proteins in the same blot membrane, the method of stripping and reprobing was used. Briefly, the membrane was submerged in stripping buffer (100mM 2-Mercaptoethanol, 2% SDS, 62.5mM Tris-HCl, pH 6.7), followed by incubation at 50°C for 30 minutes with occasional agitation. After washing the membrane 3-5 times using a large volume of TBS-Tween at room temperature, it was blocked in 5 % non-fat dried milk in TBS-Tween (0.1%) for 1 hour at room temperature. Finally, the immunodetection was repeated as described above. I have checked the adequate stripping by developing the stripped membrane directly. Basically, there was no band or very faint band detected. If the very faint band was detected , a parallel gel was run at the same time to detect the other target proteins with similar molecular weight.

2.3.4 Enzyme linked immunosorbent assay (ELISA)

Human cytokine measurements were made on cell culture supernatants using the commercially available ELISA kit for TGF-β1 (DY240, R & D system) and IL-6 (DY 206, R & D system).

TGF-β1 ELISA

TGF-β1 concentrations were determined using a commercially available ELISA development kit. The kit contains paired anti-TGF-β antibodies for capture and detection. The capture antibody is a mouse anti-human polyclonal antibody, and the detection antibody is a biotinylated chicken anti-human TGF-β1 antibody. These

show 0.3 to 0.96% cross reactivity with TGF-β2 and TGF-β3 (R & D system, summary of product characteristics). The within sample coefficient of variation was 0.27 to 6% in the data presented. Briefly, high protein binding 96 well plates (Immulon 4, Dynex Technologies) were coated with TGF-β capture antibody (2ug/ml in PBS) overnight at room temperature. Wells were washed three times with wash buffer (0.2% v/v Tween-20 in PBS) using a plate washer (Denley Wellwash 4, Thermo Life Sciences, Basingstoke, UK) at this and each subsequent wash step. The plate was incubated for 1 hour at room temperature with block buffer (5% v/v Tween-20, 5% v/v sucrose in PBS) then washed before addition of TGF-β standards and acid cell culture supernatant samples. Recombinant human TGF-β was prepared in fresh tissue culture medium according to the kits instruction to provide a standard curve of 7 concentrations from 2000pg/ml to 31.25 pg/ml. Cell culture supernatant samples were acid activated according to the kit instructions: 20ul 1M HCl was added to each 100ul sample, and the samples were incubated for 10 minutes at room temperature. The samples were neutralised with 20ul 1.2 M NaOH/0.5M HEPES. The pH of the samples following neutralisation was checked with pH indicator paper, and was in the desired range for the assay of 7.2-7.6. [302]

Following the addition of standards and samples, plates were covered and incubated for 2 hours at room temperature, then washed three times with wash buffer. Detection antibody was added at 300ng/ml in reagent diluents (1.4% w/v delipidized bovine serum albumin (Sigma) 0.05% v/v Tween-20 in PBS, 0.2um filter sterilised) and the plate incubated for 2 hours at room temperature, then washed. Streptavidin-Horseradish Peroxidase (0.5% v/v in reagent diluent) was added to the wells and the plate incubated in the dark for 20 minutes then washed. Substrate solution (1:1 H_2O_2

solution and Tetramethylbenzidine, R & D system) was added to the wells and the plate incubated for 20 minutes in the dark before the addition of stop solution (2M H_2SO_4). The plate was read immediately at 450nm. A standard curve was prepared and the amount of TGF-β1 in each sample quantified using the Plate Reader's software packages.

IL-6 ELISA

IL-6 concentrations were measured using a commercially available ELISA development kit. The kit contains anti- IL-6 antibodies for capture and detection. Firstly, the high protein binding 96 well plates (Immulon 4, Dynex Technologies) were coated with IL-6 mouse anti-human polyclonal capture antibody (2ug/ml in PBS) overnight at room temperature. Wells were aspirated and then washed three times with wash buffer (0.2% v/v Tween-20 in PBS) using a plate washer (Denley Wellwash 4, Thermo Life Sciences, Basingstoke, UK). The plate was blocked for 1 hour at room temperature with block buffer (5% v/v Tween-20, 5% v/v sucrose in PBS) then washed three times with wash buffer before adding in IL-6 standards and cell culture supernatant samples. Recombinant human IL-6 was prepared in distilled water according to the kits instruction to produce a standard curve of 7 concentrations from 2000pg/ml to 31.25 pg/ml.

Following the addition of standards and samples, plates were covered and incubated for 2 hours at room temperature, aspirated and then washed three times with wash buffer. Biotinylated goat anti-human IL-6 detection antibody was added at 200ng/ml in reagent diluent (1.4% w/v delipidised bovine serum albumin (Sigma) 0.05% v/v Tween-20 in PBS, 0.2um filter sterilised) and the plate incubated for 2 hours at room

temperature, then washed. Streptavidin-Horseradish Peroxidase (0.5% v/v in reagent diluent) was added to the wells and the plate incubated in the dark for 20 minutes then washed. Substrate solution (1:1 H_2O_2 solution and Tetramethylbenzidine, R & D system) was added to the wells and the plate incubated for 20 minutes in the dark prior to the addition of stop solution (2M H_2SO_4) with gently mixing. The plate was read immediately at 450nm. A standard curve was prepared and the amount of IL-6 in each sample quantified using the Plate Reader's software packages.

2.3.5 Fluorescence Activated Cell Sorting (FACS)

Preparation and staining of cells

Cells for FACS were grown to confluence in 6-well-plate and growth arrested in serum-free medium for 48 hours before use in experiments. Adherent cells were removed from their tissue culture plate using Trypin: PBS in 1:4 to avoid the destruction of membrane protein and transferred to tubes. The solution was centrifuged at 2000 rpm for 6 minutes. The pellets were washed with PBS, centrifuged and resuspended in 1% BSA (50mg BSA) in FACS buffer (500ml PBS, 10mM EDTA, 15mM sodium azide, pH to 7.35) with an approximate volume. The suspension was transferred to the individual wells of round-bottom 96-well-plate (Falcon) with 100ul aliquots in each well. The primary antibodies, CD44 (Rat anti-CD44 human monoclonal antibody, Calbiochem, UK) and CD44 V3 (Mouse monoclonal anti-CD44V3 human antibody, R & D, UK) were added to the samples at the manufacturers recommended dilution and incubated at 4°C for 30 minutes covered with tinfoil. Then the whole plate was spinned and washed with FACS buffer three times. Then the second antibodies (Rabbit anti-rat immunoglobulins

FITC-conjugated antibody for CD44, Goat Anti-mouse Immunoglobulins PRE-conjugated antibody for CD44 V3, DAKO, Denmark, 1:50 diluted concentration) were added in at the manufacturers recommended dilution and incubated at 4°C for 30 minutes in the dark. After spinning and washing the whole plate with FACS buffer three times, the samples were resuspended in FACS buffer prior to analysis.

Acquisition and analysis of cells

The fluorescent labelled cells were analysed in a Becton Dickinson FACScaliber (Becton Dickkinson, San Jose, CA, USA) using previously defined setting for forward scatter (FSC), side scatter (SSC), and channel fluorescence(FL1 for CD44 and FL2 for CD44 v3). A minimum of 10,000 cells was acquired for each sample. All experiments included negative controls of unlabelled cells. For the cells examined, it was possible to define a population of cells including > 50% of the cells counted in the mid-range of FSC and SSC values. These populations were used for analysis to exclude the cell debris and small cell clusters.

2.4 Immunocytochemistry

Immunocytochemistry is a method to detect localization of protein in cells using antibodies binding to target antigens in cells. In my work, expression of Smad3 in HK-2 cells was examined by immunocytochemistry. Briefly, HK-2 cells were grown in 8-well multi-chamber slides (In Vitrogen Ltd, Paisley, U.K.) until 80% confluent, and then incubated in serum free medium alone for a further 48 hours. Subsequently, cells were stimulated with different cytokines or chemical reagents under serum free conditions. At the end of each time point, cells were rinsed with PBS three times

prior to fixation. Cells were fixed by adding 1:1 (v/v) methanol/acetone at room temperature for 10 minutes. Following fixation, cells were washed with PBS and blocked with 1% BSA in PBS at 4°C for 1 hour prior to a further washing step with 0.1% BSA in PBS. Subsequently, cells were incubated with the Smad3 antibody (1: 50 diluted concentration, anti-rabbit polyclonal antibody, Santa Cruz) for 2 hours at room temperature. After washing 3 times with 0.1% BSA in PBS, the specimen was further incubated with the fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG antibody (1:1000 diluted concentration, DAKO) at room temperature for 1 hour. After washing 3 times with 0.1% BSA in PBS, slides were mounted with Vectashield fluorescent mountant (Vecta Laboratories, Peterborough, UK), and examined on a Leica Dialux 20m fluorescent microcope (Leica Microsystem (UK) Ltd., Milton Keynes, UK).

2.5 RNA extraction and analysis

2.5.1 RNA extraction and quantification

When the experiments were ended, cell monolayers were rinsed by PBS once and total RNA was extracted from cells with 1ml of Tri Reagent (Sigma) per well of 6 well plate. The lysates were incubated at room temperature for 10 minutes to disrupt nucleo-protein complexes and collected in eppendorfs. To separate RNA from the mixture of DNA and proteins, phase separation was achieved by adding chloroform (Sigma, USA) and centrifugation at 12,000rpm for 20 minutes at 4°C. The interphase and the lower chloroform phase were discarded, and the aqueous supernatant

containing predominantly RNA was carefully collected into a sterile eppendorf. To further purify RNA extracts, RNA was precipitated from the aqueous phase by mixing with isopropanol (Sigma, USA) at -20°C overnight. Following centrifugation at 12,000rpm for 20 minutes at 4°C, the supernatant was carefully aspirated and discarded, and the RNA precipitate was air-dried. The precipitated RNA pellet was washed with 75% ethanol twice. After aspiration of the final ethanol wash, the pellet was air dried for 20 minutes in a fume hood before being dissolved in 10μl sterile water. Finally the sample was stored at -70°C until further analysis.

The purity and concentration of the RNA was measured using a Beckman DU® 64 single beam spectrophotometer (Beckman Instruments, High Wycombe, UK) as follows: One microlitre of the dissolved RNA was diluted in 54 μl of sterile water and the absorbance of the resulting solution at 260nm and 280nm was measured. The RNA content was calculated as the value of the absorbance at 260nm multiplied by 40 (one A_{260} unit equals 40μg RNA/ml). The purity of total RNA was evaluated as the ratio of A_{260}/A_{280}, which should be in the range of 1.6~1.8.

2.5.2 Reverse transcription

Reverse transcription (RT) is the process where a ribonucleic acid (RNA) molecule is reversed transcribed into its DNA complement or complementary DNA (cDNA). Reverse transcription of mRNA into cDNA was performed as follows. One μg of purified RNA dissolved in 10μl sterile water was mixed with M-MLV™ reverse transcriptase (Gibco/BRL Life Technologies Ltd, Paisley, UK) in the presence of the mixture of 1μl of 100μM random hexamers (Pharmacia Biosystems Ltd, Milton

Keynes, UK), 5μl of 2.5mM dNTPs (Gibco/BRL Life Technologies Ltd, UK), 2μl of 10X PCR buffer (Applied Biosystems, Beaconsfield, UK), and 2μl of 0.1M dithiothretiol (DTT; Gibco/BRL Life Technologies Ltd, UK). The reaction mixture was kept for 5 minutes at 95 °C to denature RNA in a GeneAmp PCR system 9700 thermocycler (PE Applied Biosystems, Beaconsfield, UK), followed by cooling on ice for 2 minutes. Following the addition of 1μl M-MLV^{TM} Superscript Reverse Transcriptase (200Units; Gibco/BRL Life Technologies Ltd, Paisley, UK) and 1μl RNAase inhibitor (40Units; Gibco/BRL Life Technologies Ltd, Paisley, UK), the reaction mixture was incubated for 10 minutes at room temperature to allow annealing of random hexamers, 45 minutes at 42°C for synthesis of a single cDNA strand, and 5 minutes at 95°C to separate the strands and to halt the reaction. The product of reverse transcription was stored at –20°C until PCR amplification.

2.5.3 Quantitative Polymerase chain reaction (Q-PCR)

Polymerase chain reaction

The polymerase chain reaction (PCR) allows a small amount of a DNA template to be amplified exponentially. The principle is that PCR uses a thermostable DNA polymerase and two short oligonucleotides as primers to create millions of identical copies of DNA. In my work, I mostly used the fluorescent DNA binding dye SYBR Green to detect PCR products.

The sequences of the primers are complementary to those of the DNA template. Firstly, the double stranded DNA (dsDNA) of the template is denatured and separated to two single stranded DNA by heating to 94~98°C (step 1). Secondly, the

primers are then allowed to hybridize (anneal) to the component strands of the target DNA following the reduction of temperature to 37~65°C (step 2). Finally, in the presence of four dNTPs, each primer can be extended by a thermostable DNA polymerase at 72°C (step 3). These 3 steps, which together constitute one cycle of PCR, produce twice the number of original templates. Therefore, successive cycles of template denaturation, primer annealing, and primer extension will generate an exponentially increasing number of DNA fragments.

Detection of PCR products

Detection of PCR products can be achieved by using fluorescent DNA binding dyes like SYBR green, or oligonucleotide probes such as TaqMan. In my work, I mostly used the SYBR green except for measuring CD44, in which case Taqman primer was used (Hs00153304_ml, Applied Biosystems, USA).

The principle of TaqMan detection system is that the TaqMan probe is designed to anneal to the target sequence between the forward and reverse primers. The probe is labeled at the 5' end with a reporter fluorochrome and a quencher fluorochrome at the 3' end. As *Taq* polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of *Taq* degrades the probe, releasing the reporter fluorochrome. Thus, the amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle. SYBR green is a dye in the PCR reaction which binds to all double-stranded DNA. SYBR fluoresces when it binds double stranded DNA, the greater the fluorescence the greater the amount of DNA bound.

PCR amplification was performed in a total volume of 20μl including 1μl of reverse transcription product and 19μl of master mix (7.8μl H_2O, 0.6μl 5'-primer (20μM), 0.6μl 3'-primer (20μM), 10ul SYBR green master mix) or TaqMan assay (8μl H_2O,

1μl TaqMan probe, 10ul TaqMan master mix) by using a GeneAmp PCR system 7900HT thermocycler (Applied Biosystems). The repetition is 40 cycles.

2.5.4 Primers for QPCR

All the primers for SYBR were designed by Primer 3. The primers sequences and product sizes are listed below (see table 2.2).

Table 2.2 The sequences of the PCR amplification primers

Gene		Primers	Product size
TGF-β1	F	ACCGGCCCTTCCTGCTCCTCA	288
	R	CGCCCGGGTTATGCTGGTTGT	
CTGF	F	GGCCCAGACCCAACTATGAT	143
	R	AGGCGGCTCTGCTTCTCTA	
PAI-1	F	TCTCTGCCCTCACCAACATTC	150
	R	CGGTCATTCCCAGGTTCTCT	
Id1	F	GGCTGTTACTCACGCCTCAA	111
	R	CAACTGAAGGTCCCTGATGTAGTC	
Id2	F	CAGCCTGCATCACCAGAGAC	152
	R	CTTATTCAGCCACACAGTGCTTT	
Id3	F	CGTCATCGACTACATTCTCGACCT	179
	R	ACCTGCGTTCTGGAGGTGTC	
Arkadia	F	AGGGGCAATTTTGAGGAACT	144
	R	TTCTTCCCCATCTTGTTTGC	
Gapdh	F	GAGTCAACGGATTTGGTCGT	238
	R	TTGATTTTGGAGGGATCTCG	

2.5.5 Presentation of Q-PCR data

The threshold cycle (Ct) value is determined by identifying the cycle number at which the reporter fluorescence emission intensity rises above background noise. The Ct is at the exponential phase of the reaction and there are two methods to analyse Q-PCR data. One is absolute quantification, which determines the amount of the transcript of interest usually by relating the PCR signal to a standard curve. Another is relative quantification, which describes the change in expression of the target gene relative to some reference groups such as an untreated control.

In my study, I used the method of relative quantification and the formula $2^{-(\text{delta deltaCt})}$ to present my data. Fold change expression of target gene mRNA after treatment was calculated by DeltaDeltaCt method. The DeltaCt is the difference between the amplification threshold for target genes and Gapdh (Endogenous control). P values were calculated by t-test using excel.

2.6 Investigation of Protein: DNA interactions

2.6.1 Electrophoretic Mobility Shift Assay (EMSA)

The electrophoretic mobility shift assay (EMSA) determines the binding interaction between DNA or RNA and nucleic acid binding proteins.

2.6.1.1 Radiolabelling of double-stranded oligonucleotide probes

Annealing oligonucleotide for use as an EMSA probe

Oligonucleotides containing the transcription factor binding site of interest or region of gene promoter to be studied were annealed and labelled with [^{32}P]α-dTTP. Annealed oligonucleotides contained short 5'-overhang of 3 to 4 bases complementary to the label deoxynucleotide (A for [^{32}P]α-dTTP). The oligonucleotides are resuspended at 1μg/μl in double distilled water.

To anneal the primers, the following reaction mix was made in a 1.5ml Eppendorf (1μg/μl sense primer 10μl, 1μg/μl antisense primer 10μl, 1M NaCl 10μl, dH_2O 70μl). Afterwards, the annealing mix was heated to 95℃ for 10mins in a water bath. Then the water bath was switched off and allowed to cool to room temperature overnight. The annealed oligonucleotide probe (100ng/μl) was stored at –20℃. Before use, it was to be diluted 1:10 in dH_2O to 10ng/μl.

Labelling oligonucleotide probe

The labelling reaction mixture (25ng oligonucleotide probe 2.5μl, 2.5mM mix of 3 unlabelled dNTPs including dATP, dCTP, dGTP 1.0μl, 10X Klenow Buffer 5.0μl, 1M NaCl 5.0μl, Double distilled water 32.5μl) was centrifuged for a few seconds at 6000rpm. Then behind the Perspex shield, the radionucleotide [^{32}P}α-dTTP 3.0μl and Klenow fragment (2U/μl) 1.0μl were added in the labelling reaction mixture. Then the labelling reaction mixture was votexed and centrifuged for a few seconds at 6000rpm. Prior to stopping the reaction by adding 0.5M EDTA (pH 8) 2.0μl and STE 50.0μl, the mixture was incubated at room temperature for 10-20 minutes. Then the labelled probes were purified using probe quant micro columns (Amersham). The column end was broken off and placed in a 1.5ml eppendorf without a lid and

centrifuging 3000rpm in a microfuge for 1minute. The column was dried and placed in a fresh 1.5 ml eppendorf, added in labelling reaction. After spinning at 3000rpm for 2minutes, the labelled probe was transferred into a fresh eppendorf and stored at –20°C.

2.6.1.2 Electrophoretic Mobility Shift Assay

Nuclear protein extraction was performed as previously described. Briefly, cells were harvested in ice-cold PBS (pH 7.4) and pelleted by centrifugation. Cells were resuspended in ice-cold bufferA (10 mm HEPES-KOH [pH 7.9], 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 10 minutes. The cell pellet was collected by centrifugation, resuspended in buffer B (20 mM HEPES-KOH [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.3 mM DTT, 0.2 mM PMSF), and incubated on ice for 20 minutes followed by brief high-speed centrifugation (12000 g for 10 seconds at 4°C), and the resulting supernatants (nuclear extract) were collected. Protein concentrations were determined using the Bradford method.

Oligonucleotides containing consensus motifs for NF-κB (5' -gaTCCATGGG GAATTCCCC-3' and 3'-AGGTACCCCTTAAGGGGag-5') were annealed for use in EMSA. Smad Binding Element Oligonucleotides were bought from Santa Cruz (Sense: 5'- TCGAGAGCCAGACAAAAAGCCAGACAT TTAGCCAGACAC-3'; anti-Sense:3'-AGCTCTCGGTCTGTTTTTCGGTCTGT AAATCGGTCTGTG -5') and annealed for use in EMSA. These double-stranded fragments were labelled with

[alpha32P] dTTP (Amersham-Pharmacia Biotech, Amersham, United Kingdom) using the Klenow fragment of DNA polymerase I.

The composition of NF-κB protein/DNA complexes was determined by supershift assays using rabbit polyclonal antibodies specific for NF-κB–p50 and NF-κB–p65 (Santa Cruz Biotech Inc., Santa Cruz, California, USA).The composition of Smad binding element protein/DNA complexes was determined by supershift assays using rabbit polyclonal antibodies specific for Smad1 (New England Biolabs, USA), Smad2 (New England Biolabs, USA), Smad3 (Invitrogen, USA), Smad4 (Santa Cruz, California, USA), and Smad 5 (New England Biolabs, USA).

2.6.2 Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) assays is used to evaluate the association of proteins with specific DNA regions. The technique involves crosslinking of proteins with DNA, fragmentation and preparation of soluble chromatin followed by immunoprecipitation with an antibody recognizing the protein of interest. The segment of the genome associated with the protein is then identified by PCR amplification of the DNA in the immunoprecipitates. Here, I gratefully acknowledge the kindly help from Prof. Ray Water's Lab.

HK-2 cells were cross-linked by rotating gently with final concentration of 1% formaldehyde at room temperature for 20 min. Cross-linking was quenched by immediately washing cells twice with ice-cold PBS. Cells were scrapped into 10ml cold PBS and Centrifuged for 5 minutes at 3,000rpm. Cell pellets were resuspended in lysis buffer (50mM HEPES-KOH pH7.5, 140mM NaCl, 1mM EDTA pH 8, 1%

Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, with protease inhibitors freshly) and incubated on ice for 10 min. Cell lysates were sonicated to yield DNA fragments ranging in size from 200 to 500 bp (Bioruptor, Diagenode SA) Approximately 25ug protein of the cross-linked, sheared chromatin solution was used for immunoprecipitation.

A small portion of each IP mixture was saved as input DNA (5%). Supernatants were diluted 10-fold in RIPA buffer (50 mM Tris-HCl pH8,150 mM NaCl,2 mM EDTA pH8,1% NP-40,0.5% Sodium Deoxycholate) and immunoprecipitation was performed by rotating for 4 h at 4°C with 5ug of rabbit anti-Smad3 (Invitrogen, 51-1500). For each sample, the same amount IgG was added into the chromatin solution as a control. 60ul of protein A-Sepharose containing 1.5ug of sheared salmon sperm DNA was added to samples by rotating overnight at 4°C. The beads were centrifuged 1 minute at 13,000 rpm, and supernatant removed before washing 3× with wash buffer I (0.1% SDS, 1% Triton X-100, 2mM EDTA pH8, 150mM NaCl, 20mM Tris-HCl pH8) and 1× with wash buffer II (0.1% SDS, 1% Triton X-100, 2mM EDTA pH8, 500mM NaCl, 20mM Tris-HCl pH8). Then the DNA was eluted by adding Elution Buffer (1% SDS, 100mM NaHCO3) to the protein-A beads and rotating at room temperature for 1 hour. Following centrifugation, the supernatant was removed and incubated with 5ul protease K (20ug/ml) at 65°C overnight.

The DNA was extracted by phenol: chlorophorm and ethanol precipitated in the presence of 10ul glycogen (5mg/ml). Precipitated DNA was amplified by real-time PCR using SYBR green master mixture. Primers were designed using Primer 3 Software for the second and third Smad Binding Elements in the PAI-1 promoter,

previously shown to cooperatively regulate the transcriptional response to PAI-1 to TGF-β [303]. (2nd primers, -580CAGA, F: 5'-GGGAGTCAGCCGTGTATCAT-3', R: 5'-ACCTCCATCAAAACGTGGAA-3'), (3rd primers, -280CAGA, F: 5'-AGTCAACCTGGCAGGACATC-3', R: 5'-CACCTC CCTCTCTGGGACT-3').
Data is presented as Smad3-precipitated signal/pre-immune globulin-precipitated signal, normalized to control.

2.7 Antibodies used in the thesis: See table 2.3

2.8 Statistical analysis

The data are presented as mean ± SEM of experiments. For each individual experiment, the mean of duplicate determinations was calculated.

Comparison of groups of data was by t-test, using the 'two sample assuming unequal variances'' t-test. Two-sided tests with a hypothesised mean difference of zero and an alpha level of 0.05 were performed. Implicit in this analysis is the assumption that the data were normally distributed, although this was not formally tested.

Table 2:3 Antibodies used in the thesis:

Immunohistochemistry: Rabbit anti-Smad3 (sc-8332), Santa Cruz, USA, dilution 1:50.

ChIP: Rabbit anti-Smad3 (51-1500), Invitrogen, USA, 5ug/sample

For Western Blot, EMSA supershift, FACS, see below:

Antibodies	Supplier	dilution or working concentration		
		Western Blot	EMSA super shift	FACS
Rabbit anti-phospho-Smad3/1 (9514s)	Cell Signalling Technology,USA	1:1000		
Rabbit anti-Smad3 (51-1500)	Invitrogen,USA	1:1000		
Rabbit anti-phospho-Smad2 (3101s)	Cell Signalling Technology,USA	1:1000		
Rabbit anti-Smad1 (9512s)	Cell Signalling Technology,USA	1:1000		
Rabbit anti-Smad2 (3122s)	Cell Signalling Technology,USA		2ug/5ug nuclear protein	
Rabbit anti-Smad4 (sc-7154)	Santa Cruz,USA		2ug/5ug nuclear protein	
Mouse anti-Smad5 (9517s)	Cell Signalling Technology,USA		2ug/5ug nuclear protein	
Rabbit anti-Smad7 (ab5825)	abCam, USA	1:500		
Rabbit anti-Gapdh (ab9485)	abCam, USA	1:1000		
Mouse anti-TGF beta receptor I (sc9048)	Santa Cruz,USA	1:500		
Rabbit anti-IκB a (sc-371)	Santa Cruz,USA	1:500		
Rabbit anti-IκB-β(sc-945)	Santa Cruz,USA	1:500		
Rabbit anti-p-IκK (2687)	Cell Signalling Technology,USA	1:500		
Rabbit anti-IκK (sc-7607)	Santa Cruz,USA	1:500		
Rabbit anti-p65 (sc-109)	Santa Cruz,USA		2ug/5ug nuclear protein	
Rabbit anti-p50 (sc-7178)	Santa Cruz,USA		2ug/5ug nuclear protein	
Rat-anti-CD44(217594)	Calbiochem,UK			1:1000
Mouse-anti-CD44v3 (BBA11)	R&D, UK			2ug/ml
Mouse anti-Arkadia (H00054778-B01)	Abnova, Tai Wan	1:500		
Rabbit anti-ski (sc-9140)	Santa Cruz,USA	1:500		
Rabbir anti-SnoN (sc-9141)	Santa Cruz,USA	1:500		

Chapter Three:

The effects of IL-1β and BMP-7 on TGF-β1 signalling in human proximal tubular epithelial cells

3.1 Introduction

Increased expression of TGF-β1 is widely known as a key stimulus to the progression of renal fibrosis after renal injury. However, the mechanisms by which other environmental cues may alter response to TGF-β are not well studied. Prolonged inflammation and macrophage influx adversely affect prognosis in chronic kidney disease, whereas acute inflammation does not appear to have the same consequences. In the short term, pro-inflammatory cytokines such as IFN-γ [219] and TNF-α [304] have previously been shown to be important negative regulators of TGF-β signalling and exhibit anti-fibrotic activity [305]. More recently, sensitization to TGF-β, and hence profibrotic effects, has been described for chronic proinflammatory stimulation [220, 221]. But the differential effects of acute and chronic inflammation on TGF-β signalling are not widely studied.

On the other hand, anti-TGF-β1-induced fibrotic role in kidney has been raised as an important target to delay or even reverse renal fibrosis. BMP-7 has emerged as a key anti-fibrotic cytokine extensively demonstrated *in vivo* and *in vitro*. But the mechanism of BMP-7 on TGF-β signalling remains unclear.

As detailed in the next chapter, circumstantial evidence suggests IL-1β and BMP-7 might affect TGF-β signalling via effects on the HA/CD44 axis. The aim of the work detailed in this chapter was to determine the effects of IL-1β and BMP-7 on TGF-β signalling in proximal tubular epithelial cells.

With these experiments, I used 1ng/ml TGF-β1 stimulation based on previous extensive characterisation of TGF-β responses at this dose in our lab [306]. For IL-1β and BMP-7, I started with doses ranging from 10pg/ml to 10ng/ml for IL-1β and ranging from 5ng/ml to 2000ng/ml for BMP-7 with the reporter gene construct assay. I selected the time points from 15 minutes to 24 hours to evaluate TGF-β Smad2 and Smad3 signslling separately by Western Blot.

3.2 Results

3.2.1 IL-1β inhibits early Smad3 signalling but enhances late Smad3 signalling in response to TGF-β1

Initially, I evaluated the time-course effects of co-administration of IL-1β with TGF-β1 on Smad3 signalling response. HK-2 cells were cultured from 15 minutes up to 24 hours in the presence of 1 ng/ml TGF-β1 ±1 ng/ml IL-1β before detection of phospho-Smad3 by immunoblotting. Co-administration of IL-1β and TGF-β1 caused a minor reduction in Smad3 phosphorylation compared to TGF-β1 alone in the first 1 hour (Figure 3.1.A). Longer IL-1β and TGF-β1 co-treatment from 3 hours to 24 hours showed an obvious increased phospho-Smad3 compared to TGF-β1 alone after 12 hours (Figure 3.1.B and C).

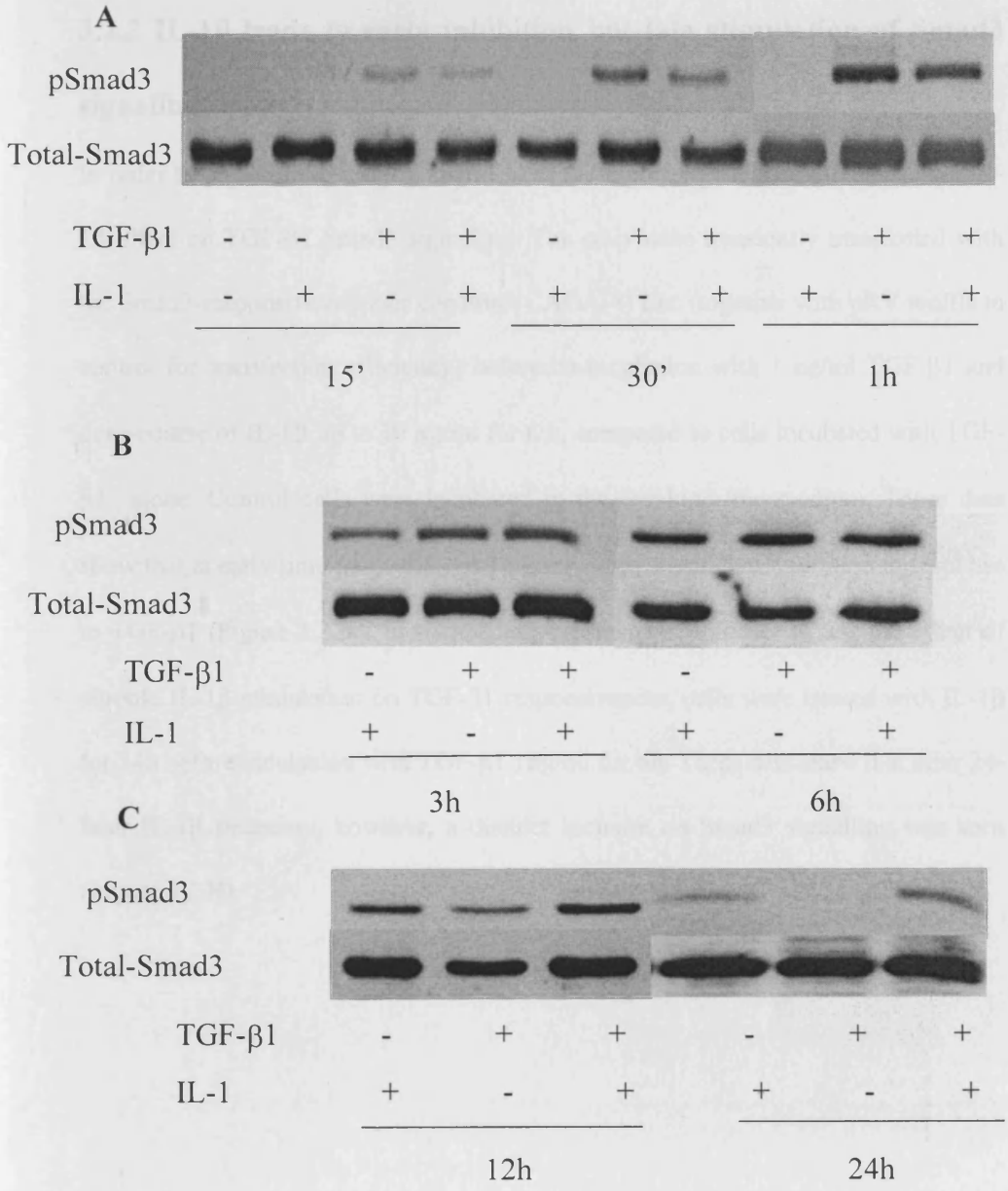


Figure 3.1: Time course of IL-1β effect on Smad3 phosphorylation

IL-1β and TGF-β1 were added to confluent monolayers of growth arrested HK-2 cells under serum free conditions. Immunoblots of phospho-Smad3 in growth arrest HK-2 cells treated with 1ng/ml TGF-β1 ± 1 ng/ml IL-1β from 15 minutes up to 24 hours, before protein extraction and immunoblotting for phospho-Smad3. Blots were stripped and reprobed for total Smad3. **(A)** Early effect of IL-1 β on TGF-β Smad3 signalling. **(B and C)** Enhancement of TGF-β1 Smad3 signalling by chronic IL-1 β stimulation. One representative experiment of three separate experiments is shown.

3.2.2 IL-1β leads to early inhibition but late stimulation of Smad3 signalling

In order to confirm the result above, I used the reporter gene assay to assess the IL-1β effect on TGF-β1 Smad3 signalling. The cells were transiently transfected with the Smad3-responsive reporter construct CAGA(4) Luc (together with pRV renilla to control for transfection efficiency) before co-incubation with 1 ng/ml TGF-β1 and dose-course of IL-1β up to 10 ng/ml for 6 h, compared to cells incubated with TGF-β1 alone. Control cells were incubated in the cytokine-free medium. These data show that at early time points, IL-1β 1 ng/ml led to significant inhibition in response to TGF-β1 (Figure 3.2.A). In subsequent experiments, in order to test the effect of chronic IL-1β stimulation on TGF-β1 responsiveness, cells were treated with IL-1β for 24h before incubation with TGF-β1 1ng/ml for 6h. These data show that after 24-hour IL-1β treatment, however, a distinct increase on Smad3 signalling was seen (Figure 3.2.B).

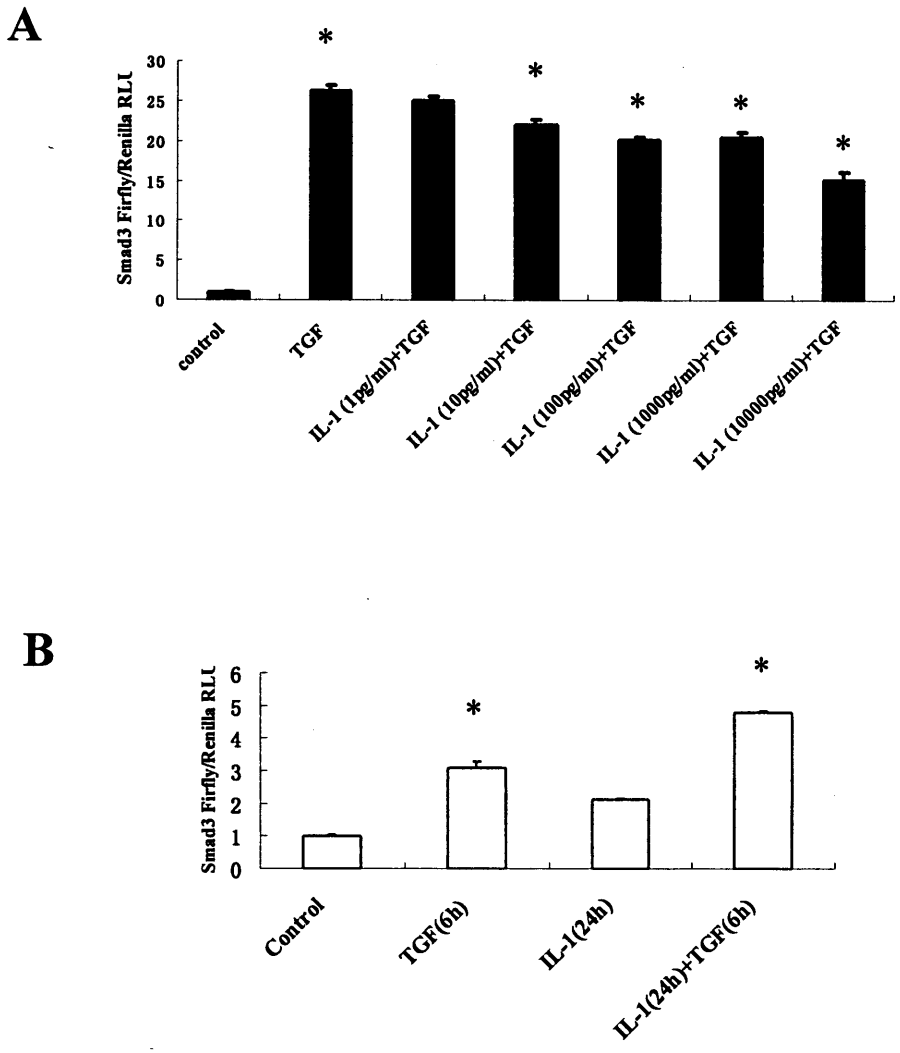


Figure 3.2: Early and late effect of IL-1β on Smad3 signalling

HK-2 cells were transfected with the Smad3 reporter construct CAGA(4) together with pRL-CMV renilla before incubation with IL-1β and/or TGF-β1 . Subsequently, firefly and renilla luciferase activities were assayed. Ratio of firefly to renilla luciferase is displayed and normalized to control. $n = 3$, mean ± SEM is plotted. **(A)** HK-2 cells were treated with 1 ng/ml TGF-β1 ± variable dose course of IL-1β for 6 hours. * $P < 0.002$ for TGF-β1 vs Control. * $P < 0.01$ for both vs TGF-β1. **(B)** Incubation with control medium or 1ng/ml IL-1β for 24h,then incubated with control medium or 1ng/ml TGF-β1 only for 6 hours. * $P < 0.01$ for TGF-β1 vs Control.* $P < 0.01$ for both vs TGF-β1. One representative experiment of three separate experiments is shown.

3.2.3 IL-1β has consistent early inhibition but late stimulation on TGF-β1 Smad2 signalling

Immunoblots suggested a similar inhibitory action on Smad2 phosphorylation when HK-2 cells were cultured for 1 h in the presence of 1 ng/ml TGF-β1 ±1 ng/ml IL-1β (Figure 3.3.A). The cells also were transiently transfected with Smad2-responsive construct ARE-luc before co-incubation with 1 ng/ml TGF-β1 and dose-course of IL-1β 0.1ng/ml to 10ng/ml for 6 h, compared to cells incubated with TGF-β1 alone. Control cells were incubated in the cytokine-free medium. It shows IL-1β inhibited the TGF-β1 Smad2-dependent signal (Figure 3.3.B). In keeping with Smad3, the transfected HK-2 cells were treated with IL-1β for a longer time of 24 hours and it shows an increase on Smad2 signalling, compared to cells incubated with TGF-β1 alone (Figure 3.3.C).

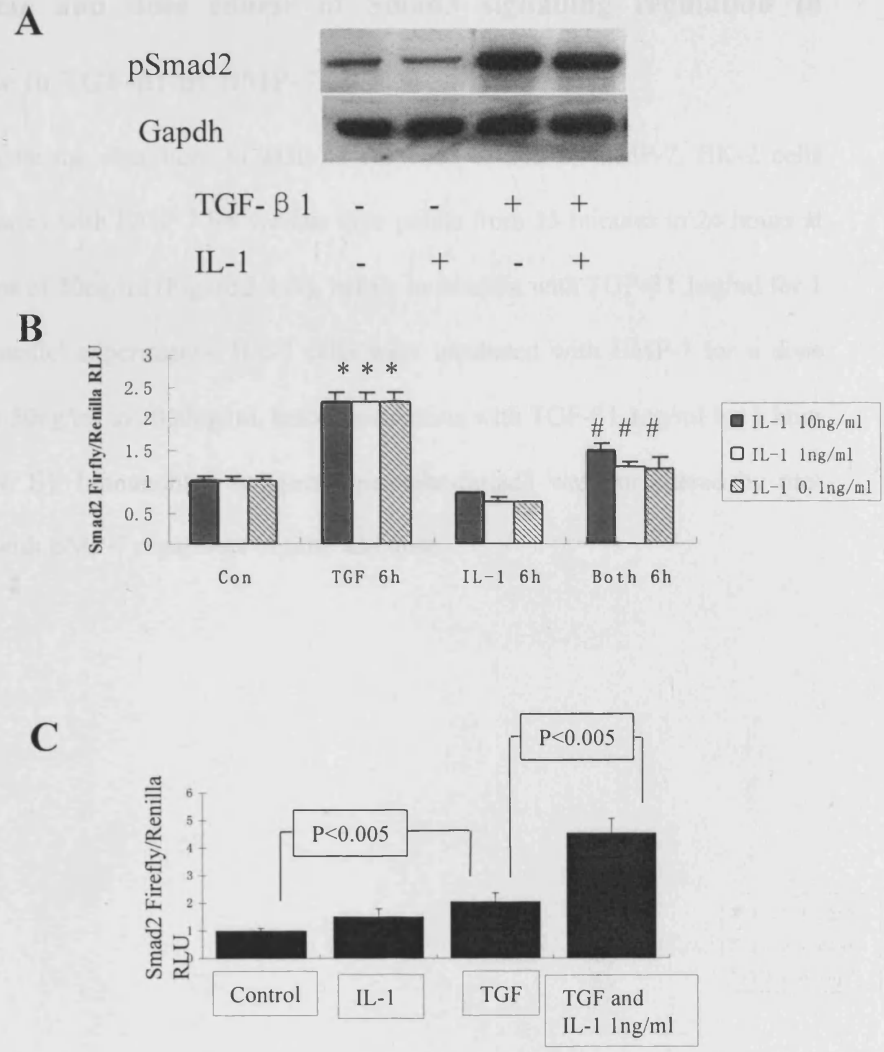


Figure 3.3: Effect of IL-1β on Smad2 signalling

(A) Immunoblots of pSmad2 in growth arrested HK-2 cells treated with 1ng/ml TGF-β1 ± 1 ng/ml IL-1β for 60 minutes, using the same experimental approach as for (3.1) above. Blots were stripped and reprobed for Gapdh. One representative experiment of three separate experiments is shown.

HK-2 cells were transfected with the Smad2 reporter ARE/MF-1 using the same experimental approach as for (3.2) above. **(B)** HK-2 cells were treated with 1 ng/ml TGF-β1 ± 1ng/ml IL-1β for 6 hours. * $P < 0.01$ for TGF-β1 vs Control.

# $P < 0.01$ for Both vs TGF-β1. **(C)** Incubation with control medium or 1ng/ml IL-1β for 24h,then incubated with control medium or 1ng/ml TGF-β1 only for 6 hours. Firefly and renilla luciferase activities were assayed. Ratio of firefly to renilla luciferase is displayed and normalized to control. Results represent mean ± SEM of 3 individual experiments.

3.2.4 Time and dose course of Smad3 signalling regulation in response to TGF-β1 by BMP-7

To investigate the alterations in TGF-β1 response caused by BMP-7, HK-2 cells were incubated with BMP-7 for various time points from 15 minutes to 24 hours at BMP-7 dose of 50ng/ml (Figure 3.4.A), before incubation with TGF-β1 1ng/ml for 1 hour. In parallel experiments, HK-2 cells were incubated with BMP-7 for a dose range from 50ng/ml to 2000ng/ml, before incubation with TGF-β1 1ng/ml for 1 hour (Figure 3.4. B). Immunoblots suggested phospho-Smad3 was not altered by pre-treatment with BMP-7 regardless of time and dose.

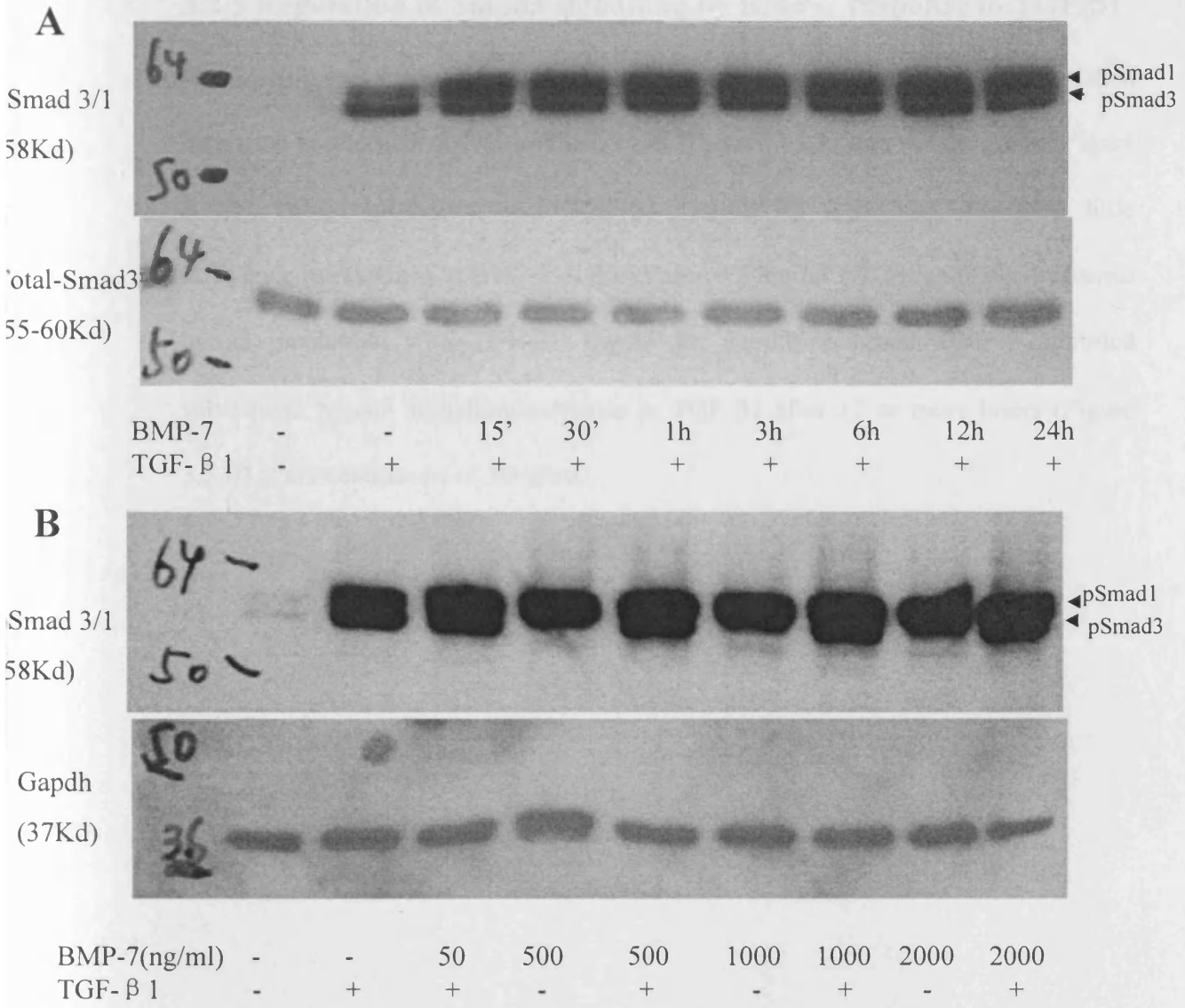


Figure 3.4: Effect of BMP-7 on Smad3 phosphorylation

(A) HK-2 cells were treated with BMP-7 50ng/ml for times up to 24 hours before incubation with TGF-β1 1ng/ml for 1 hour. After immunoblotting for phospho-Smad3, blots were stripped and reprobed for total Smad3. One representative experiment of four separate experiments is shown. **(B)** HK-2 cells were treated with BMP-7 50ng/ml to 2000ng/ml for times up to 24 hours before incubation withTGF-β1 1ng/ml for 1 hour. After immunoblotting for phospho-Smad3, blots were stripped and reprobed for total Smad3 or Gapdh. One representative experiment of three separate experiments is shown.

3.2.5 Regulation of Smad3 signalling by BMP-7 response to TGF-β1

HK-2 cells were transfected with Smad3 responsive plasmid CAGA(4), then incubated with BMP-7 for various doses (Figure 3.5.A and C) or times (Figure 3.5.B), before incubation with TGF-β1 1ng/ml for 6 hours. There was little difference in response to BMP-7 at doses above 50ng/ml for 24 hours pre-treatment before incubation with TGF-β1 1ng/ml for another 6 hours. BMP-7 inhibited subsequent Smad3 signalling response to TGF-β1 after 12 or more hours (Figure 3.5.B) at concentrations of 50ng/ml.

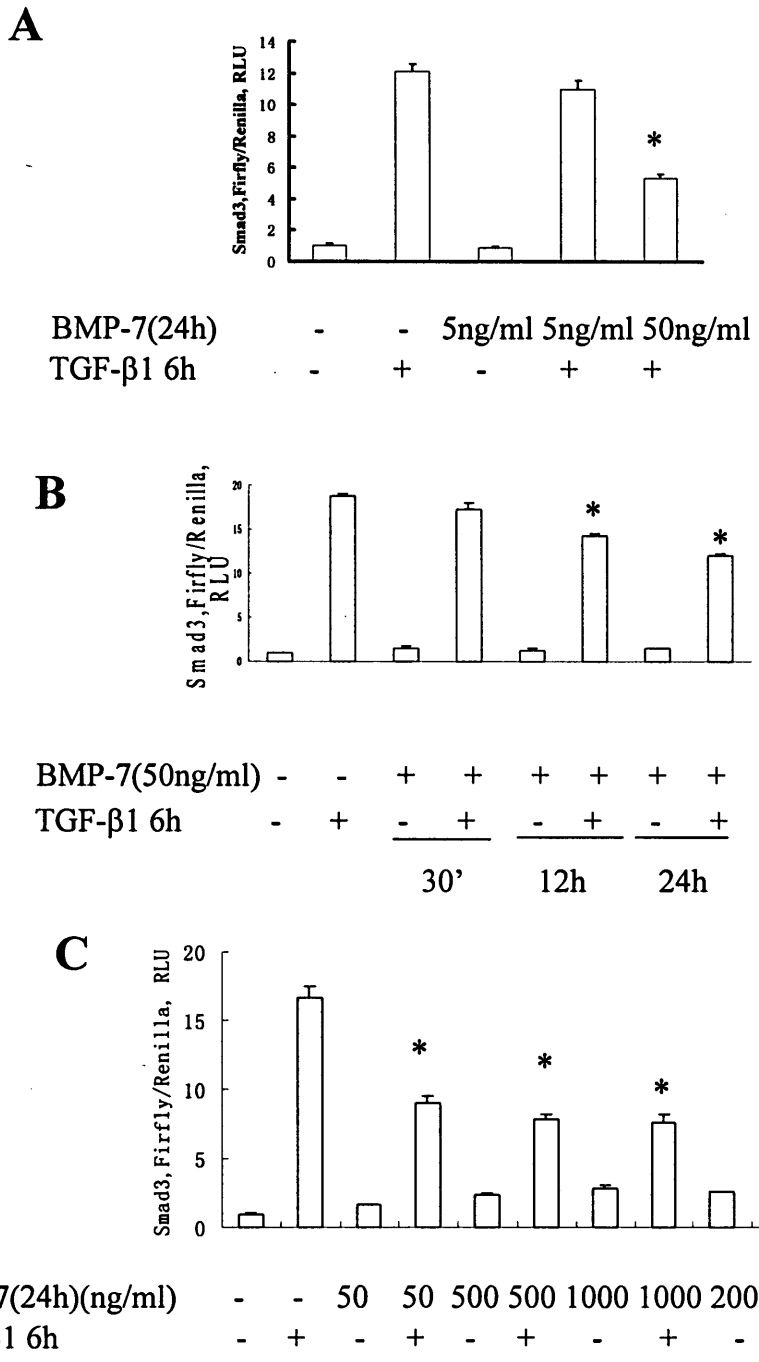


Figure 3.5: Effect of BMP-7 on Smad3 signalling

(A,B,C)HK-2 cells were transfected with Smad3 reporter construct CAGA(4) ,then incubated with BMP-7 for various time points and doses ,before incubation with serum free medium or TGF-β1 1 ng/ml under serum free conditions. $n = 3$, mean ± SEM is plotted. Subsequently, firefly and renilla luciferase activities were assayed. Ratio of firefly to renilla luciferase is displayed and normalized to control. * $P < 0.01$ compared to TGF-β1. One representative experiment of three separate experiments is shown.

3.2.6 BMP-7 has no effect on Smad2 signalling response to TGF-β1

In order to further investigate the alterations in Smad2 response caused by BMP-7, immunoblots and transient transfection of reporter genes of Smad2-responsive construct ARE/MF-1-luc was undertaken. HK-2 cells were cultured with BMP-7 50ng/ml for time points to 24h before addition of TGF-β1 1ng/ml for 1h and subsequent immunoblotting. No change in phospho-Smad2 was seen (Figure 3.6.A). Similarly, cells were transiently transfected with Smad2 reporter plasmids before incubation with BMP-7 at doses up to 2000ng/ml for 12 hours, and subsequent addition of TGF-β1 1ng/ml for 6h. No significant changes in reporter gene activity were seen (Figure 3.6 B and C).

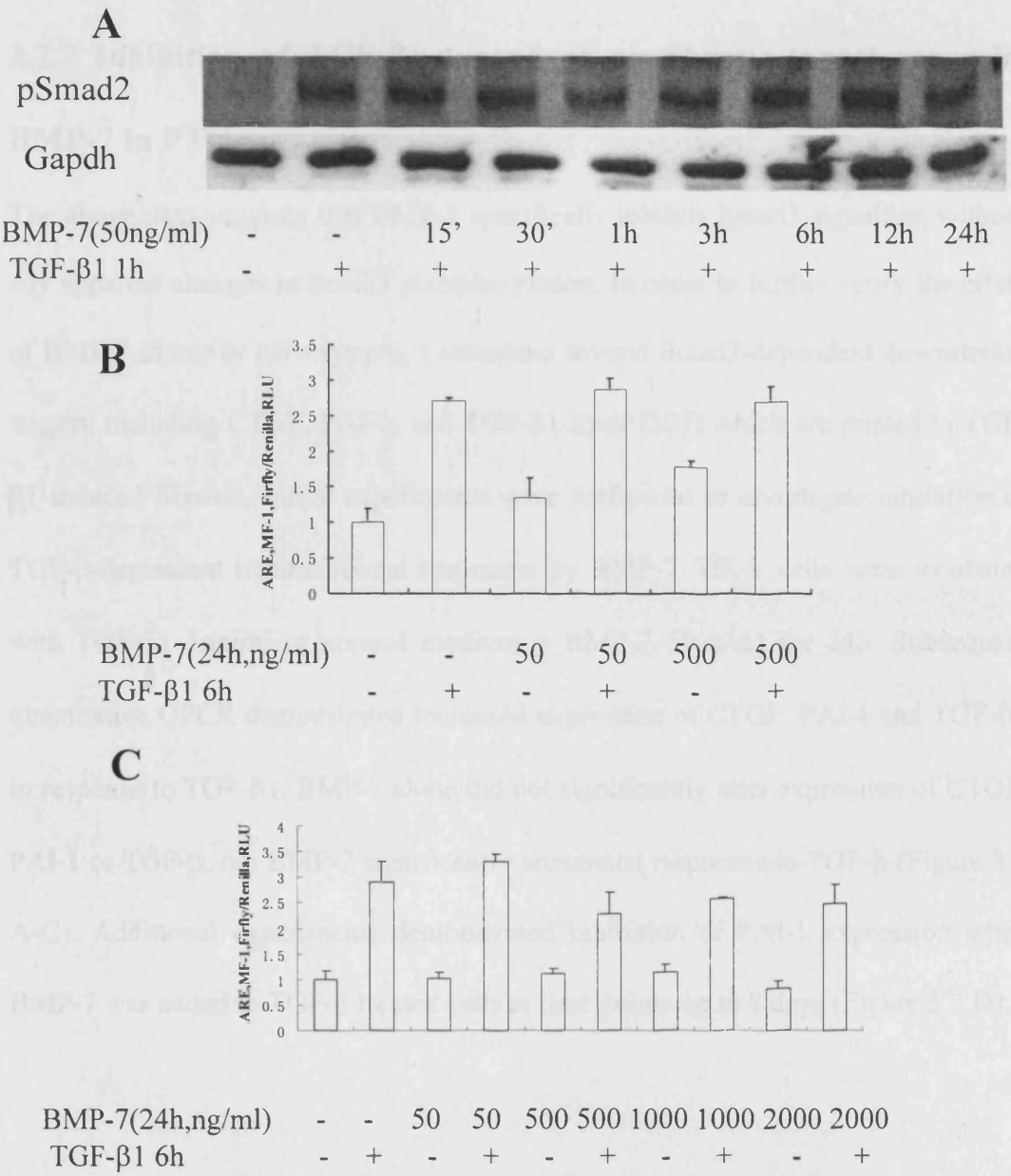


Figure 3.6: Effect of BMP-7 on Smad2 signalling

(A) HK-2 cells were treated with BMP-7 50ng/ml for times up to 24 hours before incubation with TGF-β1 1ng/ml for 1 hour. After immunoblotting for phospho-Smad2, blots were stripped and reprobed for Gapdh. One representative experiment of four separate experiments is shown. **(B,C)** HK-2 cells were transfected with Smad2 reporter construct ARE/MF-1, then incubated with BMP-7 for various doses before incubation with serum free medium or TGF-β1 1ng/ml under serum free conditions. $n = 3$, mean $\pm$ SEM is plotted. Subsequently, firefly and renilla luciferase activities were assayed. Ratio of firefly to renilla luciferase is displayed and normalized to control. One representative experiment of three separate experiments is shown.

3.2.7 Inhibition of TGF-β1-dependent profibrotic target genes by BMP-7 in PTC

The above data suggests that BMP-7 specifically inhibits Smad3 signalling without any apparent changes in Smad3 phosphorylation. In order to further verify the effect of BMP-7 in our *in vitro* system, I examined several Smad3-dependent downstream targets, including CTGF, PAI-1, and TGF-β1 itself [307] which are related to TGF-β1-induced fibrosis. Initial experiments were performed to investigate inhibition of TGF-β-dependent transcriptional responses by BMP-7. HK-2 cells were incubated with TGF-β1 1ng/ml or control medium ± BMP-7 50ng/ml for 24h. Subsequent quantitative QPCR demonstrated increased expression of CTGF, PAI-1 and TGF-β1 in response to TGF-β1. BMP-7 alone did not significantly alter expression of CTGF, PAI-1 or TGF-β, but BMP-7 significantly attenuated responses to TGF-β (Figure 3.7 A-C). Additional experiments demonstrated inhibition of PAI-1 expression when BMP-7 was added to TGF-β treated cells at time points up to 8 days (Figure 3.7 D).

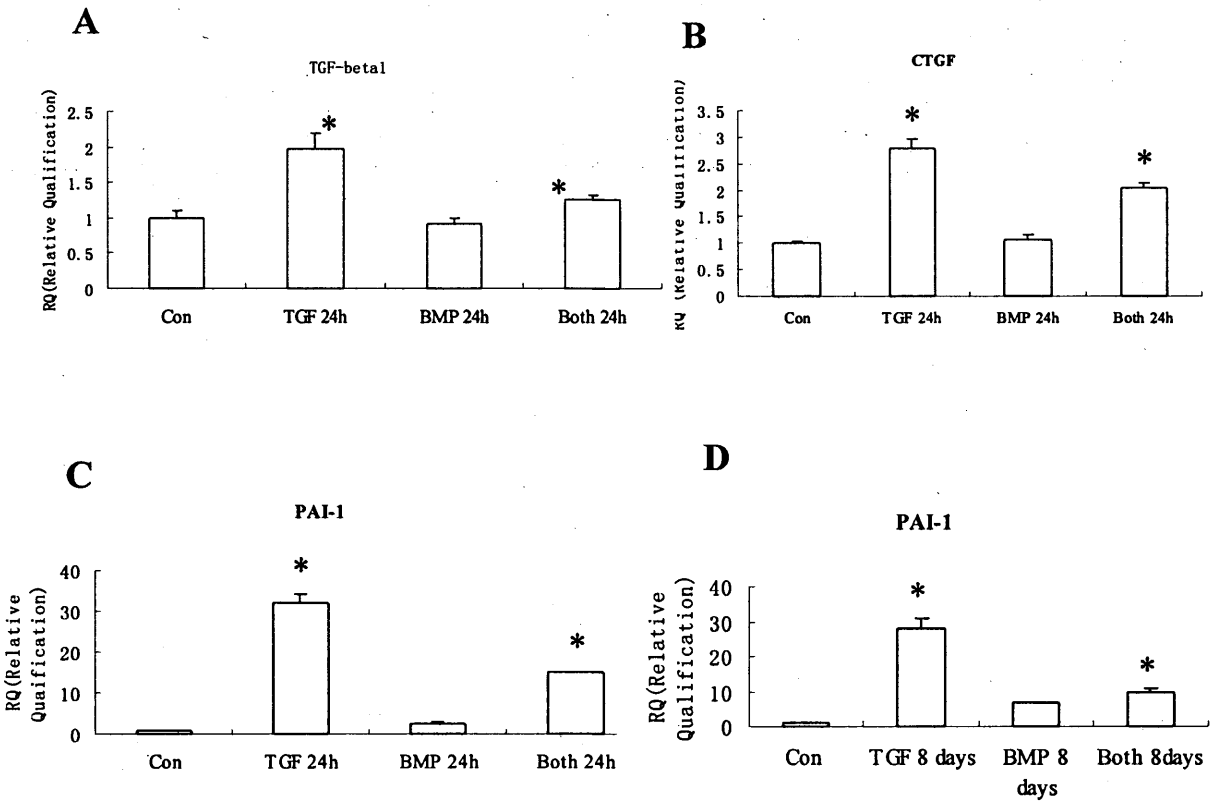


Figure 3.7 Regulation of profibrotic TGF-β1 transcriptional targets by BMP-7.

RT-qPCR analysis shows expression of profibrotic TGF beta-inducible genes in PTC after 24 hours ± 1ng/ml TGF-β1 incubation ± BMP-7 50ng/ml. (A) TGF-β1. (B) Connective Tissue Growth Factor. (C) Plasminogen Activator Inhibitor-1. (D) Plasminogen Activator Inhibitor-1 expression after 8 days of culture ± TGF-β1 1ng/ml ± BMP-7 50ng/ml. n=3, mean of duplicate determinations. mean ± SEM is plotted. * $P < 0.01$ TGF compared to control. * $P < 0.01$ both compared to TGF-β1 alone. Representative data from one of five experiments giving similar results.

3.3 Discussion

TGF-β1 is not only a key profibrotic cytokine leading to fibrosis in the kidney and other organs [56] but also plays an important role in repair following acute injury [216]. Acute inflammation is also associated with repair following injury, but more prolonged inflammation is associated with renal fibrosis [217, 218]. In this chapter, I have investigated the effects on TGF-β signalling of IL-1β and BMP-7 at various doses and times. I have found that short term IL-1β incubation inhibited TGF-β1 responsiveness, whereas chronic IL-1β incubation facilitated TGF-β1 responsiveness. In contrast, I found that BMP-7 served to limit Smad3 signalling response to TGF-β1, without apparent effect on Smad2 signalling. These observations have implications for our understanding of modulation of TGF-β1 signalling by these important signalling pathways in the kidney.

Part I: IL-1β

Previously, Ulloa *et al* have shown pro-inflammatory cytokine interferon gamma negatively regulates phosphorylation of Smad3 and its downstream events, namely, the association of Smad3 with Smad4, the Smad3 accumulation in the nucleus, and the activation of TGF-β-responsive genes in acute inflammation [219]. A number of *in vitro* studies support the inhibition of TGF-β signalling by IL-1β [227, 230, 308]. In contrast, recent work suggests that chronic inflammation may promote fibrosis by enhancing signalling responses to TGF-β1. Specifically, in the liver, bacterial lipopolysaccharide-mediated toll like receptor 4 (TLR4) activation sensitizes hepatic stellate cells to TGF-β1 signalling and leads to hepatic fibrogenesis [220]. Similar

TGF-β1 Smad3-associated effects are observed in PTC in which TLR2 is activated by leptospiral membrane proteins *in vitro* [221]. IL-1β also leads to lung fibrosis via the TGF-β Smad3 pathway [223]. In our lab, we have previously shown that another pro-inflammatory cytokine, IL-6, increases signalling response to TGF-β1 [309]. These studies indicate that IL-1β has different effects on TGF-β signalling in a cell-specific and context-specific manner, and that the participation of inflammation to the acute organ injury and chronic organ fibrosis through TGF-β signalling regulation is complicated.

With my work in this chapter, these experiments demonstrate that short-term IL-1β exposure from 15 minutes to 3 hour inhibits PTC TGF-β Smad3 phosphorylation rapidly and transiently by co-incubation with TGF-β1 by Western Blot. In keeping with this, data from the CAGA (4) Smad3 reporter gene assay shows that IL-1β inhibits Smad3 luciferase activity following 6 hours co-incubation with TGF-β1. In experiments examining Smad2, Western Blot shows an inhibition of Smad2 phosphorylation at 1 hour co-incubation, similar to Smad2 reporter gene assay at 6 hours co-incubation subsequently with various doses of IL-1β. After 6 h or more of IL-1β prolonged treatment, there is a switch from early inhibition to later stimulation on TGF-β Smad3 signalling by both approaches as well as Smad2. Taken together, these data are suggestive that IL-1β has an early inhibitory effect on TGF-β signalling, but with chronic stimulation this is reversed. This may highlight an important difference in the interaction of pro-inflammatory and TGF-β signalling in short term versus chronic inflammation, in that IL-1β may inhibit TGF-β1 dependant signalling in pathologies such as acute tubular necrosis, acute tubulointerstitial

nephritis and acute rejection following renal transplantation. In contrast, chronic IL-1β stimulation enhances TGF-β1 signalling, and may accelerate the progress of renal fibrosis in chronic kidney disease.

Part II: BMP-7

BMP-7 has emerged as a key anti-fibrotic cytokine in the kidney. BMP-7 expression and Smad1/5 phosphorylation are decreased in the acute ischemic kidneys and chronic kidney injury *in vivo* and *in vitro*. A lot of study suggests that exogenous BMP-7 can block ECM accumulation in chronic fibrosis as well as EMT. BMP-7 diminished the activation of tubulointerstitial inflammational monocyte/macrophage interstitial infiltration [247] and pro-inflammatory cytokines expression such as IL-6, IL-1β and ET-1 in PTC [246]. As for TGF-β1, a key profibrotic cytokine for renal fibrosis, BMP-7 inhibits nuclear accumulation of main signalling Smad3 response to TGF-β1 in mesangial cells. But little is known about the mechanism for TGF-β1 signalling regulation by BMP-7 in kidney diseases [260].

In terms of R-Smads, regulatory mechanisms and transcriptional responses may vary between Smad2 and Smad3 [310-312]. Some work has shown that the endogenous ratio of Smad2 and Smad3 influences the cytostatic function of Smad3 *in vitro* [311]. Also, several proteins interacting with R-Smads balances final Smad2/3 specific transcriptional initiation. The adaptor protein embryonic liver fodrin (ELF) interacts with receptor-associated Smad3/Smad4 complex, but not Smad2, after TGF-β1 stimulation. This interaction facilitates nuclear translocation of Smad3/4 and subsequent TGF-β1 transcriptional responses [313]. In contrast, TRAP-1-like protein

(TLP) differentially activates TGF-β1 Smad2-dependent responses and blocking Smad3-dependent transcription accomplished by inhibiting Smad3/Smad4 complex formation [300]. A key observation is that the Smad3 null mouse is protected from TGF-β-dependent induction of c-Jun, collagen, and inflammatory cell infiltrates, as well as autoinduction of TGF-β, indicating Smad3 plays an important role in fibrosis [215]. My work in this chapter shows that BMP-7 inhibits TGF-β Smad3 signalling in PTC without apparent effect on Smad3 phosphorylation, or on Smad2 signalling.

It was important in view of this apparently subtle effect of BMP-7 in PTC to check that BMP-7 had measurable effects on TGF-β-dependent responses, therefore I did QPCR to confirm this. Some work has shown BMP-7 reverses TGF-β1-induced EMT transition [44], extracellular matrix proteins, CTGF and PAI-1 [260, 314]. I also did QPCR to check the effect of BMP-7 on TGF-β1-induced profibrotic transcriptional target genes. All these data intimate that in PTC, BMP-7 specifically limits Smad3 signalling in PTC, without alteration in Smad2 signalling, leading to reduced activation of pro-fibrotic gene targets by TGF-β1, including PAI-1, CTGF and TGF-β1 itself.

In conclusion, my results show that IL-1β causes an early inhibition but a later stimulation of Smad2/3 signalling. In contrast, BMP-7 inhibits Smad3-dependent but not Smad2-dependent transcription without altering Smad3 phosphorylation levels.

Chapter Four:

The effects of HA-CD44 system on TGF-β1 signalling in human proximal tubular epithelial cells

4.1 Introduction

In chapter three, my results have shown the biphasic effects of IL-1β and inhibitory effects of BMP-7 on TGF-β1-induced Smad signalling. In our lab, the previous work indicated CD44 is co-localized with TGF-β1 type I receptor in the cytoplasmic membrane of PTC, leading to altered TGF-β receptor distribution and association. We have previously shown that in PTC, TGF-β receptor distribution in the plasma membrane is regulated by stimuli including HA (via CD44) and IL-6, leading to changes in receptor degradation and signalling response to TGF-β1.

As the ligand of CD44, HA attenuates TGF-β1 signalling in PTC [156]. Whereas in breast cancer cells, HA promoted TGF-β1 signalling via interaction between CD44 and type I receptor oppositely [289]. This contradiction indicated that the regulation of TGF-β/ TGF-β receptor and HA/CD44 contributes to the different biological functions in diverse cell contexts. In this chapter, the aims were to observe the possible roles of HA-CD44 system in the TGF-β1 signalling regulation by IL-1β and BMP-7.

Part I:

Previous finding demonstrated the upregulation of HAS gene expression and subsequently HA synthesis in culture medium in PTC in response to prolonged IL-1β treatment after 12 hours [294]. In breast cancer cells, HA promoted TGF-β1 signalling. Therefore I examined the potential role of HA-CD44 on the stimulatory effect of IL-1β on TGF-β1 signalling. I also investigated the possibility that IL-1β

might alter CD44 isoform expression in PTC, specifically whether it led to enhanced CD44v3 expression, as this has been linked to enhanced TGF-β signalling downstream of HA.

Part II: BMP-7

Previous work has found BMP-7 inhibits monocyte-stimulated TGF-β1 generation in PTC via disruption of HA-CD44 interaction [315]. Therefore, at the start of my work, I hypothesized that changes in HA/CD44 might help define PTC responses to TGF-β1, and might underline observed alterations in Smad signalling consequent on IL-1β and BMP-7.

In conclusion, the aims in this chapter were to investigate the potential role of changes in HA/CD44 interaction in the effects of IL-1β and BMP-7 on TGF-β1 signalling that I had already demonstrated.

4.2 Results

4.2.1 IL-1β induces PTC CD44 expression, but this is not involved in the modulation of TGF-β1 signalling by IL-1β

Previous work in our lab has shown that interaction of HA with CD44 decreases PTC sensitivity to TGF-β1 by augmenting receptor degradation. In a breast cancer cell line, in contrast, following HA stimulation, CD44v3 binds to and activates the TGF-β1 type I receptor and activates Smad signalling. Additionally, IL-1β induces PTC HA synthesis after 12h treatment. Therefore, I examined the potential role of HA/CD44 in augmentation of TGF-β1 signalling by IL-1β.

Firstly, changes in PTC CD44 expression were examined by FACS after HK-2 cells were treated with 1ng/ml IL-1β from 15 minutes to 24 hours. IL-1β increased cell surface CD44 protein expression by 24h (Figure 4.1). Then I examined CD44 protein expression at 24 hours with 1ng/ml and 10ng/ml IL-1β treatment. However, there is no significant change between 1ng/ml and 10ng/ml IL-1β stimulation (Figure 4.2A and B). Also, no difference in CD44 isoform v3 expression was seen (Figure 4.2C).

Next, I studied the effect of reduced CD44 expression on Smad signalling, and on the modulation of Smad signalling by IL-1β, using knockdown of CD44 with siRNA. Preliminary experiments showed greater than 90% knockdown of CD44 mRNA from 24-72 post-transfection (QPCR), and significant decrease in cell surface CD44 expression detectable from 24-72h post-transfection by FACS. HK-2 cells were

sequentially transfected with CD44 siRNA and the Smad3 reporter CAGA (4), as described in the materials and methods. In parallel control experiments, cells were transfected with scrambled control siRNA. However, increased Smad signalling response was still seen in CD44 knockdown cells following IL-1β pre-treatment (Figure 4.3). (Scrambled siRNA group, IL-1β+ TGF-β1 group, VS TGF-β1 group, *$p<0.01$; CD44 siRNA group, IL-1β+ TGF-β1 group VS TGF-β1 group, #$p<0.05$). Smad3 phosphorylation was also examined by immunoblotting following CD44 knockdown. Following CD44 siRNA transfection, cells were cultured in medium containing IL-1β 1ng/ml, or control medium, for 24h before change to TGF-β1 1ng/ml for 60 minutes. Cells were lysed, and phospho-Smad3 detected by immunoblotting. In parallel control experiments, cells were transfected with scrambled siRNA before culture as above. Enhanced Smad3 phosphorylation was still detectable in cells with CD44 knockdown following treatment with IL-1β (Figure 4.4).

To investigate the role of HA in the effects of IL-1β on Smad signalling, I used reporter gene analysis to examine Smad3 signalling response in IL-1β pre-treated cells in the presence of HA and hyaluronidase. Cells were transfected with CAGA (4) reporter gene, then treated with IL-1β for 24 hours. HA was added in the culture medium or removed by treatment with bovine testicular hyaluronidase for 15 minutes. Both had no effect on sensitisation by IL-1β for Smad3 signalling (Figure 4.5 and 4.6).

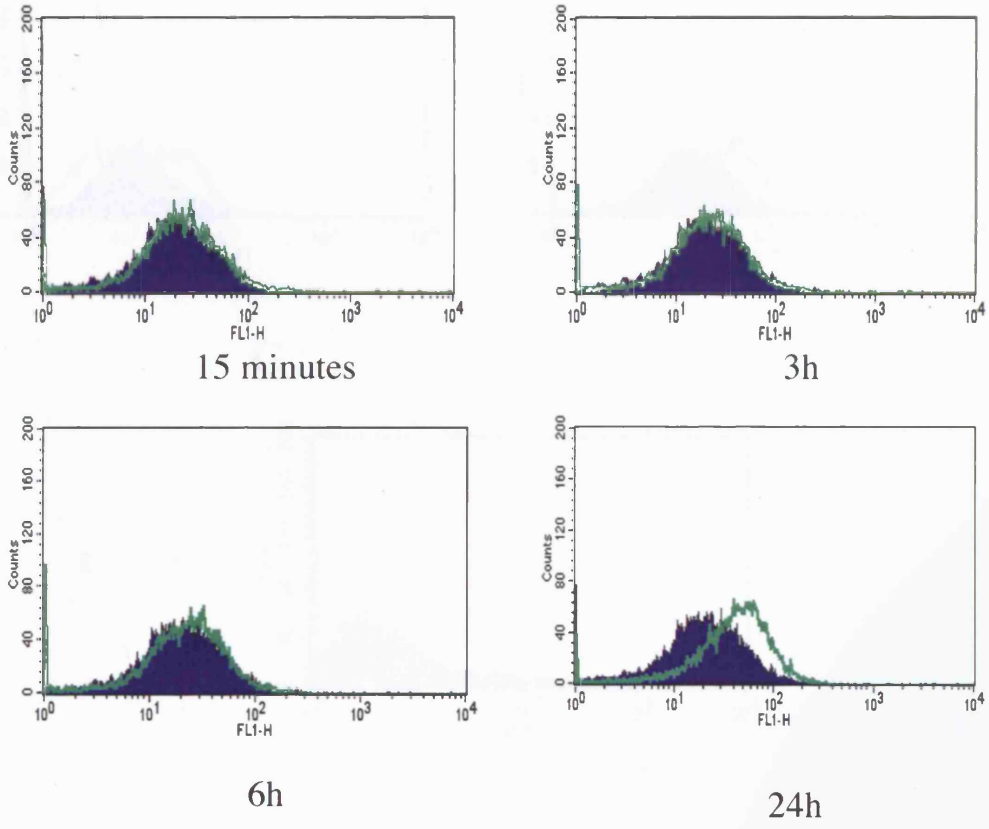
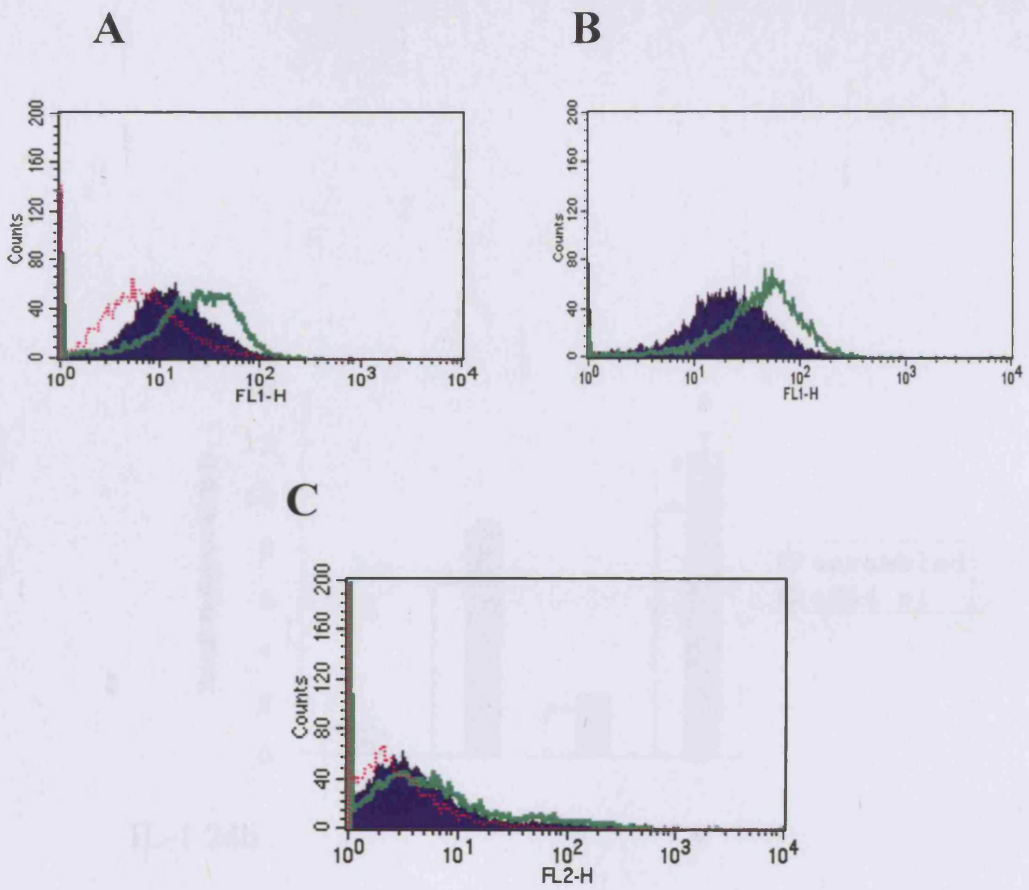


Figure 4.1 Time course of CD44 expression by IL-1 β.

IL-1 β was added to confluent monolayers of growth arrested HK-2 cells under serum free conditions. HK-2 cells were treated with IL-1 β 1ng/ml from 15 minutes to 24 hours. Cell surface expression of CD44 was analysed by FACS. Blue is control cells, green is IL-1β treated cells. One representative experiment of three separate experiments is shown.



S-AB only (pink) vs. Control (blue) vs. IL-1β (green)

Figure 4.2 Expression of CD44 and CD44v3 by IL-1β.

IL-1 β was added to confluent monolayers of growth arrested HK-2 cells under serum free conditions. HK-2 cells were treated with 1ng/ml **(A and C)** and 10ng/ml **(B)** IL-1β for 24 hours. Cell surface expression of CD44 **(A and B)** and CD44v3 **(C)** were analysed by FACS. Pink is secondary antibody only as a negative control, blue are control cells without IL-1β treatment, green are IL-1β treated cells. One representative experiment of three separate experiments is shown.

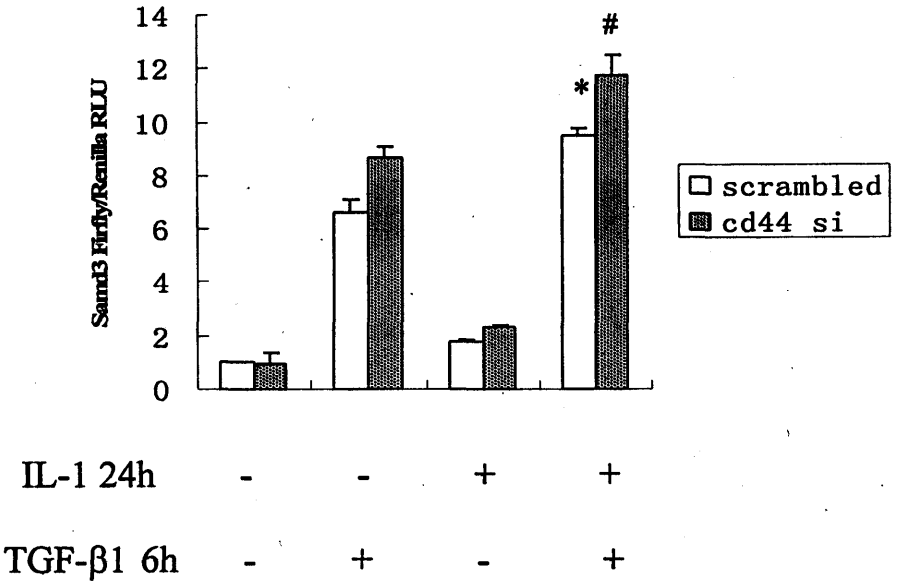


Figure 4.3 Effect of IL-1β on Smad3 signalling by CD44 knockdown.

HK-2 cells were transfected with CD44 siRNA for 24h, parallel with scrambled si RNA, then transfected Smad3 reporter construct CAGA(4)) together with pRL-CMV renilla. After incubation with control medium or IL-1β 1ng/ml for 24h, then incubated with control medium or 1ng/ml TGF-β1 for 6 hours. Results represent mean ± SEM of 3 individual experiments. $^{*}P < 0.01$ compared to TGF- β1. $^{\#}P < 0.05$ compared to TGF- β1. One representative experiment of three separate experiments is shown.

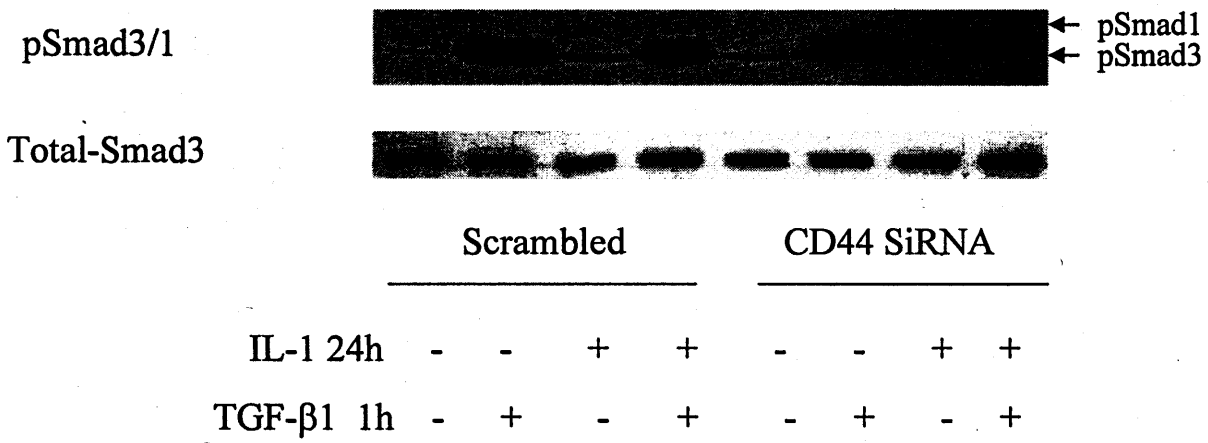


Figure 4.4 Effect of IL-1β on Smad3 signalling by CD44 knockdown.

HK-2 cells were transfected with CD44 siRNA for 24h, parallel with scrambled si RNA, then for another 24 hours under serum free. After incubation with control medium or IL-1 β 1ng/ml for 24h, then incubated with control medium or 1ng/ml TGF- β1 for 1 hour. The whole lysats were immunoblotted by phospho-Smad3. Subsequently, blots were stripped and reprobed for total-Smad3 as a loading control. One representative gel of three experiments is shown.

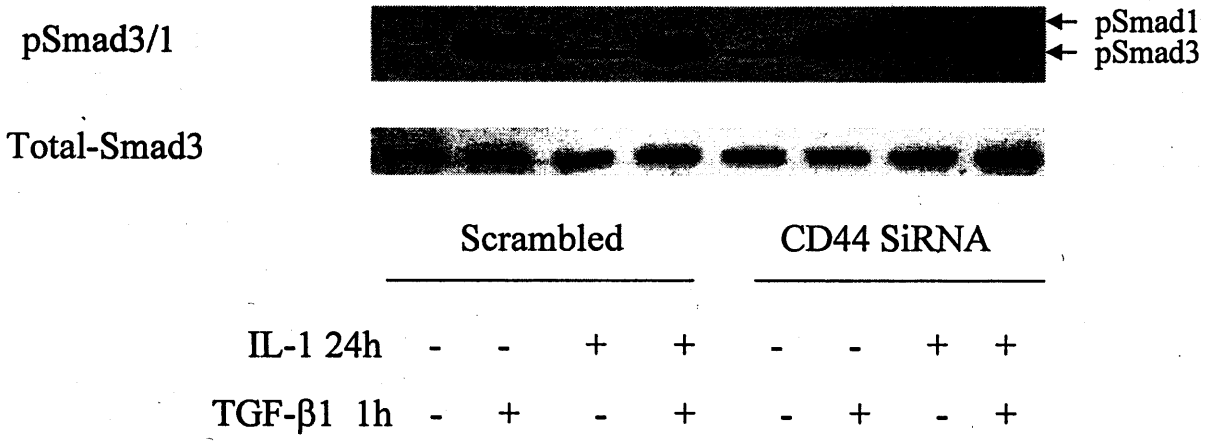


Figure 4.4 Effect of IL-1β on Smad3 signalling by CD44 knockdown.

HK-2 cells were transfected with CD44 siRNA for 24h, parallel with scrambled si RNA, then for another 24 hours under serum free. After incubation with control medium or IL-1 β 1ng/ml for 24h, then incubated with control medium or 1ng/ml TGF- β1 for 1 hour. The whole lysats were immunoblotted by phospho-Smad3. Subsequently, blots were stripped and reprobed for total-Smad3 as a loading control. One representative gel of three experiments is shown.

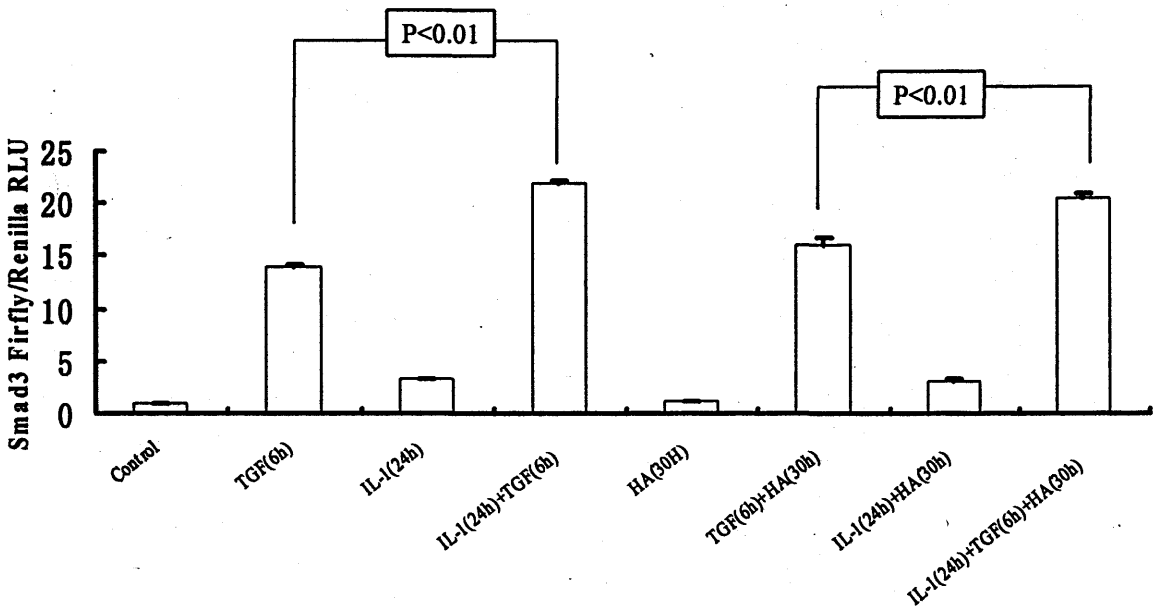


Fig 4.5 Effect of HA on IL-1β-induced Smad3 signalling augmentation.

HK-2 cells were transfected with Smad3 CAGA(4) and pRL-CMV renilla reporter constructs. After incubation with control medium or IL-1β 1ng/ml for 24h, then incubated with control medium or 1ng/ml TGF- β1 for 6 hours with or without HA all through the whole time. Data is presented as firfly/renilla luciferase activity, normalized to control. $N = 3$, mean ± SEM is plotted of three separate experiments is shown.

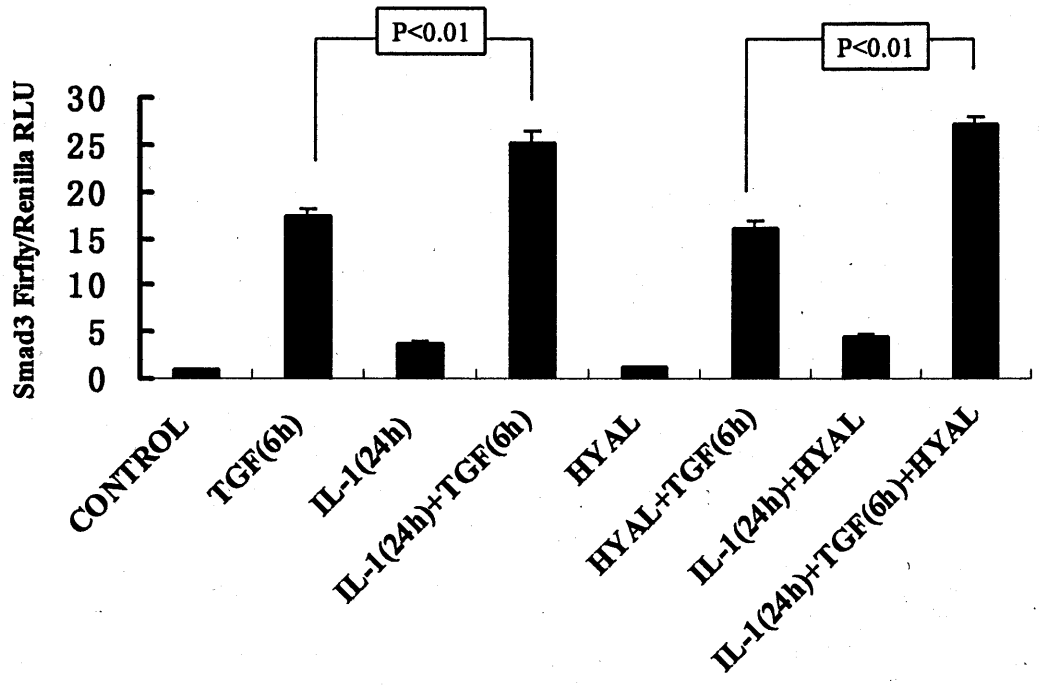


Fig 4.6 Effect of HYAL on IL-1β-induced Smad3 signalling augmentation.

HK-2 cells were transfected with Smad3 CAGA(4) and pRL-CMV renilla reporter constructs. After incubation with control medium or IL-1β 1ng/ml for 24h,then incubated with control medium or 1ng/ml TGF- β1 for 6 hours with or without HYAL to remove the coated-HA all through the whole time. Data is presented as firfly/renilla luciferase activity, normalized to control. $N = 3$, mean ± SEM is plotted of three separate experiments is shown.

4.2.2 BMP-7 has no effect on PTC CD44 expression, and disruption of CD44-TGF-β1 receptor type I receptor interaction is not involved in the reduction of TGF-β1 signalling by BMP-7

Interaction of HA decreases PTC sensitivity to TGF-β1 by augmenting receptor degradation via CD44. In my experiments, disruption of hyaluronan-CD44 interactions had little effect on the TGF-β responses; however, re-establishing CD44-hyaluronan ligation promotes a robust cellular response to BMP-7 in articular chondrocytes [316]. Thus, I examined the hypothesis that interaction of HA/CD44 is involved in the inhibition of TGF-β1 Smad3 signalling by BMP-7.

Similar to IL-1β, changes in PTC CD44 expression were examined by FACS before HK-2 cell was treated with 50ng/ml and 500ng/ml BMP-7 from 15 minutes to 24 hours. BMP-7 had no detectable effect on regulation of cell surface CD44 protein expression (Figure 4.7 and 4.8). In subsequent experiments, HA-CD44 association was interrupted by employing CD44 siRNA to reduce CD44 expression. HK-2 cells were sequentially transfected with CD44 siRNA and the Smad3 reporter CAGA (4), along with a parallel transfection of CD44 siRNA and Smad3 reporter gene. The result showed that the inhibition of BMP-7 on Smad3 signalling still remains with disruption HA-CD44 interaction (Figure 4.9). (Scrambled siRNA group, BMP-7+ TGF-β1 group VS TGF-β1 group, *$p<0.01$; CD44 siRNA group, BMP-7+ TGF-β1 group VS TGF-β1 group #$p<0.01$.) Smad3 phosphorylation was examined by immunoblotting following CD44 knockdown. Following CD44 siRNA transfection, cells were cultured in medium containing BMP-7 50ng/ml, or control medium, for

24h before change to TGF-β1 1ng/ml for 60 minutes. In parallel control experiments, cells were transfected with scrambled siRNA before culture as above. Enhanced Smad3 phosphorylation was still detectable in cells with CD44 knockdown following treatment with BMP-7 (Figure 4.10).

To further evaluate the role of HA in the effects of BMP-7 on Smad 3 signalling, I used reporter gene analysis to examine Smad3 signalling response in BMP-7 pre-treated cells in the presence of HA. Cells were transfected with CAGA (4) reporter gene, then treated with 50ng/ml BMP-7 for 24 hours before change to TGF-β1 1ng/ml for 60 minutes with or without HA in the culture medium. Similar to CD44, the effect of BMP-7 on TGF-β1 Smad3 signalling is not changed by adding in HA (Figure 4.11).

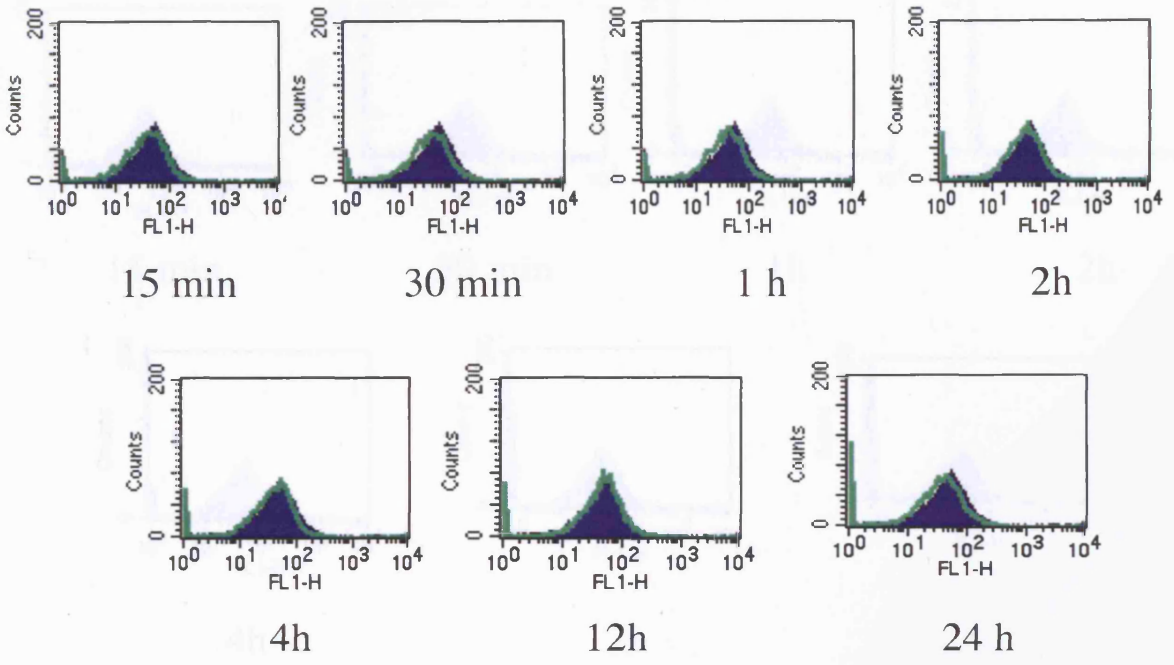


Figure 4.7 Time course of CD44 expression by BMP-7.

BMP-7 was added to confluent monolayers of growth arrested HK-2 cells under serum free conditions. HK-2 cells were treated with BMP-7 50ng/ml from 15 minutes to 24 hours. Cell surface expression of CD44 was analysed by FACS. Blue is control cells, green is BMP-7 treated cells. One representative experiment of three separate experiments is shown.

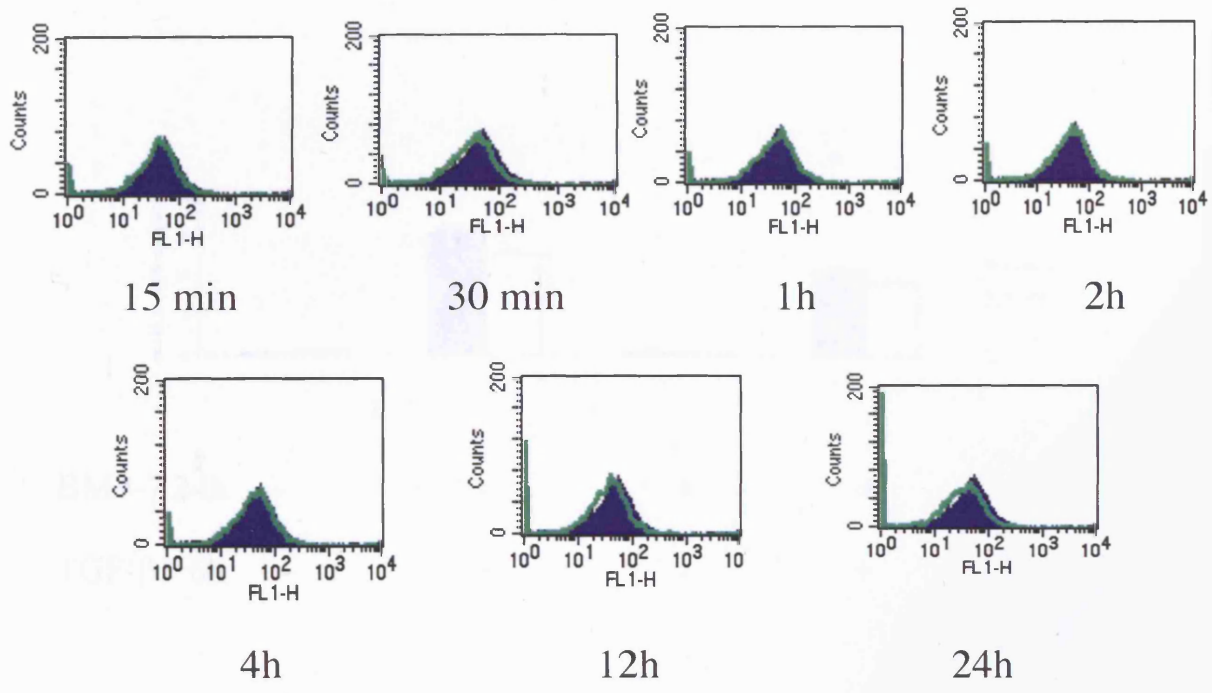


Figure 4.8 Time course of CD44 expression by BMP-7.

BMP-7 was added to confluent monolayers of growth arrested HK-2 cells under serum free conditions. HK-2 cells were treated with BMP-7 500ng/ml from 15 minutes to 24 hours. Cell surface expression of CD44 was analysed by FACS. Blue is control cells, green is BMP-7 treated cells. One representative experiment of three separate experiments is shown.

BMP-7 24h	-	-	+	+
TGF-β1 6h	-	+	-	+

Figure 4.9 Effect of BMP-7 on Smad3 signalling by CD44 knockdown.

HK-2 cells were transfected with CD44 siRNA for 24h, parallel with scrambled si RNA, then transfected Smad3 reporter construct CAGA(4)) together with pRL-CMV renilla. After incubation with control medium or BMP-7 50ng/ml for 24h, then incubated with control medium or 1ng/ml TGF- β1 for 6 hours. Results represent mean ± SEM of 3 individual experiments. *$P < 0.01$ compared to TGF-β1. #$P < 0.01$ compared to TGF- β1. One representative experiment of three separate experiments is shown.

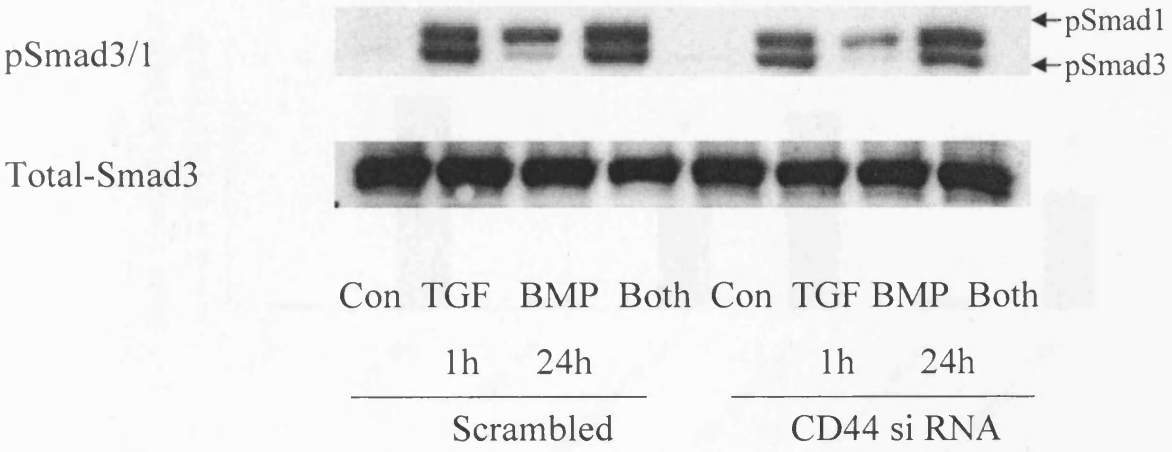


Figure 4.10 Effect of BMP-7 on Smad3 phosphorylation by CD44 knockdown.

HK-2 cells were transfected with CD44 siRNA , parallel with scrambled si RNA for 24 hours, then with fresh serum free medium for another 24 hours . After that, the cells were incubated with control serum free medium or BMP-7 50ng/ml for 24h, then incubated with control medium or 1ng/ml TGF-β1 for 1 hours. The whole lysates were immunoblotted by phospho-Smad3. Subsequently, blots were stripped and reprobed for total-Smad3. One representative gel of three experiments is shown.

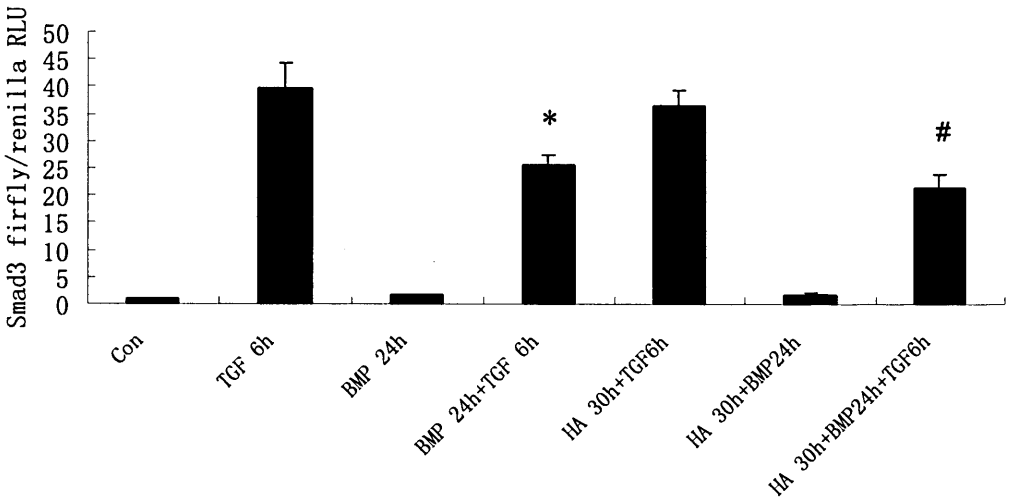


Fig 4.11 Effect of HA on BMP-7-induced Smad3 signalling reduction.

HK-2 cells were transfected with Smad3 CAGA(4) and pRL-CMV renilla reporter constructs. After incubation with control medium or BMP-7 50ng/ml for 24h,then incubated with control medium or 1ng/ml TGF- β1 for 6 hours with or without HA all through the whole time. $*P < 0.01$ compared to TGF- β1. $\#P < 0.05$ compared to TGF- β plus HA. Results represent mean ± SEM of 3 individual experiments, normalized to control. One representative gel of three experiments is shown.

4.3 Discussion

TGF-β1 is a key stimulus leading to interstitial fibrosis and hence to progression of renal fibrosis after renal injury. It elicits deposition of ECM by decreasing the synthesis of proteases and by increasing the levels of protease inhibitors [317].

HA is a ubiquitous connective tissue polysaccharide which *in vivo* is present as a high molecular mass component of ECM in normal kidney [270]. In diverse renal diseases, it is expressed around PTC following renal injury. Previous data have demonstrated that HA activates the MAPK pathway through binding to its receptor CD44, enhancing PTC migration [318].

There are two forms of HA distribution on HK-2 cell surface. One is diffusely arranged HA over the cell surface. HA is also found in cable-like structures that span several cell lengths [294, 315]. IL-1β increases the general rate of HA synthesis in HK-2 cells, whereas BMP-7 induces formation of HA cable-like structures [315]. Previous studies have indicated that formation of these cables in response to BMP-7 inhibits subsequent TGF-β1 generation in PTC bound by monocytes [261].

In a malignant breast epithelial cell line, HA binding to CD44v3 induces Smad signalling via direct Smad phosphorylation, dependent on an interaction between the variable stalk regions of CD44 coded for by the V3 exon. In contrast, in PTC, the work in our lab has previously shown that CD44v3 expression is low [294], and that HA binding to CD44 downregulates signalling response to TGF-β1 [156]. This led me to study possible changes in CD44v3 expression in PTC in response to IL-1β,

and the potential role of CD44 in IL-1β mediated changes in TGF-β1 signalling. In my results, IL-1β induced CD44 expression in PTC, but had little effect on CD44v3 expression. Knockdown of CD44, IL-1β still augmented Smad signalling response following CD44 knockdown, suggesting that changes in CD44 expression are not responsible for the alterations in TGF-β1 signalling in response to IL-1β.

In parallel experiments, HA was added in the culture medium or limited hyaluronidase digestion removed the cable-like HA structures. My data showed that both treatments did not alter modulation of TGF-β1 signalling by IL-1β.

Previous study showed that Smad1 interacts with the cytoplasmic domain of the hyaluronan receptor CD44 in chondrocytes, suggesting a functional link between the BMP-7 signal cascade and CD44 [291]. Moreover, their further study found HA promoted the chondrocytes response to BMP-7 by enhanced Smad1 phosphorylation and nuclear translocation, but not TGF-β1 [316]. This led me to study the potential mediation of HA-CD44 on BMP-7 reduction upon TGF-β1-induced Smad3 signalling.

Firstly, CD44 expression was examined and showed there was no change on CD44 expression from 15 minutes to 24 hours at 50ng/ml and 500ng/ml BMP-7 by FACS. Following CD44 knockdown to disrupt the CD44-TGF-β1 receptor I interaction, BMP-7 still diminished Smad3 signalling response, suggesting that CD44 is not required for the alterations in TGF-β1 signalling in response to BMP-7. Nystatin can disrupt cholestrerol, then preventing HA-mediated shift of TGF-β receptor into the raft fraction to inhibit signalling initiation [288]. Since the previous data implied

BMP-7 induces HA cable-like structures formation, I have used nystatin to examine if HA-medicated TGF-β receptor shift blocking is involved in BMP-7 effect, but the results are not consistent. Together with exogenous HA in the culture medium, BMP-7 reduction on TGF-β1 Smad3 signalling still remained. Above all, these findings indicate that HA-CD44-TGF-β type I receptor interaction is not implicated in TGF-β1 signalling regulation by IL-1β and BMP-7.

Taken together, in this chapter I have observed the HA/CD44 system effect on the TGF-β1 signalling regulation by IL-1β and BMP-7 in PTC. It showed HA/CD44 is not involved in Smad signalling regulation by either stimulus. Next, I further investigated the mechanisms underlying the results I obtained in chapter three.

Chapter Five:

The mechanisms of IL-1 β early inhibition and late stimulation on TGF-β1 signalling

5.1 Introduction

In the chapter three, my findings indicate that IL-1β has two distinct effects on TGF-β1 signalling: early inhibition and later a switch to enhancement of TGF-β1 signalling in PTC. In this chapter, I set out to examine potential mechanisms by which IL-1β might induce these different long-term and short-term effects, and hence might play a significant role in determining proximal tubular cell fate following renal injury.

IL-1β nuclear factor-κB (NF-κB) signalling pathway

On activation of the cell surface type I IL-1β receptor (IL-1β R) by IL-1β, a cascade of signalling events is initiated, leading to nuclear factor-kB (NF-κB) activation, which in turn eventually activates transcription of proinflammatory genes and regulates multiple aspects of cell survival, proliferation, and differentiation [319]. The NF-κB proteins family includes NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (also called p65), c-Rel, and RelB, all of which are characterized by an N-terminal Rel homology domain (RHD) responsible for homo- and heterodimerization as well as for sequence-specific DNA binding [320].

p65, c-Rel, and RelB also contain a C-terminal transcription activation domain (TAD), whereas the p52 and p50 rely on interactions with other factors to positively regulate transcription [321]. RelB preferentially heterodimerizes with p52 precursor, p100 [322] as well as p52 [323, 324], but p65 and c-Rel subunits predominantly heterodimerize with p50 [325]. The well studied major pathway by most stimuli is

the canonical NF-κB signalling pathway, which mainly depends on p65:p50 and c-Rel: p50 heterodimers [320].

Regulation of NF-κB signalling pathway

IκB proteins are the most important NF-κB-interacting proteins inhibiting NF-κB [321, 325]. IκBs including IκBα, IκBβ, and IκBε, which are classical IκBs, as well as a few "novel" IκB-like proteins, which include B cell CLL/lymphoma 3 (BCL3), IκBζ, and IκBNS, retain NF-κB dimers inactive in the cytoplasm of nonstimulated cells. Extracellular stimuli can trigger the phosphorylation, ubiquitylation and, subsequently degradation of IκB thereby releasing the NF-κB complexes. The IκB degradation allows activated NF-κB proteins to enter the nucleus and act as transcription factors to regulate a wide range of target genes expression [326-329].

The degradation of IκB is an essential step for releasing NF-κB and exerting its subsequent activation [321, 325]. A crucial regulatory step in this process is the IKK (IκB kinase) complex, consisting of several proteins — the main ones being IKKα, IKKβ and the regulatory subunit NF-κB essential modulator (NEMO; also known as IKKγ). IKK -induced phosphorylation of IκB occurs at specific amino-terminal serine residues (such as Ser32 and Ser36 for IκBα). After that, the phosphorylated IκBα is ubiquitylated at Lys21 and Lys22 by β-TRCP (β-transducin repeat-containing protein), which targets it for degradation by the 26S proteasome[330].

NF-κB is an important downstream effector for IL-1β and has been linked to inhibition of TGF-β signalling [230, 305]. The major form of NF-κB exists as a

heterodimer around p50 and p65 initiated by different pro-inflammatory cytokines, including interleukins and tumour necrosis factor-α (TNF-α) [331, 332]. NF-κB has previously been shown to inhibit TGF-β1-induced gene expression via sequestration of the transcriptional coactivator p300 by p65 NF-κB [333]. Further, NF-kB subunit of RelA (also named p65) is necessary for the inhibition of TGF-β1-induced Smads phosphorylation, nuclear translocation, and DNA binding by TNF-α [305].

IKK phosphorylation leading to degradation of IκB is the main regulation axis, which is an essential process for freeing NF-κB and initiating its subsequent activation [321, 325]. It has been well known that IκBα regulates transient NF-κB activation whereas IκBβ maintains persistent NF-κB activation [334]. Thus, as well as measuring NF-κB activation directly, in this chapter I observed the IκBα, IκBβ expression as well as IKK phosphorylation following short and long-term exposure to IL-1β. In terms of further mechanisms by which p65 antagonizes TGF-β1-induced Smads signalling by TNF-α, previous work has implicated up-regulation of Smad7 synthesis and therefore more stable association between ligand-activated TGF-β1 receptors and Smad7 [305]. In human chondrocytes, IL-1β prevents TGF-β1 response by downregulating TGF-β1 R II and up-regulating Smad7 via NF-κB/p65 pathway [228, 229]. Therefore, I have also studied Smad7 and TGF-β1 R I expression responses to IL-1β in PTC.

In chapter three, independent of the early inhibition of TGF-β1 signalling by IL-1β, I have shown that in PTC, long term IL-1β stimulation leads to augmentation of TGF-β1-induced Smad2 and Smad3 signal, and detectable Smad3 phosphorylation in the absence of exogenous TGF-β1. My results show the augmentation of IL-1β on TGF-

β1 signalling is dependent on TGF receptor/ligand interaction without altering TGF-β1 synthesis or Smad3 dephosphorylation. These outcomes suggest IL-1β enhances TGF-β1-induced Smad signalling at or before the level of TGF-β1 receptor-ligand interaction. Previous work has indicated IL-6 leads to enhancement of TGF-β1 signalling via a shift of type I receptors from lipid rafts to non-raft associated plasma membrane [309]. Thus, I assessed IL-6 generation by IL-1β treatment in PTC.

In conclusion, aims in this chapter were:

1 To characterize the mechanism of early inhibition effect of IL-1β on TGF-β1 signalling

2 To identify the mechanism of late stimulation effect of IL-1β on TGF-β1 signalling

5.2 Results

5.2.1 Early inhibition of NF-κB activity reverses the inhibitory effect on TGF-β1 Smad3 signalling by IL-1β

HK-2 cells were stimulated for time points to 24h with IL-1β 1ng/ml before cell lysis and cellular protein extraction. Then I detected Smad7 and TGF-β1 R I expression by immunoblotting. In HK-2 cells, the data indicates that neither TGF-β1 nor IL-1β stimulation up to 24 hours regulates Smad7 and TGF-β1 R I expression (Figure 5.1 and 5.2).

Since NF-κB is an important downstream effector for IL-1β and has been shown to inhibit TGF-β1 signalling, the effect of NF-κB inhibition on modulation of Smad signalling by IL-1β was investigated using the peptide inhibitor SN50, previously shown to be effective at preventing NF-κB signalling in HK-2 cells [335]. Blockade of NF-κB with SN50 prevented the inhibitory effect of IL-1β on TGF-β1 Smad3 signalling, assayed by immunoblotting (Figure 5.3 A). In related experiments, HK-2 cells transfected with CAGA(4) and renilla vectors were treated with TGF-β1+ IL-1β ±SN50 for 6 h. Inhibition of reporter gene activity by IL-1β was blocked by SN50 (Figure 5.3 B).

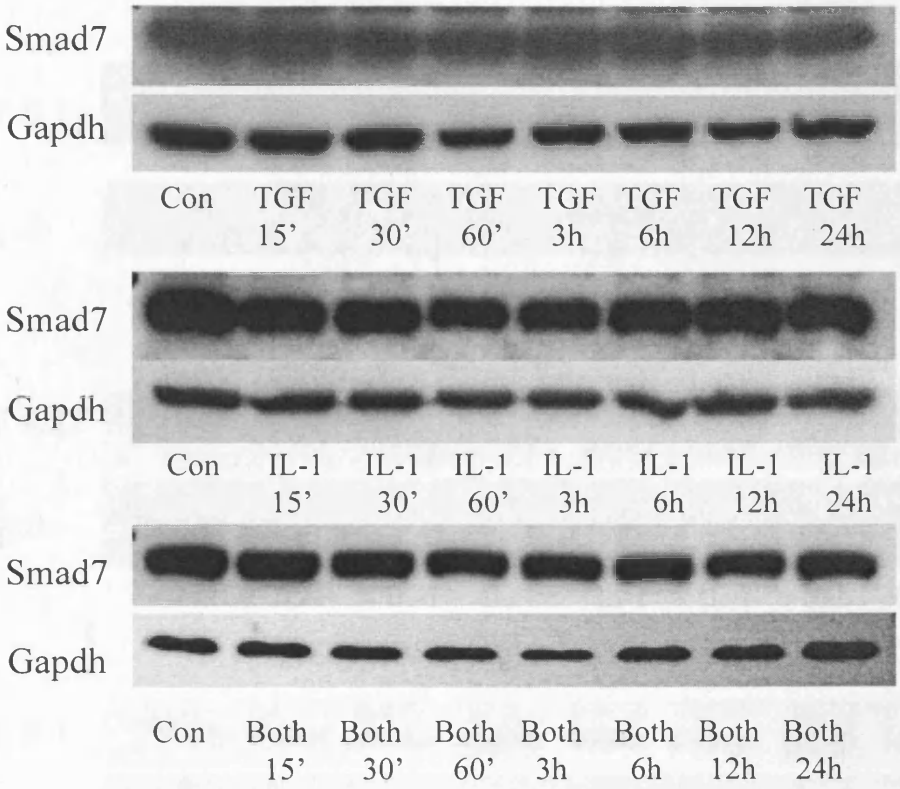


Figure 5.1: Time course of Smad7 expression by IL-1β ± TGF-β1

IL-1β and TGF-β1 were added to confluent monolayer of growth arrested HK-2 cells under serum free conditions. HK-2 cells were co-treated with 1ng/ml TGF-β1 ± 1ng/ml IL-1β for time points from 15 minutes to 24 hours, before protein extraction and immunoblotting for Smad7. Blots were stripped and reprobed for Gapdh. One representative experiment of three experiments is shown.

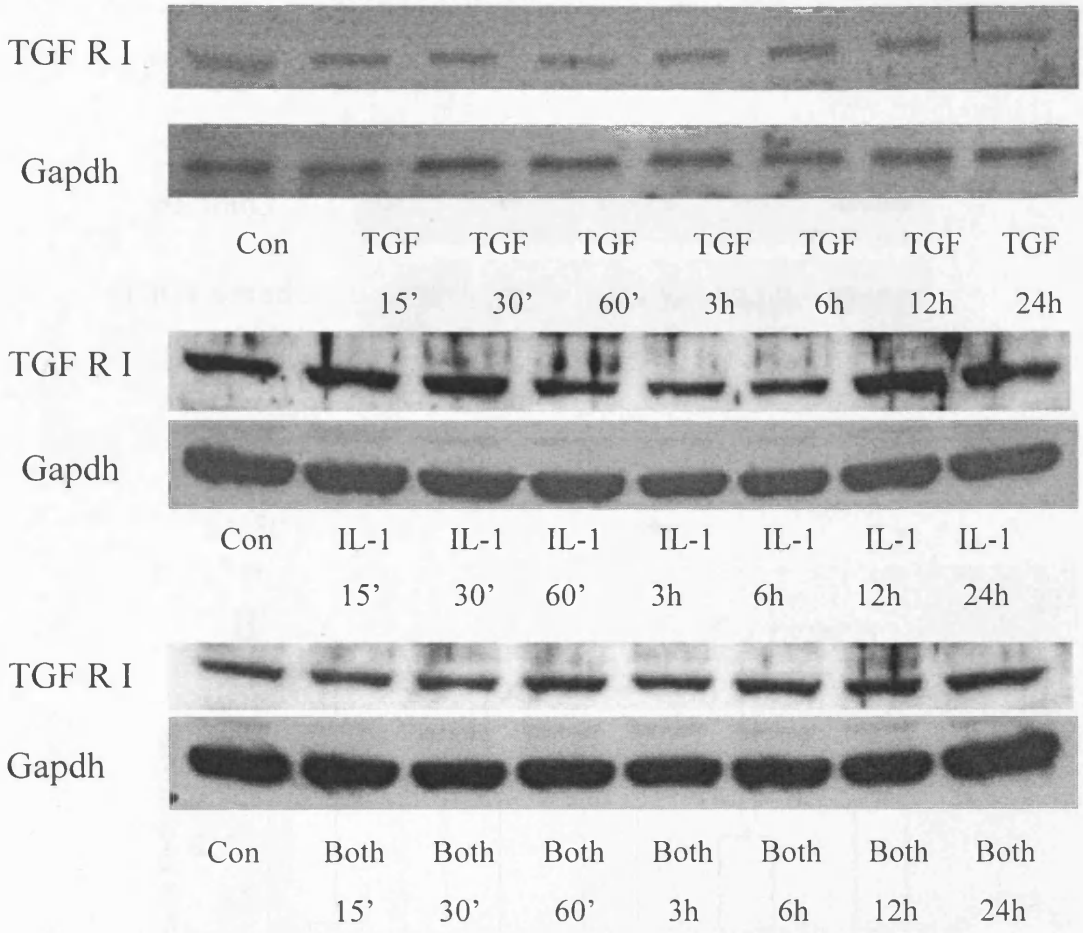


Figure 5.2: Time course of TGF-β type receptor I expression by IL-1β ± TGF-β1

IL-1β and TGF-β1 were added to confluent monolayer of growth arrested HK-2 cells under serum free conditions. HK-2 cells were co-treated with 1ng/ml TGF-β1 ± 1ng/ml IL-1β for time points from 15 minutes to 24 hours, before protein extraction and immunoblotting for TGF-β receptor I. Blots were stripped and reprobed for Gapdh. One representative experiment of three experiments is shown.

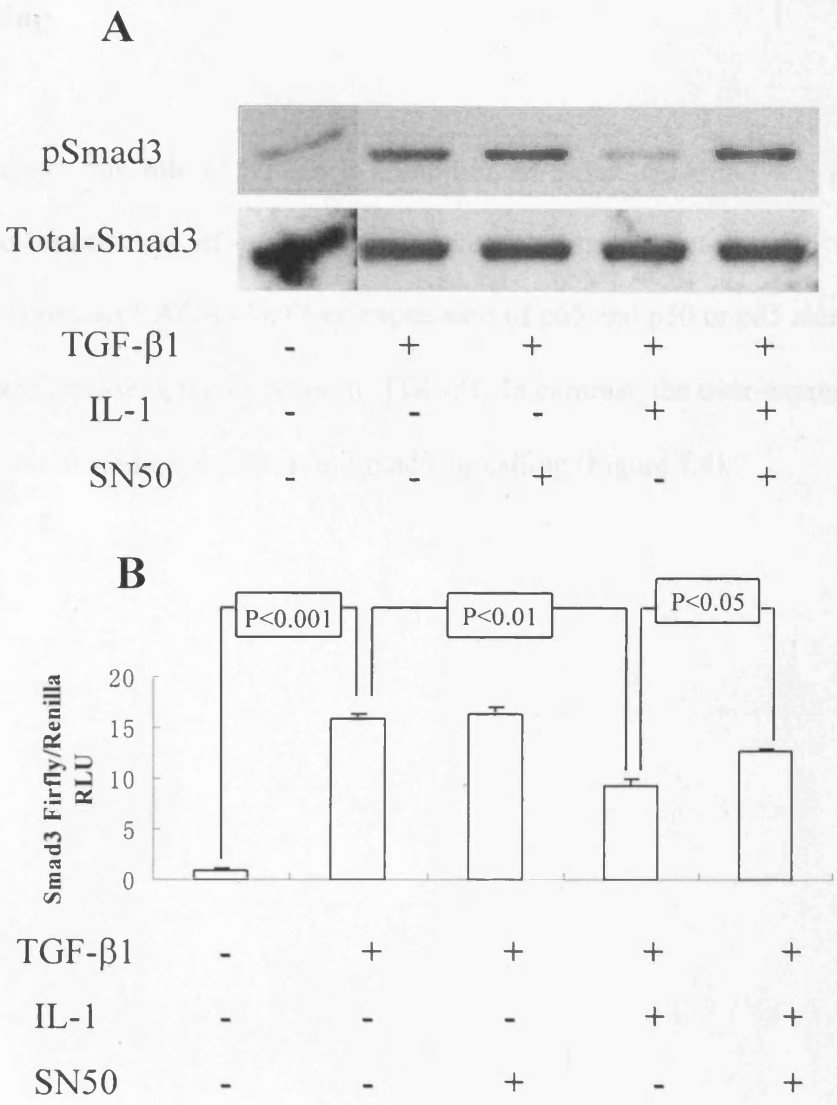


Fig 5.3: Inhibition of TGF-β1 signalling by IL-1β occurs via NF-kB.

(A) HK-2 cells were incubated with TGF-β1 , TGF-β1 and SN50, TGF-β1 and IL-1β or TGF-β1, IL-1β and SN50 for 1 hour before lysis and analysis by immunoblotting for phospho-Smad3. Subsequently, blots were stripped and reprobed for total Smad3. One representative experiment of three experiments giving similar results is shown. **(B)** HK-2 cells were transfected with CAGA(4) and pRL-CMV renilla before incubation for 6 hour with the control medium,1ng/ml TGF-β1 ± IL-1 β 1ng/ml or TGF-β1+IL-1 β +SN50. Ratio of firefly to renilla luciferase activity is shown, normalized to control cells. $N = 3$, mean ± SEM is plotted. One representative experiment of three separate experiments is shown.

5.2.2 Over-expression of NF-κB subunits inhibits TGF-β1 Smad signalling

Subsequently, the role of NF-κB in inhibition of Smad signalling was investigated using co-transfection of p65 and p50 over-expression vectors with the Smad3 reporter construct CAGA (4). Over-expression of p65 and p50 or p65 alone inhibited subsequent reporter gene response to TGF-β1. In contrast, the over-expression of the p50 subunit alone had no effect on Smad3 signalling (Figure 5.4).

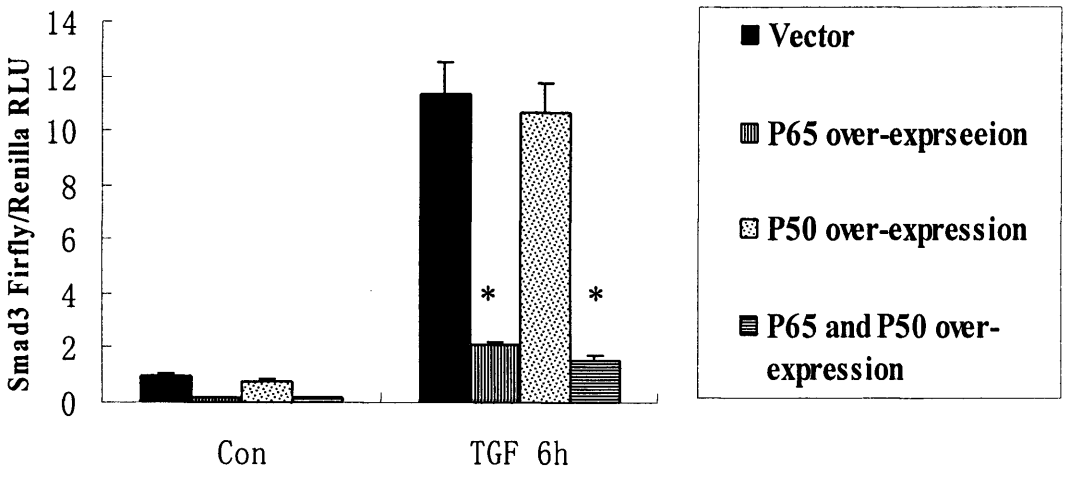


Fig. 5.4: Role of NF-κB in the TGF-β1 signalling inhibition by IL-1β.

HK-2 cells were transfected with an empty vector alone, empty vector and p65 over-expression vector, empty vector and p50 over-expression vector or p65 and p50 over-expression vectors, together with CAGA(4) and pRL-CMV renilla for 24 hour before incubation with TGF-β1 for 6 hour. Control cells were incubated in the TGF-β1-free medium. Equal amounts of DNA were transfected into all wells. $N = 3$, mean ± SEM is plotted. The empty vector plus TGF-β1 versus p65 plus TGF-β1 * $P < 0.001$, versus p65 and p50 plus TGF-β1 * $P < 0.001$, versus p50 plusTGF-β1 P = not significant. Ratio of firefly to renilla luciferase activity is shown, normalized to control cells. One representative experiment of three separate experiments is shown.

5.2.3 IL-1β stimulates NF-κB activity from early p65/p50 complex to later p50/p50 complex

The time course of NF-κB activation in IL-1β-stimulated PTC was assessed by the electrophoretic mobility shift assay. Increased probe retardation was seen within 15 min and persisted for up to 24 h (Figure 5.5). NF-κB probe retardation can be secondary to signalling p65/p50 heterodimers or to inhibitory p50/p50 homodimers [336]. Supershift experiments were performed with antibodies to p65 and p50, using PTC nuclear extracts from the cells treated for 1 h and 24 h with IL-1β (Figure 5.6). Both p65 and p50 were detectable at 1 h, while only p50 was detected at 24 h. The classical regulation of NF- κB activation pathway involves IκK phosphorylation, which leads to degradation of IκB alpha/IκB beta, and release of active NF-κB subunits. Immunoblotting of the cell extracts following IL-1β stimulation showed rapid but transient IκK phosphorylation (Figure 5.7), followed IκB alpha and IκB beta rapid degradation. However, both IκB alpha and IκB beta have been reaccumulated by 24 h (Figure 5.7).

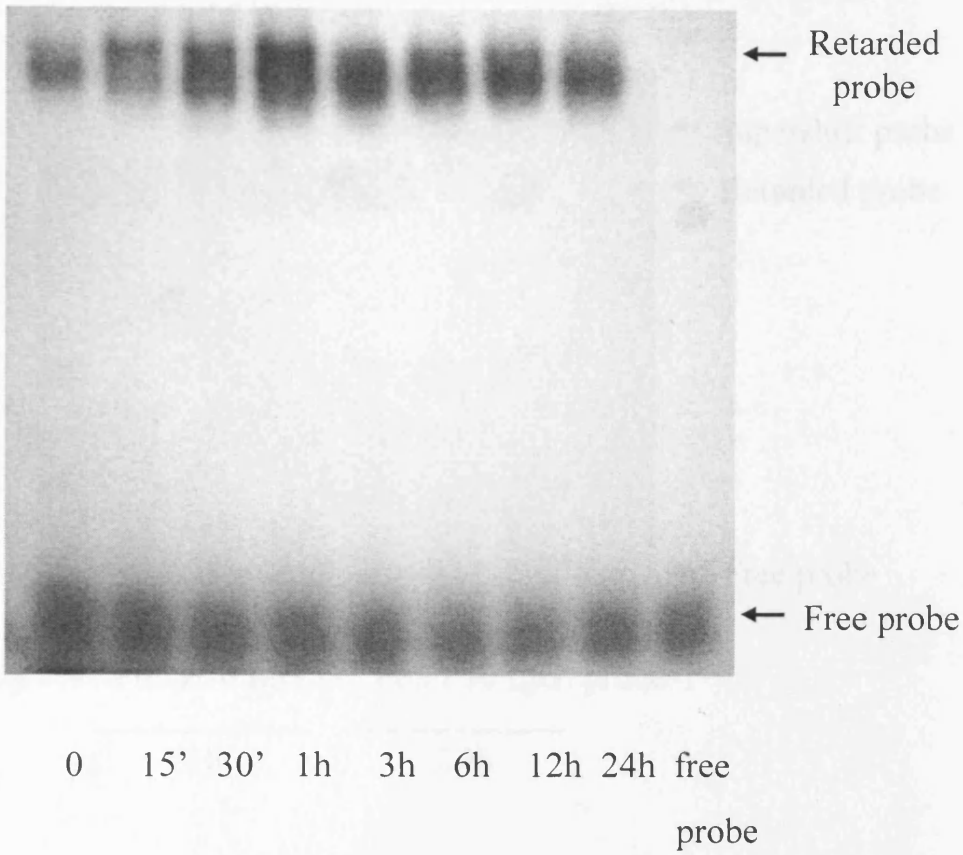


Fig 5.5: Time course of NF-kB activation by IL-1β in proximal tubular cells.

NF-kB electrophoretic mobility shift assay. PTC were stimulated with 1 ng/ml IL-1β for time points to 24 hour before nuclear protein extraction, and electrophoretic mobility shift assay with consensus NF-kB probe. Retarded probe and free probe are indicated . One representative gel of three experiments is shown.

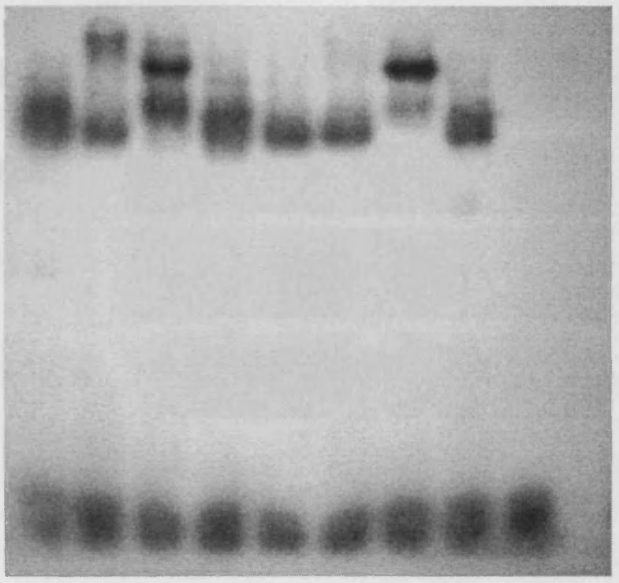


Fig5.6: Time course of NF-kB activation by IL-1β in proximal tubular cells.

Supershift assay. NF-kB EMSA with addition of p60, p50 and an irrelevant antibody to nuclear protein from cells stimulated with IL-1β for 1 hour and 24 hours. Retarded probe and supershifted bands are indicated. One representative gel of three experiments is shown.

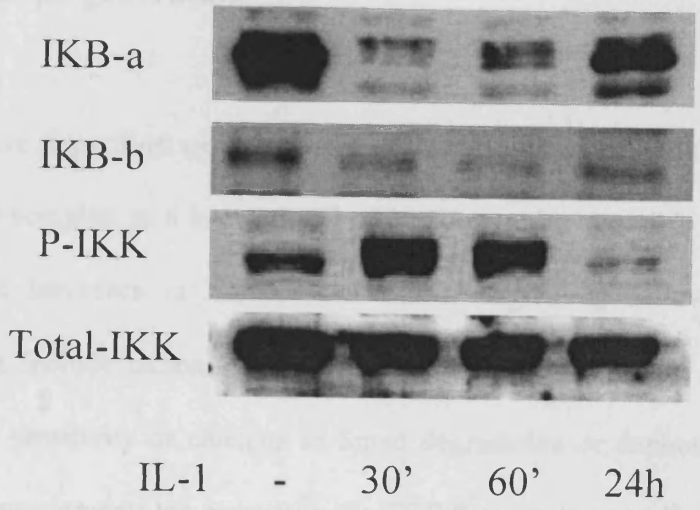


Fig 5.7: NF-kB activation by IL-1β in proximal tubular cells.

IkK and IkB activation. HK-2 cells were stimulated with 1ng/ml IL-1β for time points to 24 hours before immunoblotting of whole cell lysates for phospho-IKK. Blots were subsequently stripped and reprobed for total IKK. In separate experiments, immunoblotting for IkB-alpha and -beta was performed. One representative experiment of three separate experiments is shown.

5.2.4 The sensitizing effect of prolonged IL-1β on TGF-β1 signalling required TGF-β receptor–ligand interaction and was independent on TGF-β1 generation

The above SuperShift experiments showed there was a switch from an early NF-κB p65/p50 complex to a later NF-κB p50/p50 complex. Possible mechanisms for the observed increases in Smad2 and Smad3 signalling following chronic IL-1β exposure include increased TGF-β1 generation or activation, changes in TGF-β1 receptor sensitivity or changes in Smad degradation or dephosphorylation. NF-κB responsive elements are present in the TGF-β promoter, and IL-1β increases TGF-β generation in human alveolar epithelial cells [337]. Therefore, I quantified the release of TGF-β1 into the cell culture supernatant by PTC in response to IL-1β. HK-2 cells were cultured in with IL-1β (dose range 0–1 ng/ml) for 24 h before the assay of TGF-β1 generation by ELISA. No increase in TGF-β1 release was seen in response to IL-1β (Figure 5.8 A).

In terms of whether TGF-β1 receptor–ligand interaction is required for the sensitizing effect of IL-1β on TGF-β1 signalling, the experiments were performed in the presence of TGF-β1 receptor blocking antibody by Smad3 reporter gene assay. Addition of a blocking antibody to the TGF-β1 type II receptor inhibited IL-1β-mediated changes in Smad signalling in a dose dependent fashion (Figure 5.8 B), suggesting that the effect of IL-1β on Smad signalling was due to increased response to TGF-β1, rather than the activation of Smad signalling independent of TGF-β1 receptors.

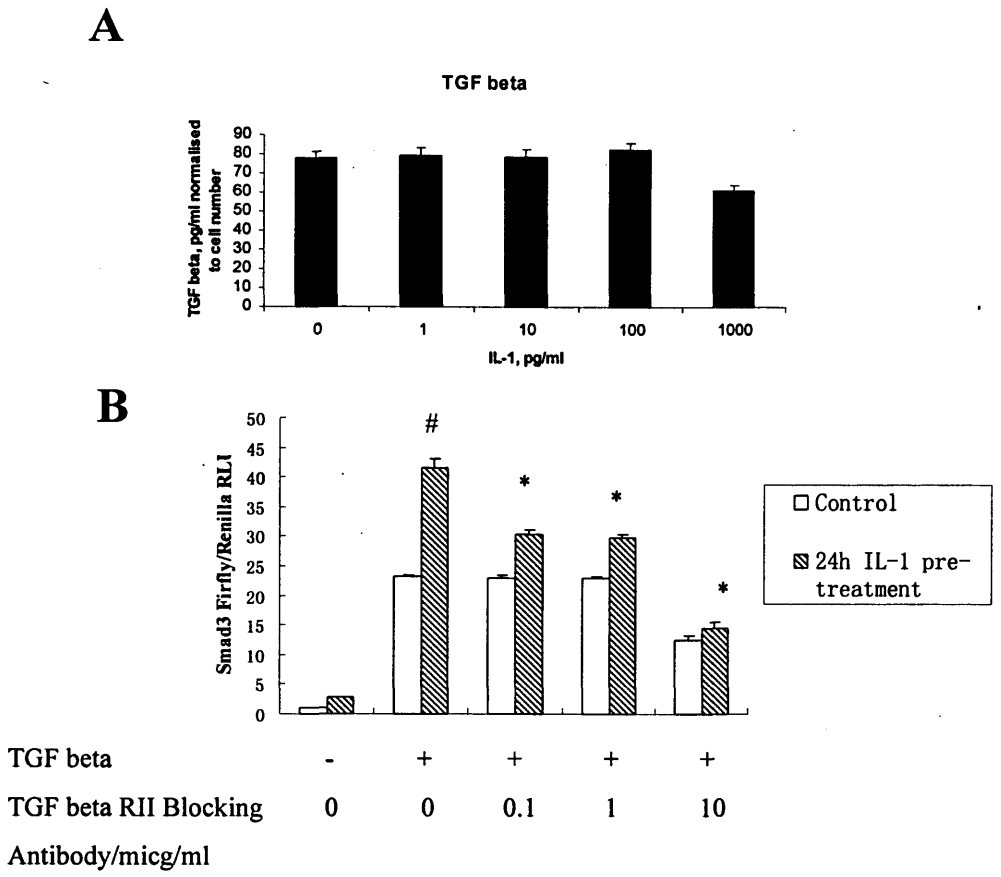


Fig. 5.8: Mechanism of sensitization to TGF β 1 by chronic IL-1β stimulation.

(A) HK-2 cells were treated with IL-1β, dose range 1–1000 pg/ml, for 24 hour before ELISA of cell culture supernatant total TGF- β 1. $N = 3$, mean ± SEM is plotted. No significant differences were found. **(B)** Blockade of IL-1β sensitization by an anti-TGF beta1 receptor antibody. HK-2 cells were transfected with Smad3 responsive (CAGA) and pRL-CMV renilla plasmids before treatment with 1ng/ml TGF β 1 ± 1ng/ml IL-1 β, in combination with a blocking antibody to the TGF-β type II receptor (dose range 0–10 ug/ml) for 24 h. Data are expressed as firefly/renilla luciferase activity, normalized to control cells. $N = 3$, mean ± SEM is plotted. TGF β 1 versus TGF-β1+IL-1β $^{\#}P < 0.01$, TGF-β 1+IL-1β versus TGF-β +IL-1β +blocking antibody $^{*}P < 0.01$ .One representative experiment of three separate experiments is shown.

5.2.5 Smad3 dephosphorylation/degradation were not altered by IL-1β

To evaluate possible changes in Smad dephosphorylation in response to IL-1β, the half-life of phospho-Smad3 was examined using the Alk5 kinase inhibitor SB431542. Similar decay rates for phospho-Smad3 were observed in control cells and cells treated with IL-1β (Figure 5.9 A and B), suggesting that phospho-Smad3 dephosphorylation was not altered in response to IL-1β.

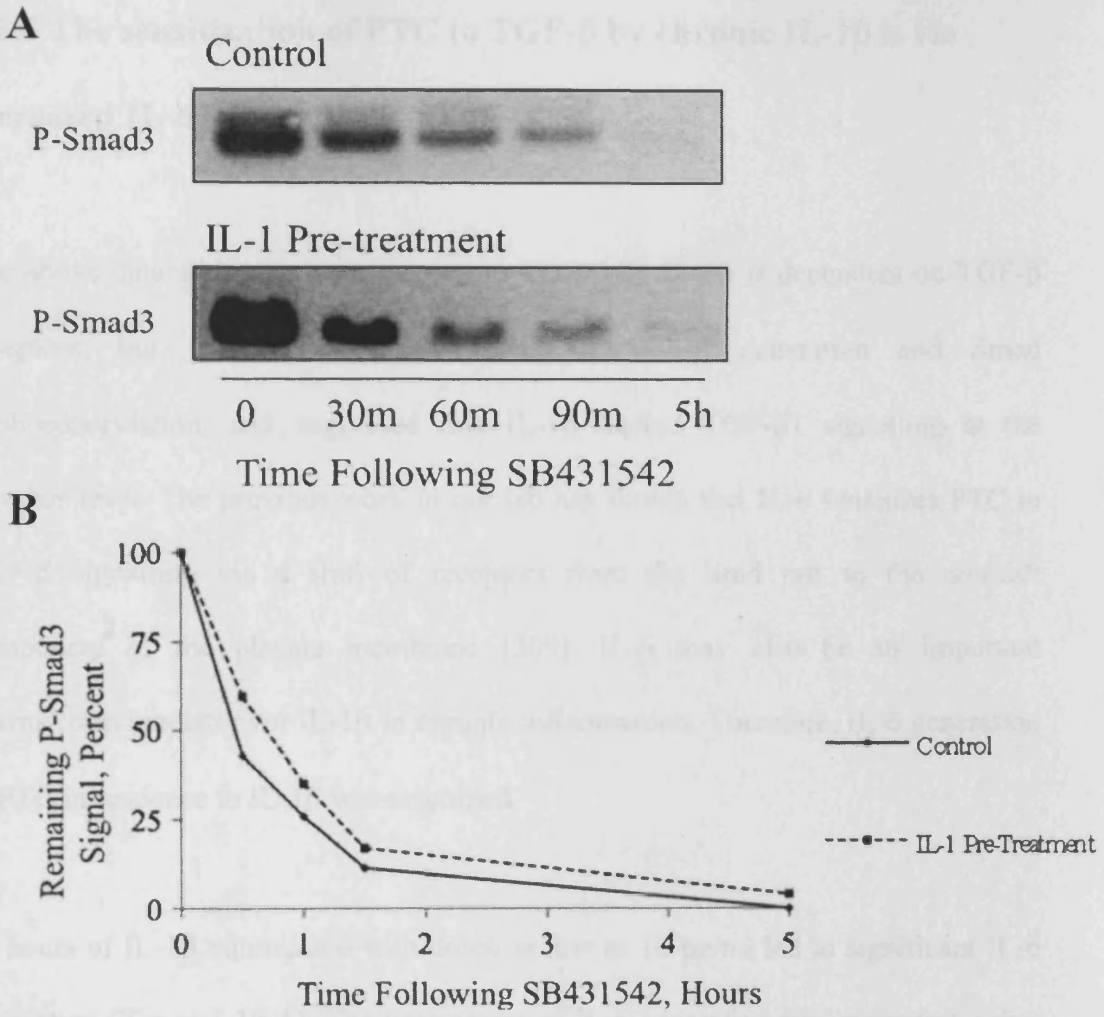


Fig 5.9: Time course of phospho-Smad3 degradation/ dephosphorylation.

(A) HK-2 cells were treated with IL-1β or the control medium for 24 hours, before TGF-β1 1ng/ml treatment for 30 minutes. Subsequently, the cells were again incubated in the medium containing the Alk5 inhibitor SB431542 10uM for time points to 5 hours. Residual phospho-Smad3 was detected by immunoblot. **(B)** Plot of densitometry results for immunoblots were shown. Phospho-Smad3 signal is plotted as a percentage of initial signals (time zero) for control and IL-1β pre-treated cells. One representative experiment of three experiments is shown.

5.2.6 The sensitization of PTC to TGF-β by chronic IL-1β is via increased IL-6 generation

The above data show that sensitization to TGF-β by IL-1β is dependent on TGF-β receptors, but not involving alterations in TGF-β generation and Smad dephosphorylation, and suggested that IL-1β altered TGF-β1 signalling at the receptor level. The previous work in our lab has shown that IL-6 sensitizes PTC to TGF-β signalling via a shift of receptors from the lipid raft to the non-raft component of the plasma membrane [309]. IL-6 may also be an important downstream mediator for IL-1β in chronic inflammation. Therefore, IL-6 generation in PTC in response to IL-1β was examined.

24 hours of IL-1β stimulation with doses as low as 10 pg/ml led to significant IL-6 generation (Figure 5.10 A). The time course of IL-6 generation was examined, using stimulation with 1 ng/ml IL-1β based on the previous experiments. Increased IL-6 generation was seen after 3 h or more IL-1β stimulation, but not at earlier time points (Figure 5.10 B).

Subsequently, the effect of a blocking antibody to the IL-6 receptor on TGF-β responsiveness in the cells stimulated with IL-1β for 24 h was determined. Phospho-Smad3 immunoblotting showed that IL-6 receptor blockade abrogated sensitization of Smad signalling by IL-1β, without inhibiting baseline TGF-β1 response (Figure 5.11).

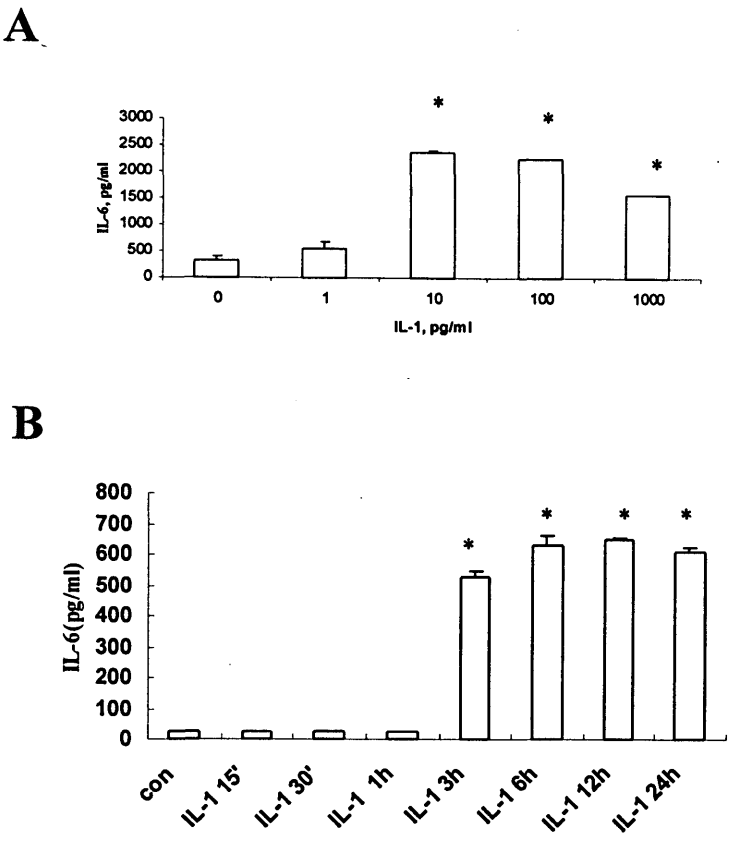


Fig 5.10: IL-6 generation by chronic IL-1β stimulation.

(A) Dose-dependent IL-6 generation following IL-1β stimulation. HK-2 cells were cultured in the medium containing IL-1β (dose range 0–1000 pg/ml) for 24 hours before the assay of IL-6 in the supernatant by ELISA. $N = 3$, mean ± SEM is plotted. $P < 0.01$ for IL-1β 10, 100 and 1000 pg/ml compared to control. **(B)** Time course of IL-6 generation following IL-1β. HK-2 cells were cultured in the medium containing IL-1β 1ng/ml for time points to 24 hours before assay of IL-6 in the supernatant by ELISA. $N = 3$,mean ± SEM is plotted. $P < 0.01$ for time points of 3, 6, 12, 24 h compared to control, P = NS for earlier time points. One representative experiment of three experiments is shown.

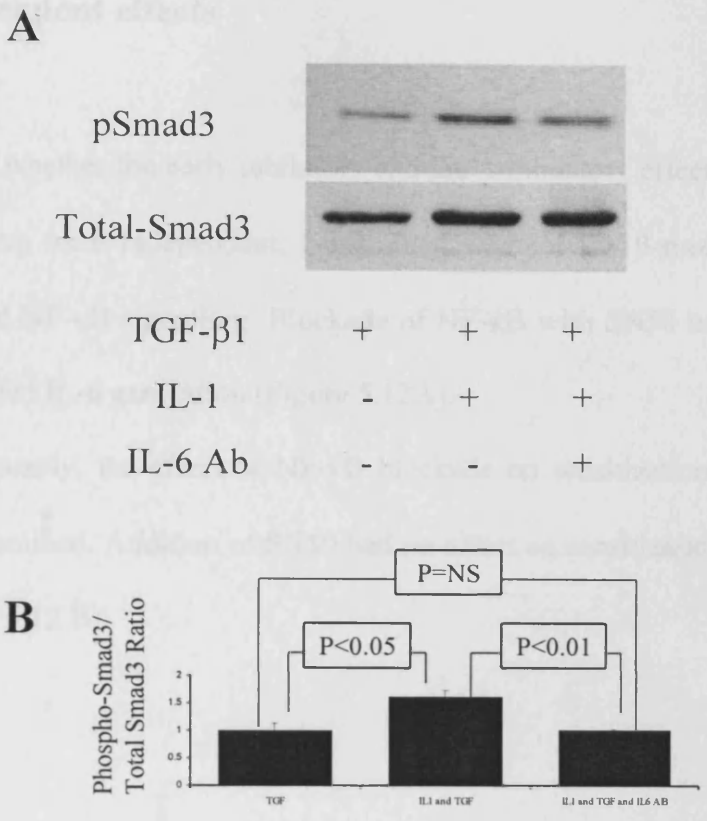


Fig 5.11: Effect of IL-6 blockade on sensitization of TGF-β1 signalling by IL-1β.

(A) HK-2 cells were cultured in the medium containing 1ng/ml IL-1β and 1ng/ml TGF-β1 ± IL-6 receptor-blocking antibody for 24 hours. Control cells were cultured in the medium containing TGF-β1 alone. Whole cell lysates were immunoblotted for phospho-Smad3 and reprobed for total Smad3. One representative blot of three experiments giving similar results is shown . **(B)** Combined densitometry results of the blots from three experiments is described . Mean phospho-Smad3/total Smad3 is blotted.

5.2.7 Early inhibition and late enhancement of Smad signalling are independent effects

To test whether the early inhibitory and late stimulatory effects of IL-1β on TGF-β1 signalling were independent, I examined whether IL-1β-mediated IL-6 generation required NF-κB signalling. Blockade of NF-κB with SN50 had no effect on IL-1β-stimulated IL-6 generation (Figure 5.12A).

Subsequently, the effect of NF-κB blockade on sensitization to TGF-β1 by IL-1β was examined. Addition of SN50 had no effect on sensitization to TGF-β1 by IL-1β (Figure 5.12 B).

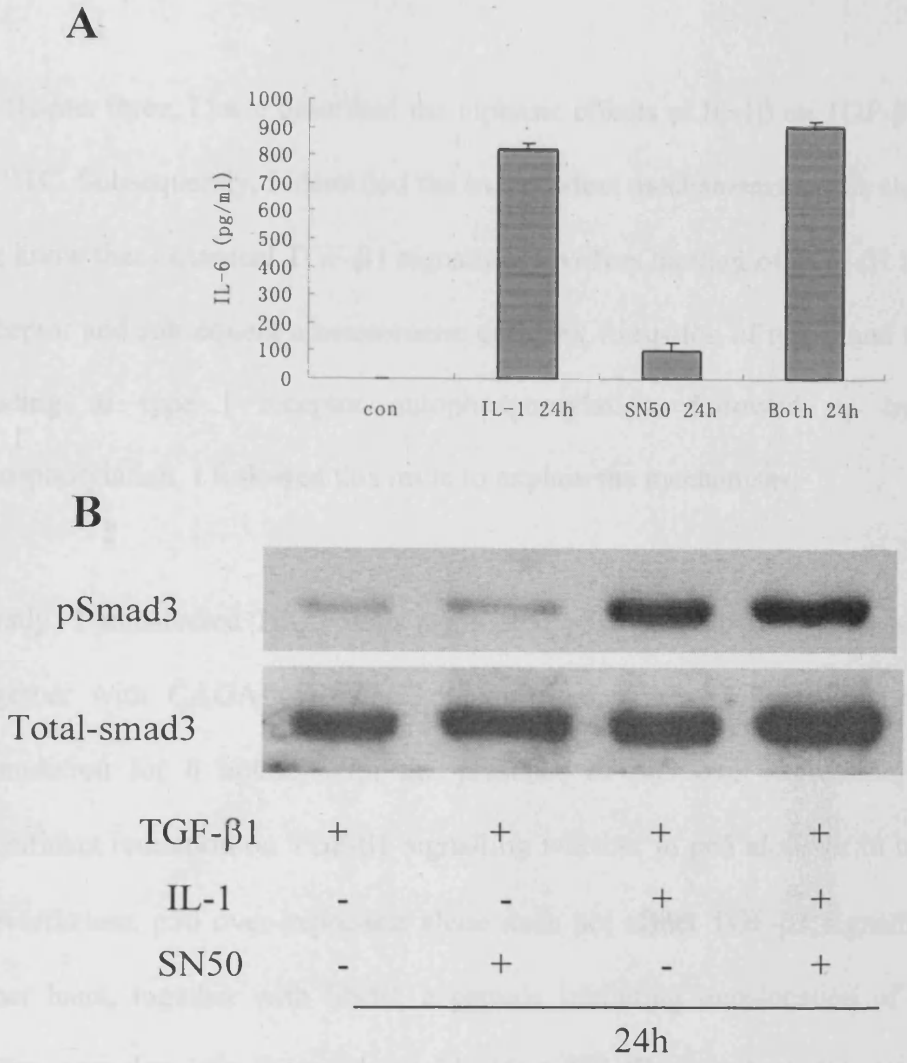


Fig. 5.12: Sensitization to TGF-β1 by chronic IL-1β stimulation is independent of NF-kB.

(A) HK-2 cells were treated with IL-1β, or IL-1β and SN50, for 24 hours. Control cells were treated with the IL-1β -free medium, or SN50 alone. Supernatant IL-6 was measured by ELISA. $n = 4$, mean ± SEM isplotted. IL-1β vs Both, P = NS. **(B)** HK-2 cells were treated with 1ng/ml TGF-β1 ±1ng/ml IL-1β ± SN50 for 24 hours; then total cell lysates were immunoblotted for phospho-Smad3 and total Smad3. One representative experiment of three experiments is shown.

5.3 Discussion

In chapter three, I have described the biphasic effects of IL-1β on TGF-β1 signalling in PTC. Subsequently, I identified the independent mechanisms in this chapter. Since we know that canonical TGF-β1 signalling involves binding of TGF-β1 to its type II receptor and subsequent a heteromeric complex formation of type I and II receptors, leading to type I receptor autophosphorylation, followed up by R-Smads phosphorylation, I followed this route to explain the mechanism.

Firstly, I transfected HK-2 cells with p65, p50, or both over-expression vectors together with CAGA (4) Smad3 reporter constructs, followed up by TGF-β1 stimulation for 6 hours. With the presence of p65 over-expression, there is a significant reduction on TGF-β1 signalling whether in p65 alone or in both groups. Nevertheless, p50 over-expressed alone does not affect TGF-β1 signalling. On the other hand, together with SN50, a peptide inhibiting translocation of the NF-κB active complex into the nucleus, blocking NF-κB activation prevents the early inhibition of IL-1β on TGF-β1 signalling at Smad3 phosphorylation and the following DNA-binding activity. Moreover, the data show clear inhibition of Smad signalling at early time points by reporter gene assay, but the effect of IL-1β on Smad phosphorylation by TGF-β1 at early time points is relatively modest, suggesting that NF-κB acts on Smad signalling predominantly beyond the level of Smad phosphorylation by the activated receptor complex.

In order to further confirm the NF-κB subunits activation by IL-1β in PTC, I assessed NF-κB activation by EMSA and it shows the activation persisted from 15 minutes to 24 hours. However, there was a change in appearance of the retarded band on EMSA that appears to coincide with the time of switch from inhibition to stimulation of TGF-β1 signalling. Subsequent supershift results show a switch from p65/p50 complex to p50/p50 complex. The interesting thing is the complex switched from p65/p50 at early 1 hour IL-1β treatment to p50/p50 at late 24 hour IL-1β treatment, accompanying with the loss of NF-κB inhibitory effect on Smad signalling. It suggests that p65 plays a crucial role on IL-1β-induced early inhibition on TGF-β1 signalling.

Then, I investigated the mechanism of how p65 inhibits TGF-β1 signalling pathway. Previous work has suggested that NFkB may limit Smad signalling through up-regulation of Smad7 and down-regulation of type I TGF-β receptors [223, 224,298]. However, I found that in HK-2 cells, neither TGF-β1 nor IL-1β stimulation up to 24 hours altered Smad7 or TGF-β1 R I expression. In addition, in the previous work quoted above, increased Smad7 protein was detected after 12 or more hours of IL-1β stimulation, whereas in my experiments, IL-1 had a detectable inhibitory effect on TGF-β1 signalling within 1 hour. Some work has shown NF-κB inhibits TGF-β1 response via sequestration of the transcriptional coactivator p300 by p65 NF-κB [333], and it would be interesting to investigate the potential role of p300 sequestration in the inhibitory effect of IL-1 that I have demonstrated.

Because of the important regulation axis of IKK- IκB on NF-κB signalling, I detected them by Western Blot and have shown that at early time point of IL-1β up

to 1 hour, IKK is phosphorylated leading to the final obvious degradation of IκBα. Following the degradation of IκBα, the NF-κB subunits are released and translocate from cytoplasm to nucleus, initiating NF-κB signalling pathway. The similar but weaker regulation is detected for IκBβ, another member of IκB family. These data support transient NF-κB activation by IL-1β in PTC [334].

Having investigated the mechanism of early effect of IL-1β on TGF-β1 signalling, I have observed how the late enhancement effect of IL-1β on TGF-β1 signalling processes.

In chapter three, I have shown that in PTC, the longer term presence of IL-1β leads to different augmentation of Smad2 and Smad3 response to TGF-β1 compared to early inhibition effect of IL-1β and to detectable Smad3 phosphorylation in the absence of exogenous TGF-β1. Previous work has shown that HA leads to the activation of TGF-β1 type I receptors via CD44 v3 and to R-Smads phosphorylation without TGF-β1 ligand–receptor interaction or formation of the conventional activated receptor complex [289]. However, the augmentation of TGF-β1 signalling by IL-1β is prevented by a type II receptor blocking antibody, suggesting that IL-1β is dependent on TGF-β receptor/ligand interaction. Also, IL-1β does not alter TGF-β1 synthesis. After formation of the activated receptor complex, the next step is R-Smads phosphorylation. It was demonstrated that the TGF-β1-regulated R-Smads, Smad2 and Smad3, constantly shuttle between the nucleus and the cytoplasm both in uninduced cells [175] and during TGF-β1 signalling, Smad nucleocytoplasmic shuttling monitored receptor activity [102]. Most importantly, nucleocytoplasmic

Smad shuttling in the presence of TGF-β1 appears to require the cycles of Smad phosphorylation and dephosphorylation. With SB 431542, an inhibiter of ALK-5 (TGF-β1 type I receptor) [338], my results show IL-1β does not affect the R-Smads dephosphorylation in response to TGF-β1. Thus, these outcomes together suggest that IL-1β enhances response to endogenous/exogenous TGF-β1 via alteration in the signalling initiation of TGF- β receptor expression or distribution.

TGF-β receptors internalize into both caveolin- and EEA1-positive vesicles. Clathrin-dependent internalization into the EEA1-positive endosome promotes TGF-β signalling. In contrast, the lipid raft-caveolar internalization pathway contains the Smad7-Smurf2-bound receptor and is essential for a rapid receptor turnover [153]. My results show Smad7 and type I receptor expression is not changed by long time treatment of IL-1β response to TGF-β1. The previous work in our lab has shown that in PTC, TGF-β receptor distribution in the plasma membrane is regulated by stimuli including hyaluronic acid (via CD44), which leads to the attenuation of TGF-β1 signalling and IL-6, which contributes to enhance TGF-β1 signalling via a shift of receptors from lipid rafts to non-raft associated plasma membrane [156, 309].

IL-6 is a multifunctional cytokine produced by a variety of cells in response to IL-1β during infection, trauma and immunological challenge [339]. In my work, IL-1β leads to increased PTC IL-6 generation after 3 hours treatment, and IL-1β doses and times required to stimulate IL-6 generation mirrored those required to sensitize PTC signalling response to TGF-β1, which matches the time point switch from signalling inhibition to signalling enhancement. Additionally, a blockade of IL-6 signalling

abrogated sensitization of TGF-β1 signalling by IL-1β, confirming that in PTC, IL-1β augments Smad signalling response to TGF- β via an autocrine IL-6 loop.

As the results described above, IL-1β functions through NF-κB signalling up to 24 hours. In order to confirm if NF-κB activation is a precondition for later IL-6 generation and TGF-β1 sensitization by IL-1β, I blocked NF-κB action with SN-50 to evaluate the Smad3 phosphorylation and IL-6 generation. The data show that blocking NF-κB activation has no influence either on IL-6 generation or Smad3 phosphorylation, suggesting that the early and late effects of IL-1β on TGF-β1 signalling are independent.

In conclusion, these results suggest that early inhibition effect of IL-1β on TGF-β1 signalling is via NF-κB, but that after 3 hours there is a switch from p65/p50 to p50/p50 complexes, Furthermore, the later prolonged enhancement effect of IL-1β on TGF-β1 signalling is via an autocrine IL-6 loop. This data scores how the relative simplicity of TGF- β signalling pathways contrasts with the complexity and diversity of epithelial cell responses to IL-1β. Changes in epithelial cell response to TGF-β secondary to important environmental cues such as inflammation provide an explanation for the apparently contradictory roles of TGF-β in the kidney, suggesting how this cytokine can be involved in controlled healing following acute injury on the one hand, yet be the principal promoter of scarring in chronic disease on the other.

Chapter Six:

The mechanisms of BMP-7 effect on TGF-β1 signalling

6.1 Introduction

In chapter three, I showed BMP-7 inhibits TGF-β1 Smad3 but not Smad2 signalling without apparent reduction Smad3 phosphorylation.

BMP-7 and TGF-β1 belong to TGF-β super family. BMP-7 signalling has its own two type receptors on the cytoplasm membrane and R-Smads.

BMP-7 signalling pathway and its regulation

Like TGF-β signalling pathway, BMP7 receptors are type I/II heterodimeric serine/threonine kinase receptors. Several different type I receptors, namely the activin receptor–like kinases (Alk) Alk2 (activin receptor I), Alk3 (BMP receptor IA), and Alk6 (BMP receptor IB), associate with the BMP type II receptor (BMPRII). Receptor activation induces intracellular recruitment and serine/threonine phosphorylation of Smad substrates. Smad1, -5, and -8 are BMP-restricted Smads, and Smad1 and/or -5 are signalling substrates for BMP-7 in different cell types [252, 314]. However, in some other cell types including endothelial cells, TGF-β ligand can phosphorylate Smad1, Smad5 and Smad8 through a receptor complex that contains the tissue-specific ALK1 type I receptor [93, 340-342].Similar to TGF-β, BMP-7 can also regulate cell type and context, dependently other signals including the tyrosine kinases ERK, JNK, and p38 [240]. In mPTC, murine mesangial cells, and murine podocytes, studies indicate that the preferred Smad signalling candidate for BMP-7 is Smad5 [240, 252]. Moreover, phosphorylation of Smad5 by BMP-7 is not inhibited by coincubation with TGF-β [252]. TGF-β– restricted Smad2 and 3 are not activated by BMP-7[252].

The bioactivity of BMP-7, is further regulated by a series of secreted, extracellular proteins that bind and neutralize peptide activity, including BMP-7 antagonists such as, noggin, follistatin, and gremlin, USAG-1 [252, 253, 343, 344]. The activity of BMP-7 also can be enhanced by endoglin and KCP [343, 344]. CTGF, another profibrotic player is not only induced by TGF-β1, but also a major enhancer of the biological activity of TGF-β1. Recent work has shown that CTGF inhibits BMP-7 signal transduction in the diabetic mice kidney, thus reducing MMP activity, and albuminuria [345]. In the human patients, pSmad1/5/8 was decreased along with increased CTGF expression. Whereas in CTGF(+/-) mice, pSmad1/5/8 were preserved compared to diabetic CTGF(+/+) mice [346]. In myoblastic L6E9 cells, endoglin inhibits TGF-β1-induced ALK-5/Smad3 signalling but enhanced the BMP-7/Smad1/Smad5 pathway [347]. Another TGF-β type III receptor, betaglycan, can specifically bind to multiple numbers of BMP family including BMP-7 *in vitro*, enhancing ligand binding to the BMP type I receptors to attenuate BMP-mediated Smad1 phosphorylation [348].

MAPKs (especially ERK1/2, GSK3-β (Glycogen synthase kinase-3)) also phosphorylate the linker of Smad1/5, creating a docking site for the E3 ubiquitin ligase, Smurf1. Smurf1 not only causes ubiquitin-proteasome degradation of the Smads but also prevents Smad complex translocation from the cytoplasm to the nucleus to block their interaction with the nuclear pore complex [222]. In some conditions BMP7 may activate JNK [349] in human mesangial cells and ERK in proximal tubular epithelial cells [252]. The canonical Wnt signalling pathway, which functions through the inhibition of Glycogen Synthase Kinase 3 activity (GSK3-β), reduces Smad1 ubiquitination and stabilizes the BMP-Smad signalling [350].

Possible mechanisms of BMP-7 effects on chronic renal injury in response to TGF-β

BMP-7 is a well known cytokine to protect TGF-β-dependent renal fibrogenesis due to various causes. We know EMT is a pivotal process contributing to fibrosis. Interestingly, some work has shown that cardiac fibrosis is associated with the participation of fibroblasts deriving from endothelial cells, suggesting an endothelial-mesenchymal transition (EndMT), which can be induced by TGF-β1. The systemic administration of recombinant human BMP-7 (rhBMP-7) significantly inhibited EndMT and the progression of cardiac fibrosis in mouse model [351]. Recent results found out that EndMT is also a novel mechanism for accumulation of fibroblasts and myofibroblasts in kidney fibrosis [22, 352].

Since massive work has shown BMP-7 can antagonize TGF-β-induced cellular and histological changes contributing to renal fibrosis, some work has been done to explore the mechanisms on TGF-β signalling pathway regulation. In BMP-7 transgenic mice, BMP-7 transgene leads to elevated phosphorylated Smad1/5 and moderately reduces p-Smad2/3 levels without affecting TGF-β levels in kidney [254]. In mesangial cells *in vitro*, BMP-7 opposes the TGF-β-dependent fibrogenesis via activation of downstream of Smad5, the inhibitory Smad6 and subsequently reduces the availability of Smad3 in the nucleus [314]. The aim in this chapter was to characterize the mechanism of inhibition effect of BMP-7 on TGF-β1 signalling in HK-2 cells.

6.2 Results

6.2.1 BMP-7 does not alter Smad3 nuclear accumulation, or dephosphorylation

The inhibition of Smad3 but not Smad2 signalling suggests that BMP-7 acts beyond the point of TGF-β ligand-receptor interaction. In our lab, the preliminary experiments showed Smad2 and Smad3 phosphorylation detectable for 24h following TGF-β treatment, with a maximal response by 30 minutes to 1h. Although Smad1 phosphorylation was readily detected following BMP-7 treatment, confirming signalling response to BMP-7, BMP-7 had no effect on Smad2 or Smad3 phosphorylation in cells pre-incubated with BMP-7 then exposed to TGF-β (Figure 6.1). Transient Smad1 phosphorylation was also evident following TGF-β treatment, in keeping with recent reports of transient Smad1/5 phosphorylation in epithelial cells in response to TGF-β1 [353].

Nuclear import and export of Smad3, together with its dephosphorylation, ubiquitinylation, and degradation, are highly regulated [354, 355]. To study possible Smad3 dephosphorylation or degradation in response to BMP-7, I examined the half-life of phospho-Smad3. HK-2 cells were incubated with BMP-7 or control medium, then Smad3 phosphorylation was stimulated with TGF-β1. After 30 minutes, further Smad3 phsophorylation was inhibited using the Alk5 Kinase inhibitor SB431542, and the rate of decline of phospho-Smad3 was evaluated with Immunoblotting. Similar rate of decline in phospho-Smad3 was observed in control cells and cells treated with BMP-7 (Figure 6.2), suggesting that phospho-Smad3 dephosphorylation

and degradation are not altered in response to BMP-7. Next, nuclear accumulation of phospho-Smad3 was assessed by immunoblotting and by fluorescence microscopy. Immunoblots of nuclear protein fractions suggested that BMP-7 did not alter nuclear accumulation of Smad3 (Figure 6.3A). Similarly, immunocytochemistry did not detect differences in TGF-β-dependent nuclear accumulation of Smad3 following BMP-7 treatment (Figure 6.3B), confirming the outcome that BMP-7 has no effect on Smad3 nuclear accumulation.

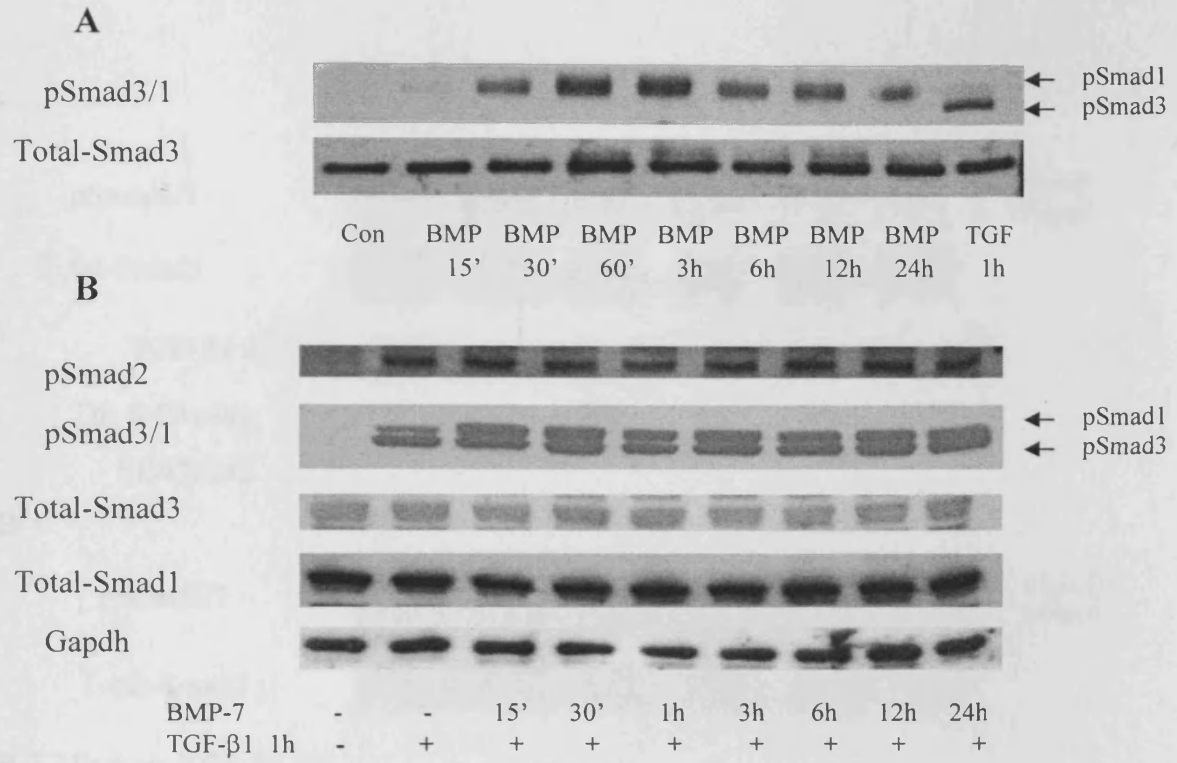


Fig 6.1: Time course of Smad phosphorylation in response to BMP-7 ± TGF-β1

(A) HK-2 cells were incubated with BMP-7 50ng/ml for time points up to 24 hours. The whole cell lysates were immunoblotted with phospho-Smad3/1. The TGF-β1 treated lane takes as a control to show the pSmad3 band. Blots were stripped and reprobed for total Smad3.

(B) HK-2 cells were incubated with BMP-7 50ng/ml for time points up to 24 hours before incubation with TGF-β1 1ng/ml for 1 hour. Whole cell lysates were immunoblotted with antibodies against phospho-Smads1,2,3, and total Smads 1 and 3. Stripping and reprobing for Gapdh was used to confirm approximately equal loading.
One representative experiment of three separate experiments is shown.

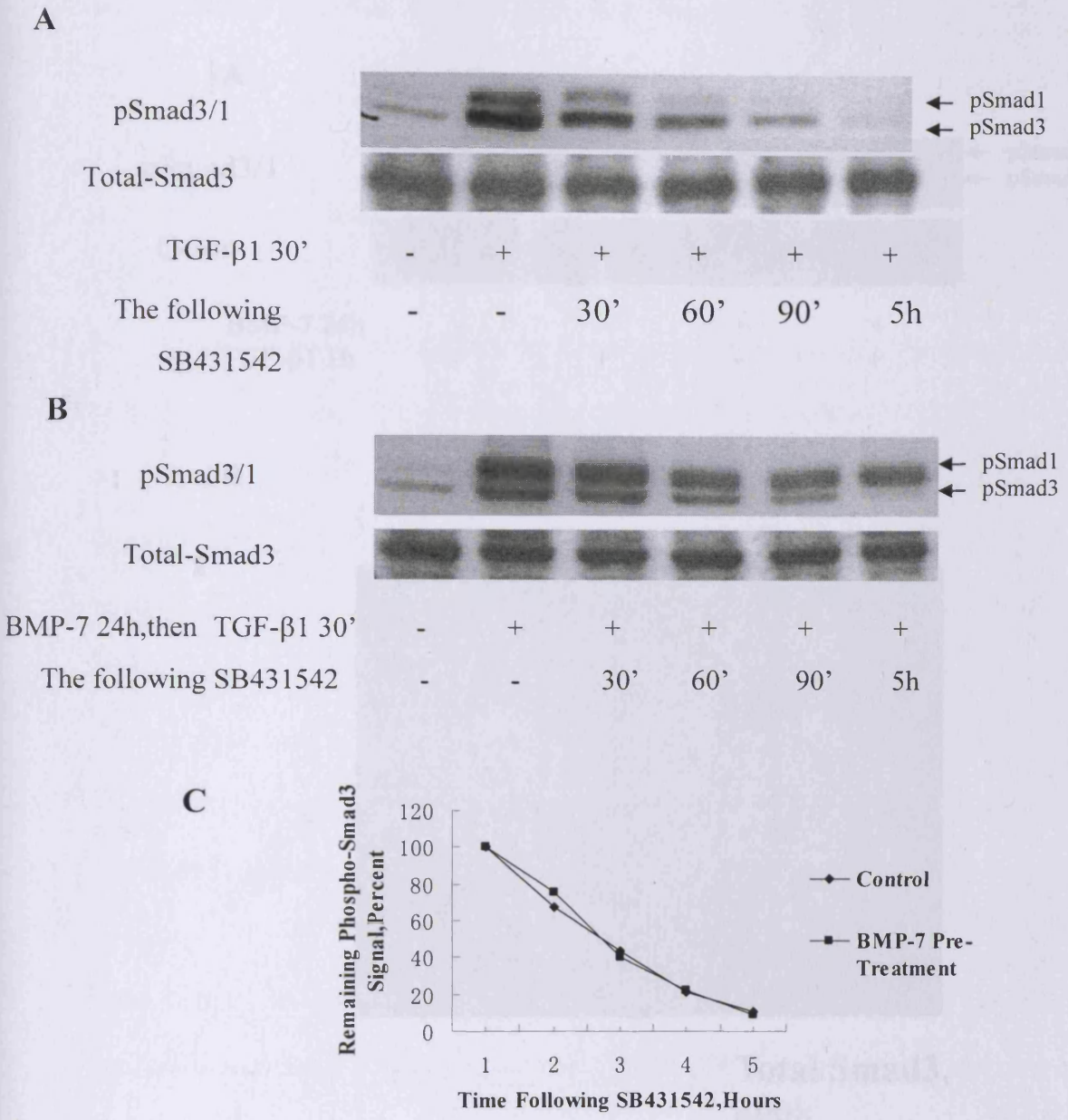
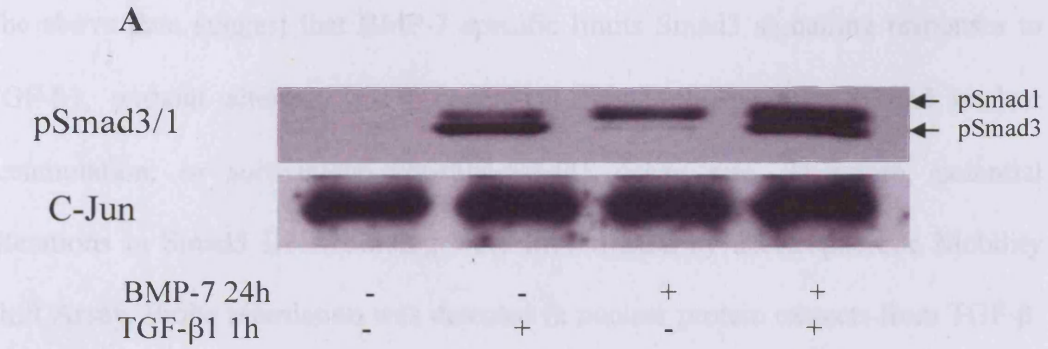


Fig 6.2: Time course of Smad dephosphorylation/degradation in response to TGF-β1 and BMP-7

HK-2 cells were incubated with control medium or BMP-7 50ng/ml for 24 hours before incubation with TGF-β1 1ng/ml for 30 minutes. **(A and B)** Cells were washed and incubated in control medium or medium containing the ALK5 Kinase Inhibitoe SB431542 for time points up to 5 hours. Residual phospho-Smad3 activity was detected by immunoblot before stripping and reprobing for total Smad3. One representative experiment of three experiments is shown. **(C)** Plot of densitometry results for immunoblots were shown in.phospho-Smad3 signal is plotted as a percentage of initial signals (time zero) for without and with BMP-7 pre-treated cells .



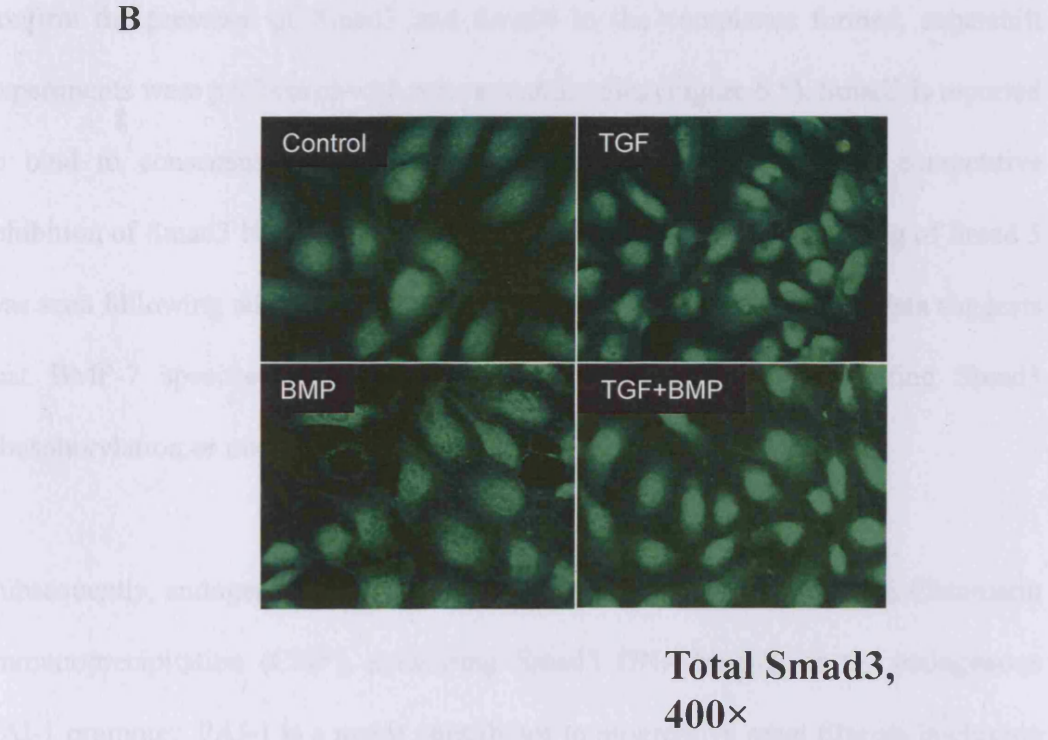


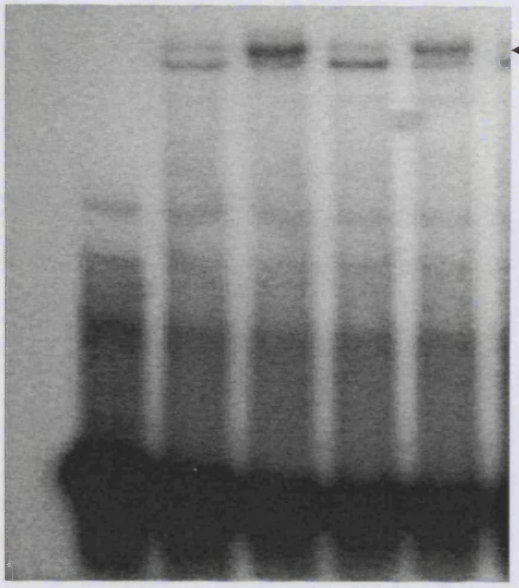
Fig 6.3: Nuclear accumulation of Smad3.

HK-2 cells were incubated with control medium or BMP-7 50ng/ml for 24 hours before incubation with TGF-β1 1ng/ml for 1 hour. **(A)** Immunoblotting of nuclear extracts for phospho-Smad3/1, and subsequently reprobing for C-Jun to confirm approximately equal nuclear protein loading. **(B)** Immunoflurorescent localization of Smad3.
One representative experiment of three separate experiments is shown.

6.2.2 BMP-7 inhibits Smad3 DNA Binding

The above data suggest that BMP-7 specific limits Smad3 signalling responses to TGF-β1, without altering TGF-β-dependent Smad phosphorylation and nuclear accumulation, or subsequent Phospho-Smad3 decay rate. Therefore, potential alterations in Smad3 DNA binding were investigated by Electrophoretic Mobility Shift Assay. Probe retardation was detected in nuclear protein extracts from TGF-β-treated cells, but this was attenuated in cells by BMP-7 (Figure 6.4). In order to confirm the presence of Smad3 and Smad4 in the complexes formed, supershift experiments were performed with relevant antibodies (Figure 6.5). Smad5 is reported to bind to consensus Smad3 elements, such that the potential for competitive inhibition of Smad3 binding by Smad5 exists [356]. However, no binding of Smad 5 was seen following addition of anti-Smad5 antibody (Figure 6.6). This data suggests that BMP-7 specifically limits Smad3 DNA-binding, without altering Smad3 phosphorylation or nuclear import.

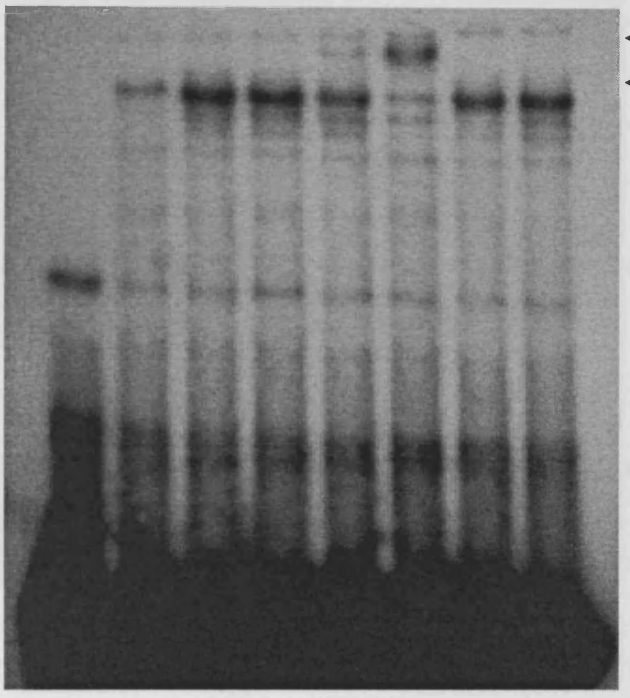
Subsequently, endogenous Smad3 DNA binding was further evaluated by Chromatin Immunoprecipitation (ChIP), measuring Smad3 DNA-binding to the endogenous PAI-1 promoter. PAI-1 is a major contributor to progressive renal fibrosis in chronic kidney diseases, its promoter element contains several Smad Binding Elements, of which two cooperatively regulate the transcriptional response of PAI-1 to TGF-β [38]. ChIP showed increased Smad3 binding to these two PAI-1 promoter Smad-responsive elements, with maximal Smad3 binding detected 6 hours post-TGF-β1 stimulation (Figure 6.7). BMP-7 alone did not significantly alter Smad3 binding to PAI-1 promoter sites, but inhibition of binding was seen when cells were co-incubated with TGF-β1 and BMP-7 (Figure 6.8).



BMP-7 24h	Probe	-	-	+	+
TGF-β1 1h	only	-	+	-	+

Fig 6.4: EMSA of consensus Smad3/4 Binding Element Probe with nuclear protein extract.

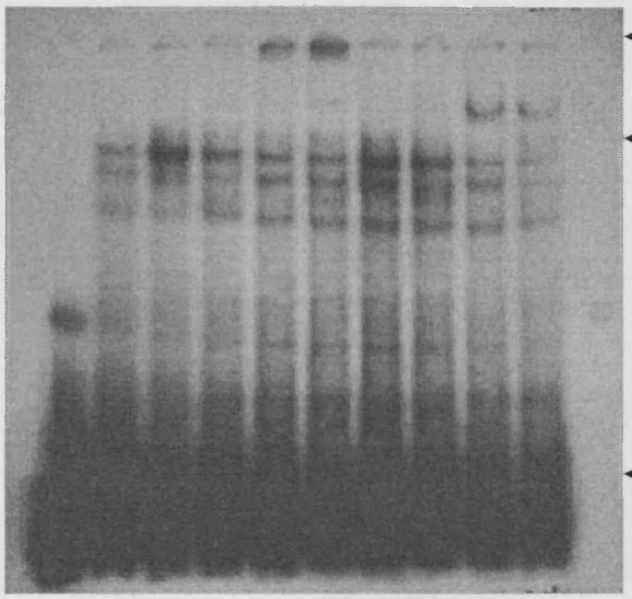
HK-2 cells were incubated with control medium or BMP-7 50ng/ml for 24 hours before incubation with TGF-β1 1ng/ml for 1 hour. Retarded probe and free probe are indicated. One representative experiment of three separate experiments is shown.



TGF-β1 1h	Probe only	-	+	+	+	+	+	+
Antibody		-	-	Smad2,3,4		IgGs	

Fig 6.5: Supershift assay with antibodies of Smad2,3,4 with nuclear protein.

HK-2 cells were incubated with control medium or TGF-β1 1ng/ml for 1 hour . **(Lane 1)** Probe only. **(Lane 2)** Control cells without antibody. **(Lane 3)** TGF-β1 treated cells without antibody. **(Lane 4-6)** TGF-β1 treated cells with Smad2,3,4 antibodies. **(Lane 7-8)** TGF-β1 treated cells with relevant IgGs. Supershift probe, retarded probe and free probe are indicated. One representative experiment of three separate experiments is shown.



BMP-7 24h	Probe	-	-	+	-	+	-	+	-	+
TGF-β1 1h	only	-	+	+	+	+	+	+	+	+
Antibody				-	-	-	Smad3	Smad5	Smad4	

Fig 6.6: Supershift assay with antibodies of Smad3,4,5 with nuclear protein extract.

HK-2 cells were incubated with control medium or BMP-7 50ng/ml for 24 hours before incubation with TGF-β1 1ng/ml for 1 hour . **(Lane 1)** Probe only. **(Lane 2-4)** Supershift without antibody. **(Lane 5-6)** Supershift with Smad3 antibody. **(Lane 7-8)** Supershift with Smad5 antibody. **(Lane 9-10)** Supershift with Smad4 antibody. Supershift probe, retarded probe and free probe are indicated. One representative experiment of three separate experiments is shown.

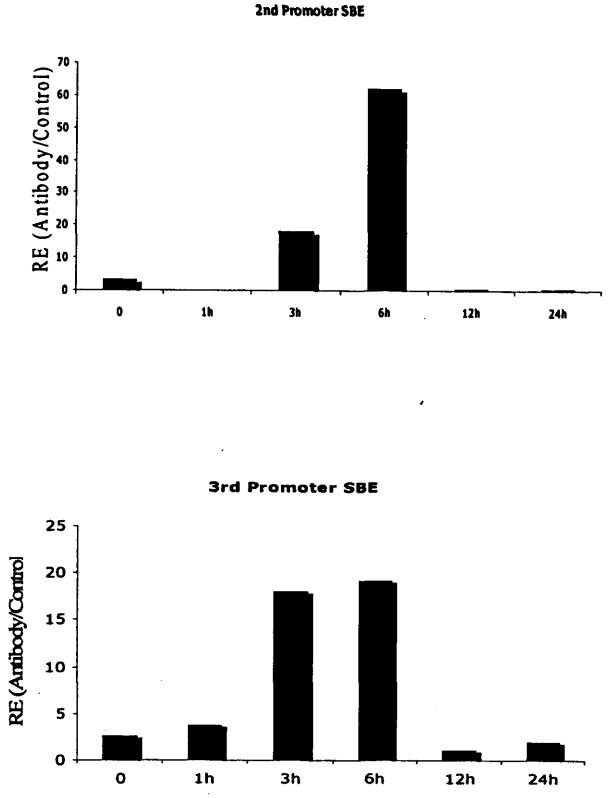


Fig 6 .7: Chromatin Immunoprecipitation (ChIP): Smad3 binding to the PAI-1 promoter.

Following chromation immunoprecipitation with Smad3 antibody or pre-immune globulin, two of PAI-1 promoter Smad3 binding elements were detected by QPCR.

HK-2 cells were incubated with TGF-β1 1ng/ml for up to 24 hours before CHIP. Data is presented as Smad3-precipitated signal/pre-immune globulin-precipitated signal, normalized to control. One representative experiment of three separate experiments for each PAI-1 Smad3 binding site is shown.

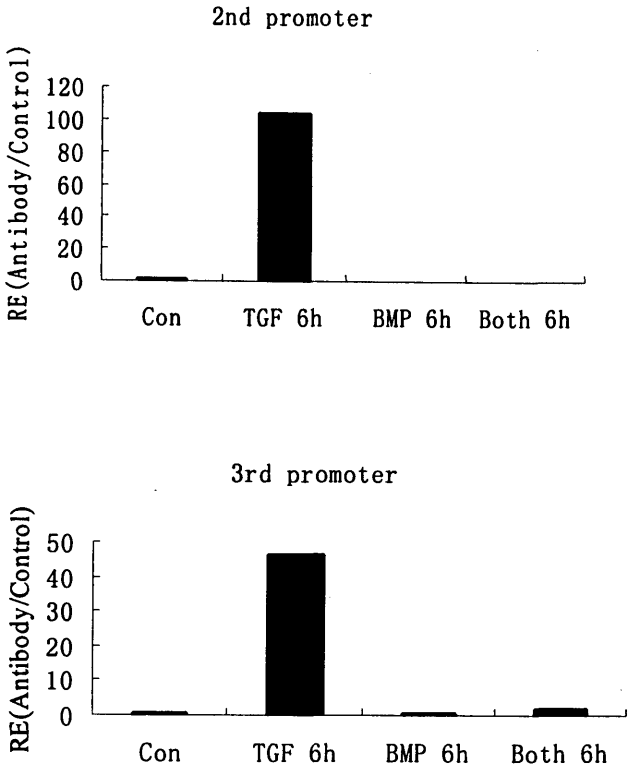


Fig 6.8 : Chromatin Immunoprecipitation (ChIP): Smad3 binding to the PAI-1 promoter.

Following chromation immunoprecipitation with Smad3 antibody or pre-immune globulin,PAI-1 promoter Smad3 binding elements were detected by QPCR.
HK-2 cells were incubated with BMP-7 50ng/ml with or without TGF-β1 1ng/ml for 6 hour. Data is presented as Smad3-precipitated signal/pre-immune globulin-precipitated signal, normalized to control. One representative experiment of three separate experiments for each PAI-1 Smad3 binding site is shown.

6.2.3 Inhibition TGF-β1-induced Smad3 signalling by BMP-7 is not via Ids

Overall, the above results show the core inhibition effect by BMP-7 is on Smad3 DNA-binding. Next, I assessed several transcriptional factors that might be involved in this process. The Id family is the helix-loop-helix (HLH) family of transcriptional factors, as an inhibitor of DNA-binding/differentiation. Id1, 2 and 3 all are BMPs target genes performing various functions depending on the cell types. Firstly, I assessed the expression of Ids in PTC by QPCR after the HK-2 cells were incubated with 1ng/ml TGF-β1 ± 50ng/ml BMP-7 for 24 hours. The data shows that BMP-7 alone dominantly increases all Ids expression, whereas a faint induction by TGF-β1 alone treatment is detected in PTC. When the cells were co-treated with TGF-β1 and BMP-7 together, all the Ids expression were reduced but still higher than the control cells (Figure 6.9). To examine the possible involvement of Ids in BMP-7 inhibition on Smad3 DNA-binding, I used Ids siRNA to knock down their expression alone or in combination. The results are not consistent with a role for Id proteins in the BMP-7 in response to TGF-β1 (Figure 6.10).

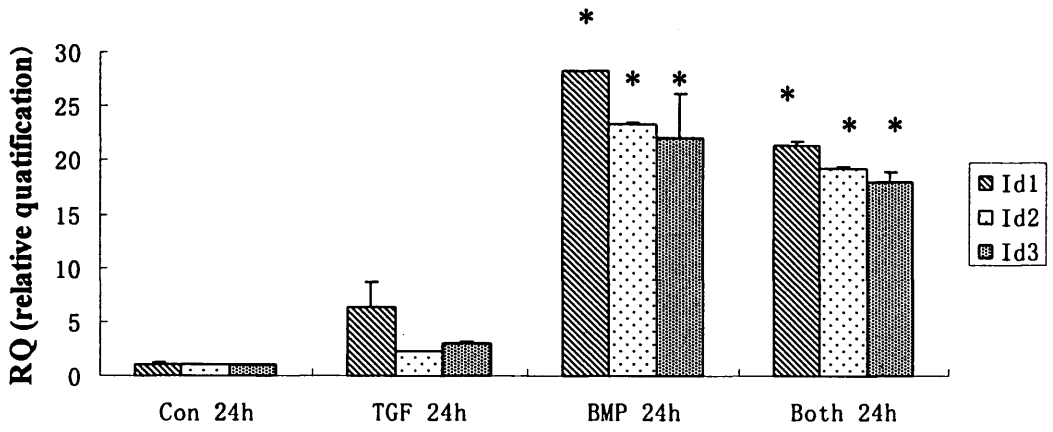
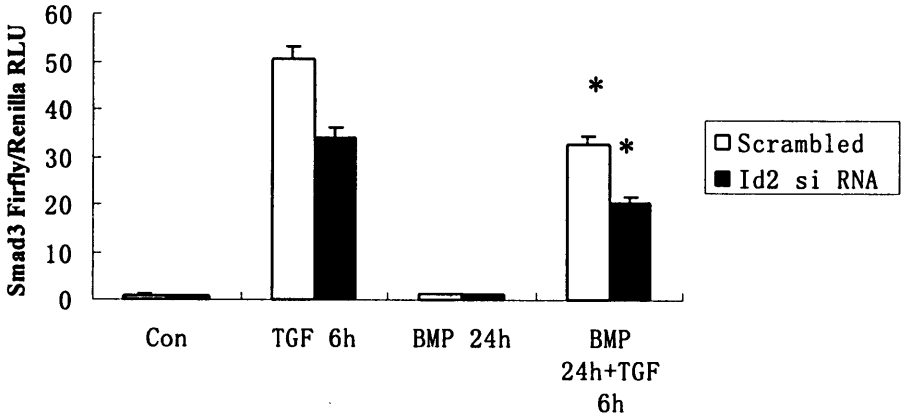


Fig 6.9: QPCR of Id1-3 expression by BMP-7 response to TGF-β1

HK-2 cells were growth arrest and exposed to BMP-7 50ng/ml with or without TGF-β1 1ng/ml for 24 hours. The relative quantification is calculated as described in the method with endogenous control Gapdh, normalized to control. $N = 3$,Mean ± SEM is plotted. $^*P <$ 0.01 for both and BMP-7 alone compared to control and TGF-β1 alone. One representative experiment of four separate experiments is shown.

A



B

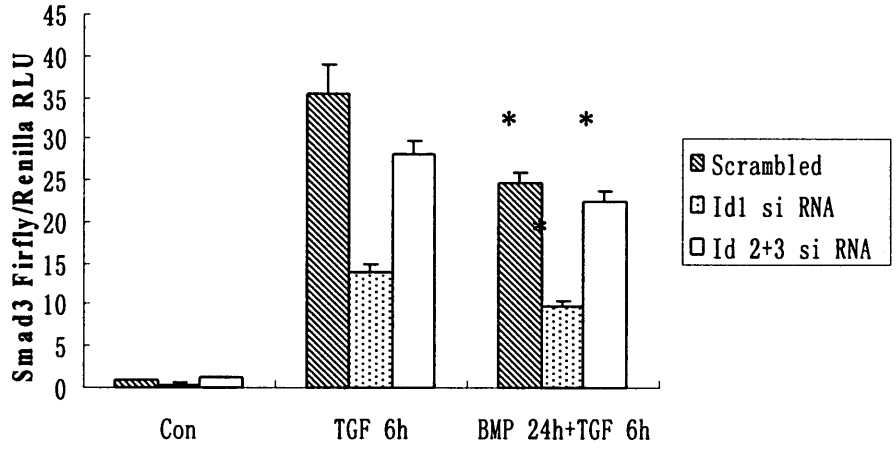


Fig 6.10: Effect of Ids on Smad3 signalling

HK-2 cells were transfected with Id1 **(B)** ,Id2 **(A)** , or Id2 and 3 siRNA together **(B)** for 24h, parallel with scrambled si RNA, then transfected Smad3 reporter construct CAGA(4)) together with pRL-CMV renilla. After incubation with control medium or BMP-7 50ng/ml for 24h,then incubated with control medium or 1ng/ml TGF-β1 for 6 hours. Ratio of firefly to renilla luciferase is displayed and normalized to control. $*P <$ 0.01 for BMP-7± TGF-β1 compared to TGF-β1 alone. Results represent Mean ± SEM of 3 individual experiments.

6.2.4 BMP-7 Inhibits Smad3 DNA Binding by preventing SnoN degradation but not Ski

Three key Smad transcriptional co-repressors, namely Ski, SnoN, and TGIF, have been identified. They are unique regulatory components within the nuclei during the final stage of TGF-β1 signalling binding to Smad proteins and to Smad binding elements in DNA, repressing Smad-dependent transcriptional activation.

Recent work demonstrates that SnoN degradation is required for Smad3-, but not for Smad2-dependent transcription [357]. Accordingly, Ski and SnoN expression were examined in PTCs following TGF-β stimulation. Immunoblot showed reduced SnoN expression from 15 minutes to 6 hours post-TGF-β stimulation, but from 12 or more hours post-TGF-β stimulation, SnoN was reaccumulatd (Figure 6.12A). No changes in Ski expression were seen in response to TGF-β over the time course examined (Figure 6.11 A). Subsequently, cells were incubated for 24h with 50ng/ml BMP-7 or control medium, followed by addition of TGF-β 1ng/ml for 30 minutes. Pre-incubation with BMP-7 prevented loss of SnoN expression seen with TGF-β alone (Figure 6.12 B).

The above data is consistent with reduction in Smad3 DNA binding due to a failure to degrade SnoN in BMP-7 treated cells. Accordingly, the effect of SnoN and Ski knockdown on TGF-β signalling by BMP-7 was examined. In subsequent experiments, PTCs were co-transfected with siRNA and with the Smad3 reporter vector CAGA (4). Basal Smad3 reporter activity was not altered by SnoN knockdown. BMP-7 inhibited reporter response in control transfected cells, but not

in those transfected with SnoN siRNA (Figure 6.13). In contrast, Ski knockdown did not prevent inhibition of reporter activation by BMP-7 (Figure 6.11 B).

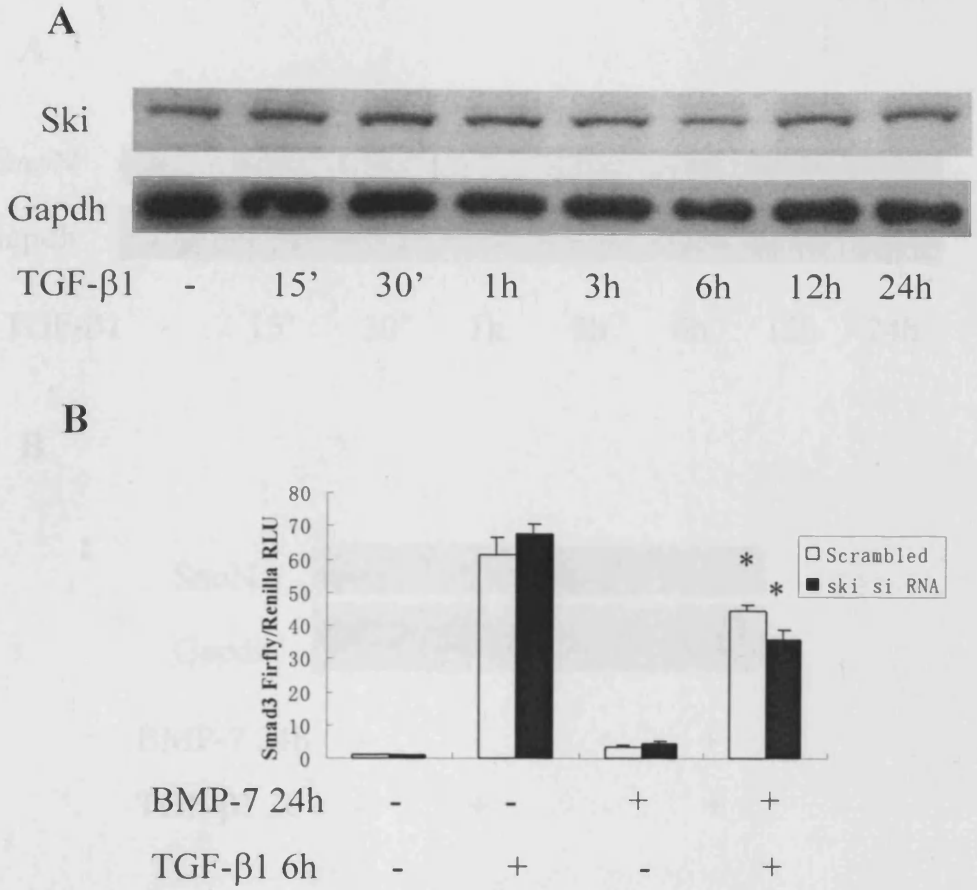


Fig 6.11: Effect of ski on Smad3 signalling

(A) TGF-β1 1 ng/ml was added to growth arrest HK-2 cells under serum free conditions for time points to 24 hours, before protein extraction and immunoblotting for ski. Blots were stripped and reprobed for Gapdh. **(B)** HK-2 cells were transfected with ski siRNA for 24h, parallel with scrammbled si RNA, then transfected Smad3 reporter construct CAGA(4) together with renilla as above. $*P < 0.01$ for BMP-7 ± TGF-β1 compared to TGF-β1 alone. Results represent Mean ± SEM of 3 individual experiments.

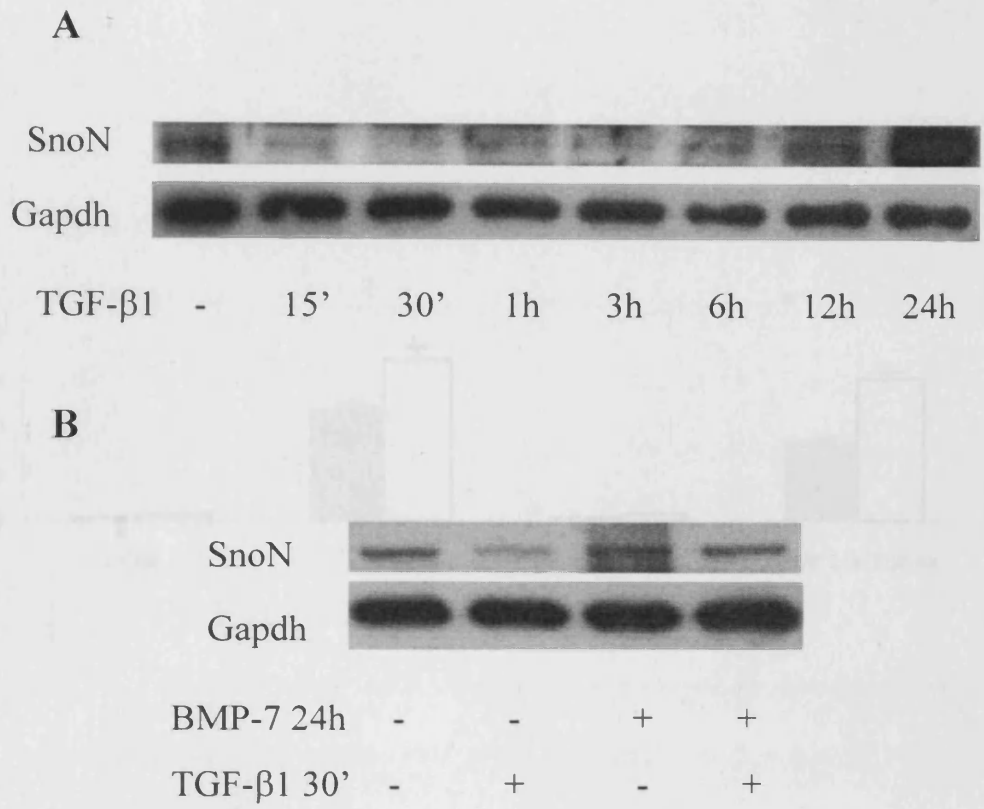


Fig 6.12: SnoN expression levels in response to TGF-β1 and BMP-7

(A) TGF-β1 1 ng/ml was added to growth arrested HK-2 cells under serum free conditions for time points to 24 hours, before protein extraction and immunoblotting for SnoN. Blots were stripped and reprobed for Gapdh. **(B)** HK-2 cells were treated with BMP-7 50ng/ml for 24 hours before incubation with TGF-β1 1ng/ml alone for 30 minutes. After immunoblotting for SnoN, blots were stripped and reprobed for Gapdh. One representative experiment of three separate experiments is shown.

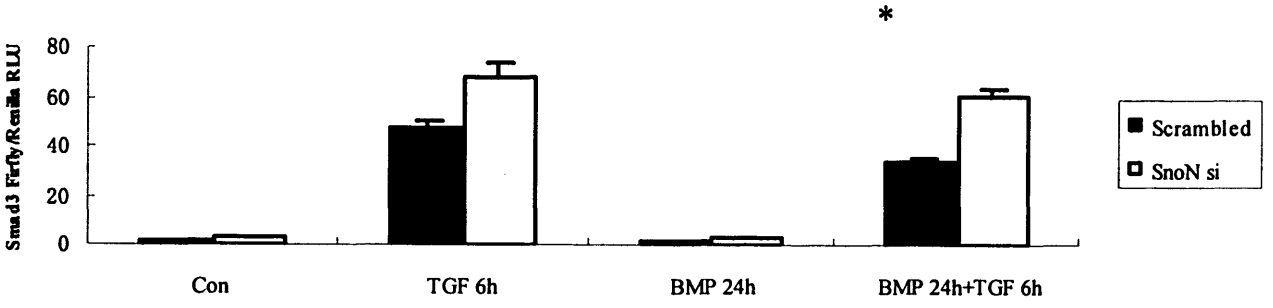


Fig 6.13: Effect of SnoN on Smad3 signalling

HK-2 cells were transfected with SnoN siRNA for 24h, parallel with scrammbled si RNA, then transfected Smad3 reporter construct CAGA(4) together with renilla as above. $*P < 0.01$ for BMP-7± TGF-β1 compared to TGF-β1 alone within scrammbled group. P=NS for for BMP-7± TGF-β1 compared to TGF-β1 alone within SnoN si RNA group. One representative experiment of three separate experiments is shown.

6.2.5 BMP-7 inhibition effect is reproduced by MG132

Degradation of SnoN may be prevented by the proteasome inhibitor MG132 [358]. The effect of MG132 on SnoN expression and Smad3 signalling in PTC was studied instead of BMP-7. Immunoblotting showed that MG132 did not alter basal SnoN expression, but that TGF-β-induced SnoN degradation was prevented by prior treatment with MG132 (Figure 6.14 A). MG132 treatment of cells transfected with CAGA (4) reporter did not alter reporter activity. However, reporter activity in response to TGF-β was diminished in cells pre-treated with MG132 (Figure 6.14 B). Subsequently, induction of PAI-1 by TGF-β was studied in MG132-treated PTC by qRT-PCR. PAI-1 induction by TGF-β was diminished by MG132 (Figure 6.15). Taken together, this data shows that prevention of SnoN degradation reproduces the effect of BMP-7 on Smad3 signalling.

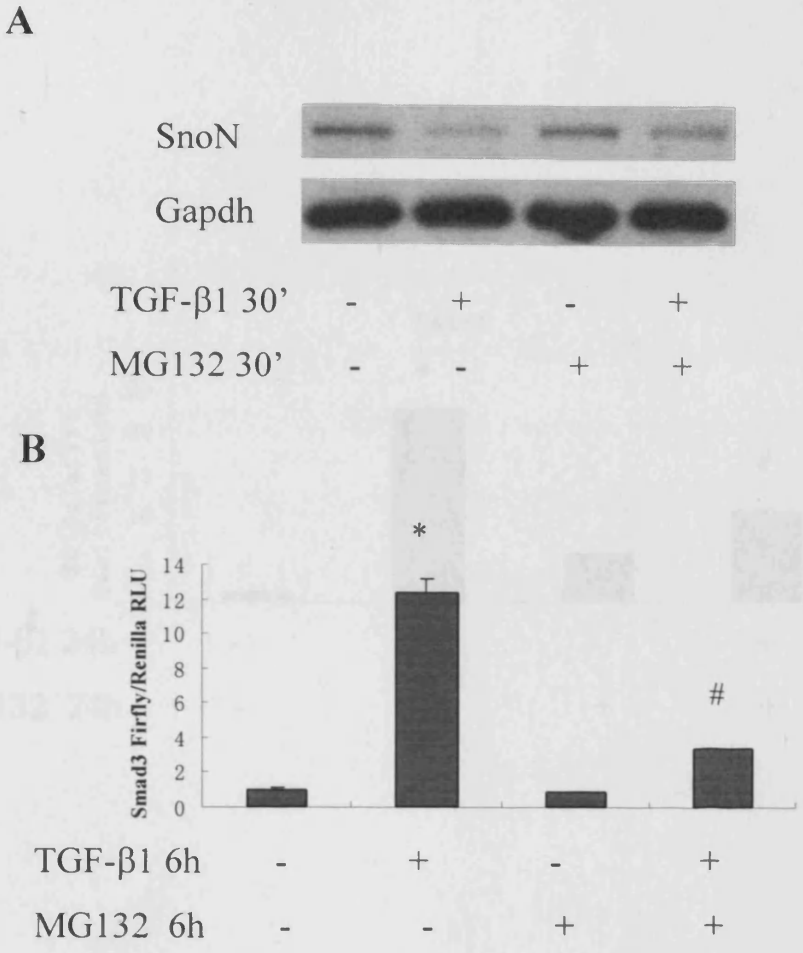


Fig 6.14: Effect of proteasome inhibitor MG132 on SnoN expression, Smad3 signalling

(A) HK-2 cells were incubated with control medium or TGF-β1 1 ng/ml ±MG132 10um for 30 minutes, and immunoblots of whole cell extracts for SnoN. Then blots were reprobed for Gapdh. **(B)** HK-2 cells were transfected with Smad3 and renilla reporter constructs, before incubation with control medium or TGF-β1 1 ng/ml ±MG132 10um for 30 minutes. *$P < 0.01$ for TGF-β1 vs Control, #$P < 0.01$ for MG132± TGF-β1 vs TGF-β1 alone. Data is presented as firfly/renilla luciferase activity, normalized to control. Result represented is from one of three individual experiments .

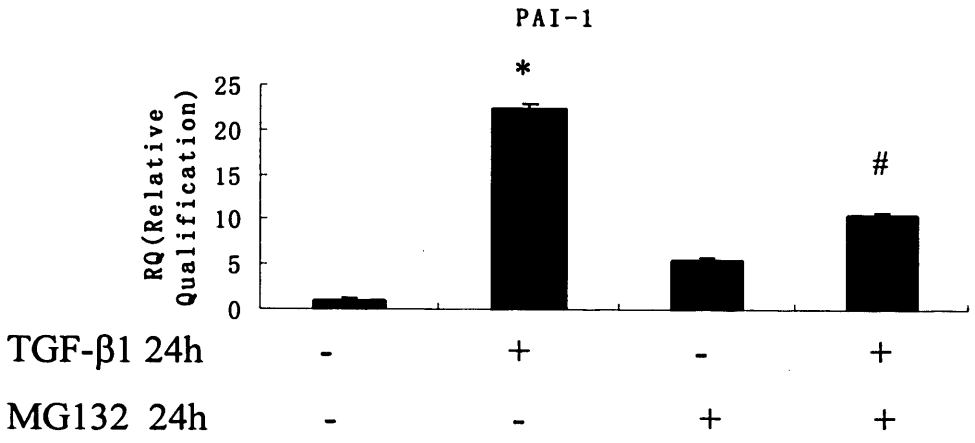


Fig 6.15: Effect of proteasome inhibitor MG132 on TGF-β1 -induced PAI-1 expression.

HK-2 cells were incubated with control medium or TGF-β1 1 ng/ml ±MG132 10um for 30 minutes , before detection of PAI-1 mRNA by QPCR, $^{*}P < 0.01$ for TGF-β1 vs Control, $^{\#}P < 0.01$ for MG132± TGF-β1 vs TGF-β1 alone. Results represent Mean ± SEM of 3 individual experiments.

6.2.6 Initial results about Arkadia effect on BMP-7 inhibition effect in response to TGF-β1

Recently, several groups have showed Arkadia, an E3 ubiquitin ligase, targets SnoN for degradation[357, 359]. Thus, I checked the Arkadia expression with TGF-β1 treatment from 15 minutes to 24 hours, and pre-treat BMP-7 for up to 24 hours prior 30 minutes TGF-β1 in PTC. No change was observed by immunoblotting (Figure 6.16 A-B). With QPCR, there is no difference at transcriptional change by TGF-β1 with or without BMP-7 treated for 24 hours (Figure 6.17).

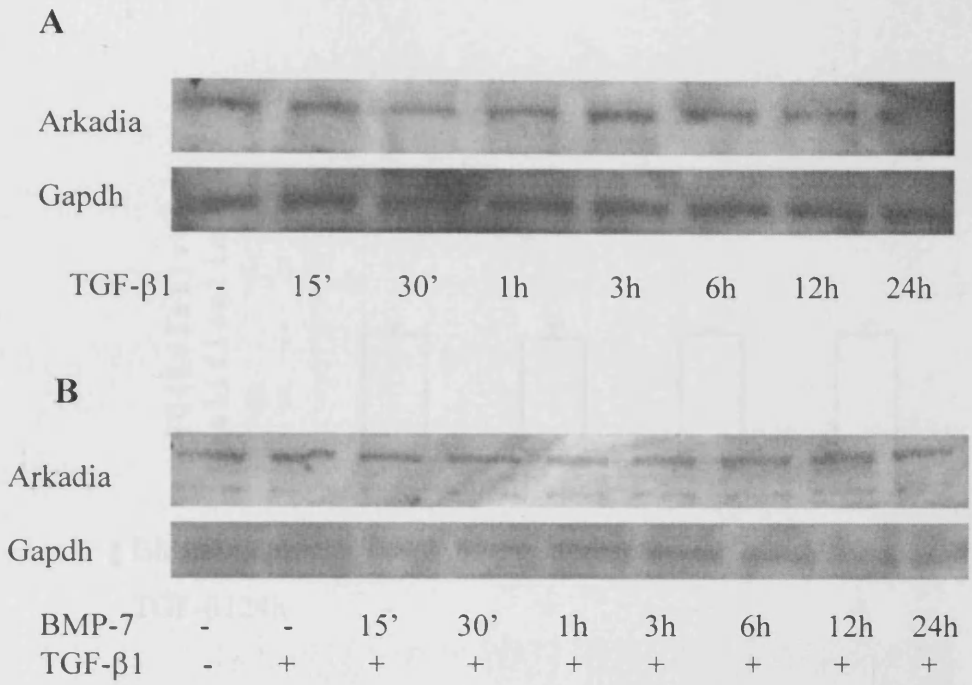


Fig 6.16: Time course of Arkadia expression in response to TGF-β1 ± BMP-7

(A) TGF-β1 1 ng/ml was added to growth arrest HK-2 cells under serum free conditions for time points to 24 hours, before protein extraction and immunoblotting for Arkadia. Blots were stripped and reprobed for Gapdh. **(B)** Growth arrest HK-2 cells were treated with BMP-7 at 50ng/ml up to 24 hours , before incubation with 1ng/ml TGF-β1 for 30 minutes. The whole lysate was probed with Arkadia. Blots were stripped and reprobed for Gapdh. One representative experiment of three separate experiments is shown.

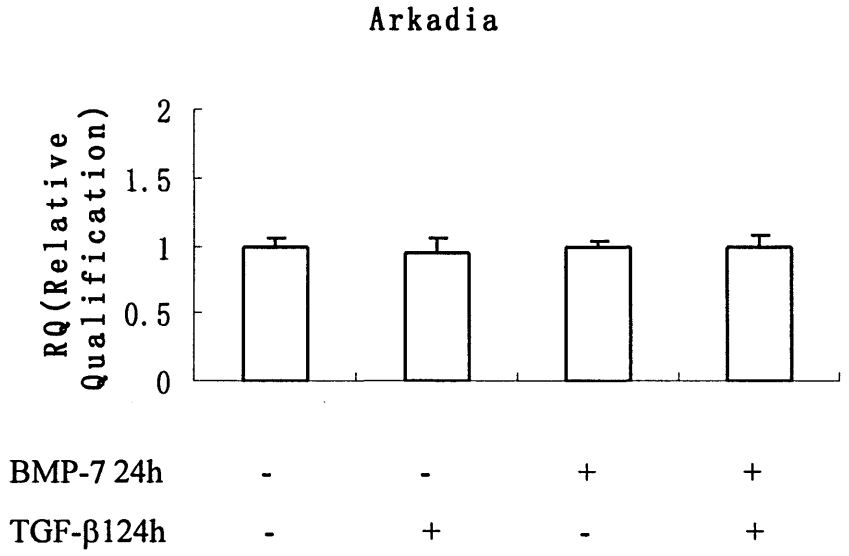


Fig 6.17: Arkadia expression in response to TGF-β1 and BMP-7

Growth arrest HK-2 cells were incubated with BMP-7 50ng/ml with or without TGF-β1 1ng/ml for 24 hour under serum free conditions. qRT-PCR analysis shows expression of Arkadia. Results represent Mean ± SEM from one of three individual experiments.

6.3 Discussion

In chapter three, I have observed that BMP-7 exclusively lessens Smad3 signalling without alteration Smad2 in PTC via DNA-binding with EMSA but not Smad3 phosphorylation. In this chapter, I examined the mechanism further for this.
After Smad3 phosphorylation, the nuclear import and export of Smad3, together with dephosphorylation, ubiquitination, and degradation will be regulated.

Firstly, my initial results have shown BMP-7 decreases TGF-β1 Smad3 signalling with CAGA (4) reporter gene assay. Then I attempted to find out the upstream mechanisms leading to attenuation of this DNA-binding. On the basis of the TGF-β1 signalling pathway, shuttling of phospho-Smad3 between the cytoplasm and nucleus monitors TGF-β1 receptors activity. I examined the phospho-Smad3 half-life or degradation by BMP-7 in response to TGF-β1 with ALK5 kinase inhibitor SB431542. The rate of decline of phospho-Smad3 in response to TGF-β1 is not altered by BMP-7.

Together with the unchanged immunoblotting of phospho-Smad3 by BMP-7 in response to TGF-β1, these data suggest that the regulation of Smad3-DNA-binding occurs beyond the R-Smads phosphorylation, mainly in the nucleus.

Following the route of TGF-β1 signalling, after Smad3 phosphorylation, the R-Smad-Smad4 complex moves into the nucleus. In mesangial cells, previous work has shown BMP-7 leads to the loss of TGF-β1 signalling via BMP-7-induced Smad5

downstream inhibitory Smad6 induction following the failure of Smad3 nuclear accumulation [314]. Compared to this, whereas my results demonstrated no difference in TGF-β1-induced phospho-Smad3 nuclear accumulation and BMP-7 treated cells.

My EMSA and ChIP results show that BMP-7 inhibits Smad3 DNA binding. Previous work suggests Smad5 may compete with Smad3 at consensus Smad binding elements [356]. However, my SuperShift experiments showed no evidence for this.

As outlined in chapter one, Smads have DNA-binding capability, but do not act as transcriptional inhibitors in isolation. Instead, co-activators and repressors take part in transcriptional regulation progress. Therefore, I studied the role of known transcriptional co-repressors of TGF-β1 signalling in the reduction of Smad3 DNA-binding by BMP-7.

Activated R-Smads-Smad4 complex achieves target genes promoter to initiate transcription by association with diverse DNA-binding factors. There are some transcriptional repressors such as Ski, SnoN, TGIF, and inhibitors of differentiation (Ids) to inhibit Smads DNA-binding.

There are four known members of the Id family in vertebrate (called Id1, Id2, Id3 and Id4) that constitutes the helix-loop-helix (HLH) family of transcription factors, which lack a DNA-binding domain, and then fail to bind DNA specifically or form an active heterodimers [360]. And its activity as an inhibitor of DNA-binding is cell

type dependent. Id1 is a well known BMPs target gene and regulates differentiation switches in osteoblasts, fibroblasts, epithelial cells, and endothelial cells[361], also associated with cell dedifferentiation including tubular epithelial cells by suppressing E-cadherin and ZO-1 [362]. Id2 and Id3 expression is also induced by BMPs, but their physiological roles remain unknown [361]. TGF-β1 can repress or induce Id1 expression, in a cell type specific manner [361, 362]. My results show that both BMP-7 and TGF-β1 induce Id1-3 mRNA expression in PTC, with substantially greater induction of Ids by BMP-7 than by TGF-β1. However, siRNA-mediated repression of Id expression did not affect the inhibitiory action of BMP-7 on TGF-β1 signalling.

Ski and SnoN are important transcriptional repressors of TGF-β1-induced Smads signalling. Both are markedly reduced in the fibrotic kidney induced by UUO mice [363]. In addition to disruption of Smad complexes activity, Ski or SnoN also prevents R-Smads from binding to transcriptional co-activators and recruits transcriptional co-repressors to integrate with R-Smads to the targeted promoters DNA-binding elements [364]. Thus, my finding shows that Ski is not involved in the inhibitory role of BMP-7 on TGF-β1 signalling. As for SnoN, my data show that BMP-7 repressed specific TGF-β1 Smad3 signalling via preventing SnoN degradation.

The previous work has shown that Ski, SnoN are involved in the downregulation of Smads signalling [363, 365]. In the current study, I have found that BMP-7 specifically limits Smad3 dependent transcriptional activation in PTC, *via* preventing

SnoN degradation, which is required for Smad3 but not Smad2 dependent transcriptional events.

SnoN is a member of the *ski* family of oncoproteins that binds to the consensus Smad3 binding element (GTCTAGAC) and represses transcription [366]. Interestingly, SnoN specifically inhibits Smad3 DNA binding activity , and SnoN degradation is required for transduction of the Smad3, but not Smad2 signal cascade[357]. SnoN expression is progressively reduced in the kidney following unilateral ureteric obstruction in mice, and loss of SnoN expression sensitizes renal tubular epithelial cells to EMT [363]. SnoN may be degraded by recruitment to the proteasome by Smad3 [358], and loss of SnoN expression in animal models of progressive renal fibrosis occurs *via* enhanced ubiquitin-mediated degradation [367, 368]. Interestingly, Hepatocyte Growth Factor exerts an anti-fibrotic effect in the kidney by enhancing SnoN expression in PTCs [43, 369].

Arkadia, an E3 ubiquitin ligase, is involved in the ubiquitin-dependent proteasomal degradation of Smad7 [173, 174], amplifying TGF-β signalling. In addition, Arkadia interacts with SnoN in their free forms as well as in the forms bound to Smad proteins complex, and constitutively down-regulates levels of their expression [359]. Moreover, Arkadia promotes specifically transcription via Smad3/Smad4 binding sites by degrading the transcriptional repressor, SnoN, in response to TGF-β1 signalling [357]. Smad7 is an important negative regulator in TGF-β1 signalling in the cytoplasm especially at TGF-β receptor level [370], and recently it has been shown that Smad7 disrupts the formation of the TGF-β-induced functional Smad-DNA complex in the nucleus independent of TGF-β type I receptor [161]. I have

shown that the regulation level of BMP-7 on TGF-β1 signalling is in the nucleus, and then the possible mechanisms are the involvement on Arkadia-Smad7 DNA-binding interaction or Arkadia-SnoN interaction on Smad3-DNA-binding. Firstly, I examined the expression of Arkadia in PTC by TGF-β1 and/ or BMP-7, and the data show there is no change on mRNA and protein level expression by Immunoblotting and QPCR. The next possibility is the association change between Arkadia and SnoN or Smad7. This is a possible future direction for this work.

In summary, my current data demonstrate that preservation of SnoN expression underlies antifibrotic actions of BMP-7, loss of SnoN expression may be a key element of progressive renal fibrosis.

Chapter Seven:

General Discussion

TGF-β1 is a vital cytokine for organ development, maintaining the balanced tissue homeostasis. TGF-β1 has critical functions in embryogenesis, tissue repair, inflammation and immunity, as well as the generation of cancer and fibrosis. It is also a well known key pro-fibrotic factor in fibrotic progress including renal fibrosis. In the kidney, the degree of tubulointerstitial fibrosis is correlated well with the rate of renal function loss, leading to ESRD. And TGF-β1 plays important roles in the development of tubulointerstitial fibrosis. TGF-β1 acts through its Smads-related signalling pathway. Thus the regulation of TGF-β1 signalling pathway is a main point to determine the final response of TGF-β1 in renal fibrosis without affecting its other key functions.

Since renal inflammation is thought to be an important determinant of progressive interstitial fibrosis injury involving infiltration of the tublo-interstitum by macrophages, and consequent release of cytokines including interleukin-1, the first stimulus on TGF-β1 signalling regulation I selected was IL-1β. I have focused on the effect of IL-1β on TGF-β1 signalling in the proximal tubular cells. HK-2 cells were incubated with TGF-β1±IL-1β from a short-time point 15 minutes to a long-time point up to 24h. I have demonstrated a switch from an inhibitory to a sensitising effect of IL-1β on Smad signalling after approximately 3 hours with immunoblot of phospho-Smad3. I obtained the similar results for phospho-Smad2. In order to confirm these findings, the transient transfection of reporter gene constructs confirmed these two contrary effects of IL-1β for both Smad2 and Smad3 signalling. The second stimulus I selected is BMP-7 because it is highly expressed in the kidney, and can block fibrogenic actions of TGF-β1 including epithelial to mesenchymal transition (EMT). In my work of this part, I have shown that BMP-7 significantly

decreased Smad3 signalling specifically but not Smad2 in PTC with the transient transfection of reporter gene constructs, whereas phospho-Smad3 is not lessened by BMP-7 in response to TGF-β1 by immunoblot.

In our lab, we have previously shown that in PTC, TGF-β receptor distribution in the plasma membrane is regulated by stimuli including HA (via CD44). CD44 is co-localized with TGF-β1 type I receptor in the cytoplasmic membrane of PTC, leading to altered TGF-β receptor distribution and association in response to HA-CD44 interaction. This relationship led to a hypothesis that HA-CD44 was a potential mediator by which both IL-1β and BMP-7 might affect TGF-β1 signalling. I have found that IL-1β induced CD44 expression at 24h but found no change in the isoform of CD44 previously shown to alter TGF-β1 signalling, CD44v3. Furthermore, following siRNA-mediated repression of CD44, increased Smad signalling response was still seen following chronic incubation with IL-1β. Similarly, I did not detect any change in PTC CD44 expression following incubation with BMP-7, and repression of CD44 expression with siRNA did not alter the effect of BMP-7 on TGF-β1 signalling. Moreover, adding in or removing HA in the medium dId not change the effects of IL-1 or BMP-7 on TGF-β1 signalling. The above results showed HA/CD44 is not involved in both TGF-β1 Smad signalling regulation by these stimuli.

NF-κB is an important downstream effector stimulated by IL-1β and has been linked to inhibition of TGF-β signalling. One subunit of NF-κB, p65, is showed to inhibit TGF-β1-induced Smads phosphorylation, nuclear translocation, and DNA binding by TNF-a. The previous work in PTC has indicated IL-6 leading to enhancement of

TGF-β1 signalling via a shift of type I receptors from lipid rafts to non-raft associated plasma membrane. To explore the possible roles of P65 and IL-6 in the early inhibitory effect and later stimulatory effect of IL-1β respectively on TGF-β1 signalling, I sought to determine the P65 activation and IL-6 generation during the whole time points of IL-1β. The data demonstrate that early inhibition effect of IL-1β on TGF-β1 signalling is via NF-κB activation, a switch from p65/p50 to p50/p50 complexes, consistent with the time curve of upstream regulators, IKK and IKB. Furthermore, the later prolonged enhancement effect of IL-1β on TGF-β1 signalling is via an autocrine IL-6 loop.

In terms of the further mechanism of how P65 regulates IL-1β inhibition TGF-β1 signalling, some work has previously been shown to inhibit TGF-β1-induced gene expression via sequestration of the transcriptional coactivator p300. This will be a possibility to do for the future work. As for IL-6, it will be interesting to examine if it is a mediator for other proinflammational stimuli and if it is the same story *in vivo*.

A lot of work *in vivo* and *in vitro* has shown BMP-7 has a protective effect on renal fibrosis, but the mechanism is not clear. My data has indicated that BMP-7 inhibits TGF-β1 Smad3 but not Smad2 signalling via DNA-binding without apparent reduction in Smad3 phosphorylation and translocation into the nucleus. This led to a hypothesis that the regulation site is in the nucleus around DNA-binding. Thus, I examined the role of transcriptional co-repressors such as Ski, SnoN and Id proteins in this process.

SnoN binds to the consensus Smad3 binding element and represses transcription. Interestingly, SnoN has a specific inhibitory action on Smad3 DNA binding, and SnoN degradation is required for transduction of the Smad3, but not Smad2 signal to recruit to the proteasome. My data showed that BMP-7 prevented the SnoN degradation in response to TGF-β1, thus disturbed the Smad3 DNA-binding. With SnoN siRNA, the effect of BMP-7 on TGF-β1 Smad3 signalling reduction is at least partly lost, suggesting SnoN degradation is involved in BMP-7 role on Smad3 signalling.

Arkadia, an E3 ubiquitin ligase, degrades ubiquitin-dependent Smad7, thus amplifying TGF-β signalling. In addition, Arkadia interacts with SnoN and constitutively down-regulates SnoN expression. Moreover, Arkadia promotes specifically transcription via Smad3/Smad4 binding sites by degrading SnoN, in response to TGF-β1 signalling [357]. In order to investigate if Arkadia is involved in SnoN degradation by BMP-7, I examined the expression of Arkadia preliminary in PTC and it showed no change on its expression. The next possibility is the interaction between Arkadia and SnoN or Smad7, this will be a possible further direction to do.

In addition to BMP-7, there is another well known anti-fibrotic cytokine, HGF. Some work have shown HGF blocks EMT by antagonizing TGF-β1's action via upregulating Smad transcriptional co-repressor SnoN expression in HKC cells [371]. The investigation has shown that HGF induces SnoN expression in a cell type specific manner coordinating with CREB activation and Sp1 [369]. This will lead to

another area of how SnoN and Arkadia take part in HGF performance in renal fibrosis *in vivo* and *in vitro*.

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