The Contribution of Methyltransferases / Demethylases to Renal Fibrosis
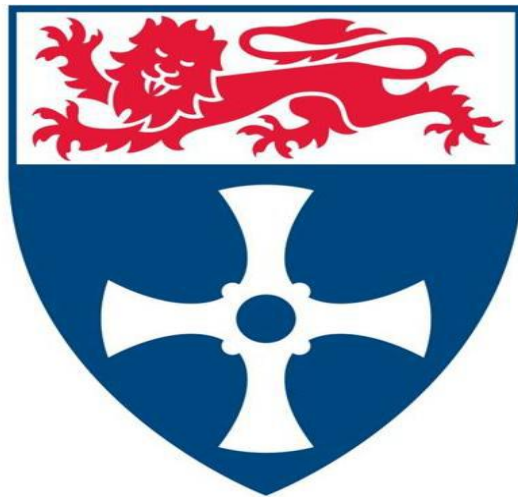
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In the name of Allah, the Most Gracious and the Most Merciful

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ABBREVIATIONS

AdoMet	S-Adenosyl methionine
AngII	Angiotensin II
CKD	Chronic kidney disease
COLI	Collagen type I
COLIII	Collagen type III
CCL5	Chemokine (C–C motif) ligand 5
CTGF	Connective tissue growth factor
DMEM	Dulbecco''s Modified Eagle''s Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethlyenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
EMT	Epithelial mesenchymal transition
ECM	Extra cellular matrix
EMT	Epithelial–mesenchymal transition
FGF2	Basic fibroblast growth factor
FN	Fibronectin
H3K4	Lysine 4 of histone H3
HMTs	Histone methyltransferases
LAP	Latency-associated peptide
LTBP	Latent TGF-β binding protein
MCP1	Monocyte chemotactic protein 1
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem cells

NLS	Nuclear localisation signal
PBS	Phosophate buffered saline
PBST	Phosophate buffered saline-Tween 20
PCR	Polymerase chain reaction
PAI-1	Plasminogen activator inhibitor 1
PDGF	Platelet-derived growth factor
PTMs	Post transitional modifications
siRNA	Small interfering ribonucleic acid
α-SMA	Alpha-smooth muscle actin
SMAD	SMA/MAD homology. SMA is one of a class of C. elegans genes that give a "small" phenotype, and some of them are homologous to MAD
TBM	Tubular basement membrane
TGF-β	Tumour growth factor-beta
TH	T helper
THMC	Transformed human mesangial cells
TNF	Tumor necrosis factor
tPA	Tissue-type plasminogen activator
PKMATs	Protein Lysine Methyltransferases
UUO	Unilateral ureteric obstruction

ABSTRACT

TGF β-1 signalling regulates many cellular processes, including proliferation, differentiation, apoptosis, immune responses, and fibrogenesis. The essential role of TGF β-1/SMAD signalling in stimulating fibrogenic cells to produce extra cellular matrix proteins and promoting proliferation of myofibroblasts is widely recognised. SMAD3 is known as a mediator in TGF β- 1-induced fibrosis in the kidney. Upon activation of TGF-β receptors, SMAD2 and SMAD3 are phosphorylated and form cytoplasmic heteromeric complexes with SMAD4. These complexes translocate to the nucleus where they regulate expression of TGF β-1 target genes. Tissues undergoing fibrosis exhibit markedly increased expression of a-SMA and interstitial matrix components, such as collagen and fibronectin which are TGF β-1/SMAD3-responsive genes. The mechanism by which SMADs mediate transcriptional regulation of these genes is incompletely understood, however SMAD3-null mice are protected against renal tubulointerstitial fibrosis, glomerular sclerosis, and also fibrosis in other organs.

Data from this thesis has demonstrated for the first time that methylation might be important in the regulation of SMAD3 transcriptional activity; indeed the introduction of siRNAs targeting demethylases/methyltransferases led to changes in SMAD3 transcriptional activity. The introduction of siRNAs targeting both methyltransferases and demethylases resulted in changes in α-SMA and fibronectin expression at the protein level. The work in this dissertation again confirms that TGF β-1 signalling is a SMAD3- dependent pathway. SET9 is a methyltransferase enzyme that can methylate non-histone protein substrates including the transcription factors p53, Stat3, Rb, TAF10, E2F1, ERα, NF-κB, and DNMT1. I show that SET9 plays a central role in regulating SMAD3 activity, as shown by SET9 knockdown. I also show that wild type SET9 overexpression results in increased SMAD3 activity, in the presence of TGF β-1. Conversely, expression of a mutant SET9, which lacks methyltransferase activity,

failed to increase SMAD3 activity, even in the presence of TGF β-1. Furthermore, SET9 gene silencing with siRNAs significantly attenuated TGF β-1–induced ECM gene expression. These novel effects of SET9 warrant further evaluation of SET9 as a target in the treatment of fibrotic diseases such as CKD.

Screening a demethylase siRNA library showed that the putative demethylase HSPBAP-1 is also involved in TGF β/SMAD signalling. Interestingly, I show that HSPBAP-1 interacts with SMAD3, and suppresses the transcriptional activity of SMAD3-driven reporter-genes. This is the first report of such an interaction, and the first data implicating a potential demethylation event in TGF β-1/SMAD3 signalling.

Taken together, the work in this study defines novel roles of SET9 and HSPBAP-1 in fibrosis by mediating TGF β-1/SMAD3 signalling.

On the basis of my work, future examination of SET9/HSPBAP-1 in whole organism models of renal fibrosis should be considered.

1 INTRODUCTION

1.1 The urinary Tract

1.1.2 Anatomy of the Kidney

The kidneys are paired bean-shaped organs, located either side of the vertebral column along the posterior muscular wall of the abdominal cavity, with the left kidney higher than the right. In adults, the kidneys contribute approximately 0.5 percent of total body weight, with each kidney weighing approximately 150 to 160 grams. Each kidney is surrounded by a capsule, formed by a thin layer of fibrous connective tissue. The kidney is divided into two main regions, the renal cortex and the renal medulla. The medulla is made of renal pyramids that project into the renal pelvis (Figure 1.1).
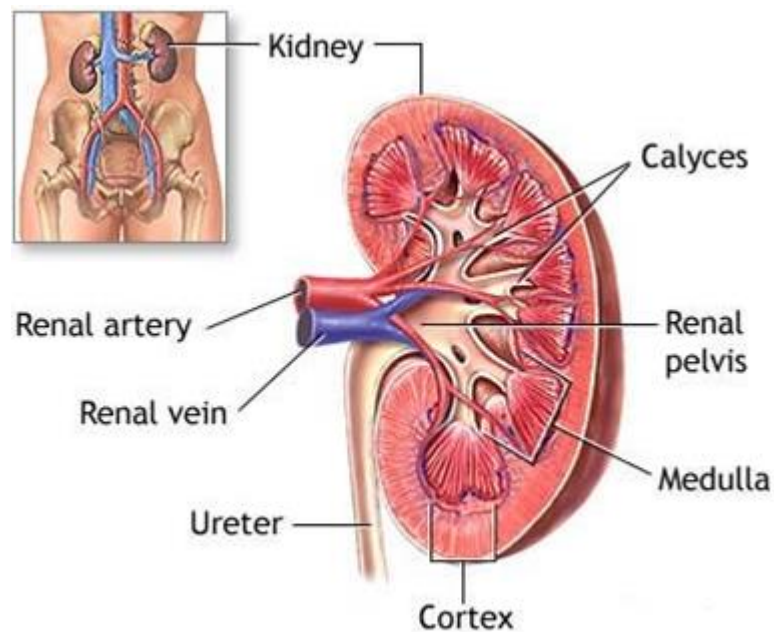
Figure.1.1.General anatomy of kidney

Taken from http://www.kidneystone911.com/kidney-anatomy.html

1.2 The nephron

In the human kidney there are approximately one million nephrons, which are the functional subunits of the kidney. Based on their location and length of the Loop of Henle, they are classified into cortical and juxtamedullary nephrons. The glomerulus is a dense network of capillaries with a high surface area allowing blood to contact the capillary walls. Glomerular capillaries are surrounded by special epithelial cells, known as podocytes that work together with the endothelium and the glomerular basement membrane of the capillaries to form a filter, separating blood passing through the glomerulus from the urinary space. Water and small molecules pass through this filter due to relatively high hydrostatic pressure within the glomerular capillaries. The filtrate subsequently enters the tubular network and is modified by reabsorption and secretion of water and soluble ions. Urine is further concentrated as it passes through the collecting duct, before being carried the renal pelvis and the ureter (Figure 1.2 and 1.3).
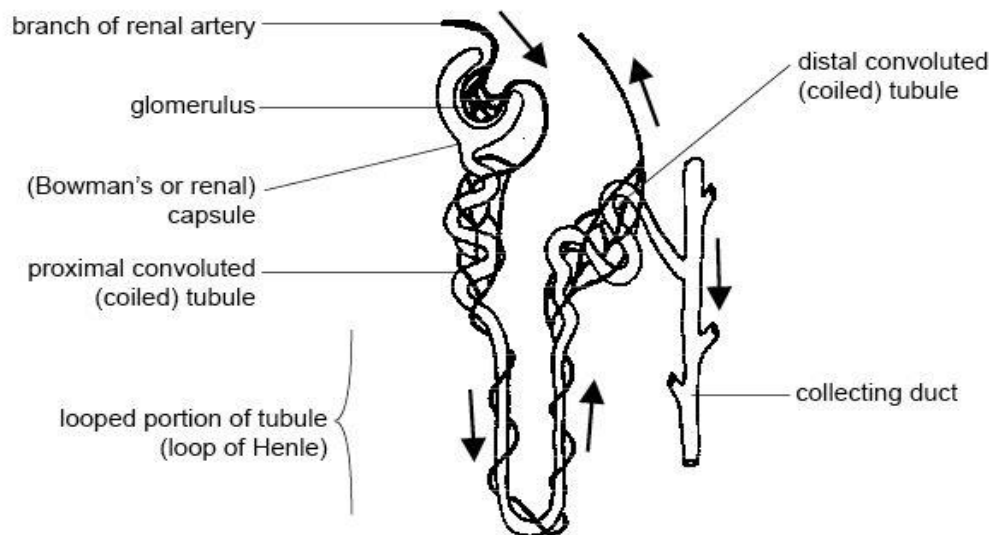
Figure 1.2. Structure of an individual nephron

Taken from http://wikieducator.org
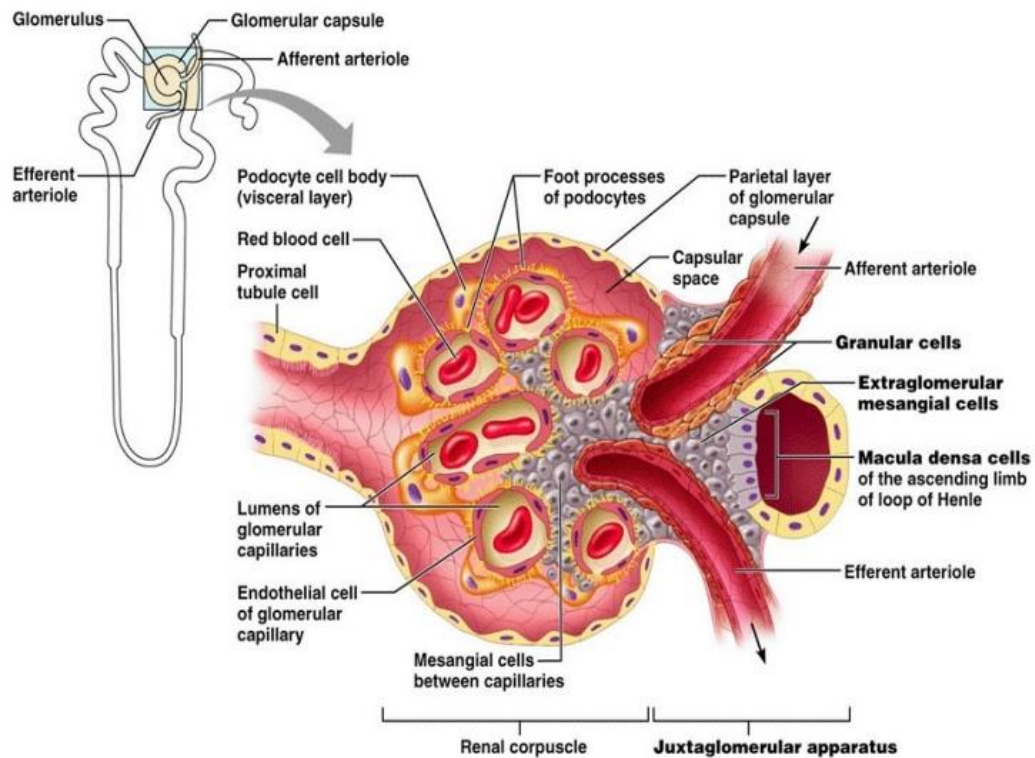
Figure 1.3. Anatomy of the glomerulus

Taken from http://classes.midlandstech.edu/carterp/Courses/bio211/chap25/chap25.htm

1.1.1 The ureters and The urinary Bladder

The ureters are approximately 12 inch long hollow muscular tubes lined with transitional epithelial cells. Two ureters are present, one attached to each kidney. They originate from the renal pelvis and facilitate the passage of urine from the kidney to the urinary bladder (Figure 1.1).

The bladder, located in the pelvis between the pelvic bones, is the site of short-term storage of urine before micturition. The bladder wall consists of smooth muscle bundles and the lumen is lined by a multi-layered transitional epithelium connected to the bladder wall through a thin basement membrane.

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1.1.3 Renal Function

The kidneys perform a critical role in regulating fluid and electrolyte homeostasis. This is achieved by ultrafiltration of water and small molecules, reabsorption of solutes from the filtrate, and tubular secretion from blood into the urine. In health, the kidneys function to remove water and wastes from blood, control blood pressure. Furthermore, the kidneys are involved in the hormonal production of erythrocytes, via the synthesis of erythropoietin, and vitamin D metabolism to maintain bone structure.

1.2 Renal Fibrosis and Chronic Kidney Disease

1.2.1 Chronic Kidney Disease (CKD)

Chronic kidney disease (CKD) is a worldwide public health problem. It is associated with an increased risk of cardiovascular disease and end stage renal failure. CKD is defined by the presence of blood or protein in the urine or a decline in glomerular filtration rate (GFR) to less than 60 ml / min/$1.73m^2$ for at least 3 months (National Kidney Foundation (NKF), 2002).

Despite an improved awareness of CKD, delays in diagnosis, as well as very limited therapeutic options, mean that potential opportunities to prevent renal failure are limited. The economic importance of CKD should not be underestimated, the expenditure on which was approximately £1.45 billion between 2009 and 2010 in England alone. For the patient, the main consequences of CKD include progression to kidney failure and the complications of poor kidney function, such as increasing cardiovascular risk, bone disease, and anaemia. Progression of kidney disease to end-stage renal failure can be prevented in some cases by recognising and treating kidney disease in its early stages (Kerr, Bray et al. 2012).

1.2.2 Renal Fibrosis

Fibrosis refers to the excessive formation and deposition of scar tissue, and is considered as a major cause of morbidity and mortality. It is associated with organ failure in a variety of chronic diseases, affecting the kidneys, heart, lungs, eyes, liver, and skin (Gordon and Blobe 2008). Fibrosis is initiated by a diverse range of pathological, physiological, biochemical and physical factors. Regardless of the variant aetiologies, there are common pathogenetic processes, such as activation of the key pro-fibrotic cytokine transforming growth factor-beta1 (TGF β-1). This cytokine plays a critical role in increasing the synthesis of extracellular matrix (ECM) proteins, such as collagen, which characterises fibrosis (Wynn 2008) .

Renal fibrosis is common to most, perhaps all, progressive kidney diseases that lead to the need for dialysis or kidney transplantation (Boor, Ostendorf et al. , Boor, Ostendorf et al. 2010). Fibrosis can occur in either the filtering unit (glomerulus) or around the tubules of the nephron, referred to as glomerulosclerosis and tubulointerstitial fibrosis, respectively. Tissue capillary rarefaction, a decrease in the number of capillaries, is also a typical feature of chronic progressive renal disease. A comprehensive understanding of the pathogenesis of renal fibrosis after kidney injury remains the main challenge in designing effective therapeutic strategies to slow the progression to renal failure and dialysis or transplantation (Cho, Hwang et al. 2010).

1.2.3 Cellular and Molecular Mechanism of Renal Fibrosis

The pathogenesis of renal fibrosis involves all of the cell types within the kidney, including fibroblasts, tubular epithelial cells, pericytes, endothelial cells, vascular smooth muscle cells, mesangial cells and podocytes, as well as infiltrating cells such as lymphocytes, macrophages and fibrocytes, highlighting the complexity of this process (Boor, Ostendorf et al. 2010) (Zeisberg and Neilson 2010). Key cellular events during tubulointerstitial fibrosis, the final outcome of all progressive kidney diseases, include fibroblast activation and expansion,

infiltration of inflammatory cells, production and deposition of extracellular matrix (ECM) components, and tubular atrophy and microvascular rarefaction (Figure1.6) (Liu 2011). These events together lead to a replacement of normal renal parenchyma with fibrotic tissue and subsequent loss of kidney function (Liu 2006).

In the injured kidney, inflammation, provoked by pro-inflammatory factors, occurs before fibrosis. A concentration gradient of chemotactic cytokines create a directional signal driving the infiltration of inflammatory cells to the sites of injury within the kidney(Chung and Lan 2011). Renal fibrosis is also characterised by the infiltration of inflammatory cells, including lymphocytes, monocytes/macrophages, dendritic cells and mast cells. These cells become active, producing molecules that destroy tissues, such as reactive oxygen species (ROS), and promote the expression of fibrogenic cytokines and growth factors (Vielhauer, Kulkarni et al. 2010). Sustained pro-fibrotic cytokine synthesis and activation within the local tissue microenvironment induces fibroblasts and tubular epithelial cells to undergo phenotypic change and activation and to produce large quantities of ECM components. Hence, continued inflammation or injury functions as a precursor to tissue fibrogenesis (Liu 2011).
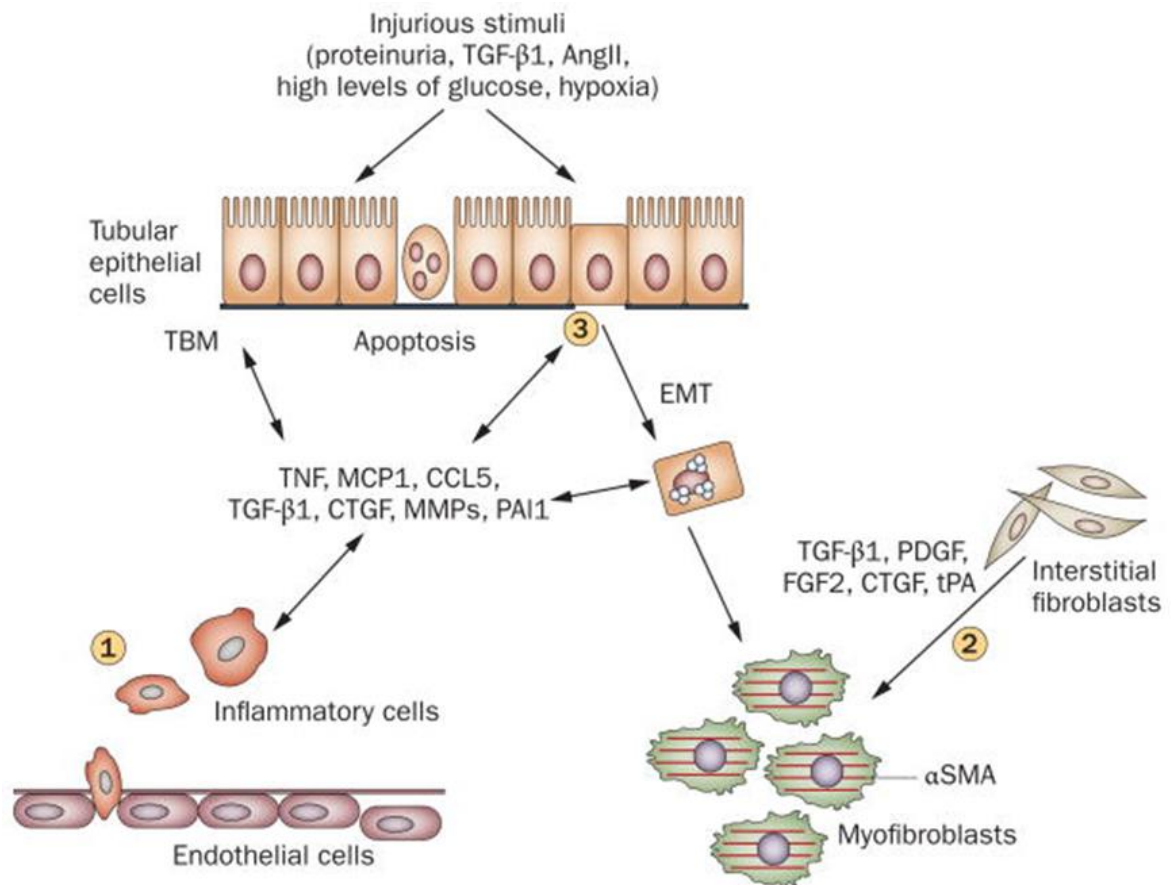
Figure 1.4. Major events during renal interstitial fibrogenesis. (1) An early event that initiates the activation and expansion of matrix-producing cells, is caused by peritubular infiltration of inflammatory cells, particularly T cells and macrophages. (2) Activation and expansion of myofibroblast. The main stream of the matrix-producing myofibroblasts are possibly generated from local activation of interstitial fibroblasts. (3) Tubular atrophy caused by tubular cell apoptosis and EMT (Liu 2011).

1.2.3.1 Renal Fibroblasts and Myofibroblasts

Fibroblasts are mesenchymal cells that exhibit a spindle-shaped morphology and are present in tissues throughout the body. They are the source of extracellular matrix (ECM) and therefore vital for the maintenance of normal tissue structure. Furthermore, they produce proteolytic enzymes and inhibitors, which allows them to regulate the formation and turnover of the ECM (Lemley and Kriz 1991). It is challenging to study fibroblasts as they lack cell type –specific markers (Boor, Ostendorf et al. 2010). However, fibroblasts can be distinguished from other

interstitial cells by their prominent endoplasmic reticulum, high capacity for protein synthesis, prominent F-actin cytoskeleton, and by ecto-5′-nucleotidase in their plasma membrane (Kaissling and Le Hir 2008). Fibroblasts synthesis of collagen and other matrix proteins, for example collagens type I, II and III is controlled by paracrine signalling by growth factors (Fujigaki, Muranaka et al. 2005).

Myofibroblasts are thought to be the primary effector cells of fibrosis. The term "myofibroblast" is used for fibroblasts that develop contractile characteristics (Tomasek, Gabbiani et al. 2002). Originally, myofibroblasts were recognised as the cells responsible for wound contraction (Majno, Gabbiani et al. 1971). In renal fibrosis, myofibroblasts play a vital role as the activated fibroblast responsible for the deposition of ECM in the tubulointerstitial space and its subsequent contractility, resulting in tissue retraction that characterises fibrotic, scar tissue. These cells express α-smooth muscle actin (α-SMA), but α-SMA may be expressed by other cells of mesenchymal lineage such as vascular pericytes (Kalluri and Zeisberg 2006).

1.2.3.1.1 The Cellular Origin of Myofibroblasts in Fibrosis

Following tissue injury, it was originally thought that ECM components are produced by the local resident tissue (myo) fibroblasts. It is now recognised that (myo) fibroblasts are derived from various sources (Quan, Cowper et al. 2006). The process of epithelial–mesenchymal transition (EMT), that involves epithelial cells altering their phenotype to a mesenchymal type, is one possible source of myofibroblasts (Willis, duBois et al. 2006). Recently, it has also been suggested that myofibroblasts can be derived from a similar process occurring with endothelial cells, termed endothelial–mesenchymal transition (EndMT) (Kalluri and Neilson 2003). Additionally, it has been suggested that a circulating fibroblast-like cell originating from bone marrow stem cells might somehow contribute to the kidney fibroblast population (Bucala, Spiegel et al. 1994). These circulating mesenchymal stem cell progenitors have

fibroblast/myofibroblast-like phenotypes and are known as fibrocytes (Ebihara, Masuya et al. 2006). Furthermore, pericytes are believed to be a source of myofibroblasts, as has been shown in the ureteral obstruction model of kidney fibrosis (Lin, Kisseleva et al. 2008). Pericytes are stromal cells that are associated with blood vessels, and they are the collagen type IαI producing cells in normal kidney (Schrimpf and Duffield 2011). In renal injury, pericytes migrate from the capillary wall to the interstitial space, where they are activated and differentiate to myofibroblasts. Thus, fibrosis and loss of capillaries during renal injury are strongly associated (Kida and Duffield 2011). Loss of Tgfbr2 in αSMA+ cells showed the importance of TGF-β pathway in the recruitment of myofibroblasts via differentiation, whereas deletion of Tgfbr2 prevented the bone marrow derived mesenchymal stem cells (MSCs) from sustaining a TGF β-1 induced α-SMA expression and conversion to myofibroblasts, further supporting the notion that TGF β-1 may play a role in differentiation of MSCs into myofibroblasts in kidney fibrosis. (LeBleu, Taduri et al. 2013).

In summary, it is possible that activated myofibroblasts could originate from nay of the lineages indicated in figure 1.7 (Figure 1.7; (Barnes and Gorin 2011)), again making this a difficult disease to study.
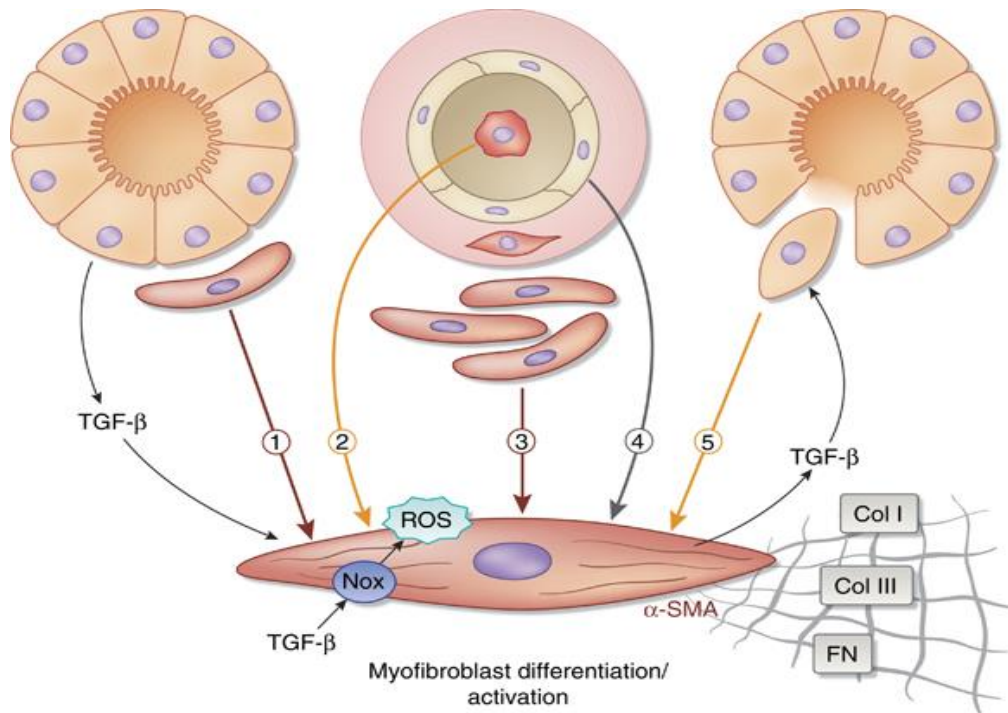
Figure 1.5. Proposed origin(s) of interstitial myofibroblasts during fibrosis. Myofibroblasts are functional contributor of profibrotic genes production and kidney fibrosis. The origin of myofibroblasts has been a potential source of argument in the recent years with different proposals for their origin. Accumulation of myofibroblasts occurs predominantly from different sources: (1) activation of resident fibroblasts or pericytes, (2) infiltration of circulating bone marrow-derived fibrocytes, (3) expansion of perivascular adventitial fibroblasts, (4) endothelial–mesenchymal (EndoMT) transition, and/or (5) epithelial-to-mesenchymal transition (EMT). Transforming growth factor-beta (TGF-β) released via paracrine or autocrine pathways induces (myo)fibroblast differentiation identified by the acquisition of an alpha-smooth muscle actin (α-SMA) and consequent synthesis of mesenchymal matrix proteins collagen type I (Col I), collagen type III (Col III), and fibronectin EIIIA (FN) (Barnes and Gorin 2011).

1.2.4 Inflammatory Cells

Several inflammatory cell types are believed to contribute to renal fibrosis, as described below

1.2.4.1 Lymphocytes

Lymphocytes play important roles in the genesis of renal fibrosis, particularly in tubulo-interstitial fibrosis (IF). CD4+, but not CD8+, lymphocyte reconstitution caused more severe IF in recombination-activating genes knockout mice and CD4+ depletion reduced IF (Tapmeier, Fearn et al. 2010). Studies using various cytokine-deficient mice have shown that fibrogenesis is related to the development of a T helper 2 (T_h2) CD4+ T-cell response,

implicating interleukin-4 (IL-4), IL-5 and IL-13. Although inflammation increases when T_h1 CD4$^+$ T cells predominate, the progression of tissue fibrosis is attenuated (Wynn, Cheever et al. 1995). Recent microarray experiments have shown the opposing effect of T_H1- and T_H2-cytokine responses in fibrosis (Sandler, Mentink-Kane et al. 2003). Various programs of gene expression are promoted when inflammatory responses are predominantly controlled by T_H1 or T_H2 cytokines, as shown by the investigations of gene-expression profiles of diseased tissues (Hoffmann, McCarty et al. 2001). Inhibition of the T_H2 cytokine IL-13 has identified it as a dominant effector cytokine during fibrosis in several models (Kumar, Herbert et al. 2002). Evidence has shown that the ECM proteins, types I and III collagen and fibronectin, are synthesised by fibroblasts upon stimulation with IL-13 (Fertin, Nicolas et al. 1991, Tiggelman, Boers et al. 1995, Doucet, Brouty-Boye et al. 1998).

CD4$^+$ T cells have also been shown to contribute to the activation and recruitment of macrophages and fibroblasts (Hesse, Modolell et al. 2001). Activation of macrophages was initially described as a T_H1 cell–IFN-γ-mediated process; however, it is now known that macrophages differentiate into at least two functionally different populations depending on exposure to T_H1 or T_H2 cytokines (Wynn 2004). The T_H2 cytokines, IL-4 and IL-13, promote arginase-1 (ARG1) activity in 'alternatively activated' macrophages, whereas the T_H1 cytokines activate the expression of nitric-oxide synthase 2 (NOS2) in 'classically activated' macrophages (Munder, Eichmann et al. 1998, Gordon 2003). The pro-fibrotic activity of IL-13 (Chiaramonte, Donaldson et al. 1999) and the anti-fibrotic activity of IFN-γ are possibly explained by the preferential activation of ARG1 compared with NOS2 in macrophages and/or fibroblasts (Hesse, Cheever et al. 2000).

1.2.4.2 Monocytes /Macrophages and Dendritic Cells

The majority of interstitial and glomerular renal diseases are associated with accumulation of macrophages in the kidney. Traditionally, these macrophages are regarded as transient cells in the glomerular or interstitial regions to control immune responses and/or process debris and apoptotic cells produced by renal injury. However, resident macrophages may constantly generate wound-healing growth factors, such as transforming growth factor beta (TGFβ). This may connect wound healing and pathological, irreversible fibrosis that occurs in progressive chronic kidney disease (Sunderkotter, Nikolic et al. 2004).

In the case of tissue damage, blood monocytes are recruited to the site of injury and then they differentiate in response to the environment including oxidative stress, hypoxia, toxins, or activation by pathogen-associated molecular pattern receptors (Anders and Ryu 2011). *In vitro* studies have described the conditions that lead to the development of classically activated M1 pro-inflammatory macrophages and the distinct subtypes of M2 alternatively activate macrophages. It has been shown that, immune components and lipopolysaccharide induce M2b, IL-4 and IL-13 prompt M2a 'wound-healing' macrophages and IL-10, transforming growth factor-β1 (TGF β-1), and glucocorticoids promote M2c 'regulatory macrophages (Martinez, Sica et al. 2008).

Dendritic cells originate from the same bone marrow myeloid progenitors as macrophages and they are normally abundant in kidney interstitium (Teteris, Engel et al. 2011). Dendritic cells can contribute to inflammation in the kidney. Renal dendritic cells, in a mouse model of proteinuric disease, detect and present antigens to T cells, resulting in the expression of pro-inflammatory cytokines. Moreover, the disease progression is attenuated as a result the depletion of dendritic cells (Heymann, Meyer-Schwesinger et al. 2009, Hochheiser, Engel et al. 2011). They can also form antigenic peptides from albumin via a proteasome-dependent pathway in a remnant kidney model, which then results in an activation of syngeneic CD8+ T cells (Macconi, Chiabrando et al. 2009).

In kidney fibrosis, members of the TGF-β superfamily are the most widely studied macrophage-derived growth factors (Border and Noble 1994). TGF-β is synthesised by not only by macrophages, but also tubular epithelial cells, and myofibroblasts, at various phases during the progression of kidney fibrosis (Eddy 2005). Nevertheless, knocking down macrophage TGF-β production significantly decreases fibrosis suggesting that macrophages are among the predominant source of this growth factor (van Goor, van der Horst et al. 1992).

1.3 Transforming Growth Factor Beta (TGFβ)

The TGF-beta family of cytokines are abundant, multifunctional and critical for homeostasis. They play essential roles in cell proliferation and differentiation, inflammation and repair and host immunity. Mammalian TGF-beta exists in three isoforms called TGF β-1, TGF β-2 and TGF β-3. These proteins are secreted as latent precursors and have multiple cell surface receptors of which at least two mediate signal transduction (Massague 1990). The other members of the superfamily include the bone morphogenetic proteins (BMPs) which control embryonic patterning (Miyazono, Maeda et al. 2005), the activins (Acts) and inhibins (Inhs) which regulate the release of pituitary hormones (Woodruff and Mather 1995), and growth and differentiation factors (GDFs) which regulate the development of cartilage and bone (King, Storm et al. 1996) (Figure 1.8 (Hinck 2012)).
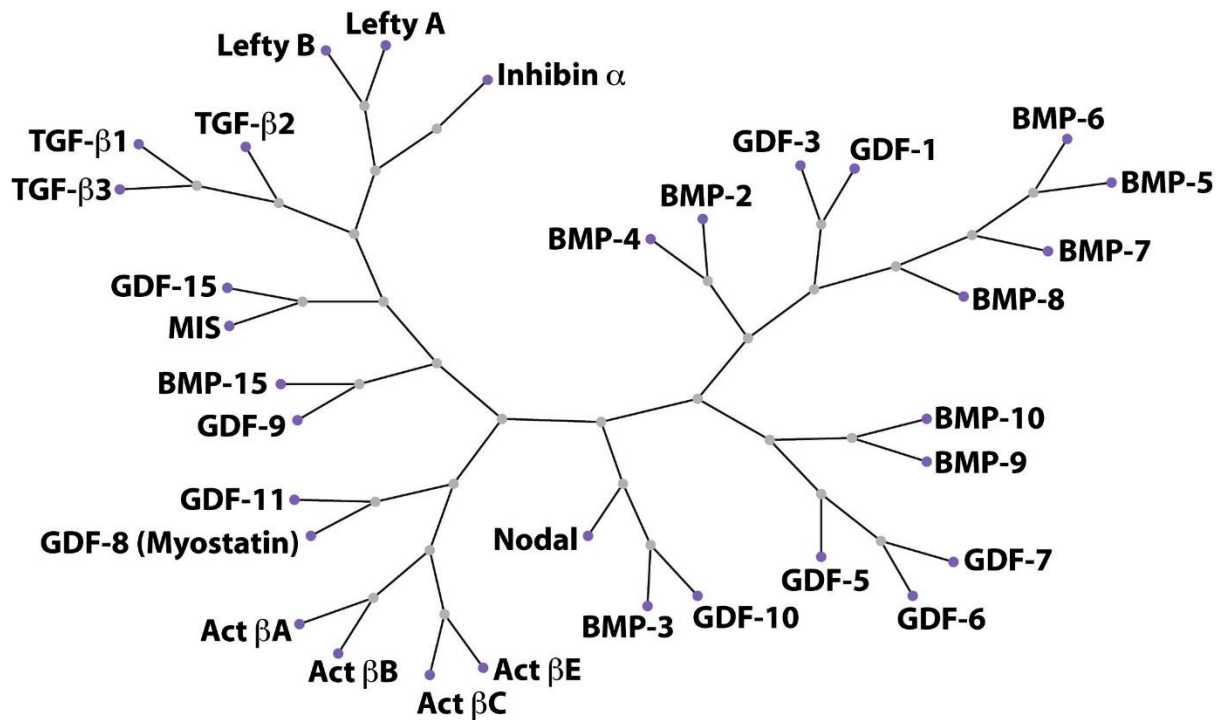
Figure 1.8. Phylogeneic tree of TGF-β superfamily proteins in humans. The construction of the tree was created on an alignment of the mature C-terminal domains (Hinck 2012).

I*n vitro*, TGF-β1, 2, and 3 stimulate mesenchymal cells to proliferate and produce extracellular matrix components and induce a fibrotic response in different tissues. Conversely, TGF β-1, 2, and 3 inhibit proliferation in many types of cells and induce the apoptosis of epithelial cells.(Leask and Abraham 2004). TGF β-1, TGF β-2 and TGF β-3 are all synthesised as precursor proteins with a propeptide region in addition to the TGF-β homodimer (Stern, Krieger et al. 1985). After it is synthesised, the TGF-β homodimer interacts with a Latency Associated Peptide (LAP) leading to a formation of a complex termed Small Latent Complex (SLC). This complex stays in the cell until it is bound by another protein termed Latent TGF-β-Binding Protein (LTBP), resulting in a larger complex termed Large Latent Complex (LLC), which is secreted into the ECM (Rifkin 2005). Generally, before the secretion of LLC, the TGF-β precursor is cleaved from the pro-peptide but stays connected to it via non-covalent bonds. Activation of TGF-β happens when LTBP is removed extracellularly by proteolytic

cleavage. In the assembly of the latent TGF-β complex, disulfide linkages are made between cysteine residues of latent TGF-β through its LAP and LTBP (Annes, Munger et al. 2003). As a result, latent TGF-β is unable of binding to its receptors. The LTBP/TGF-β complex is basically located in the matrix; the amino-terminal region of LTBP-1 is covalently cross-linked to ECM proteins by transglutaminase. Activation of TGF-β activators needs cleavage of the carboxyl-terminal pro-region, to which LTBP is bound, from the amino-terminal portion of the protein (Annes, Munger et al. 2003). The thus far known TGF-β activators are proteins associated with the wound healing process. The plasmin proteases MMP-2 and -9 that promote matrix degradation, for instance, are able to activate TGF-β (Yu and Stamenkovic 2000). Thrombospondin-1 (TSP) is another activator of TGF-β. TSP modulates cell adhesion, promotion of angiogenesis, and reconstruction of the matrix (Breuss, Gallo et al. 1995). Integrin $\alpha_v\beta6$, which is normally expressed at low levels only in epithelia cells, is additionally considered as an activator of TGF-β, and it is induced during wounding or inflammation (Miller, Barnett et al. 2001). Using knockout animal approaches have sustained the thought of the involvement of TSP-1 and αvβ6 integrin in TGF-β activation. Integrin β6−/− are extremely resistant to lung fibrosis initiated in response to the profibrotic drug bleomycin (Munger, Huang et al. 1999), and TSP-1 null mice show a partial phenotypic overlap with TGF β-1knockout animals (Crawford, Stellmach et al. 1998). Therefore, TGF-β activation happens in response to mediators promoted during the wound healing process *in vivo*.

TGF-β is not only involved in wound healing and during a fibrotic response, but also *TGF β-1* gene knockout mice have a high mortality (50%), as a result of uncontrolled inflammation soon after birth. *TGF β -2 and TGF β-3* gene knockout mice have an impairment of central synapse function (Heupel, Sargsyan et al. 2008) and cleft palate and exhibit abnormal lung development respectively (Kaartinen, Voncken et al. 1995). TGF-β genes also play fundamental roles in the immune system by maintaining tolerance through the regulation of

lymphocyte proliferation, differentiation, and survival. TGF-β also controls the initiation of inflammatory responses via the regulation of chemotaxis, activation, and survival of lymphocytes, natural killer cells, dendritic cells, macrophages, mast cells, and granulocytes (Taylor 2009). The regulatory function of TGF-β is modified by the state of cell differentiation and by the presence of other inflammatory cytokines and co-stimulatory molecules. The overall effect of TGF-β on the immune system is to prevent the development of immunopathology due to the recognition of self-antigens without affecting the immune responses to pathogens (Taylor 2009).

TGF-β is a major pro-fibrotic growth factor involved in renal tubulointerstitial fibrosis and collagen I producing interstitial myofibroblasts are assumed to be the mediators of TGF-β-dependent fibrosis. However, a selected deletion of TβRII in mice using Cre driven by the COL1A2 and tenascin C promoters, which would abolist TGF-β signalling in myofibroblasts, did not alter the level of fibrosis after either UUO or aristolochic acid-induced injury (Neelisetty, Alford et al. 2015). Therefore, abolishing TGF-β signalling alone in matrix-producing interstitial cells is not sufficient to reduce fibrosis after renal injury. These cells have multiple functions and may still contribute to injury on a TGF-β independent manner or have a critical role in repair that is lost in the absence of TGF-β signalling (Cappellesso-Fleury, Puissant-Lubrano et al. 2010, Boor and Floege 2012).The deletion of TβRII in cells that mediate fibrosis could also lead to compensatory enhanced autocrine signaling of other growth factors such as; PDGF, recognised to increase proliferation and collagen synthesis in fibroblasts (Gao, Li et al. 2008, Wu, Chiang et al. 2013). Namely, abrogating just TGF-β signaling may not be sufficient as many growth factors have pro-fibrotic effects on fibroblasts (Wu, Chiang et al. 2013).

1.3.1 Transforming Growth Factor Beta (TGFβ)-SMAD Signalling

Among the three TGF-β isoforms (TGF β-1, 2 and 3), that share approximately 80% structural homology in their active regions, TGF β-1 is the prototypical member and has a key role in fibrosis (Yanagita 2012). In addition to enhancing fibrosis, TGF β-1 works as an anti-inflammatory cytokine (Wynn and Ramalingam 2012), suggesting that inhibition of TGF β-1 could result in adverse effects by potentiating severe inflammation (Yanagita 2012). Overexpression of TGF β-1 driven by the albumin promoter in transgenic mice results in an abnormal phenotype characterised by hepatocyte apoptosis and liver fibrosis, and also causes glomerulonephritis, renal failure, arteritis, myocarditis, and atrophic changes in pancreas and testis (Sanderson, Factor et al. 1995). This inconsistent effect of TGF β-1 on inflammation between tissues may partially be as a result of an alteration in its concentration in particular tissues (Pakyari, Farrokhi et al. 2013). This shows that the amount of TGF β-1 may determine the switch from an inflammatory to an immunoregulatory phase during the course of the healing process (Daley, Brancato et al. 2010).

There is no available clinical therapy that blocks fibrosis and restores tissue homeostasis. Many strategies have been proposed to inhibit the TGF-β signalling pathway. These include soluble receptors, small-molecule inhibitors for receptor serine/threonine kinases and neutralising antibodies (Pohlers, Brenmoehl et al. 2009, Decleves and Sharma 2010). The most studied approach is using neutralising antibodies against TGF-β, which have been successfully used and well tolerated in animal models of fibrosis (Sharma, Jin et al. 1996, Ziyadeh, Hoffman et al. 2000). In one study, the administration of the anti-TGF-β antibody prevented or even reversed the early stages of diabetic nephropathy (Chen, Iglesias-de la Cruz et al. 2003). These positive outcomes obtained from animal studies directed researchers to develop clinical trials with anti-TGF-β antibodies and other TGF-β blockers in fibrotic diseases. A TGF β-1 neutralizing antibody (CAT-192, metelimumab), for instance, resulted in serious adverse

events such as, progression of skin involvement, gastrointestinal manifestations, weight loss, or death, but provided no efficacy in patients with systemic sclerosis (Denton, Merkel et al. 2007). Recently, a nuclear receptor subfamily 4 group A member 1 (NR4A1), a transcription factor, has been characterised as an endogenous inhibitor of TGF-β signalling and as a possible target for anti-fibrotic therapies (Palumbo-Zerr, Zerr et al. 2015). NR4A1 is upregulated in leiomyoma in response to TGF-β (Wu, Luo et al. 2009), and has pleiotropic regulatory effects on glucose and lipid metabolism (Maxwell, Cleasby et al. 2005, Pei, Waki et al. 2006), vascular homeostasis (Zeng, Qin et al. 2006), and inflammatory responses (Fassett, Jiang et al. 2012). Interestingly, NR4A1 recruits a repressor complex including SP1, SIN3A, CoREST, LSD1, and HDAC1 to TGF-β target genes, thus restricting pro-fibrotic TGF-β effects (Palumbo-Zerr, Zerr et al. 2015). Moreover, modulating TGF β-1 activity with a TGF β-1-specific, humanized, neutralizing monoclonal antibody (TGF β-1 mAb) did not slow renal function loss in patients with diabetic nephropathy on chronic stable renin-angiotensin system inhibitor treatment (Voelker, Berg et al. 2016). On the other hand, to elucidate the efficacy and feasibility of targeting TGF-β in fibrosis, the strategy of global blocking of TGF β-1 has been studied. TGF-β signalling was interrupted in in fos-related antigen 2 (Fra-2) transgenic mice (a murine model that manifests three important lung pathological features of SSc: fibrosis, inflammation, and vascular remodelling), by a pan-TGF-β blocking antibody, 1D1, that blocks the signalling of all TGF-β isoforms. The systemic blockade of TGF-β with 1D1 partially protects mice from pulmonary vasculopathy in the Fra-2 tg model of scleroderma, but this protection arises at the substantial cost of exaggerated pulmonary inflammation. Thus, more caution is needed in attempts to globally block TGF-β to treat diseases characterized by tissue fibrosis in the setting of ongoing inflammation (Tsujino, Reed et al. 2016).

TGF β-1 is activate when it is disassociated from the latency-associated peptide (LAP) and latent TGF-β binding protein (LTBP) through proteolytic cleavage by plasmin, reactive oxygen

species, thrombospondin-1, binding to αvβ6 integrin and acid (Wang, Huang et al. 2005) (Figure 1.9) (Saha and Doran 2014). Once activated TGF-β binds to specific transmembrane receptors. Cell surface receptors for TGF-β family ligands are characterised by their specificity for phosphorylation of serine or threonine, rather than tyrosine residues as in the receptors of other growth factors and cytokines. Receptor complexes are heterotetrameric, comprising of two `type II' receptors (75–85 kDa), which bind ligand, and two signal transducing `type I' receptors (50–60 kDa) which, in most cases, are considered to act downstream of the type II receptor, because they cannot bind ligand directly (Massague 1998). The formation of the heteromeric complex is initiated by ligand binding and stabilised by interactions between the cytoplasmic domains of the type II and type I receptors. It appears that, for all three isoforms of TGF-β, signals are mediated by a single type II receptor called TβR-II and one type I receptor known either as TβR-I or ALK-5 (activin receptor-like kinase) (Wieser, Wrana et al. 1995) (Figure 1.10 (Hinck 2012)) . This pathway of signaling, including the need for a type I and type II receptor and the type II receptor-mediated activation of the type I receptor, is common in all proteins of the superfamily (Hinck 2012).
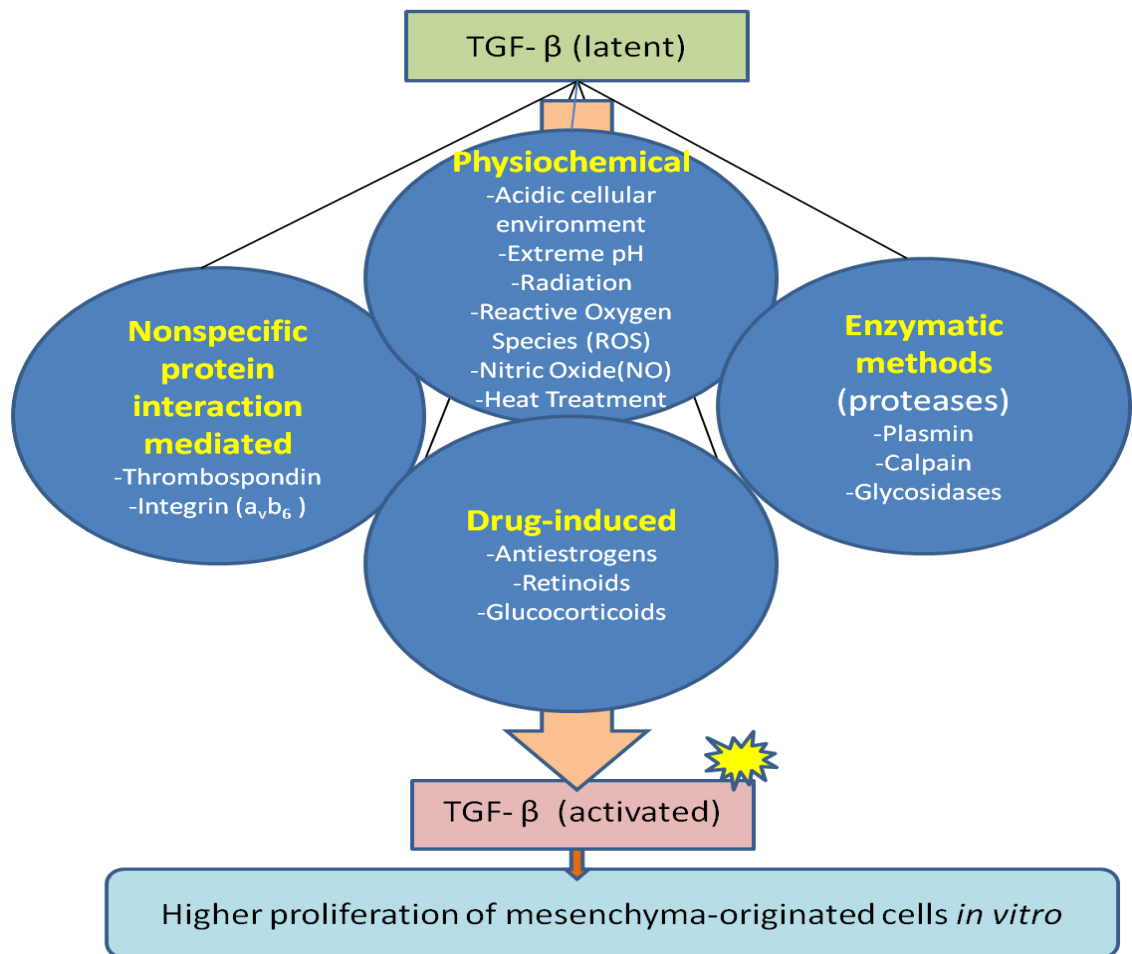
Figure 1.9. The different proposed proteolytic and non-proteolytic mechanisms of activation of TGF-β in vitro (Saha and Doran 2014).
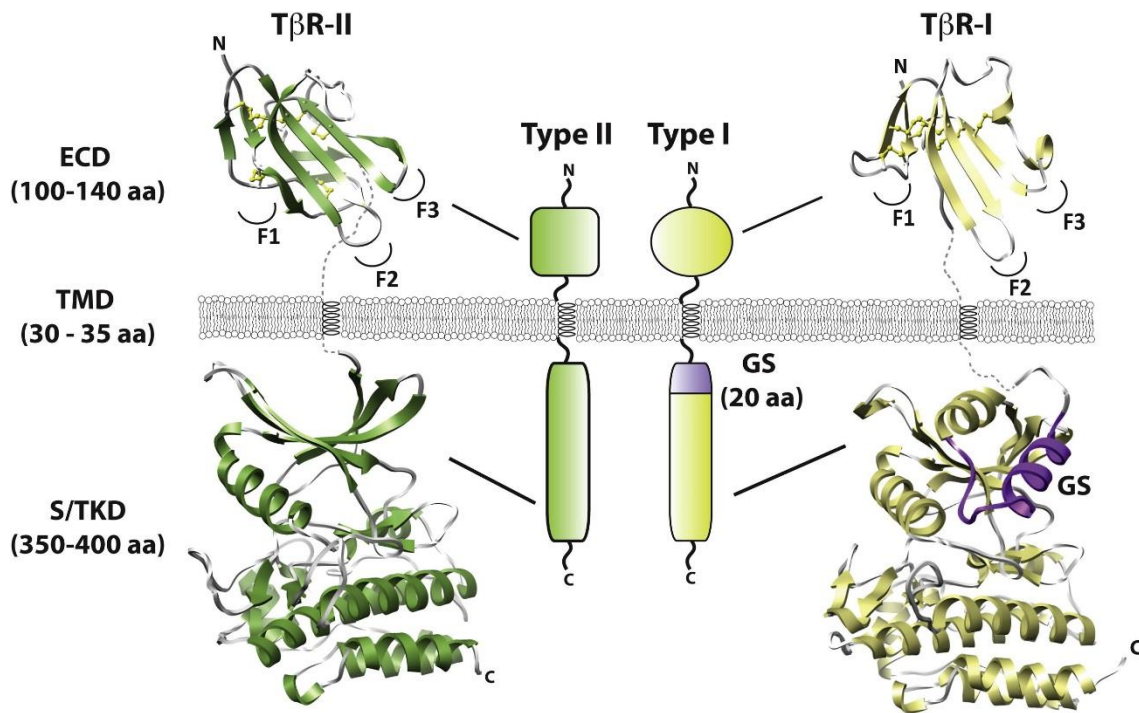
Figure 1.10. The TGF-β type I and type II receptors, TβR-I and TβR-II. The extracellular domains (ECDs) of TβR-I and TβR-II are small (101 and 136 residues, respectively), heavily disulfide-bonded (five and six disulfides, respectively), and adopt a three-finger toxin fold (F1, F2, and F3 designate the three fingers of the receptor three-finger toxin fold). The transmembrane domain (TMDs) and cytoplasmic serine-threonine kinase domains (S/TKD) are also shown. The type I receptor includes a ~ 20 amino acid juxtamembrane glycine-serine rich regulatory domain, known as the GS box (GS, purple). The structurally disordered residues between the structured portion of the ectodomain or kinase domain and the transmembrane domain are shown by dashed lines (14, 22, 8, and 26 residues for the TβR-I ecto, TβR-I kinase, TβR-II ecto, and TβR-II kinase domains, respectively) (Hinck 2012).

Active TGF-β binds its receptors and signals via SMAD-dependent and independent signalling pathways (Derynck and Zhang 2003). In renal disease, the SMAD-dependent pathway has been widely studied and regarded as the main pathway (Bottinger 2007), as SMAD signalling proposed to represent the final common pathway for renal fibrosis, irrespective of the initial causes of the disease (Wang, Koka et al. 2005) . SMAD proteins are intracellular proteins responsible for the transduction of extracellular signals from TGF-β ligands to the nucleus where they activate downstream gene transcription (Attisano and Wrana 2000). SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8 act as substrates for the TGF-β family of receptors; these are generally referred to as receptor-regulated SMADs, or RSMADs (Figure 1.11)

(Massague, Seoane et al. 2005). SMADs 1, 5, and 8 have more critical roles in the signalling pathways of the bone morphogenetic proteins (BMPs), the other member of TGF-β superfamily (Ueki and Reh 2012). SMAD proteins form a trimer of two receptor-regulated SMADs, such as SMAD2 and 3, and one co-SMAD, such as SMAD4. The RSMAD3 and co-SMADs contain preserved amino- and carboxyl-terminal MAD-homology domains (MH), the N-terminal MH1 domain and the C-terminal MH2 domain. The RSMADs, but not the co-SMADs, contain a characteristic SXS motif at their C termini. Upon ligand stimulation, R-SMAD proteins are phosphorylated in the SXS motif by TGF-β receptor 1(TGF-βRI) serine kinase, leading to the activation of further downstream events (Massague, Seoane et al. 2005). Furthermore, SMAD2 and 3 have other potential phosphorylation sites (C-terminal, linker area and N-terminal), which might be phosphorylated through other signalling pathways, such as C-Jun terminal kinase (JNK), MAPK and MAPK-P38 pathways (Funaba, Zimmerman et al. 2002). Further studies are needed to understand the functional and physiological importance of these sites for phosphorylation. Inhibition of the nuclear translocation of SMAD1, SMAD2 and SMAD3 might occur upon the phosphorylation of these sites by Ras-activated ERK1/2 in the linker region (Thr-220, Ser-245, Ser-250, Ser-255) (Kretzschmar, Doody et al. 1999). SMAD2-dependent transcriptional activity occurs independent of TGF β-1 by the activation of mitogen-activated protein kinase/ERK kinase-1 (MEKK-1) in cultured endothelial cells (Brown, DiChiara et al. 1999). SMAD6/7 are inhibitory proteins that negatively regulate SMAD2 and SMAD3 activation and by targeting the TGFβRI and other SMADs to the ubiquitin proteasome pathway (Ebisawa, Fukuchi et al. 2001). Acetylation of SMAD7 in the nucleus, due to interaction with the transcriptional co-activator p300 (Gronroos, Hellman et al. 2002), results in ubiquitination blockade on the same residues, leading to a higher concentration of SMAD7 available to bind to receptors and block receptor SMAD2 and SMAD3 binding (Liu and Feng 2010).

The binding of TGF β-1 to its receptor II (TβRII) can activate the TGF-β receptor type I (TβRI)-kinase, leading to phosphorylation of SMAD2 and SMAD3, two receptor-associated SMADs (R-SMADs). Consequently, phosphorylated SMAD2 and SMAD3 bind to the common SMAD4, resulting in formation of the SMAD2/3/4/ complex, which translocates into the nucleus to control target gene expression (Wang, Huang et al. 2005) (Figure 1.12). The identification of SMADs accelerated the study of TGF-β signaling, however, it also led to difficulty reconciling the different functions of the TGF-β family and the simplicity of the SMAD signaling pathway. Recently, it has been shown that the diversity of TGF-β signaling response is achieved by the combination of essential SMAD pathway components (including receptors, ligands, SMADs, and SMAD-interacting transcription factors) and 'cross-talk' with other signaling pathways, that regulate downstream cellular responses (Derynck and Zhang 2003, Moustakas and Heldin 2005). These non-SMAD pathways include different branches of MAP kinase (MAPK) pathways, Rho-like GTPase signaling pathways, and phosphatidylinositol-3-kinase (PI3K)/ AKT pathways (Moustakas and Heldin 2005). This thesis is solely devoted to the SMAD pathways that are activated by the TGF-β receptors through phosphorylation.
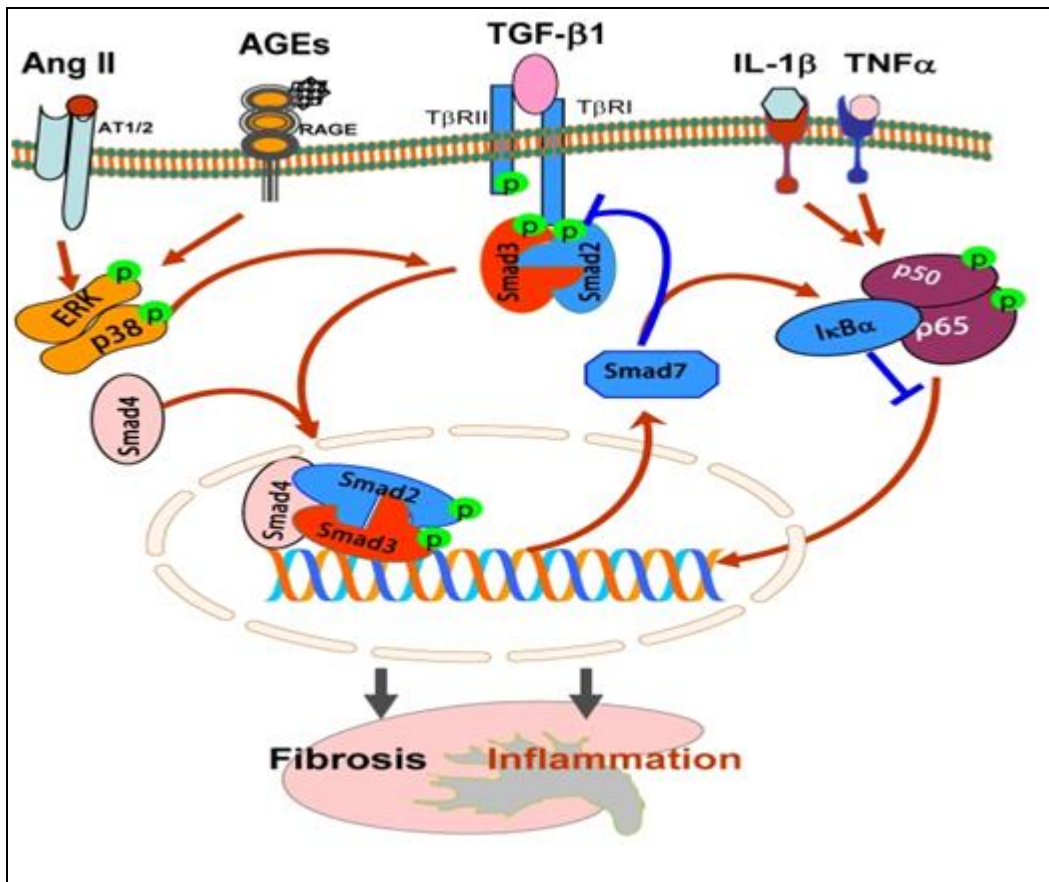
Figure 1.11. TGF-β/SMADS and crosstalk pathways in renal fibrosis. A Binding of TGF β-1 to its cognate receptor complex results in SMAD2 and SMAD3 phosphorylation. The phosphorylated SMAD2 and SMAD3 then bind toSMAD4 and form the SMAD complex, which can translocate into the nucleus and regulates the target gene transcription (Wang, Huang et al. 2005).
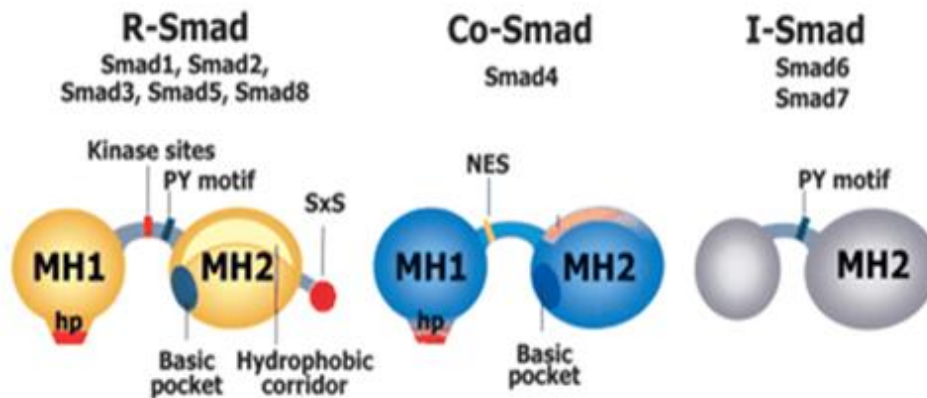
Figure 1.12. SMAD proteins and their structural elements. SMAD proteins are the central mediators for TGF-β superfamily signalling and are classified into three groups. The first group is the R-SMADs, of which SMAD1, 5 and 8 are primarily activated by the BMP-specific type I receptors, while SMAD2 and SMAD3 by the TGF-β subfamily type I receptors. The second group has the common mediator SMAD (Co-SMAD, e.g. SMAD4 in mammals). The third SMAD group includes inhibitory SMADs (I-SMAD, e.g. mammalian SMAD6 and SMAD7). SMAD proteins consist of two conserved globular domains—the MH1 and MH2 domains—and coupled by a divergent linker region. The seven mammalian SMAD proteins are shown accordingly to their classes. MH1 domain, is highly conserved in all RSMADs and SMAD4 but not in SMADs 6 and 7. MH2 domain is conserved in all SMAD proteins. MH1 domain is a DNA-binding module. The contact with DNA is initiated by a β-hairpin structure, which is conserved in all the RSMADs and SMAD4.MH1 domain is absent in I-SMADs. The MH1 domain is followed by the linker region, a flexible segment with binding sites for Smurf (SMAD ubiquitination-related factor) ubiquitin ligases, phosphorylation sites for several classes of protein kinases, and, in SMAD4, a nuclear export signal (NES).The MH2 domain contains a basic pocket for interaction with activated type I receptors in the case of the RSMADs, and in both the RSMADs and SMAD4 for interaction with the pS–x–pS motif (red ball) of RSMADs. In RSMADs, on the surface of the MH2 domain, an attached group of hydrophobic patches forming a site for multiple interactions (Massague, Seoane et al. 2005).

1.3.2 SMAD3 is a Therapeutic Target in Renal Fibrosis

Although TGF-β activates various intracellular signalling pathways, investigations have shown that SMAD proteins are the basic elements of the intracellular signalling cascade transferring TGF-β signals from the cell surface to the nucleus. SMAD3-null mice are protected against tubulointerstitial fibrosis following unilateral ureteral obstruction (UUO) suggesting a significant role of SMAD3 in kidney fibrosis (Sato, Markiewicz et al. 2003). Another study by Inazaki.I et al.2004, has also shown that SMAD3 deficiency attenuated renal fibrosis, inflammation, and apoptosis after UUO, suggesting that SMAD3 is a key molecule

for cellular and molecular events involved in UUO and a putative therapeutic target for renal fibrosis (Figure 1.13) (Inazaki, Kanamaru et al. 2004). Animal models of other fibrotic disease such as scleroderma, cystic fibrosis and cirrhosis also involve SMAD3 (Flanders 2004). Inhibition of SMAD3 by overexpression of the inhibitory SMAD7 protein or by treatment with the small molecule, halofuginone, an inhibitor of SMAD3 phosphorylation, significantly decreases disease severity in animal models of kidney, lung, liver and radiation-induced fibrosis (Flanders 2004). This suggested that small molecule inhibitors of SMAD3 might have clinical benefit in the treatment of human fibrotic diseases (Flanders 2004). Furthermore, a recent study that focused on the function of SMAD3 acetylation in kidney fibrosis suggested that deacetylation of SMAD3 might be an innovative therapeutic target for fibrotic disease (Li, Qu et al. 2010).
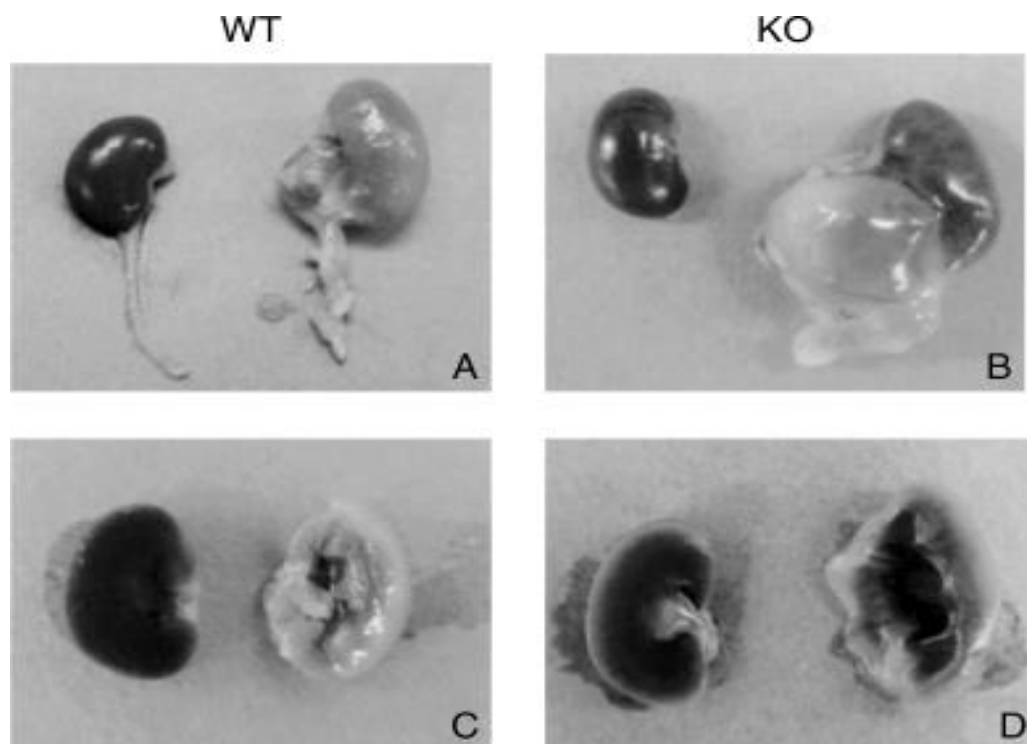
Figure 1.6. Gross appearance of the mouse kidney 14 days after unilateral ureteral obstruction (UUO). The left obstructed kidney or right nonobstructed kidney of SMAD3 (+/+) and SMAD3 (-/-) mice were removed 14 days after UUO. Representative pictures of the kidneys from SMAD3 (+/+) mice (A and C) and from SMAD3 (-/-) mice (B and D) were shown. (C and D) sections of the left and right kidneys. Note that the obstructed kidney of SMAD3 (-/-) mice showed relatively intact kidney tissue (much less whitish scar area than that of SMAD3 (+/+) mice). Abbreviations are: WT, wild-type; KO, knockout (Inazaki, Kanamaru et al. 2004).

1.3.2.1 Regulation of SMAD3 Nuclear Import

The activation and translocation of SMAD3 into the nucleus is a key process in TGF β-1 signalling. Detection of factor(s) regulating SMAD3 nuclear import would be significant in developing novel treatments to down-regulate TGF β-1 signalling in renal fibrosis and other diseases (Shi and Massague 2003). Upon the activation of TGF β-1, SMAD2 and 3 are phosphorylated, form complexes with SMAD4, and are translocated into the nucleus. However, the cellular mechanism by which SMAD3 is imported into the nucleus is not completely understood (Reguly and Wrana 2003). To identify the component(s) involved in SMAD3 import Xu L and others performed a genome-wide siRNA screen in *Drosophia Melanogaster.* This study identified moleskin (MSK) as a critical factor for nuclear import of Drosophila SMAD and depletion of the human MSK orthologues, importin7 and importin8, impaired SMAD3 nuclear localisation in response to TGF β-1 (Xu, Yao et al. 2007) (Figure 1.14). Because the inhibition of importin7 or 8 would disrupt not only SMAD3 nuclear import but also that of numerous other proteins implicated in homeostasis, the potential for their being specific targets in fibrosis is still unclear (Jakel and Gorlich 1998).
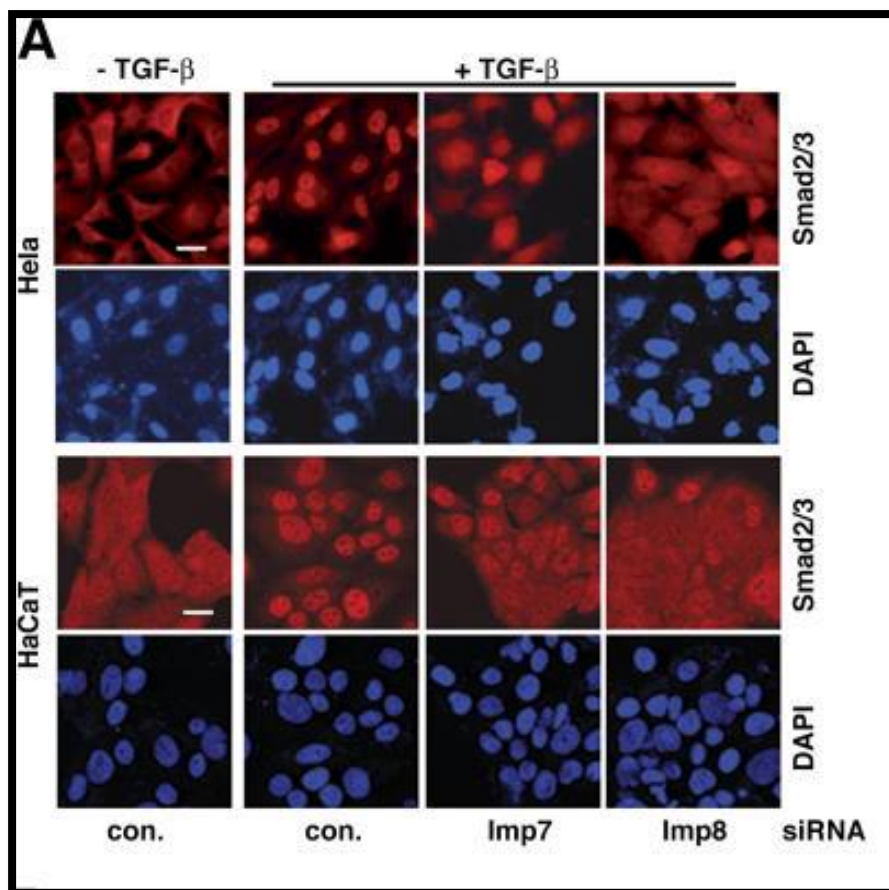
Figure 1.7. The importance of importin 7 and importin 8 in TGF-β–activated SMAD2/3 to translocation into the nucleus. HeLa or HaCaT cells transfected with indicated siRNAs (40 nM) were analysed by immunostaining using anti-Smad2/3 antibody, with or without prior TGF-β stimulation as indicated (100 pM, 30 min). The nuclei were slowed with by DAPI bars (Jakel and Gorlich 1998)**.**

A lysine-rich KKLKK (K denotes lysine) nuclear localisation signal (NLS) has been identified between residues 40-44 (Lys[40]-Lys-Leu-Lys-Lys[44]) in the N-terminal region of human SMAD3 (Xiao, Liu et al. 2000). Mutation of lysine residues 40 and 41 or 43 and 44 inhibits TGF β-1-induced SMAD3 nuclear import. However, the KKLKK motif itself does not act as a classical NLS because fusion of this sequence to a heterologous protein does not result in nuclear localization of the new protein. This raises a question of whether SMAD3 has an undisclosed NLS that will facilitate nuclear import, or whether SMAD3 needs to form a complex with an as yet unidentified NLS-containing factor(s) to allow nuclear import (Yao, Chen et al. 2008).

1.4 Protein Post Translational Modifications (PTMs)

Protein post translational modifications perform a major role in various cellular processes such as cellular differentiation (Grotenbreg and Ploegh 2007), signalling and regulatory processes (Morrison, Kinoshita et al. 2002), protein degradation (Geiss-Friedlander and Melchior 2007), protein-protein interactions and regulation of gene expression. PTMs can occur at the protein's C or N termini or on the amino acid side chains, where the chemical repertoire of the 20 standard amino acids can be extended by introducing new functional groups such as phosphate, acetate, amide groups or methyl groups (Pratt, Parker et al. 2006). Methylation of proteins is a common form of post-translational modification observed (Nesterchuk, Sergiev et al. 2011).

Histones are proteins that assemble and form the DNA of eukaryotic cell nuclei into components termed nucleosomes. They condense DNA and regulate chromatin structure, and in this way impact gene regulation (Hauschteck-Jungen and Hartl 1982). (Marino-Ramirez, Kann et al. 2005). Histone protein sequence variation, PTMs and interactions with chromatin remodelling elements effect DNA replication, transcription, repair and recombination (Arents and Moudrianakis 1995). Histones exist in five different families; H1/H5, H2A, H2B, H3 and H4. Histones H2A, H2B, H3 and H4 are the core histones, they come together to form one nucleosome, while histones H1 and H5 are the linker histones (Bhasin, Reinherz et al. 2006). The nucleosome core is formed of two H2A-H2B dimers and a H3-H4 tetramer, and this represents the first level of chromatin organisation (Luger, Mader et al. 1997).. In association with this octamer, there are about 147 bp of DNA surrounded by 1.7 superhelical turns. Nucleosomes are joined by a DNA linker of variable length that produces a 10-nm beads-on-a-string array (Olins and Olins 1974). Core histones have an important structural roles in the assembly of chromatin by forming the nucleosome. Each of the core histones have a histone fold domain, comprising three α-helices linked by two loops (Baxevanis, Arents et al. 1995). This allows heterodimeric interactions between core histones that are known as the handshake

motifs. The core histone tails have critical roles in the stability of nucleosome (Brower-Toland, Wacker et al. 2005). Furthermore, each core histone also contains an N-terminal tail that is subjected to covalent modifications, including acetylation, phosphorylation, methylation, glycosylation, and ubiquitination. Although these modifications were identified some time ago, their functions are only now being studied. Figure 1.13 shows the PMTs in the nucleosome core particle near the protein-DNA interface. (Figure 1.15(Marino-Ramirez, Kann et al. 2005)). Measurement of histone modifications may allow researchers to discover novel epigenetic mechanisms controlling cellular processes in health and disease. Abnormal modifications, for instance, have been associated with various different diseases, from cancer to autoimmune and inflammatory diseases and neurological disorders (Araki and Mimura 2017). Control of chromatin structure via histone PTMs is a crucial driver of transcriptional responses in many cell types. Likewise, histone writers, readers, and erasers the proteins device that remove, adds or recognise these PTMs have emerged as important factors in transcriptional control (Rothbart and Strahl 2014). Post- PTMs of histones can act to induce or repress the transition of chromatin from "closed" to an "open" state (figure 1.16 (Gillette and Hill 2015)). Enzymes that add PTMs to histones are known as writers, and based on the specific PTM they effect, they are divided into classes. Likewise, erasers, enzymes which reverse specific PTMs, are divided into PTM-specific classes. Furthermore, readers are protein factors that distinguish either particular post-translational marks on histones or a mixture of marks and histone variants to lead to specific transcriptional changes.
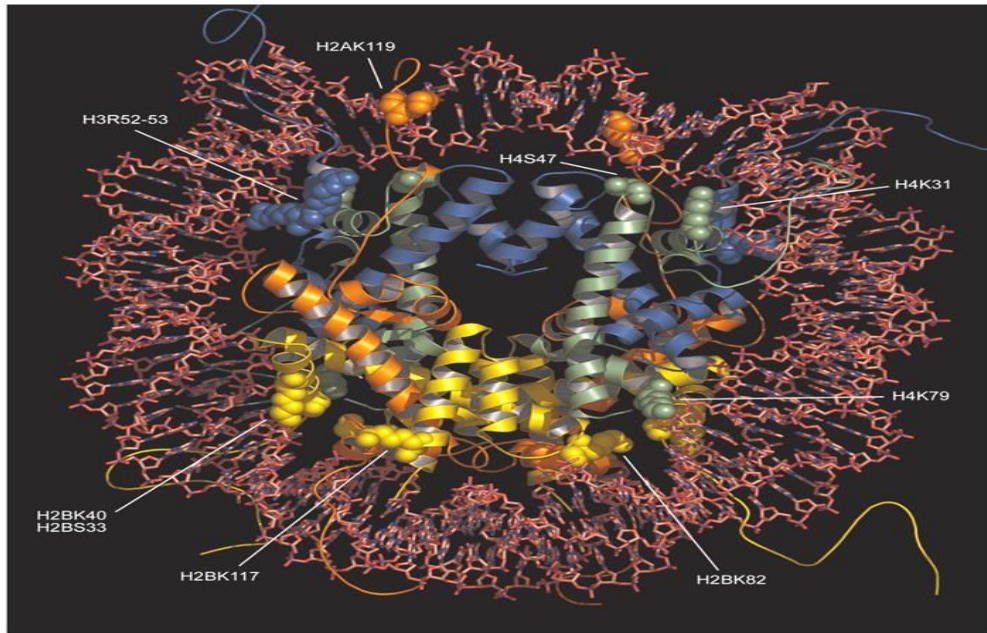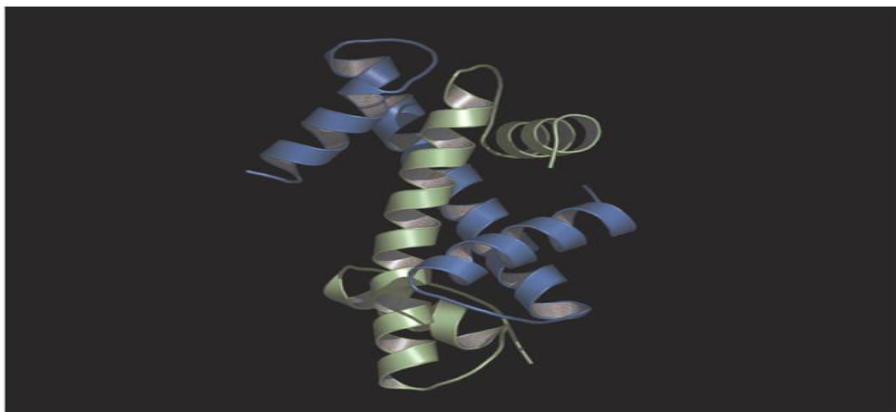
Figure 1.15. Post-translational modifications in the nucleosome core particle near the protein-DNA interface. **(A)** The nucleosome core particle structure (Protein Data Bank accession code 1KX5) with the histone modifications that might be involved in enhancing the movement of nucleosome. The colours represent different histones H3 (blue), H4 (light green), H2A (orange) and H2B (yellow); the histone modifications are shown as spheres. **(B)** The histone fold domain of H3and H4 interacting in the characteristic handshake motif (Marino-Ramirez, Kann et al. 2005).
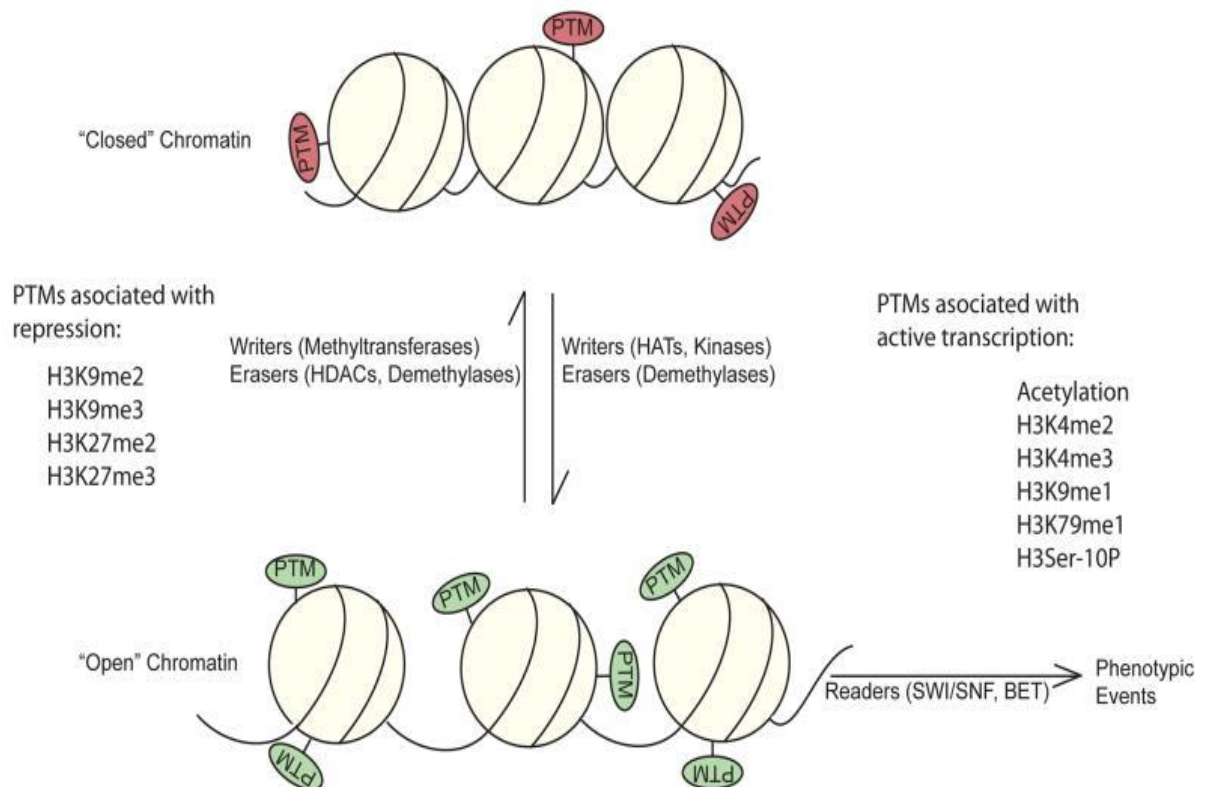
Figure 1.16. The protein machinery that adds, removes or recognizes PTMs. Histone modifications control gene transcription and chromatin structure in a context-particular manner, in what has emerged to be known as the histone code. For example, histone acetylation is commonly assumed to promote transcription, whereas methylation at specific lysine may act to activate or repress transcription. This figure indicates that PTMs of histones can work to enhance (green) or repress (red) the transition of chromatin to an open state. Combined with the action of histone PTM "readers", these changes culminate in an increase or repression of the transcription of target genes (Gillette and Hill 2015).

1.4.1 Methylation

Methylation involves the addition of a methyl group to a substrate or the replacement of an atom or group by a methyl group. In biological systems, methylation is catalysed by enzymes and are involved in regulation of gene expression, RNA processing and regulation of protein function. In vertebrates, DNA methylation usually occurs at CpG residues (cytosine-phosphate-guanine sites), and it is associated with the formation of heterochromatin and silencing of genes (Bird 2002). This methylation leads to the change of the cytosine to 5-methylcytosine, catalysed by the enzyme DNA methyltransferase. Human DNA has about 80–90% of CpG positions methylated, however, there are specific regions, identified as CpG

islands, that are GC-rich, where none are methylated. These are associated with the promoters of 56% of mammalian genes, including all ubiquitously expressed genes. 1 to 2% of the human genome is CpG groups, and there is an opposite relationship between CpG methylation and transcriptional activity (Kuzmichev and Reinberg 2001). Hence, methylation of CpG-islands located in promoter regions is vital in gene repression in genomic imprinting and during X-chromosome inactivation (Takai and Jones 2002).

Histone protein methylation is controlled by a series of histone methyltransferases (HMTs) containing highly conserved core SET, cysteine-rich pre- and post-SET domains. SET domains were termed according to the initials of the three genes initially found which express such domains, namely, Suppressor of variegation 3-9 (Su(var) 3-9), Enhancer of zeste (E(z)) and Trithorax (Trx) (Thakur, Malik et al. 2003, Binda 2013). Two main types of histone methyltransferases exist, lysine-specific (which can be SET domain containing or non-SET domain containing) and arginine-specific (Feng, Wang et al. 2002).

1.4.1.1 Arginine Methylation

Protein methylation occurs on arginine or lysine amino acid residues. Protein methylation is involved in regulation of protein-protein interactions such as those involved in regulation of transcriptional events, ageing and development, T-cell activation, nuclear transport, neuronal differentiation, ion channel function, and cytokine signalling (Zhang and Reinberg 2001). Arginine can be methylated once, known as monomethylated arginine, or twice, with either both methyl groups on one terminal nitrogen (asymmetric dimethylarginine) or one on both nitrogens (symmetric dimethylarginine) by peptidylarginine methyltransferases (PRMTs). These enzymes have been classified into two types; type I enzymes catalyse the formation of N^G -monomethylarginine and asymmetricN^G,N^G -dimethylarginine residues, whereas the type II enzyme catalyses the formation of N^G -monomethylarginine and symmetric$N^G,N^{'G}$-

dimethylarginine residues (Figure 1.13A). Several RNA-associated proteins such as, hnRNP A1, fibrillarin, and nucleolin have been identified as substrates of type I PRMTs, whereas the only substrate known to date for type II PRMT is the myelin basic protein(Gary and Clarke 1998).

1.4.1.2 Lysine Methylation

ε-N-methyl-lysine was primarily identified in a bacterial flagellar protein in 1959 (Ambler and Rees 1959) and, few years after, this PTM was also found in histone proteins (Murray 1964). For many year, the physiological significance of protein lysine methylation was unknown, but various protein lysine methyltransferases (PKMTs) have now been recognised, and their physiological importance, mainly in the area of epigenetics, has started to be clarified (Rea, Eisenhaber et al. 2000, Tachibana, Sugimoto et al. 2001, Hamamoto, Furukawa et al. 2004). The SET-domain proteins form a major group of S-Adenosyl methionine, AdoMet-dependent Protein Lysine Methyltransferases PKMTs, with about 50 human proteins characterised as members of this family, although not all of these proteins have confirmed PKMT activity (Feng, Wang et al. 2002). Several non-SET-domain proteins, including DOT1-like histone H3K79 methyltransferase (DOT1L), are also reported to have PKMT activity (Feng, Wang et al. 2002). Because the half-life of the histone methylation was approximately equivalent to the half-life of histones themselves, protein lysine methylation was thought to be irreversible (Bannister, Schneider et al. 2002). However, in 2004 the first protein lysine demethylase lysine-specific demethylase 1 (LSD1/KDM1A) was discovered (Shi, Lan et al. 2004) and subsequently the protein demethylase activity has been reported to be found in Jumonji C (JmjC)-domain-containing family (Tsukada, Fang et al. 2006), which suggest that protein lysine methylation are dynamically regulated. Lysine can be methylated once, twice or three times by protein lysine methyltransferases in an AdoMet-dependent manner (Smith and Denu 2009) (Figure 1.17B) (Gary and Clarke 1998).

Protein methylation has been widely studied in histones. The transfer of methyl groups from S-adenosyl methionine to histones is catalysed by group of enzymes known as histone methyltransferases. Methylation of histones on specific sites can act epigenetically to suppress or stimulate gene expression (Grewal and Rice 2004). Therefore, to understand the dynamic regulation of histone methylation, it would be helpful to take a general view of regulation of chromatin modification.
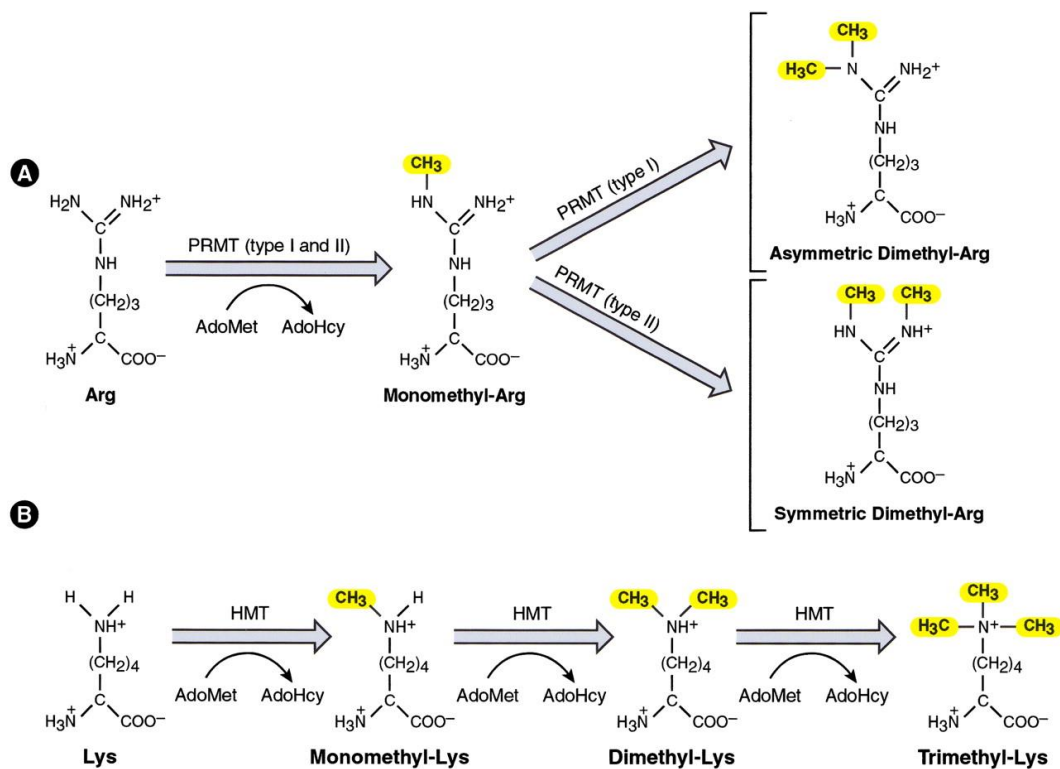
Figure 1. 8. Chemistry of arginine and lysine methylation. (*A*) Molecular structure of arginine, and mono- and di-methylarginine. Type I and II protein arginine methyltransferases catalyse asymmetric and symmetric dimethylation, respectively. (*B*) Molecular structure of lysine and mono-, di-, and tri-methyl-lysine (Gary and Clarke 1998).

1.4.1.3 Histone Methyltransferases

Histones have long been considered as substrates for methylation (Murray 1964). Initial research using metabolic labelling followed by sequencing of histones revealed that a number

of lysine residues, including lysines 4, 9, 27, and 36 of H3 and lysine 20 of H4, are sites of methylation (Ausio and Van Holde 1988, Strahl, Ohba et al. 1999). Furthermore, arginine methyltransferases can also methylate histones *in vitro* (Gary and Clarke 1998). Yet, evidence directly linking histone methylation to gene activity was not available until recently. Depending on the site and status of methylation, histone arginine methylation is usually associated with transcriptional activation, whereas histone lysine methylation can be associated with either transcriptional activation or repression (Margueron, Trojer et al. 2005).

Lysine methyltransferases are the most widely studied methylation enzymes (Schneider, Bannister et al. 2002). These enzymes contain the evolutionarily conserved catalytic SET domain. They catalyse the transfer of methyl groups AdoMet to e-amino group of target lysine residues. SET-domain-containing lysine methyltransferases can be categorised into subgroups like KMT1 (H3K9), KMT2 (H3K4), KMT3 (H3K36), KMT4 (H3K79), KMT5 (H4K20), and KMT6 (H3K27) methyltransferases (Schneider, Bannister et al. 2002). There are 7 known subfamilies of methyltransferases. These include EZ, SET1, SET2, SMYD, SUV39, SUV4-20, RIZ as main subfamilies and SET8/PR-SET7 and SET7/9 as other unclassified members (Mohan, Herz et al. 2012). The SET-domain amino acid sequence and the flank motifs of SET domain are highly similar for members of each subfamily (Zhao, Zheng et al. 2015). There are 30 unique SET domain containing proteins that are capable of histone methylation and belong to one of the above mentioned subfamilies (Table1.3).). These enzymes, depending on the site that is methylated, can either activate transcription (H3K4me) or repress transcription (H3K27me, H2K9me). They also play important role in various cellular processes such as cell cycle progression, DNA replication, cytokinesis, transcriptional regulation, DNA damage response, replication stress response, energy homeostasis, and X chromosome inactivation (Qian and Zhou 2006).

Myofibroblasts activation by TGF β-1 results in methylation of lysine 4 of histone H3 (H3K4) through the up regulation of SET domain-containing lysine methyltransferase 9 (SET9), which is important for the transcriptional activation of fibrotic genes *in vitro* (Sun, Reddy et al. 2010). A study done by Wang H et al, suggested that SET9 is partially responsible for H3-K4 methylation *in vitro* (Wang, Cao et al. 2001). This study used a methyl-K4- specific antibody to assess the H3-K4 methylation status. The antibody recognised endogenous histone H3 purified from human embryonic kidney cells, 293T cells, transfected with a vector that expresses wild-type SET9, but did not recognise an equal amount of recombinant H3 purified from E. coli, demonstrating that the antibody is methyl- K4-specific (Wang, Cao et al. 2001). However, *in vivo* studies using an experimental model of renal fibrosis are needed to develop therapeutic approaches that target SET9.

Histone Lysine methyltransferases		
Enzyme (UniprotKB recommended name)	**Coding gene/s**	**Site of histone modification**
Histone-lysine N-methyltransferase EZH1	EZH1	H3K27me1, H3K27me2
Histone-lysine N-methyltransferase EZH2	EZH2	H3K27me1, H3K27me2, H3K27me3, H1K25me1
Histone-lysine N-methyltransferase MLL	MLL	H3K4me3
Histone-lysine N-methyltransferase MLL2	MLL2	
Histone-lysine N-methyltransferase MLL3	MLL3	H3K4me3
Histone-lysine N-methyltransferase MLL4	Unknown	H3K4me3

Histone Lysine methyltransferases		
	53	
Enzyme (UniprotKB recommended name)	**Coding gene/s**	**Site of histone modification**
Histone-lysine N-methyltransferase MLL5	MLL5	
Histone-lysine N-methyltransferase NSD3	WHSC1L1	H3K4me2, H3K27me2, H3K27me3
Histone-lysine N-methyltransferase PRDM9	PRDM9	H3K4me3
Histone-lysine N-methyltransferase SETD1A	SETD1A	H3K4me3
Histone-lysine N-methyltransferase SETD1B	SETD1B	H3K4me3
Histone-lysine N-methyltransferase SETD2	SETD2	H3K36me3
Histone-lysine N-methyltransferase SETD7	SETD7/9	H3K4me1
Histone-lysine N-methyltransferase SETD8	SETD8	H4K20me1, H4K20me2
Histone-lysine N-methyltransferase SETDB1	SETDB1	H3K9me3
Histone-lysine N-methyltransferase SETDB2	SETDB2	H3K9me3
Histone-lysine N-methyltransferase SETMAR	SETMAR	H3K36me2
Histone-lysine N-methyltransferase SUV39H1	SUV39H1	H3K9me3
Histone-lysine N-methyltransferase SUV39H2	SUV39H2	H3K9me3
Histone-lysine N-methyltransferase	SUV420H1	H4K20me2, H4K20me3
Histone-lysine N-methyltransferase SUV420H2	SUV420H2	H4K20me2, H4K20me3

Histone Lysine methyltransferases		
54		
Enzyme (UniprotKB recommended name)	**Coding gene/s**	**Site of histone modification**
Histone-lysine N-methyltransferase, H3 lysine-36 and H4 lysine-20 specific	NSD1	H3K36me2, H4K20me2
Histone-lysine N-methyltransferase, H3 lysine-79 specific	DOT1L	H3K79me1, H3K79me2, H3K79me3
Histone-lysine N-methyltransferase, H3 lysine-9 specific 3	EHMT2	H3K9me1, H3K9me2, H3K27me1, H1K186me1, H1K25me1
Histone-lysine N-methyltransferase, H3 lysine-9 specific 5	EHMT1	H3K9me2, H3K27me1, H1K25me1, H1K186me1
N-lysine methyltransferase SMYD2	SMYD2	H3K36me2
PR domain zinc finger protein 2	PRDM2	H3K9me2
Probable histone-lysine N-methyltransferase ASH1L	ASH1L	H3K36me1, H3K36me2
Probable histone-lysine N-methyltransferase NSD2	WHSC1	H3K36me3, H4K20me1, H4K20me3
Histone-lysine N-methyltransferase 2D	MLL4	H3K4me
Histone-lysine N-methyltransferase 1A	LSD1	H3K4me, H3K9me
Histone-lysine N-methyltransferase 1B	LSD2	H3K4me

Table 1. 1. An overview of histone methyltransferases. Taken from:

http://www.actrec.gov.in/histome/enzymes.php?enzyme=Lysine_methyltransferases

1.4.1.4 Histone Demethylases

The discovery of histone-demethylating enzymes established the reversible nature of this histone modification. BHC110/LSD1 (lysine-specific demethylase-1), a nuclear amine oxidase homolog was characterised in 2004 as the first histone demethylase (Shi, Lan et al. 2004), followed by a study that identified LSD2 as the second histone demethylase (Karytinos, Forneris et al. 2009). The deletion of LSD1 or LSD2 has demonstrated their critical role in the development (Ciccone, Su et al. 2009, Wang, Hevi et al. 2009) Many studies showed that the biological role of LSD1 was due to its ability to demethylate histone methyl-lysine residues H3K4me2/1 and H3K9me2/1 in an FADH-dependent reaction (Kooistra and Helin 2012). This appears to be the main role of LSD1 in embryonic development (Kerenyi, Shao et al. 2013) and cancer stem cell biology (Schenk, Chen et al. 2012). LSD1 can be found in many chromatin complexes, which include components such as HDAC/CoREST, BRAF35, BHC80 and noncoding RNA (Tsai, Wang et al. 2010). Further studies were carried out to identify histone demethylases in addition to the LSD1 and 2 proteins. As a result, based on the mechanism used by DNA repair demethylases such as AlkB (a novel class of demethylases), JHDM (JmjC domain containing histone demethylase), were reported by Yamane et al in 2006. More recent studies demonstrated that the JHDM enzymes form an evolutionarily conserved histone demethylase family (Tsukada, Fang et al. 2006). The JmjC domain is found in 31 human proteins, of which 17 have been shown to act as histone demethylases (Kooistra and Helin 2012). These histone lysine demethylases can be classified into seven subfamilies, depending on the homology of the JmjC domain. They are able to demethylate of lysine in all three methylation states (mono-, di- and trimethylated lysine), (Cloos, Christensen et al. 2006, Klose, Yamane et al. 2006, Whetstine, Nottke et al. 2006). Identifying these enzymes creates new challenges to understand histone methylation and the complex nature of chromatin regulation. In the following years numerous subfamilies all containing the interacting active JmjC domain

were identified. Table 1.4 gives an overview of histone demethylases, their partners and contribution to disease.

Histone demethylases can influence many biological processes, including stem cell maintenance and differentiation, X chromosome inactivation and imprinting, genome integrity, tissue development, cell cycle regulation, and differentiation (Shi and Whetstine 2007). Furthermore, identifying the genomic sites targeted by these demethylases using chromatin immunoprecipitation (ChIP) will clarify their biological roles and subsequently facilitate tools to study the molecular mechanisms by which demethylases regulate chromatin structure and gene transcription *in vivo* (Shi and Whetstine 2007). Chromatin regulation occurs as a result of multicomponent protein complexes. Thus, ChIP assay is the most common method for studying chromatin-related modifications. It is a process by which a protein of interest binds to a specific genomic DNA region providing epigenetic researchers with important information about the interaction between specific proteins or protein modifications, including lysine demethylation, and a specific DNA sequence (Marcon, Ni et al. 2014).

Name	Synonyms	Specificity	Contribution in disease
KDM1B	LSD2, AOF1	H3K4me2/me1	
KDM1A	LSD1, AOF2	H3K4me2/me1, H3K9me2/me1	Overexpressed in prostate cancer), bladder cancer (Kauffman et al., 2011) ER-negative breast cancer (Lim et al., 2010) neuroblastoma (Schulte et al., 2009)Inhibition in animal models of engrafted acute myeloid leukaemia (Schenk et al., 2012)

KDM2B	FBXL10B, JHDM1B	H3K36me2/me1, H3K4me3	Required for initiation and maintenance of acute myeloid leukaemia (Li et al., 2011)
KDM2A	FBXL11A, JHDM1A	H3K36me2/me1	
KDM4A	JMJD2A, JHDM3A	H3K9me3/me2, H3K36me3/me2	Required for proliferation of breast cancer cells (Lohse et al., 2011), attenuated expression in bladder cancer (Kauffman et al., 2011), required for latency and replication of viruses that cause cancer (Chang et al., 2011)
KDM3B	JMJD1B, JHDM2B	H3K9me2/me1	
KDM4D	JMJD2D	H3K9me3/me2/me1, H3K36me3/me2	Required for cell proliferation and survival in colon cancer cells (Kim et al., 2012)
KDM4B	JMJD2B	H3K9me3/me2, H3K36me3/me2	Overexpressed in gastric cancer (Li et al., 2011), required for proliferation and formation of metastasis in breast cancer cells (Kawazu et al., 2011)
KDM4C	JMJD2C, GASC1	H3K9me3/me2, H3K36me3/me2	Overexpressed in breast cancer (Liu et al., 2009), esophageal cancer (Yang et al., 2000), MALT lymphoma (Vinatzer et al., 2008), acute myeloid leukemia (Helias et al., 2008) and lung sarcomatoid cancer (Italiano et al., 2006)
KDM8	JMJD5, FLJ13798	H3K36me2	
KDM6A	UTX, MGC141941	H3K27me3/me2	Tumor-suppressive function (Tsai et al., 2010)
KDM4E	JMJD2E	H3K9me3/me2	
KDM5B	Jarid1B, PLU1	H3K4me3/me2	Tumor-suppressive function in metastatic melanoma cells (Roesch et al., 2006), pro-proliferative in breast cancer (Mitra et al., 2011) and overexpressed in prostate cancer (Xiang et al., 2007)

KDM5A	Jarid1A, RBP2	H3K4me3/me2	Involved in drug resistance (Sharma et al., 2010)
KDM5D	Jarid1D, SMCY	H3K4me3/me2	
KDM5C	Jarid1C, SMCX	H3K4me3/me2	
KDM6B	JMJD3, KIAA0346	H3K27me3/me2	Overexpressed in Hodgkin's Lymphoma (Anderton et al., 2011)
	PHF8, KIAA1111, ZNF422	H3K9me2/me1, H4K20me1	
KDM7	KIAA1718	H3K9me2/me1, H3K27me2/me1	
PASS	HSPBAP1		Candidate gene for intractable epilepsy (IE)

Table 1. 2. **An overview of histone demethylases, their partners and contribution in disease.** Adapted from (Kooistra and Helin 2012).

1.4.2 Ubiquitination

Ubiquitination is the addition of ubiquitin, a 8.5KDa regulatory protein, to a target protein. It may influence proteins in several different ways; it can alter their cellular location, stimulate or avert protein interactions, signal their degradation via the proteasome, and influence their enzymatic activity (Glickman and Ciechanover 2002, Schnell and Hicke 2003, Mukhopadhyay and Riezman 2007). Ubiquitination occurs in three steps which are catalysed consecutively by ubiquitin activating enzyme (E1), ubiquitin conjugase (E2), and ubiquitin ligase (E3), leading to attachment of ubiquitin to lysine residues of substrate proteins (Pickart and Eddins 2004, Komander and Rape 2012). Among these enzymes, the E3 ubiquitin ligases play a key role in substrate recognition. Like other post-transitional modifications, ubiquitination is reversible by de-ubiquitination enzymes (DUBs) that cleave ubiquitin moieties from the target protein (Reyes-Turcu and Wilkinson 2009). Classification of ubiquitin modifications is based on the number of ubiquitin moieties attached to substrates and the position of lysine residues for the

ubiquitin chain linkage. For instance, poly-ubiquitination, with the addition of ubiquitin at the K48 position, usually targets substrates for degradation by the 26S proteasome (Hershko and Ciechanover 1998). However, mono- and oligo-ubiquitination or poly-ubiquitination through K63 linkage mediate non-degradative events controlling DNA repair, kinase activation, and endocytosis (Haglund and Dikic 2005). Many TGF-β pathway elements are modified specifically by poly-ubiquitination and proteasome-mediated degradation therefore decreasing signalling (Izzi and Attisano 2006, Inoue and Imamura 2008). However, it has subsequently been shown that some pathway elements do not undergo degradative ubiquitin but instead mono-, oligo-, or even poly-ubiquitination, under certain conditions, which can result in pathway activation (Bai, Yang et al. 2004). It has been demonstrated that SMAD3 undergoes various mono-ubiquitination, but this type of modification has no influence on SMAD3 stability or phosphorylation. However, mono-ubiquitination of SMAD3 controls its transcriptional activity (Inui, Manfrin et al. 2011). Multiple SMAD3 lysines, including K33, K53, and K81 have been identified as recipients of mono-ubiquitin in HEK293T kidney epithelial cells. Ubiquitination at these specific residues influences DNA-binding of SMAD3 as unmodified SMAD3 was pulled down by oligonucleotide probes containing the SMAD-binding sites (Inui, Manfrin et al. 2011). Although the direct interaction between SMAD3 and DNA at SMAD3 DNA-binding domains is inhibited by mono-ubiquitination it has no effect on indirect binding of SMAD3 to DNA through other transcriptional factors.

SMAD3 mono-ubiquitination is promoted by E3 ligase SMURF2. Four different lysine residues such as, K333, K341, K378, and K409, located in the MH2 domain of SMAD3, were recognised as target sites of SMURF2. SMURF2-induced multiple mono-ubiquitination of SMAD3 was shown to inhibit formation of homotrimeric SMAD3 and heterotrimeric SMAD3-SMAD4 complexes, and, therefore, limit SMADs from binding to (Inui, Manfrin et al. 2011). Furthermore, mono-ubiquitination of SMAD3 can be reversed by the de-ubiquitin enzyme,

USP15, and the recruitment of TGF-β-activated SMAD complex to chromatin was abolished by the knockdown of USP15 (Figure1.18). Therefore, deubiquitinstion by USP15 promotes SMAD3 transcriptional activity in response to TGF β-1 (Tang and Zhang 2011).
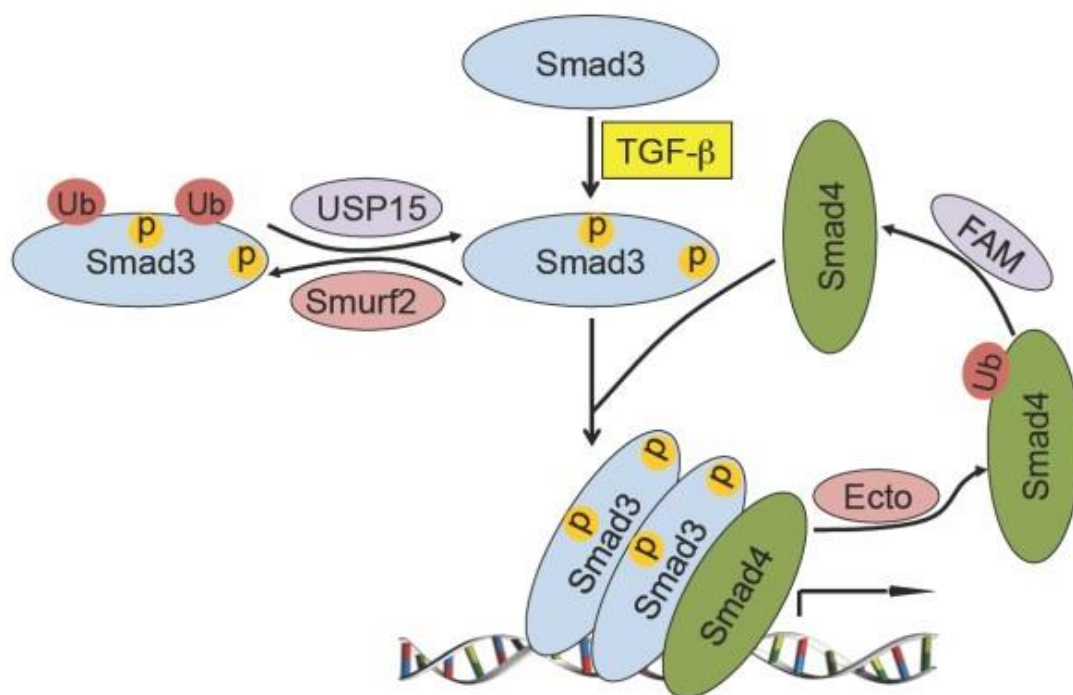
Figure 1.9. A model for mono-ubiquitination in TGF-β signalling. Upon TGF-β stimulation, SMAD3 is phosphorylated at sites in both the linker and the C-terminal tail. Phosphorylation of T179 in the linker region potentiates Smurf2 binding and the subsequent mono-ubiquitination. SMAD3 mono-ubiquitination can be reversed by USP15. On the other hand, mono-ubiquitination of SMAD4 is induced by Ecto/Tif1γ, and reversed by FAM/USP9x. The unmodified SMAD3 and SMAD4 form a DNA binding complex that regulates target gene expression whereas mono-ubiquitinated SMAD3 or SMAD4 inhibits or disrupts the SMAD complex formation (Tang and Zhang 2011).

1.4.3 Acetylation

Acetylation is the action of introducing an acetyl group into organic compounds, resulting in an acetoxy group (the replacement of hydrogen by an acetyl group). Revealed influences of histone acetylation on nucleosome structure, include temporary unwrapping of DNA from the edge of the nucleosome, decline in the linking number of nucleosomal DNA, and changed hydrodynamic behaviour (Norton, Marvin et al. 1990). Protein acetylation is a critical modification in cell biology and thousands of acetylated mammalian proteins have been identified by proteomics studies (Choudhary, Kumar et al. 2009). Proteins can be acetylated by enzymatic or non-enzymatic processes. A group of acetyltransferases catalyse the transfer of an acetyl group from acetyl-CoA to the terminal amine on the side chain of lysine residues, commonly called histone acetyltransferase (HATs), because their best-known substrates are histones. Due to their ability to acetylate lysine on various proteins, they are also referred to as lysine acetyltransferases (KATs). The alteration of the positively charged lysine to acetyl-lysine alters protein structure and function. For example, acetylation of histones enhances the recruitment of other proteins, relaxation of chromatin conformation, and an activation of transcription. Acetylation is reversible by histone deacetylases (HDACs/ KDACs), which are a small group of evolutionarily conserved enzymes (Hasan and Hottiger 2002, Freiman and Tjian 2003). Acetyltransferases have been reported to modify other proteins in addition to histones, including general transcription factors such as E2F-1, p53, GATA-1 and MyoD. The reported consequences of acetylation include increased DNA binding (p53, GATA-1 and MyoD) (Gu and Roeder 1997, Boyes, Byfield et al. 1998, Sartorelli, Puri et al. 1999) , decreased DNA binding, increased stability (E2F-1) (Martinez-Balbas, Bauer et al. 2000), inhibition of nuclear export and changes in protein–protein interactions.

Furthermore, it has been shown that SMAD3 is a direct target of the transcriptional co-activators p300/CBP. SMAD3 undergoes acetylation by p300/CBP, which is enhanced by

TGF-β. SMAD3 acetylation by p300/CBP occurs in the MH2 domain, which is important for transcriptional activity, underscoring acetylation of SMAD3 as being important for its transcriptional activation (Inoue, Itoh et al. 2007).

1.4.4 Phosphorylation

Phosphorylation is a reversible PTM that regulates protein function. Protein phosphorylation plays a critical role in intracellular signal transduction and is involved in regulating cell cycle progression, differentiation, transformation, development, peptide hormone response, and adaptation, and it is the most commonly studied post-translational modification (Hubbard and Cohen 1993, Pawson and Scott 1997, Hunter 2000, Cohen 2002). It is the addition of a phosphate group to a protein, which occurs on the side chains of three amino acids, serine, threonine and tyrosine, in eukaryotic cells (Cohen 2000).The phosphate group is added by a kinase. Not all proteins are phosphorylated, and phosphorylation can activate or inactivate a protein. Phosphorylation can be reversed. (dephosphorylation) by the removal of a phosphate group by a phosphatase enzyme (Manning, Whyte et al. 2002). Phosphorylation has an important role in TGF-β signalling. TGF-β activation initiates a kinase cascade that results in the phosphorylation of SMAD3, followed by its heteromerization with SMAD4 resulting in translocation into the nucleus. Once in the nucleus, phosphorylated SMAD3 can interact with other transcription factors, such as P300 to activate transcription from TGF-β target genes (Feng, Zhang et al. 1998).

1.5 SMAD3:SET9 Interaction

Protein methyltransferases play various roles in the epigenetic regulation of gene transcription, chromatin structure, gene silencing, maintenance of higher-order chromatin structures, DNA

repair, and replication. SET9 is a 41 kDa lysine-specific SET-domain methyltransferase encoded by a gene conserved in vertebrates and methylates Lysine-4 (K4) of histone H3 in yeast (Sanders, Portoso et al. 2004). It was originally isolated from HeLa nuclear extracts (Wang, Cao et al. 2001). Methylation of H4-K20, interestingly, is not implicated in the regulation of gene expression or heterochromatin formation. Instead, SET9 contributes to the DNA damage response by providing a 'histone mark' needed for the recruitment of the checkpoint protein Crb2, a homolog of the mammalian checkpoint protein 53BP1, to sites of DNA damage. The importance of SET9 is underscored by the fact that loss of SET9 activity or mutation of H3-K4 inhibits cell survival upon genotoxic treatment (Sanders, Portoso et al. 2004). Subsequently, SET9 has been shown to have broad target specificity *in vitro*, including transcriptional regulators such as AR, TAF10, p53, ER, p65, STAT3, Rb, Mypt, Tat, and Foxo3 (Table 1.5) (Gaughan, Stockley et al. 2011, Calnan, Webb et al. 2012). Moreover, it has been shown that half of the SET9 knockout mice die prior to birth, indicating the importance of SET9 in development. Mouse embryonic fibroblasts (MEFs) from SET9 heterozygous and null mice are more susceptible to transformation than wild-type MEFs, indicating that SET9 functions as a tumor suppressor (Kurash, Lei et al. 2008).

SET9-interaction partners	The signal to which the partner respond to	The effect of SET9
Androgen receptor (AR)	Androgens	Enhanced transcriptional activity in cell lines originating from the kidney and prostate (Gaughan, Stockley et al. 2011)
p53	DNA damage	Promotes cell death via methylation of p53 at lysine-372,which stabilizes the protein during DNA damage (Chuikov, Kurash et al. 2004)

STAT3	Cell growth and development	Methylates STAT3 at Lys140, leading to inhibition of STAT3 activity by suppressing the level of phospho-Tyr705 (Yang, Huang et al. 2010)
E2F1	DNA damage	Methylates E2F1 at lysine-185,which prevents E2F1 accumulation during DNA damage and activation of its proapoptotic target gene p73 (Xie, Bai et al. 2011)
TAF10	RNA polymerase II transcription initiation and in chromatin modification	Confers transcription stimulation in a promoter-specific manner (Kouskouti, Scheer et al. 2004)
RelA	Inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis	Negatively regulates the functions of NF-Kb by monomethylated RelA at lysines 314 and 315 in vitro and in vivo in response to stimulation (Yang XD et al.2009)
FOXO3	Oxidative stress-induced neuronal cell death	Inhibition of DNA-binding activity and transactivation(Qi Xie et al.2012)

Table 1.3. An overview of SET9 interactions, the signal to which the partner responds to and the effect of SET9 on these proteins.

1.5.1 Structure and Role of SET9

As previously mentioned, SET9 is a protein lysine methyltransferase that methylates histone H3 at Lysine 4 (H3K4) and a number of other non-histone proteins such as the tumour suppressor p53, the TATA box-binding protein (TBP) complex component TAF10 and oestrogen receptor α (ERα) (Wang, Cao et al. 2001, Nishioka, Chuikov et al. 2002, Wilson, Jing et al. 2002, Chuikov, Kurash et al. 2004, Kouskouti, Scheer et al. 2004, Subramanian, Da

et al. 2008). SET9, similar to many other protein lysine methyltransferases, contains S-(5′-adenosyl)-L-methionine (AdoMet) as the cofactor for the enzyme in its catalytic SET domain (figure 1.16) (Niwa, Handa et al. 2013). SET9 transfers the methyl group of the AdoMet to the ∈-amino group of a specific lysine residue of the substrate protein within its substrate-binding cavity. It is assumed that the proton separation from the targeted lysine residue proximal to the AdoMet of SET9 occurs before methylation by AdoMet, and a water channel is thought to be made in the presence of AdoMet to allow the proton to move towards the solvent (Zhang and Bruice 2007). Figure 1.20 shows the structure determination and architecture of SET9 (Figure 1.20) (Wilson, Jing et al. 2002).

The nature of its substrates has implicated SET9 in many molecular pathways including inflammation, cancer, and metabolism. Mice with a genetically deleted SET9 have no clear developmental abnormalities and do not develop cancer (Campaner, Spreafico et al. 2011, Lehnertz, Rogalski et al. 2011) . Therefore, the precise functional role of SET9 in disease or normal biology is still an open question (Keating and El-Osta 2013). SET9 has strong monomethyltransferase activity on H3K4 N-terminal peptides *in vitro*, however limited enzymatic activity when H3 is used as the substrate (Nishioka, Chuikov et al. 2002). Additionally, depletion or knockdown of SET9 has no effect on the global cellular levels of the H3K4me1 (Lehnertz, Rogalski et al. 2011), questioning whether H3 is the main methylation target (Keating and El-Osta 2013). SET9 also has distinctive features that differentiate it from most other PKMTs, in addition to its broad substrate specificity. SET9 has neither canonical nuclear localisation signals nor nuclear export signals is found in both cytoplasm and nucleus (Li, Reddy et al. 2008, Okabe, Orlowski et al. 2012). Therefore, nuclear localisation of SET9 may be regulated through interaction with other cellular elements. For example, the recruitment of SET9 to the promoters of target genes is accomplished by its interaction with transcription factors such as NFκB (Li, Reddy et al. 2008). Also, SET9 is the only methyltransferase that

has membrane occupation and recognition nexus (MORN) repeats that link the membrane to cytoskeleton in proteins (Garbino, van Oort et al. 2009). This suggests a critical cytoplasmic role for SET9. Given the varied interaction targets and lack of distinct SET9-associated phenotypes recognised to date, it is probable that SET9 function may be modulatory and dependent on the type of cell and/or physiological conditions being researched (Barsyte-Lovejoy, Li et al. 2014).
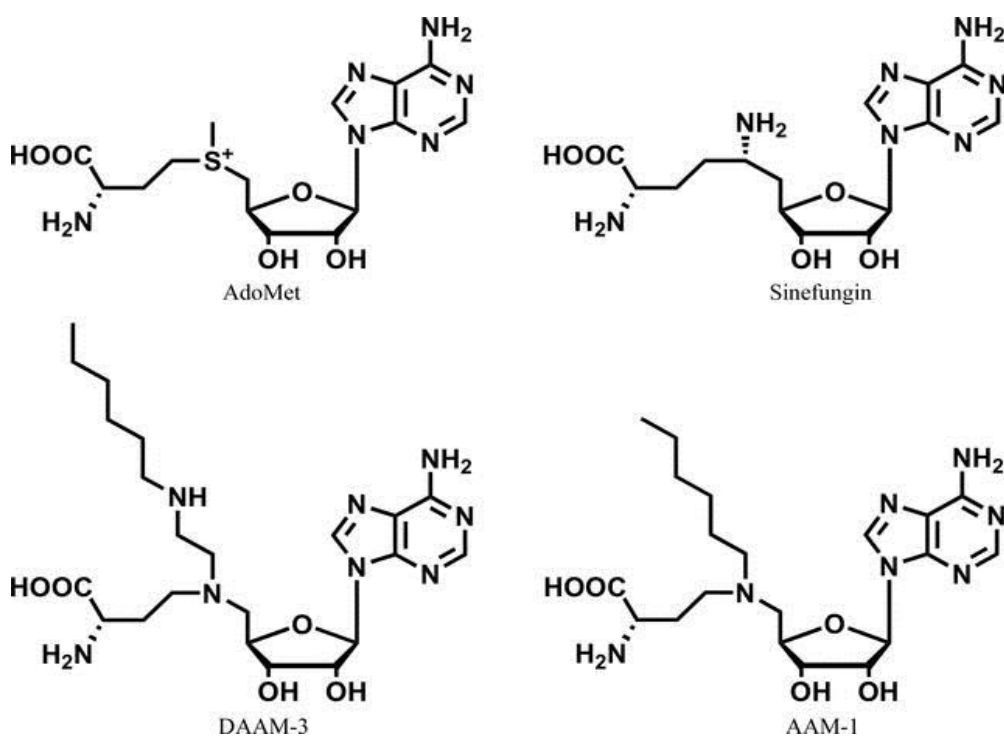
Figure 1.19. Structures of AdoMet and AdoMet analogues. DAAM-3 and AAM-1 have an n-hexylaminoethyl group and an n-hexyl group, respectively, attached to the N atom that replaces the S atom of AdoMet (Niwa, Handa et al. 2013)
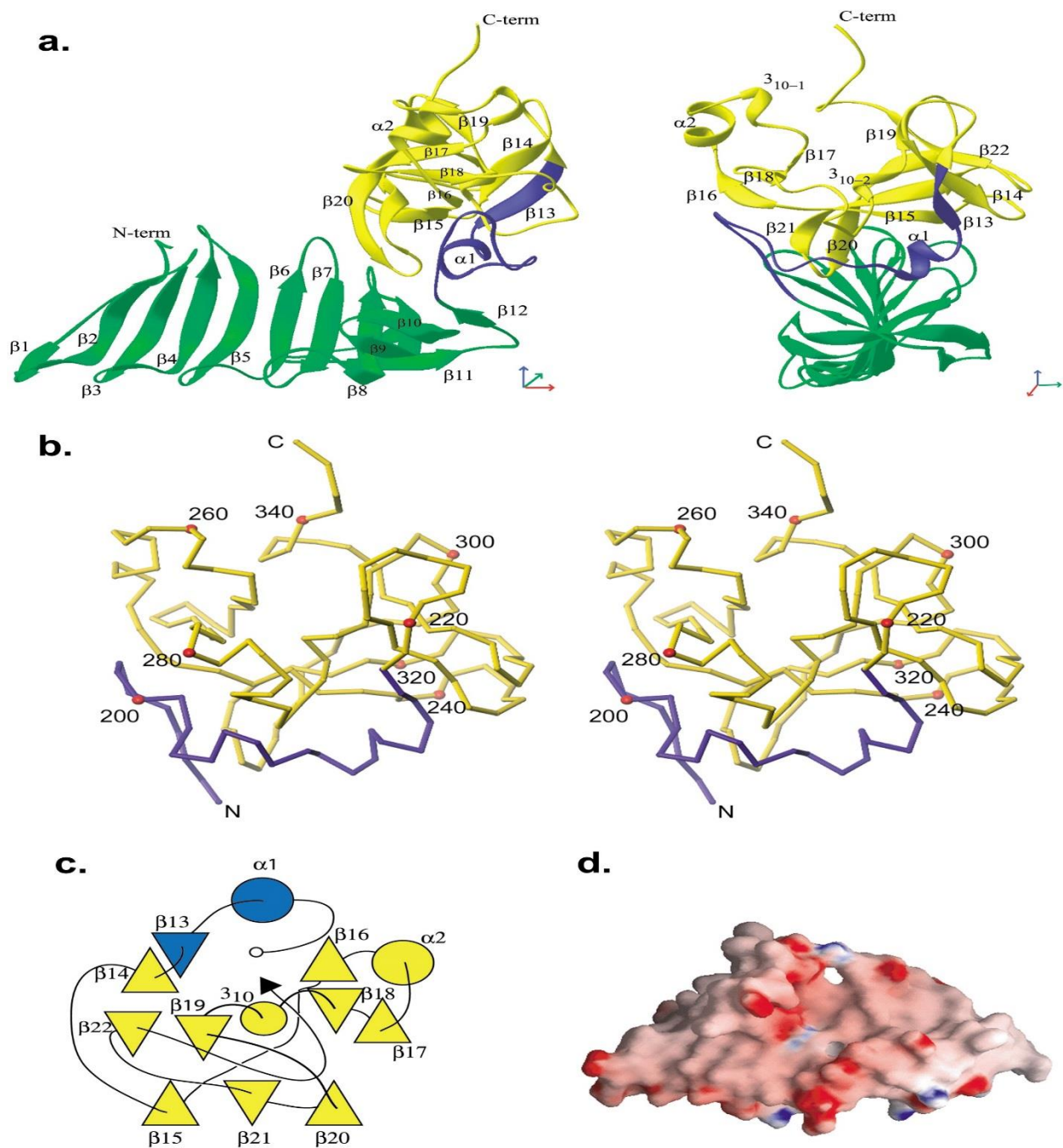
Figure 1.20. The Structure of SET9. *(A)* Two orthogonal views of the structure are shown in ribbon representation. The N domain (residues 52–192) is coloured green, the loop connecting the N domain with the conserved core of the SET domain is coloured blue, and the SET domain is coloured yellow. The secondary structure elements are labelled.*(B)* A stereographic representation of the Cα trace of the SET domain coloured and oriented as the right-hand panel of *(A)*; every 20th residue is labelled. *(C)* Schematic representation of the topology of the SET domain coloured as in *(A)*. β-strands are shown as triangles and helical segments as circles, the N terminus of the SET domain (residue 193) is indicated by an open circle, and the last residue in the crystal structure (344) by the solid arrowhead. The C terminus forms a threaded loop through the central 310-β20 connection. *(D)* The molecular surface of the N domain of SET9 is coloured according to its electrostatic potential as calculated using GRASP. Negative electrostatic potential is red, with positive electrostatic potential blue. The molecule is oriented as in the left panel of *(A)* (Wilson, Jing et al. 2002).

1.5.2 Inhibitors of SET9

To date, several histone methyltransferase inhibitors have been developed (Jones 2012). For instance, Chaetocin and BIX-01294 have been proposed as selective inhibitors of SUV39 and G9a, respectively (Greiner, Bonaldi et al. 2005, Kubicek, O'Sullivan et al. 2007), and DZNep, one of the most effective AdoHcy hydrolase inhibitors, potently diminishes the cellular levels of PRC2 molecules and inhibits histone methylation of H3K27 (Tan, Yang et al. 2007). Moreover, analogues of the methylation-reaction coenzymes have also been shown to be inhibitors, with AdoHcy, one of the methylation-reaction derivatives, being a nonselective inhibitor of many methyltransferases (Yao, Chen et al. 2011). The identification of these chemical inhibitors has enabled the investigation of the functional biology of these enzymes, as well as the examination of their potential as therapeutic targets. However, only a small number of such inhibitors are known and structural information is fairly limited. This is particularly true for the methyltransferase SET9, for which only a few selective inhibitors have been reported. Recently, the discovery of (R)-PFI-2 (Figure 1.21), a potent and selective inhibitor of SET9, and its 500-fold less active enantiomer (S)-PFI-2 provides a chemical probe tool kit to interrogate the biology of SET9. (R)-PFI-2 exposes an unusual cofactor-dependent and substrate-competitive inhibitory mechanism through occupying the substrate peptide binding groove of SET9, including the catalytic lysine-binding channel, and via directly contacting with the donor methyl group of the cofactor, S-adenosylmethionine. Treatment with (R)-PFI-2 reproduced the effects of SET9 deficiency on Hippo pathway signaling (that controls cell growth and organ size), through modulation of the transcriptional co-activator Yes-associated protein (YAP) and regulation of YAP target genes in murine embryonic fibroblasts. In breast cancer cell line (MCF7), (R)-PFI-2 altered YAP localisation, suggesting regulation of YAP by the methyltransferase activity of SET9 (Barsyte-Lovejoy, Li et al. 2014).
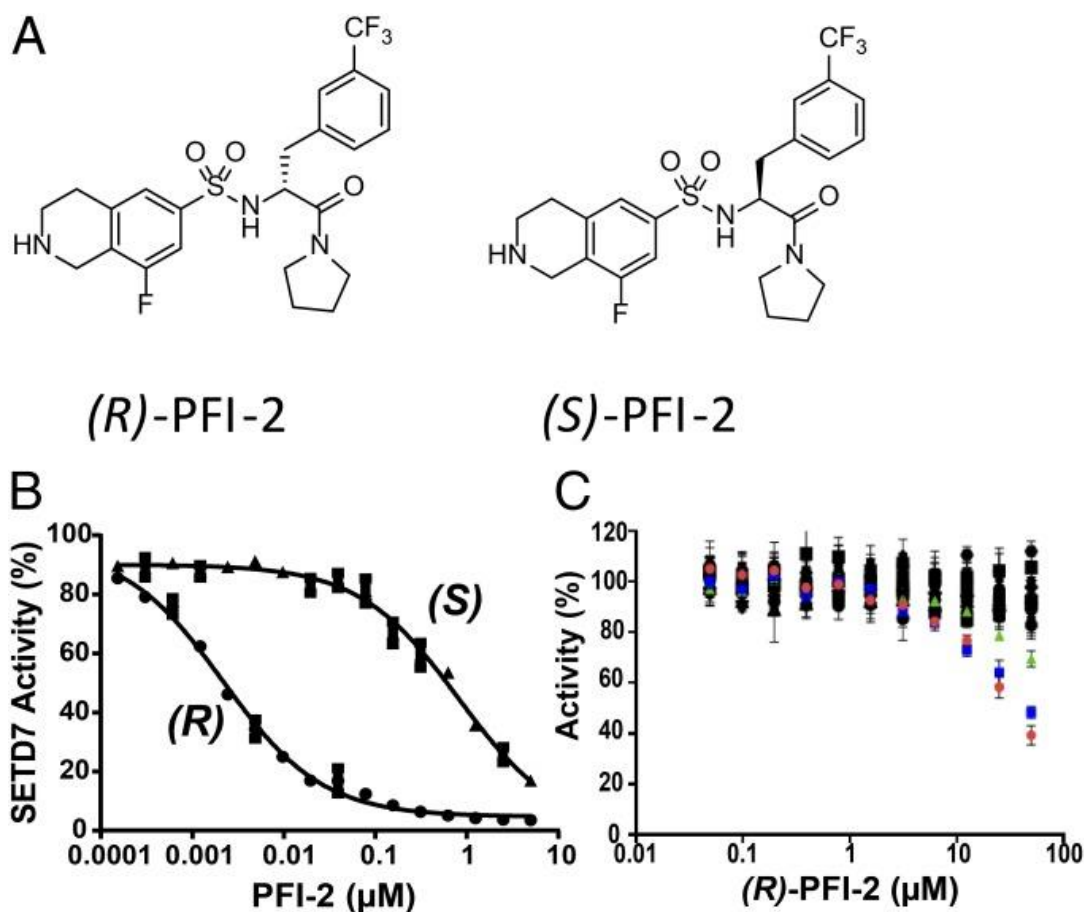
Figure 1.21. (R)-PFI-2 is a potent inhibitor of SET9. *(A)* Chemical structures of SET9 inhibitors (R)-PFI-2 and its less-active enantiomer (S)-PFI-2. *(B)* The effect of (R)-PFI-2 (●) and (S)-PFI-2 (▲) on methyltransferase activity of SETD7. Compounds inhibited SETD7 activity with IC50 values of 2.0 ± 0.2 nM (Hill slope, 0.8) and 1.0 ± 0.1 µM (Hill slope: 0.7), respectively. All experiments were performed in quadruplicate. (C) Effect of (R)-PFI-2 on activity of 18 different protein methyltransferases [(red filled circle) G9a, (blue filled square) EZH2, (green filled triangle) EHMT1, SUV39H2, EZH1, SUV420H1, SUV420H2, SETD8, SETD2, PRMT1, PRMT3, PRMT5, PRMT8, SETDB1, MLL1, DOT1L, WHSC1, and SMYD2] and DNMT1 was assessed using as high as 50 µM (R)-PFI-2 (Barsyte-Lovejoy, Li et al. 2014).

More recent studies demonstrated that TGF β-1 stimulates SET9 and H3K4me1-dependent transcriptional activation of fibrotic genes in rat mesangial cells under diabetic conditions (Sun, Reddy et al. 2010). SET9 has therefore been labelled as an epigenetic modifying enzyme that stimulates ECM protein production (Sun, Reddy et al. 2010). Further studies were carried out to examine TGF β-1-induced SET9 expression and whether inhibition of SET9 suppresses renal fibrosis in unilateral ureteral obstruction (UUO) and kidney cell lines. These studies revealed that SET9 was upregulated on days 3 and 7 in UUO mice (Figure1.22 (Sasaki, Doi et al. 2016)). This remarkable upregulation was suppressed by TGF β-1 neutralizing antibody.

TGF β-1 induced SET9 expression via SMAD3 in normal rat kidney (NRK)-52E cells. Moreover, human kidney biopsy specimens from patients diagnosed with IgA nephropathy and membranous nephropathy showed that the degree of interstitial fibrosis was positively correlated with SET9 expression. Interestingly, a small molecular inhibitor of SET9, sinefungin, led to a suppression of TGF β-1-induced fibrogenesis in *vivo* and in *vitro* (Sasaki, Doi et al. 2016).
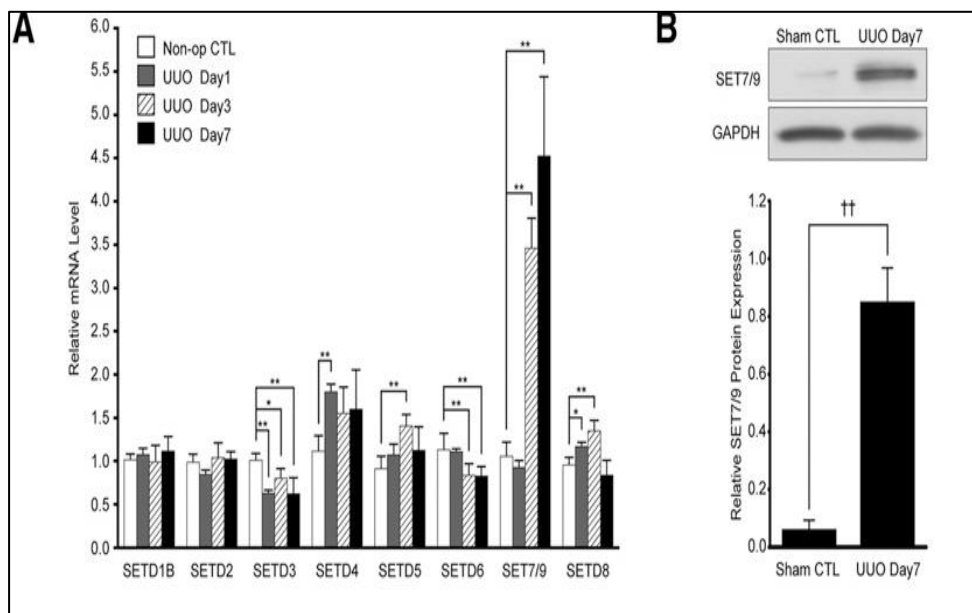
Figure 1.22. SET9 is upregulated in the kidney after obstructive injury. (A) Among genes encoding epigenetic modification enzymes containing the SET domain, SET9 was highly expressed in UUO mice. On day 1 (grey bars), day 3 (hatched bars), and day 7 (black bars) compared with control (white bars). (B) Elevation of SET9 protein in whole kidney extracts at day 7 after UUO compared with sham-operated control. Typical results of western blot analysis are shown in the upper panel. Band intensity was normalized to GAPDH. Relative levels of SET7/9 expression are shown in the lower panel (Sasaki, Doi et al. 2016).

It has been shown that SET9 plays a central role in cancer as well as fibrosis. SET9 interacts directly and methylates the androgen receptor (AR), a member of the nuclear hormone receptor family of transcription factors that regulate the expression of genes involved in progression and transformation of prostate cancer (Gaughan, Stockley et al. 2011). It has been suggested that

SET9 recognises a conserved K/R-S/T/A motif located before the lysine substrate, and has a preference to bind aspartate (D) and asparagine (N) on the C-terminal side of the lysine substrate; yet, this conserved motif does not apply to each SET9 target proteins. For example, in RelA or PCAF, methylated lysines within these proteins do not exist in such a conserved motif, suggesting that SET9 has the ability to recognize diverse sequences for lysine methylation (Couture, Collazo et al. 2006) (Figure 1.23). A lysine–rich motif, KKLKK, exists in all R-SMADs, including SMAD3, and functions as a NLS (Xiao, Liu et al. 2000). Interestingly, SET9 preferentially methylates target lysine residues within sequences very similar to the KKLKK NLS within SMAD3.

Figure 1.23. Sequences located around methylation sites of SET9 interacting proteins. Sequences surrounding the methylation sites of human histone H3, TAF10, p53, ERα, DNMT1, RelA and PCAF were aligned with methylation sites (red) located in the middle. The sequences containing the reported SET9 consensus recognition motif (asterisks) are shown in the upper panel; those which do not contain the consensus recognition motif are shown in the lower panel (Couture, Collazo et al. 2006).

Given the similarity between the SMAD3 KKLKK motif and other SET9-interacting partners, an investigation was carried out to study whether TGF-β1-induced phosphorylation

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of SMAD3 (p-SMAD3), which is the most studied pathway for TGF β-1 signalling is controlling SET9 expression. Thus, TGF β-1-induced SET9 mRNA and protein levels were significantly reduced in a cell line derived from rat kidney proximal tubules, NRK-52E cells, transfected with SMAD3 siRNA. Similarly, SMAD3 siRNA treatment potentially inhibited the expression of SMAD3 and TGF-β1-induced p-SMAD3 (Figure 1.24) (Sasaki, Doi et al. 2016).
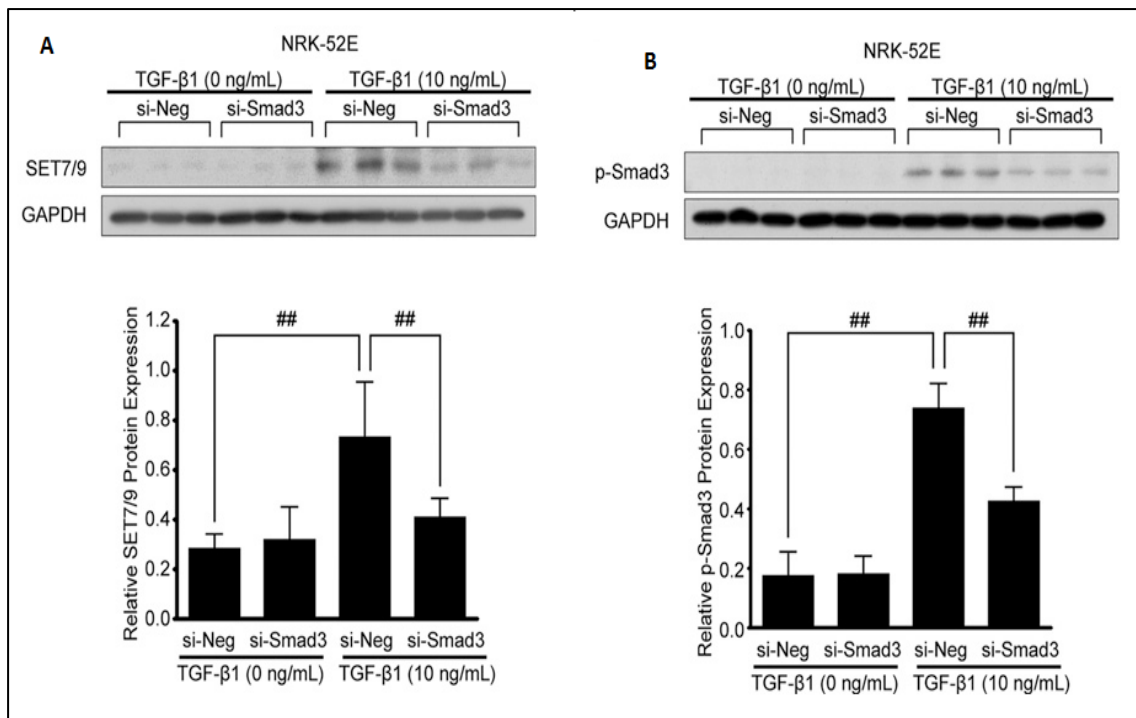
Figure 1.24. Knockdown of SMAD3 in NRK-52E cells inhibits TGF-β1-induced SET9 expression. NRK-52E cells were transfected with SMAD3 siRNA (si-SMAD3) or negative control (si-Neg) oligonucleotides. (A) Western blot analysis shows less SET9 expression in response to SMAD3 knockdown. (B) Phosphorylated SMAD3 (p-SMAD3) increased in response to TGF β-1but decreased in the absence of TGF-β1/SMAD3 (Sasaki, Doi et al. 2016).

1.5.2 SET9 and the Expression of the Pro-fibrotic Genes

Matrix deposition by cultured cells is promoted by TGF β-1 via increasing expression of ECM genes and inhibition of genes such as matrix metalloproteinases, which degrade the ECM

(Varga and Jimenez 1986). Previous work has shown that collagen induction by TGF β-1 requires the action of SMAD proteins (Chen, Yuan et al. 1999). It has been also shown that SMAD3 plays a key role in the deposition of matrix proteins via the induction of pro-fibrotic genes such as type I collagen, α-SMA, and fibronectin (Holmes, Leask et al. 2001, Verrecchia, Vindevoghel et al. 2001). The α-SMA gene, for example, contains SMAD3 binding sites within the gene promoter (Sato, Markiewicz et al. 2003) and SMAD3-null mice have considerably less α-SMA expression (Yao, Chen et al. 2008). SET7/9 has been described as an epigenetic enzyme that promotes ECM protein production *in vitro*. Consequently, further studies using SET9 siRNA in UUO models were performed in order to evaluate whether SET9 is responsible for fibrogenesis *in vivo*. These studies have revealed that a-SMA as well as collagen I were significantly decreased in mice transfected with SET9 siRNA (Figure 1.25) (Sasaki, Doi et al. 2015).

Furthermore, apart from α-SMA, SET9 knockdown was shown to attenuate TGF-β1–induced expression of ECM-associated genes such as connective tissue growth factor CTGF, collagen type I, and plasminogen associated inhibitor PAI-1 in rat mesangial cells (RMCs); demonstrating that SET9 has a functional influence upon mechanisms that are recognised to mediate fibrosis (Figure 1.26) (Sun, Reddy et al. 2010).
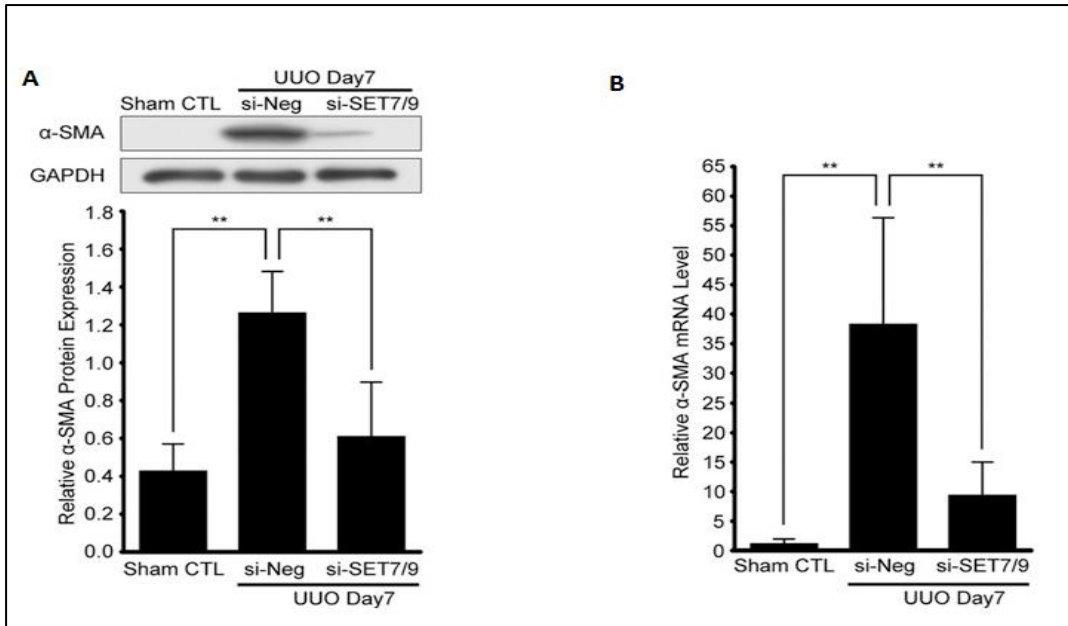
Figure 1.25. SET9 is needed for expression of the pro-fibrotic ACTA2 gene encoding α-SMA. *(A)* Typical results of western blot analysis showing the levels of α-SMA expression. Quantification is shown in the lower panel. *(B)* Expression levels of α-SMA determined by qRT-PCR upon injection with si-Neg/SET9 siRNA, indicating that α-SMA is downregulated in response to SET9 knockdown (Sasaki, Doi et al. 2016).
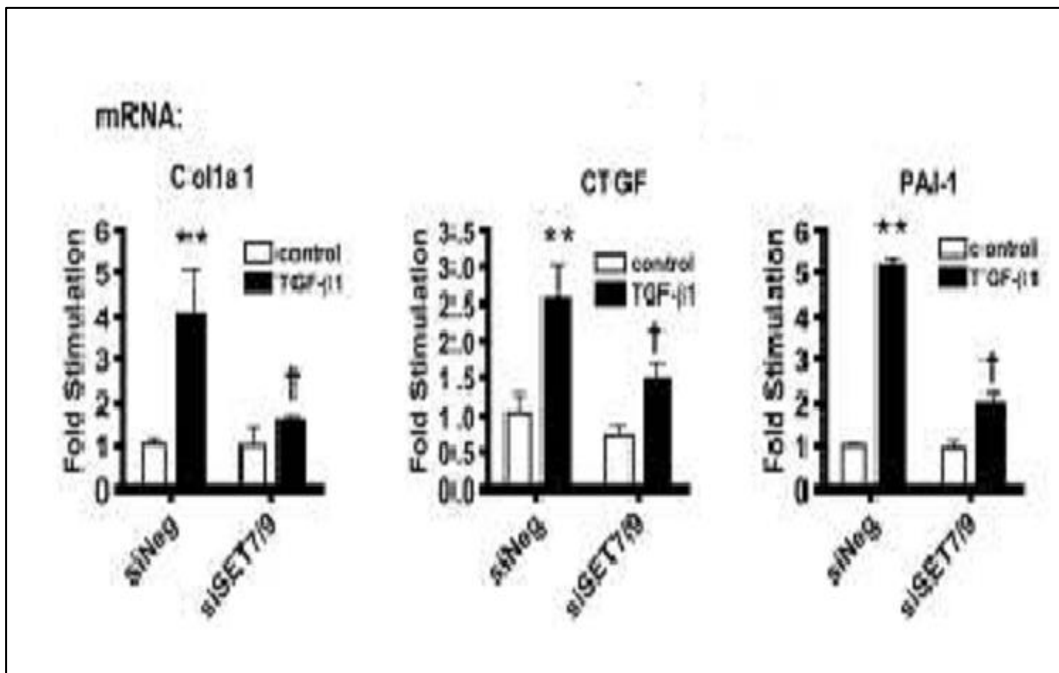
Figure 1.26. The involvement of SET9 in TGF-β1–induced regulation of ECM-associated genes in RMCs. Levels of pro-fibrotic genes(Col1a1, CTGF, and PAI-1) are decreased in RMCs transfected with various concentrations of SET9 siRNA (siSET7/9) or control (siNeg) oligonucleotides, in response to TGF-β1 (Sun, Reddy et al. 2010).

1.6 Aims

Part 1:

A new interaction between SMAD3, a transcription factor involved in renal fibrosis, and a protein called SET9 has previously been identified. I hypothesize that SET9 up-regulates the transcriptional activity of SMAD3 and this is dependent upon the catalytic activity of the SET9 enzyme. I intend to elucidate the contribution of SET9 to renal fibrosis, the common pathological process of all chronic diseases affecting the kidney. Specifically, aims of part 1 hypothesis are:

1.   Improve the understanding of the effect of SET9 on the TGF β-1 / SMAD3 signalling axis.

2.   Develop an in vitro system to study the effect of methyltransferases such as SET9 in renal fibroblast cells.

3.   Investigate the effect of methyltransferase-deficient SET9 upon SMAD3 activity in renal fibroblasts.

Part 2:

It has been shown that epigenetic alterations are implicated in fibrosis. Generally, methylation of Arg and Lys residues has been introduced as a stable, irreversible modification as a result of the slow turnover of methyl groups in chromatin. However, the discovery of a number of histone Lysine demethylases (HDMs, belonging to either the amino oxidase or the JmjC family) changed this view and proposed an innovative role for dynamic histone methylation in biological processes. Therefore, since overexpression, alteration, or mutation of a number of

HDMs has been found in many types of cancers and fibrosis, targeting such enzymes identify novel therapeutic targets to reduce fibrosis. Specifically, aim of part 2 hypothesis is:

1. To screen a demethylases siRNA library for effects on pro-fibrotic responses, identify further targets and validate the hits from that screen.

2   MATERIALS AND METHODS

2.1 Buffers

The majority of reagents were purchased from (Sigma, UK) unless otherwise stated.

10x Tris-glycine Running Buffer

30.3g of Tris-base (FLUKA), 144g of glycine, 10g of SDS, was dissolved in distilled water to a total volume of 100ml. For electrophoresis, this buffer was diluted to 1x with distilled water.

10x Transfer Buffer

30.3g of Tris-base and 144g of glycine were dissolved in distilled water to a total volume of 500ml. To make 1x of Tris-glycine transfer buffer, 100ml of 10x Tris-glycine transfer buffer was added to 200ml of Methanol (ANALAR). The volume was then adjusted to 1000ml with 700ml distilled water.

10x TBS

87.6g of NaCl and 12.1g of Tris-base were dissolved in distilled water to a total volume of 850ml. PH was adjusted to 8.0, and the volume adjusted to 1000ml by adding distilled water.

1x TTBS

To make 1x TTBS solution, 10x TBS was diluted to 1x using distilled water and Tween20 added to a final concentration of 0.1%.

5%BSA

5g of BSA was dissolved in 100ml of TTBS, and stored at 4^0C.

2.2 Cell Culture

The renal proximal tubular epithelial cell line, HK C-8 and transformed human renal mesangial cell line, THMC were maintained in DMEM F12 (Lonza) supplemented with 10% (v/v) foetal bovine serum (Australian, incubated 30 minutes at 56 degrees Celsius to inactivate enzymes and the complement cascade, and it is irradiated with a dose of 25 –40 kGy to provide the highest level of assurance of viral inactivity, Lonza), and 1% (v/v) penicillin streptomycin, hereby referred to as full media (FM). 10% FBS-supplemented media contain 1,000 – 2,000 pg/ml of latent TGF-β. This may explain the response of cells treated without TGF β-1. pCAGA12-luc reporter stably transfected HK C-8 cells were maintained in DMEM F12 supplemented with 10% (v/v) FBS, 1% (v/v) penicillin streptomycin and 300uM hygromycin B from Streptomyces hygroscopicus. Cell lines were routinely sub-cultured before reaching 80% confluence, generally with a splitting ratio of 1:4.

2.3 Freezing Cells

Frozen cell stocks were prepared in FCS containing cryoprotective dimethyl 1sulphoxide (DMSO). Cells were trypsinised, rinsed, and then re-suspended in 10%DMSO in FCS. 2ml aliquots were transferred to cryovials and cooled at rate of 1^0C per min using an alcohol bath located in a -80^0C freezer. When the bath reached -80^0C, vials were transferred to liquid nitrogen for long-term storage.

2.4 Stable Cell Cloning

HK C8 cells were co-transfected with 1ug of pDR2 plasmid and 10ug of pCAGA12-luc (acquired from Caroline Hill, London) plasmid using Lipofectamine transfection reagent (Life Technologies). 48hrs post-transfection, cells were selected in FM containing 400ug/ml

hygromycin B (Sigma), with medium changes every 4 days. After two weeks, colonies were selected and expanded in FM containing 300ug/ml hygromycin B.

2.5 Transformation of Bacterial Cells using Heat Shock Method

Competent E.coli bacterial cells were removed from -80°C and thawed on ice for 5minutes. 0.1ug of plasmid DNA was added to the cells and mixed with a sterile pipette tip. Cells were then incubated on ice for a further 30 minutes prior to heat shock at 42°C for 20 seconds. Cells were then returned back onto ice for 2 minutes and 1ml of warmed 37C LB Broth added followed by and incubation for 1 hour at 37°C. 100-150ul of the resulting culture was spread on warmed agar plates containing 50ug/ml ampicillin and incubated overnight at 37^0C to allow bacterial colonies to grow.

2.6 Plasmids

FLAG-SMAD3 and PAI-luc were obtained from Peter ten Dijk, Leiden, Nederlands. Wild type and mutant SET9 were obtained from Luke Gaughan, Newcastle University.

2.7 Plasmid DNA Maxi prep

To obtain endotoxin-free, transfection quality plasmid DNA, a a Chargeswitch-Pro filter plasmid Maxiprep Kit (Invitrogen, UK) was used, according to manufacturer's instructions, from a 100ml overnight culture that was setup by inoculating a single bacterial colony into 100ml LB culture medium, supplemented with 50ug/ml Ampicillin.

2.8 siRNA

Scrambled (SCR), SMAD3 and SET9 siRNAs were purchased from Eurofins with the following sense sequences 5'-UUCUCCGAACGUGUCACGUTT-3', 5'-CUGUGUGAGUUCGCCUUCATT-3' and 5'-AUU CCG UGAUCGUCCAGGUGC-3',

respectively. Cells were transfected using Lipofectamine RNAiMAX (LIfe Technologies) according to manufacturer's guidelines to a final concentration of between 25-50nM.

2.9 Proliferation Assays

Proliferation studies were carried out using WST-1 assay (Roche Applied Science, Germany). Cells were seeded into the well of a 96-well culture plates (Greiner bio-one, Cell star, Austria) at a density of 10000 cell per well in 100ul medium. They were then starved by washing in PBS and replacing the culture medium with serum-free medium. Some cells were left in a full medium to use as a control. Cells were incubated for 24hrs in serum free medium before treatment with TGFβ-1 for a further 24 hrs. After the direct addition of WST-1 reagents to the culture plate, the absorbance at 450 nm was measured by use of a micro-plate reader.

2.10 Measurements of Protein Concentration

Protein concentration from HK C-8 cell lysates was carried out using BCA assay (Thermo Scientific, USA) as described by manufacturer. A serial dilution of Albumin (BSA) Standards was generated to obtain a final concentration of 2000ug/ml, 1000ug/ml, 500ug/ml, 250ug/ml, and 125ug/ml in PBS. Absorbance of samples and standards was measured at 490nM using a micro-plate reader. Protein concentrations in samples were calculated using the standard curve as described in the manufacturers protocol.

2.11 Transfections and Reporter Gene Assays

pCAGA12-luc reporter stably transfected HKC8 cells were seeded in 48 well plates, reverse transfected with either control or a library of pooled demethylase siRNAs (3 siRNAs per target gene, acquired from Dr. Luke Gaughan, Northern Institute of Cancer Research, Newcastle University) at a final concentration of 25nM then treated with 1ng/ml TGFβ-1 for 24hrs. Each siRNA pool was transfected in quadruplicate. Media was removed, then the cells were washed

in PBS, lysed in 55ul Reporter lysis buffer (Promega) and subject to one freeze thaw cycle to aid complete lysis. Lysates were transferred to micro-centrifuge tubes, debris pelleted by centrifugation at 14,000 rpm for 5min, then 30ul of soluble supernatant mixed with 40ul of luciferase Assay Reagent (Promega). Luciferase activity was analysed using a plate reader. Luciferase readouts were corrected for protein concentration from the previously described BCA assay data presented as fold change in luciferase activity with standard deviation across the quadruplicate.

2.12 Western Blotting (WB)

Proteins from lysates were subject to SDS PAGE in 4-12% precast polyacrylamide gels (Life Technologies) and were electro-transferred to nitrocellulose membrane (Hybond, Amersham). Blots were then blocked in 1 x TBS (10x stock containing 87.6g NaCl, 12.1g Tris, made up to 1l ddH2O pH8.0) containing 4% bovine serum albumin (BSA) (Sigma) for 1h. Primary antibodies were next applied at 1:500 to 1:1000 dilution in 1 x TBS (PH 8.0) containing 1% BSA, overnight at 4°C. Blots were next washed 3 times in 1x 0.1% tween-TBS then exposed to secondary antibodies (anti-mouse and anti-rabbit horseradish peroxidase-conjugated, Biotin) diluted 1:1000 in 1 x TBS (PH 8.0) containing 1% BSA for 1h. After 3 washes in 1x 0.1% tween-TBS, the blots were developed with ECL (Pierce) and were exposed on X-ray film (Agfa).

2.13 Wound Healing Assay

Cells were seeded into chambers (Ibidi) to create reproducible wounds in 12 well plates. The following day, cells were treated with or without TGFβ-1 and in some experiments with or without SMAD3 siRNA/SET9 inhibitor (Tocris) 5-30uM. After 24hrs the chambers were removed and the cells imaged at time intervals for 24hrs using Zeiss Axiomager microscope.

Wound closure was presented as fold change in cell surface coverage using NIS and Image J software.

2.14 Nuclear and Cytoplasmic Fractionation

Nuclear and cytoplasmic fractions were isolated by sequential centrifugation steps, using Hypotonic Buffer Solution (20mM Tris-HCl pH7.4, 10mM NaCl and 3mM $MgCl_2$) and Cell Extraction Buffer (100mM Tris pH7.4, 2mM $NaVO_4$, 100mM NaCl, 1% Triton-X-100, 1mM EDTA, 10% glycerol, 1mM EGTA, 0.1% SDS, 1mM NaF, 0.5% deoxycholate and 20mM $Na_4P_2O_7$). Fractions were then subjected to western blotting.

2.15 Immunofluorescence (IF)

Cells were seeded on glass coverslips in 6 well plates and treated with TGFβ-1. They were then washed in PBS, fixed with ice cold 100% methanol, dried at room temperature and blocked in PBS containing 5% goat serum for 1hr. Primary antibodies were applied at a dilution of 1:100 in PBS overnight and then secondary antibodies (Alexa Fluors 488 Life Technologies) applied at 1:200 in PBS for 2hrs. DAPI nuclear stain was applied at 0.5ug/ml DAPI in PBS for 10mins. The coverslips were washed 3 times in PBS, mounted onto slides and imaged with Zeiss Axioimager microscope. All IF images were analysed by a software called ImageJ (the protocol is provided in a separate sheet).

2.16 Immunoprecipitation (IP) with Cell Lysates

Immunoprecipitation lysis buffer (50mM Tris pH7.4, 150mM NaCl, 1mM EDTA, 1% Triton-X-100 and 1 protease inhibitor tablet) was added directly onto cells and incubated on ice for 5mins. Cells were then scraped, transferred into Eppendorf tubes and sonicated to aid lysis and shearing of genomic DNA. Lysates were then centrifuged for 25mins at 20,000 rpm and

supernatants incubated with protein G dynabeads (Life Technologies) cross-linked to SMAD3/HSPBAP-1 antibodies (Abcam) or control IgG antibody (Abcam) overnight at 4°C with rotation. The following day, samples were washed 3 times with wash buffer (50mM Tris pH7.4, 150mM NaCl, 1mM EDTA, 1% Triton-X-100, protease inhibitor tablet (Roche) and phosphatase inhibitor (100ul in 10ml, Sigma)). After the final wash, samples were centrifuged to remove any remaining wash buffer and re-suspended in SDS-sample buffer (10ml 3x stock: 2.4ml 1M Tris pH 6.8, 3ml 20 SDS, 3ml glycerol, 1.6ml β-merceptoethanol with bromophenol blue) and subjected to western blotting.

2.17 Statistical Analyses

Data in this project were analysed using SPSS statistical software version 19 (IBM, Armonk, NY, USA). The mean values of the different groups were analysed using one way ANOVA that determines whether any of those means are statistically significantly different from each other, and a p-value cut-off of 0.05 was used as a cut-off for significance. One way ANOVA was followed by Tukey's honestly significant difference (HSD) post hoc test. The analysis of immunofluorescence staining was performed using Velocity software Columbus™ Image Data Management and Analysis System.

3 DEVELOPMENT OF IN VITRO MODELS TO STUDY TGF β-1 / SMAD3 SIGNALLING

3.1 Introduction

Delineating the key regulatory processes involved in SMAD3 signalling is likely to be critical to our understanding of this pathway in CKD. Phenotypic transition of differentiated epithelial cells has been studied widely as a mechanism involved in pathological tissue fibrosis. Although it is controversial, the transition of epithelial phenotype to a mesenchymal phenotype (EMT) is regarded as one of the sources of matrix secreting fibroblasts in fibrosis in various organs, including the kidney (Vongwiwatana, Tasanarong et al. 2005). TGF β-1 upregulates the expression of markers of EMT by activating SMAD3 signalling in human renal proximal tubular epithelial cell models (Phanish, Wahab et al. 2006). As such, we know that the HK C-8 cell line expresses the required components of the TGF β-1 signalling pathway to make it a useful model of TGF β-1 signalling. Thus, the specific aims of this chapter were:

1- To develop an *in vitro* reporter gene assay in which to study SMAD3 regulatory factors.

2- To understand what effect SMAD3 depletion has upon reporter gene activity.

3- To generate a stably HK C-8 CAGA-Luciferase transfected cell line.

4- To screen a methyltransferase/demethylase siRNA library for further targets.

3.2 *CAGA*-luciferase Reporter Gene System to Study the TGF β-1 / SMAD3 Signalling Axis *in vitro*

To devise a screening system in which to study SMAD3 regulatory factors, an *in vitro* reporter gene assay was developed.

A SMAD3-responsive reporter gene, termed *CAGA*-luciferase, containing 12 repeats of the consensus *CAGA* SMAD binding element driving luciferase expression was used (a gift from Caroline Hill, London).

HK C-8 cells were transfected with *CAGA*-luciferase at differing concentrations (Figure 3.2 A, B). Cells were then starved prior exposure to TGF β-1 or left in full medium. Figure 3.2 demonstrates a robust induction of luciferase expression from the *CAGA*-luciferase transfected cells in response to exogenous TGF β-1. In the absence of exogenous TGF β-1, reporter gene activity was minimal. Importantly, the two graphs show a dose-dependent increase in luciferase activity upon transfection of *CAGA*-luciferase. These data indicated that the HK C-8 cell line contains an intact TGF β-1 signalling pathway.
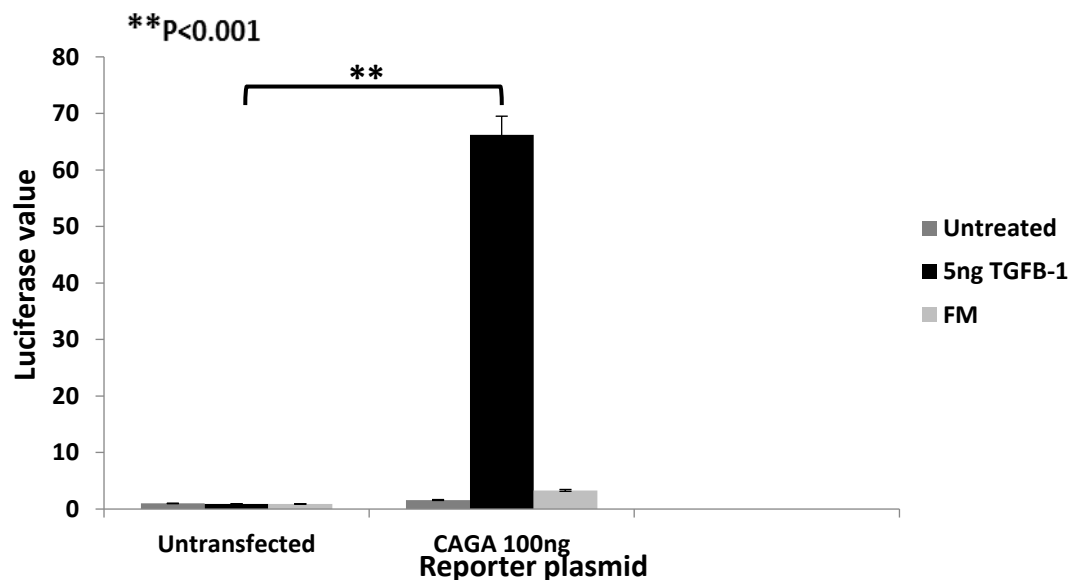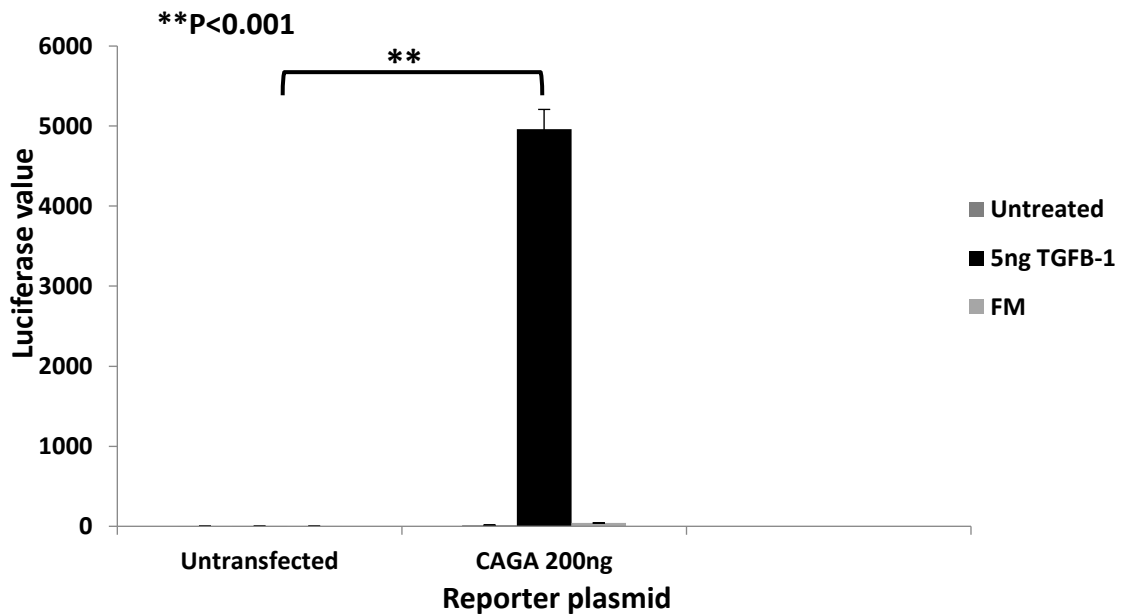
Figure 3.2.*A*, *B*. Reporter gene analysis. The activity of luciferase is promoted in response to TGF β-1. *A*. HK C-8 were transfected without or with100ng of *CAGA* luciferase, starved in serum free medium and treated without or with 5ng/ml TGF β-1 or full medium for 24hr. Cells were lysed in 1xlysis buffer, and luciferase activity measured. *B.* Cells were treated as in *A*, but transfected without or with 200ng/ml of CAGA luciferase. (n=3 Mean +/- SD).

3.3 *CAGA* Reporter Gene Activity in Response to TGF β-1 is Reduced Upon SMAD3 Knockdown

To determine whether or not the enhancement of *CAGA*-luciferase expression in response to TGF β-1 was dependent upon the activity of SMAD3, a second series of experiments were performed using a SMAD3 siRNA. HK C-8 cells were transfected with 25nM SMAD3 siRNA prior to sequential transfection with the *CAGA*-luciferase reporter gene. Cells were then starved prior to treatment with TGF β-1 as indicated in Figure 3.3. Compared to cells transfected with a control siRNA, SMAD3 knockdown produced a significant reduction in reporter gene activity

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even in the presence of exogenous TGF β-1. These data indicate that the HK C-8 cell line contains a functional TGF β-1 and SMAD3 signalling pathway.
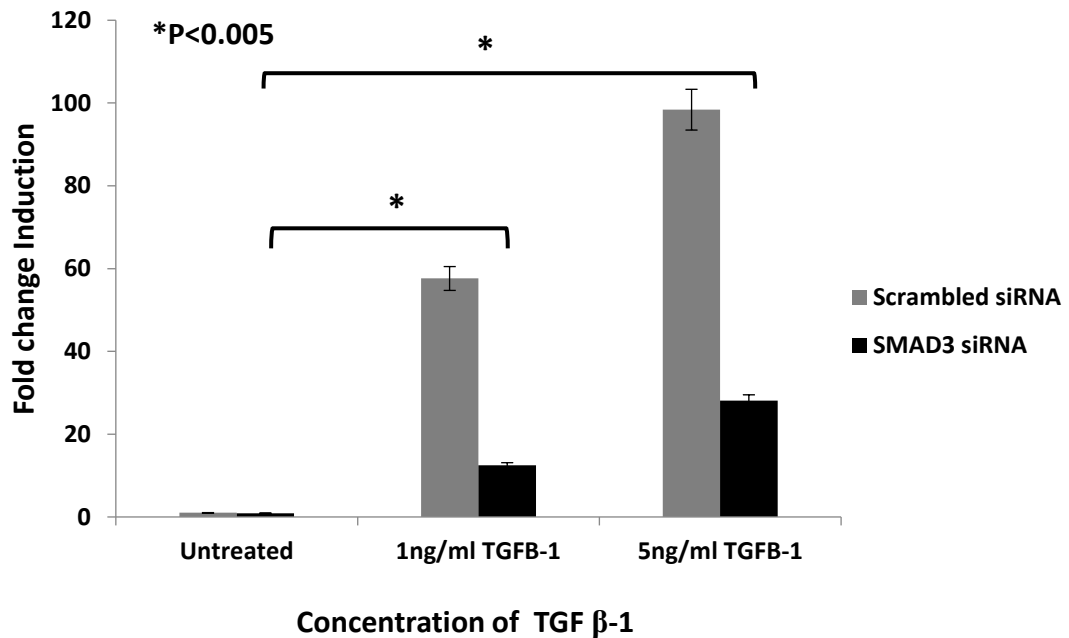
Figure 3.1. The effect of silencing SMAD3 on the expression of *CAGA* luciferase. HK C-8 cells were transiently transfected with 25nM of SMAD3 or scrambled (SCR) siRNA for 24hr followed by a transfection with 200ng *CAGA* luciferase plasmid. After 24hr, cells were incubated in serum free medium then treated without or with 1ng/ml or 5ng/ml TGF β-1. The baseline of untreated cells was used to calculate the fold change induction. (n=3 Mean +/- SD).

3.4 The Generation of HK C-8 *CAGA*-luciferase Stable Transfectants

The previous experiments applied transient transfection protocols that provide peak gene expression between 24-96 hours post-transfection. Stable cell lines provide a genetically homogenous and clonal population that overcomes the inherent variability between transient transfection experiments thereby making them suitable for screening purposes. To generate a stable reporter gene cell line, HK C-8 cells were co-transfected with 2ug pDR2 plasmid, which carries a hygromycin-resistance gene, and 10ug of pCAGA12-luc plasmid, which carries the gene of interest. 48h post-transfection, cells were selected in FM containing hygromycin B at

400ug/ml (see materials and methods). Hygromycin resistant clones were screened for luciferase activity in the presence of TGF β-1. Clone 2 showed the highest luciferase activity with approximately a 12 fold induction of activity in the presence of TGF β-1, compared to untreated cells, and was therefore further expanded to be used for subsequent experiments (Figure 3.4). Other colonies were discarded.
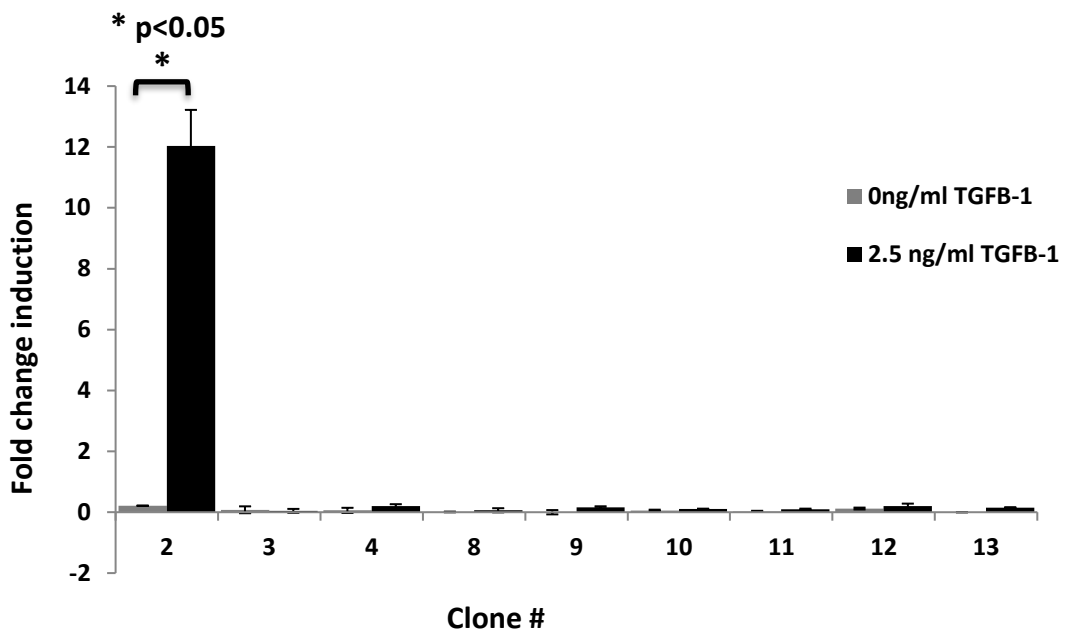
Figure 3.2. *CAGA* **luciferase activity in hygromycin resistant clones**. Stable hygromycin-resistant cell lines were established by transfection of HK C-8 cells with *CAGA* luciferase plasmid. Clones were stimulated with 2.5ng/ml TGF β-1 and Luciferase activity measured after 24 hours. Clone 2 demonstrated the greatest induction of luciferase activity (n=3, Mean +/ SD). Luciferase values, corrected for protein concentration, are expressed as fold change compared to that for the control. The baseline of untreated cells was used to calculate the fold change induction.

3.5 TGF β-1 / SMAD Signalling in *CAGA*-luciferase Stably Transfected Cells

To be certain that the effect of TGF β-1 in the stable reporter gene cell line remained dependent upon SMAD3, the stable cells (clone 2) were transfected with 25nM SMAD3 siRNA, serum starved, and then treated with TGF β-1 (Figure 3.5). Transfection of SMAD3-specific siRNA resulted in abrogation of TGF β-1-induced luciferase expression whereas transfection of

control siRNA (SCR) did not affect TGF β-1 induction of the reporter gene in these cells (Figure3.5). Taken together, the data show that HK C-8 cells contains afunctional TGF β-1 – SMAD3 signalling pathway and that the stable cell line can be used to study this pathway further.
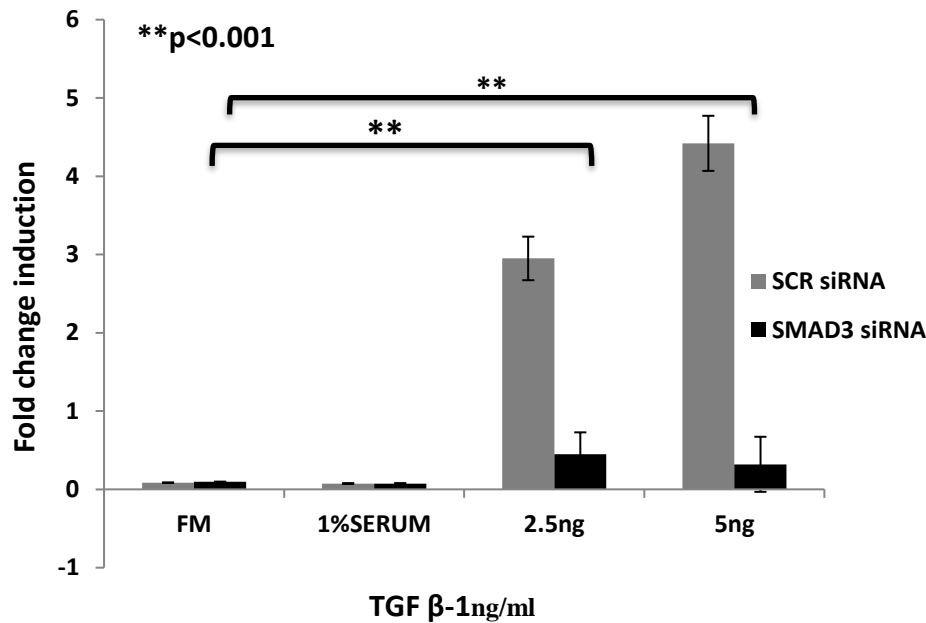
Figure 3.3. SMAD3-specific siRNA inhibits the activity of *CAGA* reporter gene induced by TGF β-1. Luciferase assays were carried out using the described reporter gene assay. HK C-8 cells were seeded and transfected with 25nM SCR/SMAD3 siRNA. Cells were treated with or without TGF β-1. Lysates were harvested next day, after 24hrs. Luciferase values, corrected for protein concentration, are expressed as fold change compared to that for the control. . The baseline of SCR siRNA untreated cells was used to calculate the fold change induction. (n=3 Mean +/- SD).

3.6 Investigating Lysine Demethylase Function *CAGA* Reporter Cell Lines

The above reporter gene assay was used to examine whether or not demethylase enzymes could alter the activity of the SMAD3 transcriptional activity. These enzymes are known to play a key role in the regulation of numerous transcription factors, gene expression and their role in disease is becoming more apparent (Dimitrova, Turberfield et al. 2015). Thus far however, there is no link between demethylase enzymes and TGF β-1–SMAD3 signalling.

25 known demethylases were targeted with a pool of three individual siRNA oligonucleotides designed against different regions of target mRNA using the Rosetta Algorithm (Sigma-Aldrich) to ensure robust target knockdown and reduced off-target effects. These siRNAs were transfected into HK C-8 cells stably transfected with the pCAGA12-luc in 48 well plates. (Figure 3.6A), and then treated with TGF β-1. As expected the addition of TGF β-1 lead to an induction in luciferase activity from stably-integrated *CAGA*-luciferase reporter gene. Interestingly, transfection with siRNA against demethylases enzymes resulted in varying changes to reporter gene expression. Whilst the introduction of some siRNAs lead to a decrease in reporter gene expression, the overall trend was more towards greater reporter gene expression after the introduction of demethylase siRNAs (Figure 3.6A), compared to control siRNA sequences. In particular, siRNAs targeting, HSPBAP1, JMJD2C, and PHF2 lead to statistically significant increases in reporter gene expression. Similar results were obtained when transiently transfected parental HK C-8 cells were co-transfected with these selected siRNAs alongside *CAGA*-Luciferase, supporting the observations from the stably transfected cells (Figure 3.6B). Overall, the data show, for the first time, a role for demethylase enzymes in the regulation of TGF β-1 – SMAD3 signalling HSPBAP1 is further characterised later in this thesis (see chapter 5).
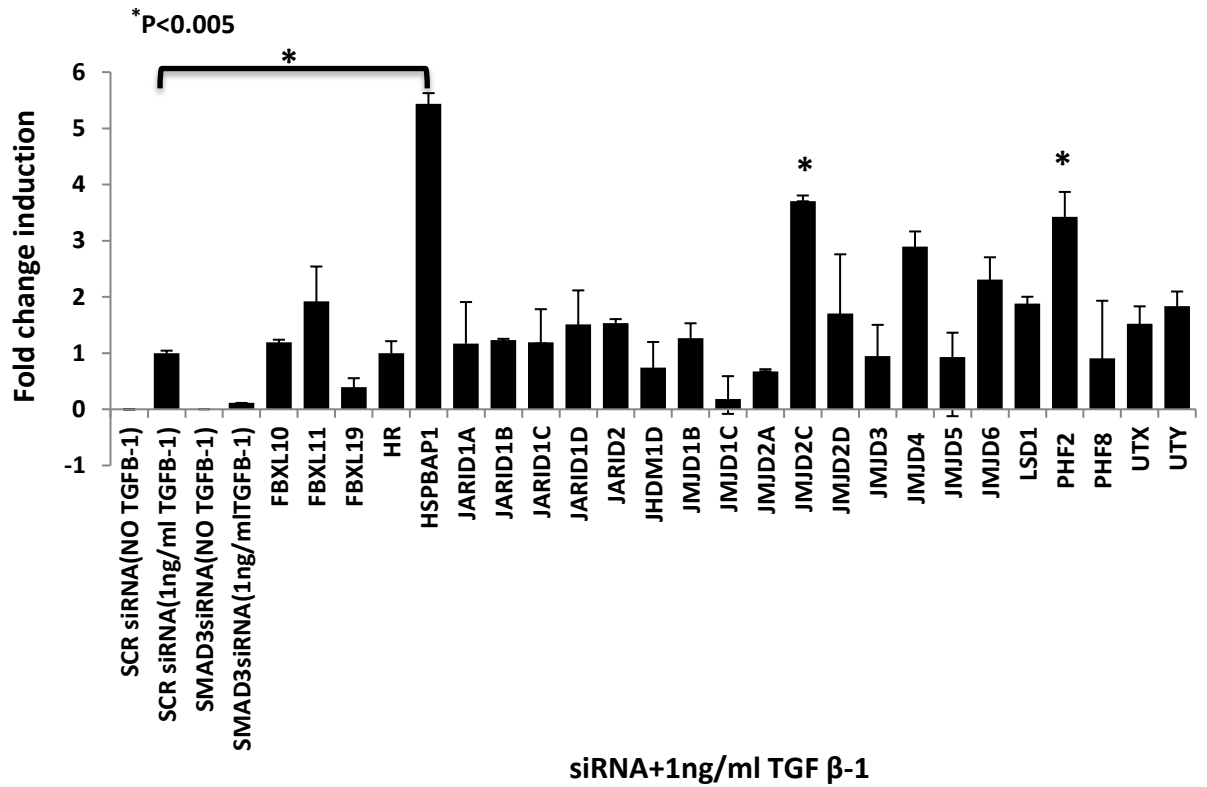
Figure 3.4A. Level of reporter gene is significantly enhanced upon the transfection of siRNA against specific demethylases. *CAGA*-luciferase stable cell line was seeded and transfected with 25nM siRNA towards the shown demethylases. Cells were treated with or without 1ng/ml TGF β-1. Cell lysates were harvested next day, after 24hrs. Luciferase values, corrected for protein concentration, are expressed as fold change compared to that for the control. . The baseline of SCR siRNA cells was used to calculate the fold change induction. (n=3 Mean +/- SD).
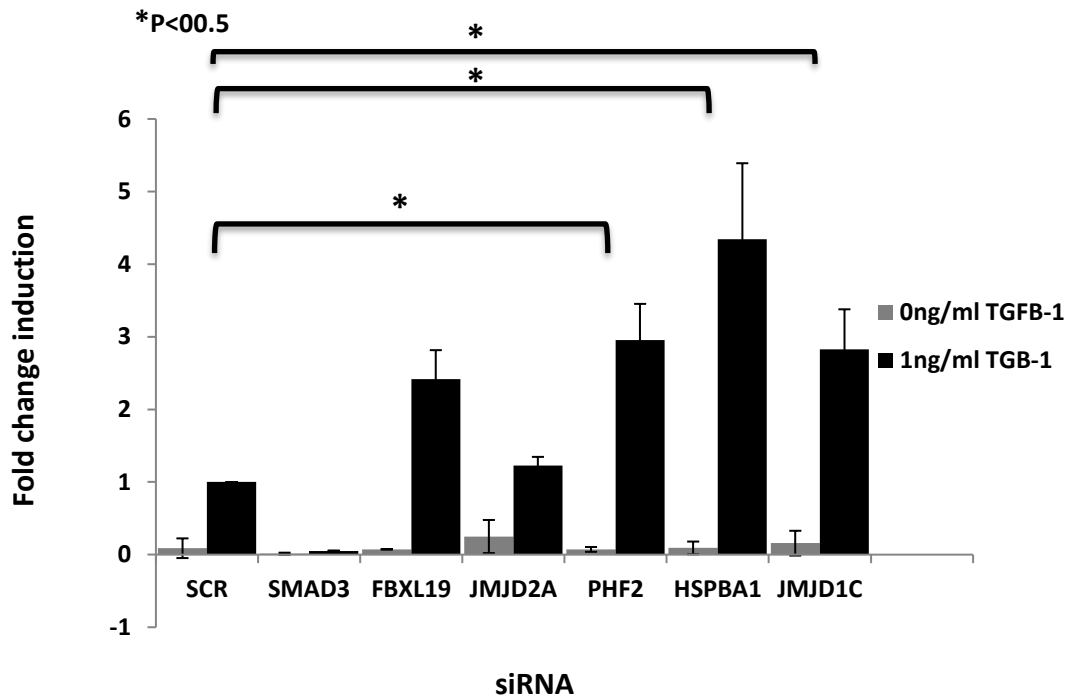
Figure 3.6B. Cells transiently transfected with *CAGA* reporter gene have identified the same targets obtained using stable cell lines. HK C-8 cells were seeded and transfected with 25nM siRNA towards the stated demethylases. Next day, cells were transfected with 300ng *CAGA* reporter gene, and left to incubate for 24hr.Then, cells were treated with or without 1ng/ml TGF β-1. Cell lysates were harvested next day, after 24hrs Luciferase values, corrected for protein concentration, are expressed as fold change compared to that for the control. . The baseline of SCR siRNA cells was used to calculate the fold change induction. (n=3 Mean +/- SD).

3.7 The expression of α-SMA and Fibronectin is controlled by Enzymes that Alter Methylation Status

To gain further insight into whether or not lysine methylation is broadly important in the expression of some of the keys markers of tissue fibrosis and CKD, the protein levels of both α-SMA and fibronectin were examined by western blotting. HK C-8 cells were transfected with siRNAs directed towards numerous different methyltransferases or demethylases. Cells were then starved and treated with TGF β-1 before α-SMA and fibronectin levels were assessed by Western blotting on whole cell lysates.

Whilst there appeared to be some minor differences in GAPDH levels, suggesting that some siRNAs either control the expression of GAPDH or result in reduced cell viability, reflected in protein loading, this initial experiment does show that cellular levels of α-SMA and fibronectin proteins changed in response to enzymes controlling methylation. Whilst some enzymes appeared to promote α-SMA / fibronectin expression, others did not. This suggests a degree of selectivity over the control of α-SMA / fibronectin expression by the different methyltransferase and demethylase enzymes (Figure 3.7).
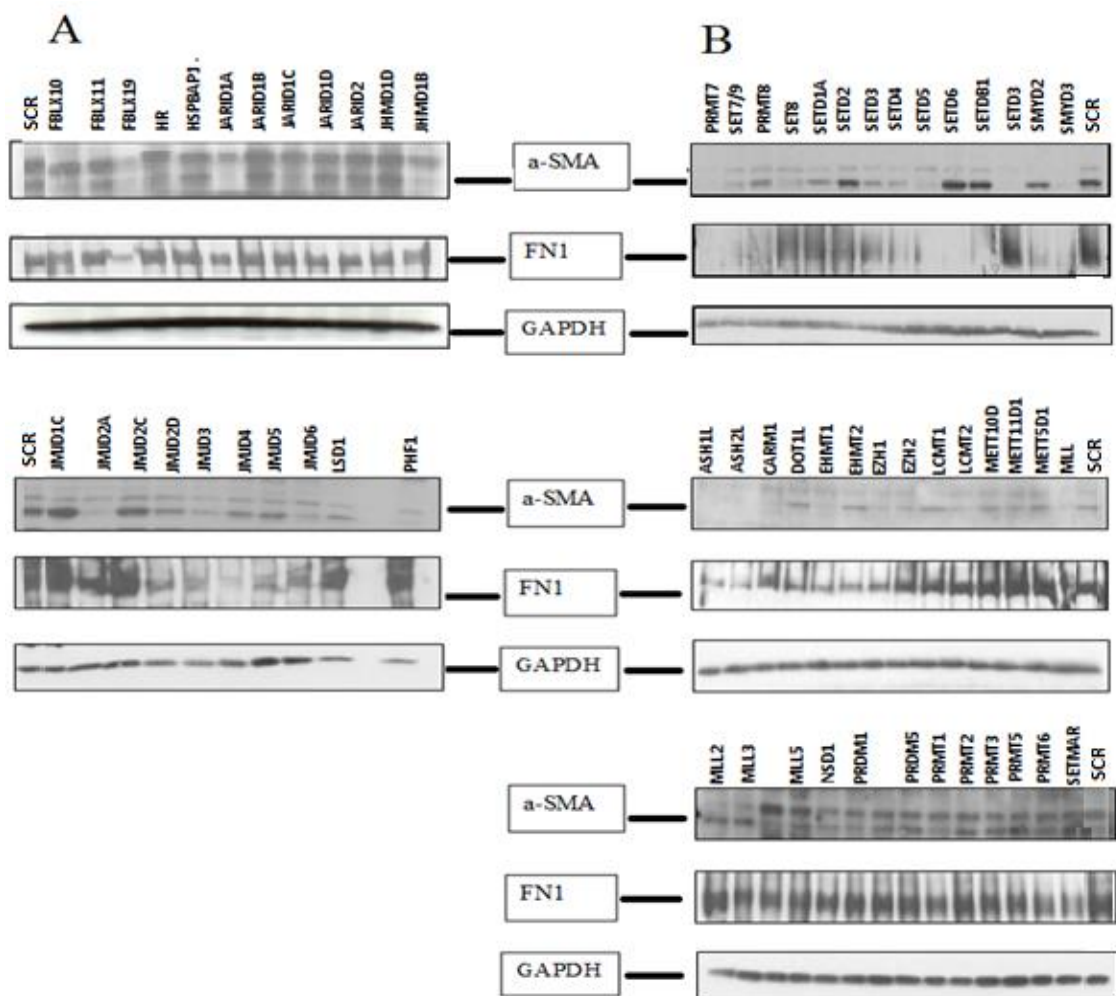
Figure 3.5. Methylation status controls the expression of α-SMA/fibronectin. Panel *A and B*. HK C-8 cells were transfected with 25nM siRNAs directed towards different demethylases/methyltransferases encoded by the human genome. Cells were starved for 24 hrs and then treated with 2ng /ml TGF β-1. Cells were lysed in RIPA buffer. Proteins then resolved on SDS PAGE were subjected to Western blotting. a-SMA was detected using mouse monoclonal anti a-SMA Fibronectin was detected using a rabbit polyclonal anti-fibronectin antibody. Blots were reprobed with GAPDH to verify equal loading of total protein in each lane.

3.8 Discussion

Data from the SMAD3 -/- mice demonstrate that loss of SMAD3 prevents fibrosis within the kidney and other organs. However, no therapeutic inhibitor of SMAD3 is currently available (Inazaki, Kanamaru et al. 2004). For this reason, a more thorough understanding of the pathophysiology of TGF β-1 – SMAD3 signalling in fibrosis is needed so that therapeutic targets might be identified. Inhibition of SMAD3 by overexpression of the inhibitory SMAD7 protein or by treatment with the small molecule, halofuginone, significantly decreases disease severity in animal models of kidney, lung, liver and radiation-induced fibrosis (Flanders 2004). Therefore small molecule inhibitors of SMAD3 might have clinical benefit in the treatment of pathological fibrotic diseases (Flanders 2004). Moreover, disruption of the TGFβ/SMAD signalling cascade can diminish renal fibrosis in animal models (Pines 2008), confirming the TGF β-1 pathway as a *bona fide* target in renal fibrosis, the histological equivalent to chronic kidney disease. Oxymatrine (OMT), the natural component extracted from the roots of traditional Chinese herb named Radix Sophorae flavescentis (Kushen), has been reported to exert prominent protective effects on hepatic (Zhang, Wu et al. 2014) and myocardial (Shen, Yang et al. 2011) fibrosis, as well as an increasing protective effect against kidney injury (Jiang, Liu et al. 2015). Recently, *in vivo* studies have shown that OMT blocks the activation of myofibroblasts by inhibiting the TGFβ/SMAD3-signaling pathway. This indicate that OMT-attenuates renal fibrosis and inflammation (Wang, Shi et al. 2016). In addition, Elsholtzia ciliata ethanol extract (ECE), an annual herb, has been shown to prevent the development and progression of renal interstitial fibrosis in UUO models. This may be mediated by blocking the activation of TGFβ and inflammatory cytokines resulting in a subsequent degradation of the ECM accumulation pathway (Kim, Kim et al. 2016). Yet, no SMAD3-inhibiting compounds have been developed to the point of clinical testing. Identification of factors involved in the

regulation of TGFβ/SMAD3 signalling could, therefore, provide a novel therapeutic target to ultimately abrogate TGFβ signalling in fibrosis.

Based on the results obtained from luciferase assays, SMAD3 knockdown produced a dramatic reduction in luciferase activity, confirming a dependency of reporter gene activity upon SMAD3, and showing that the *CAGA*-luciferase reporter gene assay system is a valid methodology to study the TGF β -1 / SMAD3 signalling axis *in vitro*. This system can therefore be used to screen for novel factors regulating TGF β-1 – SMAD3 signalling.

Post transitional modifications play a major role in essentially all cellular processes such as cellular differentiation (Grotenbreg and Ploegh 2007), signalling, the cell division cycle (Morrison, Kinoshita et al. 2002), protein degradation (Geiss-Friedlander and Melchior 2007), protein-protein interactions and regulation of gene expression. PTMs can occur at a protein C or N termini or on the amino acid side chains, whereby the chemical repertoire of the 20 standard amino acids can be extended by the addition of functional groups such as phosphate, acetate, amide groups, or methyl groups (Pratt, Parker et al. 2006). Protein methylation is a common PTM and can occur on either lysine or arginine residues within both nuclear and cytoplasmic proteins (Nesterchuk, Sergiev et al. 2011). However, to date no published data support a role for methylation / demethylation in TGF β-1 or SMAD3 signalling. To determine if methyltransferases/demethylases can broadly regulate TGF β-1 / SMAD3 signalling, siRNA screening was performed using luciferase reporter gene assays and Western blotting. A similar methodology was previously published in order to identify other SMAD3-regulating genes (Mauviel 2009). Importantly, the data demonstrate for the first time that methylation might be important in the regulation of SMAD3 transcriptional activity; indeed the introduction of siRNAs targeting demethylases lead to both increases and a decreases in SMAD3 transcriptional activity. Whilst the relative silencing efficacy of the siRNAs was not assessed, the broad range of reporter gene activity including both positive and negative changes suggests

a complex role for methylation in SMAD3 transcriptional activity that should be explored further in the context of chronic kidney disease and other fibrotic disorders.

In a second analysis, the introduction of siRNAs targeting both methyltransferases and demethylases demonstrated for the first time changes in α-SMA and fibronectin expression at the protein level. It is currently unclear whether these changes arise from underlying alterations in gene expression or whether methylation might have a secondary role in the regulation of protein turnover and stability in the case of α-SMA and fibronectin expression. Moreover, the different outcome resulted from the siRNA transfection could be related to the studies that α-SMA and fibronectin expression are regulated by different signalling pathways (Jeon, Moon et al. 2006, Jia, Hu et al. 2017). It has been argued that TGF β-induced α-SMA expression is regulated by the coordinated activation of a complex system of parallel MAPK and SMAD signalling pathways in renal proximal tubular cells during epithelial-mesenchymal transdifferentiation. It is assumed that TGF β-1 regulates α-SMA expression through the SMAD family of signaling proteins, and through the p38 MAPK, where p38 might be involved in regulating mRNA stability. Another possible regulator being β-catenin, which was shown to be involved in splicing events (Sebe, Leivonen et al. 2008). Fibronectin has also been fonud to be induced by PI3K/Akt pathways assuming a mutual stimulation of fibronectin production via PI3K/Akt pathway (Carayol, Campbell et al. 2002, Beier, Holtmeier et al. 2007). The central role of both α-SMA and fibronectin in fibrosis warrant further investigation into the role of methylation in their expression.

Given the robust, statistically significant changes observed with transfection of demethylase HSPBAP-1, this gene was taken forward for further investigation.

4 STUDYING THE FUNCTIONAL INETRACTION BETWEEN SMAD3 AND THE LYSINE METHYLTRANSFERASE SET9

4.1 Introduction

With the identification of TGF-β as a key mediator in the progression to fibrosis, it is vital to study downstream events that may explain how extracellular events result in fibroblast phenotype alteration and matrix gene regulation. The active form of TGF-β binds to the type II TGF-β receptor (TGF-βRII) leading to a TGF-βRII forming a complex with TGF-βRI, both of which are transmembrane serine/threonine kinase receptors. TGF-βRI is also known as activin-like kinase 5 (ALK5), and within the heterotetrameric complex, the kinase domain of ALK5 is phosphorylated by TGF-βRII. Consequently, ALK5 phosphorylates and activates the intracellular receptor-activated (R) SMADs (SMAD2 and SMAD3). These then interact with the co-mediator, SMAD4, and the complex translocates to the nucleus and activates a series of genes involved in matrix production and cell differentiation and proliferation (Gauldie, Kolb et al. 2006).

SET9 was originally purified as a H3K4 histone methyltransferase from HeLa nuclear extracts (Nishioka, Chuikov et al. 2002). Structural and biochemical studies suggest that SET9 catalyses mono-methylation of H3K4 (Xiao, Jing et al. 2003, Zhang, Yang et al. 2003). Moreover, SET9 has been shown to methylate some nonhistone proteins such as TAF10 (Kouskouti, Scheer et al. 2004), p53 (Chuikov, Kurash et al. 2004) and ER (Subramanian, Da et al. 2008). Methylation of ER and p53 results in the stabilisation of these proteins and increase in their transcriptional activity. Methylation of TAF10 also increases the binding to RNA polymerase II. These results suggest that SET9-mediated methylation of proteins other than histones may be common and have multiple consequences (Ea and Baltimore 2009).

The similarity between the SMAD3 KKLKK motif and other SET9-interacting partners directed us towards the possibility that SET9 could be involved in TGF β-1 / SMAD3 signalling pathway. In our lab, an investigation was carried out by Dr. Ian Logan to study the putative interaction between endogenous SET9 and SMAD3 in HK C-8 kidney cells. An interaction between the two proteins, in the presence of TGF β-1, was revealed by co-immunoprecipitation (Figure 4.1A, unpublished data). This experiment was confirmed by FLAG immunoprecipitation of an ectopically expressed FLAG-tagged SET9 (Figure 4.1B), and by performing the reciprocal experiment using an SMAD3 antibody to immunoprecipitate, under stringent high-salt conditions, the SMAD3-SET9 complex (Figure 4.1C). Moreover, GAPDH protein was not precipitated, representing a selective interaction between SMAD3 and SET9 rather than promiscuous binding of SMAD3. Thus, the enrichment and the increase of SMAD3:SET9 interaction in cells that were observed with the treatment of TGF β-1 supports the hypothesis that SET9 may be involved in TGF β-1 signalling. However, the relevance of HK C-8 cells as a model of kidney scarring or fibrosis is questionable and therefore the role of SET9 in this setting was studied here. A cell line derived from human renal mesangial cells isolated from the glomerulus of normal human kidneys (THMCs) was used (gift from Jane Norman, London) (Wilson and Stewart 2005). Mesangial cells reside around blood vessels in the glomerulus and their role is to strucutrally support the capillary tuft and to aid in controlling glomerular filtration. They have characteristics of a modified smooth muscle cell, but are also capable of a variety of other functions including synthesis of prostaglandins (PGs) and mediators of inflammation; production and breakdown of basement membrane and other biomatrix material; synthesis of cytokines; and uptake of macromolecules, including immune complexes (Schlondorff 1987). Additionally, mesangial cell proliferation and matrix overproduction are the predominant pathological features of glomerular sclerosis, a key

histological feature present in numerous kidney diseases that result in end stage renal failure, for example diabetic nephropathy. Therefore, the specfic objectives of this chapter were:

1- To characterise another cell model,THMCs, in which to study renal scaring.

2- To understand what effect SET9 depletion/overexpression has upon SMAD3 activity.

3- To examine the effect of depletion of SMAD3/SET9 on the expression of TGF β-1–induced ECM-associated proteins in THMCs.

4- To determine whether the methyltransferase activity of SET9 is required for the nuclear import of SMAD3

5- To examine the effect of SET9 inhibition in wound healing assays in THMC cells.

6- To assess the effect of SET9 inhibition on cell proliferation.
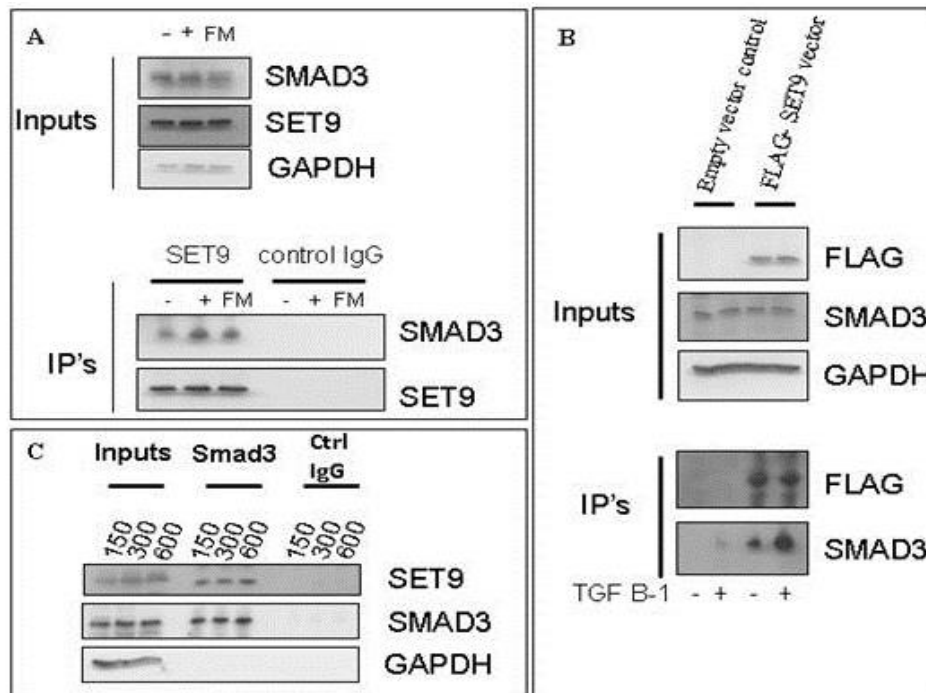
Figure 4.1. A novel interaction between SET9 methyltransferase and SMAD3. *A* HK C-8 cells were starved in serum-free medium then treated with 2ng/ml TGF β-1, where indicated, vehicle control, or full medium for 24 hr. Cells were lysed in RIPA buffer, centrifuged to remove debris then immunoprecipitated with protein G dynabeads cross-linked to antibodies to SET9 or rabbit immunoglobulin G (IgG) control. Proteins then resolved on SDS PAGE were subject to western blotting with antibodies as indicated. *B*, HK C-8 cells were transfected with human FLAG-SET9 or empty vector control over 12 hours. Cells were then starved in serum-free medium for 24hr before treatment with either 2ng/ml TGF β-1 or vehicle control. Lysates were prepared as in *A* then subject to immunoprecipitation with FLAG M2 antibody cross-linked to protein G dynabeads. Proteins were resolved and subject to western blotting as shown. *C* Cells treated with TGF β-1 were lysed in RIPA buffer then sodium chloride levels adjusted to 150 mM, 300mM or 600mM as shown. Lysates were subject to immunoprecipitation with SMAD3 antibody or control immunoglobulin then material subject to western blotting as indicated. Pilot data performed by Dr. Ian Logan.

4.2 SET9 Overexpression Enhances SMAD3 Transcriptional Activity

The reporter gene assay described in chapter 3 was used to determine whether SET9 influences SMAD3 transcriptional activity, in the presence or absence of TGF β-1. Upon transfection of HK C-8 cells with the *CAGA*-luciferase reporter, luciferase expression was enhanced approximately 8-fold in response to exogenous TGF β-1. Notably, co-transfection of wild-type SET9 produced a significant dose-dependent increase in luciferase expression that reached 12 fold induction. There was only minimal enhancement by SET9 in the absence of exogenous

TGF β-1 that could be because of the effect of the endogenous TGF β-1 (Figure 2.4). These data suggest for the first time that SET9 acts as a co-activator for SMAD3 and the first methyltransferase enzyme described as being involved in TGF β-1 / SMAD3 signalling.

Interestingly, co-transfection of a SET9 variant that lacks methyltransferase activity did not produce an enhancement in luciferase activity. In fact, this SET9 mutant seemed to act in a dominant negative fashion, and led to a reduction in luciferase activity to below baseline values of cells transfected with the control empty vector and treated with TGF β-1 (Figure 4.2). Although the expression of wild-type and mutant forms of SET9 was not assessed, this result suggests that SET9, but not the methylase-inactive mutant SET9, facilitates SMAD3 function, possibly due to methylation altering allosteric interactions within the SMAD3. Inter-domain communication between the N- and C-termini of SMAD3 is vital for enabling its full transcriptional activity, and transfection with the mutantSET9 might interfere with this (Figure 4.2).
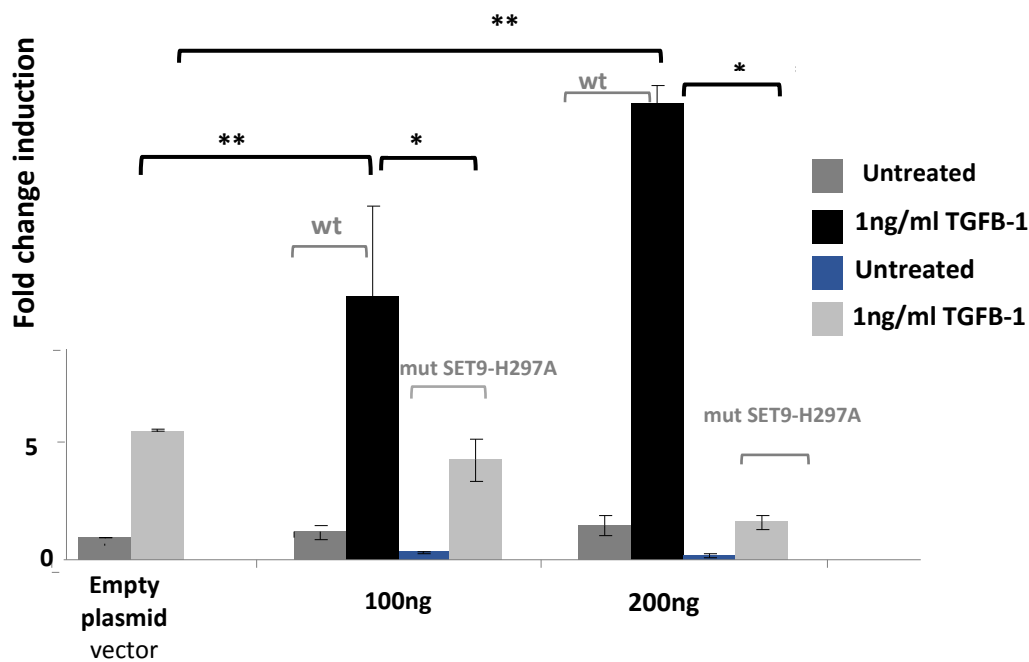
Figure 4.2. SET9 overexpression increases luciferase expression in response to TGF β-1. HK C-8 cells were transfected with wild type (wt) SET9 or mutant (mut) SET9 for 24hrs. Cells were then transfected with *CAGA*-luciferase, then starved with serum free medium for 2hrs and treated without or with TGF β-1. Cell lysates were collected to measure the luciferase activity. Luciferase values were corrected for protein and the baseline of cells transfected with empty plasmid and untreated with TGF β-1 to calculate the fold change induction. (n=3 Mean +/- SD). **P<0.001, *P<0.05.

4.3 Silencing SET9 Disrupts SMAD3 Transcriptional Activity

Having demonstrated that SET9 can act as a SMAD3 co-activator the effect of siRNA-mediated SET9 knockdown on SMAD3 signalling was examined. The reporter gene assay system was again employed as in section 4.2, except that SET9 was depleted from cells using a specific siRNA, prior to sequential transfection of the *CAGA*-luciferase reporter gene. As before, addition of TGF β-1 lead to an induction in luciferase activity from the *CAGA*-luciferase reporter gene (Figure 4.3).

Interestingly, transfection of the SET9 siRNA lead to a significant reduction in luciferase activity, even in the presence of exogenous TGF β-1, compared to a non-silencing control siRNA (Figure 4.3). The degree of reduction in luciferase activity upon SET9 silencing was similar to that observed upon SMAD3 silencing, although activity of luciferase was not significantly reduced as SMAD3 was not sufficiently supressed to affect the assay (Figure 4.3). In addition, although there was a numeric difference upon transfection with SMAD3 siRNA and in response to 5ng/ml TGF β-1, this change did not achieve statistical significance, in part explained by the large standard deviations. These are the first data describing an important role for a methyltransferase in TGF β-1 – SMAD3 signalling.
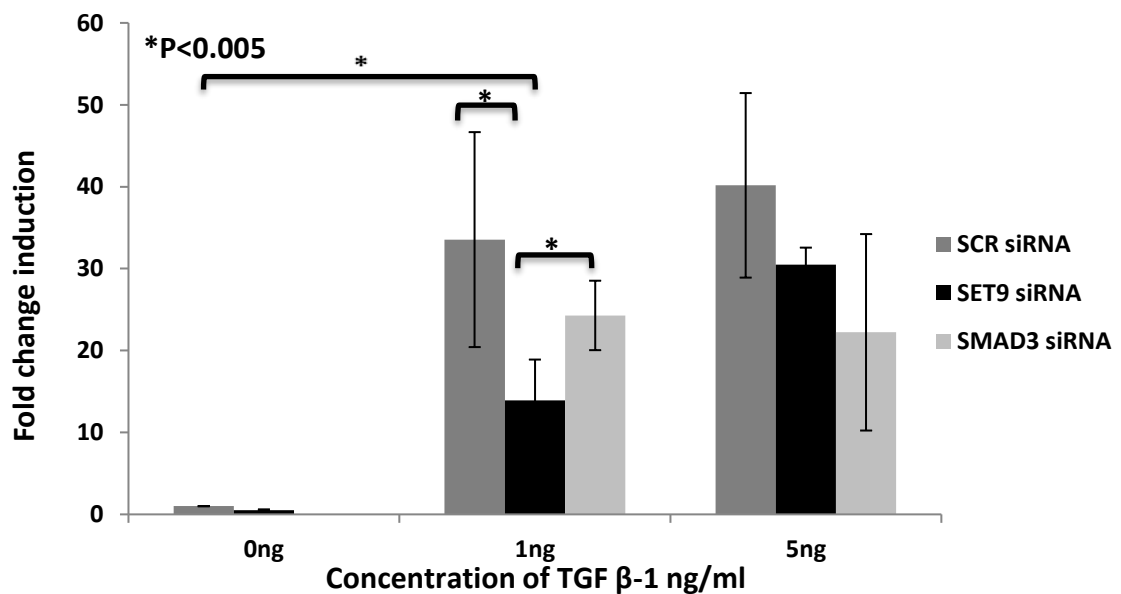
Figure 4.3. SET9 regulates the activity of SMAD3 in response to TGF β-1. HK C-8 cells were transfected with 50nM of, SCR, SET9 or SMAD3siRNA for 24hrs. Next, cells were transfected with the appropriate luciferase reporter plasmid (200ng of *CAGA* luciferase), treated without or with 1ng/ml or 5ng/ml of TGF β-1 for 24hr, and then lysates subjected to Luciferase assy. The baseline of cells transfected with SCR siRNA and untreated with TGF β-1 was used to calculate the fold change induction. (n=3 Mean +/- SD).

4.4. The Expression of Pro-fibrotic Proteins in Response to TGF β-1 in Human Mesangial Cells

 Initially, to characterise THMCs as being suitable to study renal scarring immunofluorescence was performed for the pro-fibrotic markers collagen I (COLI), collagen III (COLIII), fibronectin (FN1), PAI-1 and α-SMA. Cells were seeded in 6 well plates on glass coverslips and treated with or without TGF β-1 for 24, 48 or 72hrs prior to indirect immunofluorescence as decribed in methods section 1.15. All pro-fibrotic markers were expressed by these cells and showed an increase in expression upon treatment with 10ng/ml TGF β-1 for 72hrs, although not at 24 or 48hrs (Figure 4.4). These observations suggest that expression of the pro-fibrotic proteins are upregulated after prolonged treatment with TGF β-1 and that THMC cells can be used to study TGF β-1 induction of pro-fibrotic markers. Treatment for 72hrs with TGF β-1 was chosen as an appropriate time point in all subsequent immunofluorescence staining for profibrotic proteins. Staining was analysed by ImageJ software.

C FN-1

0ng/ml TGFB-1 — 10ng/ml TGFB-1

D PAI-1

0ng/ml TGFB-1 — 10ng/ml TGFB-1
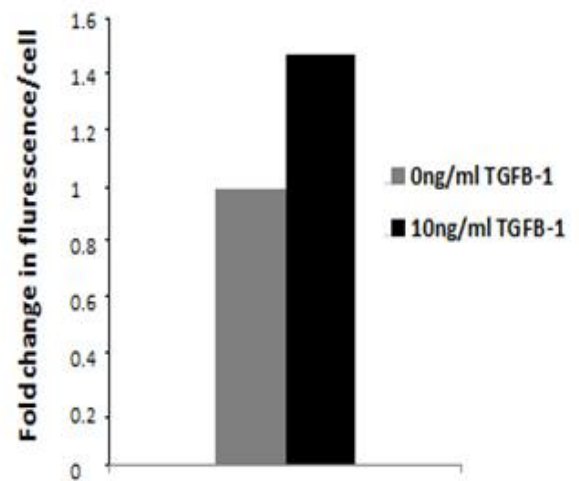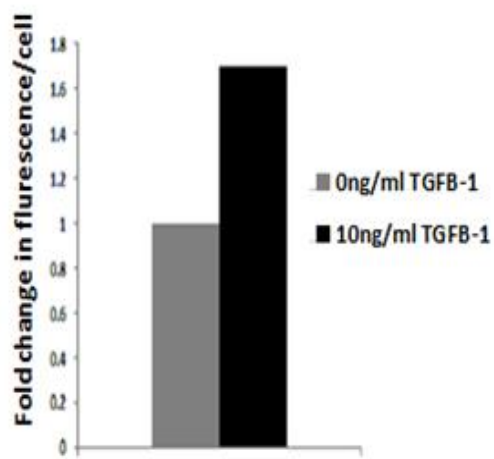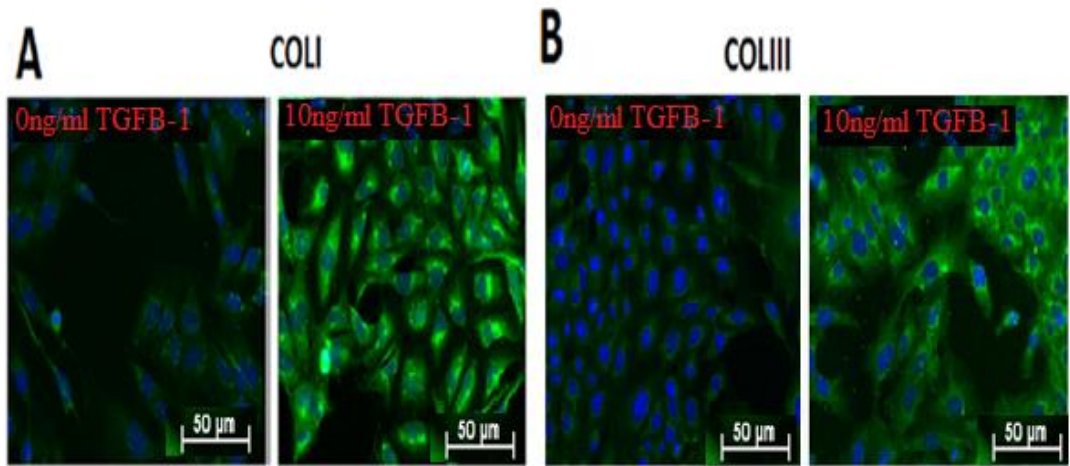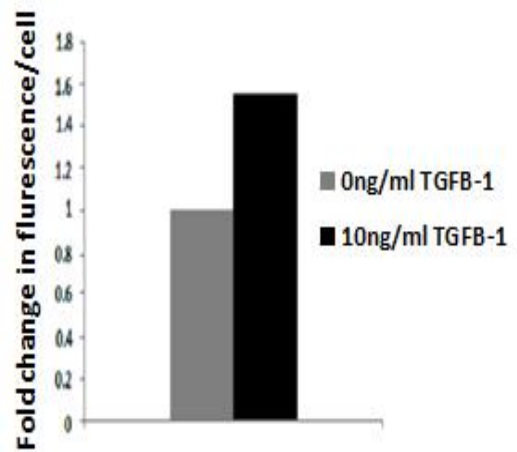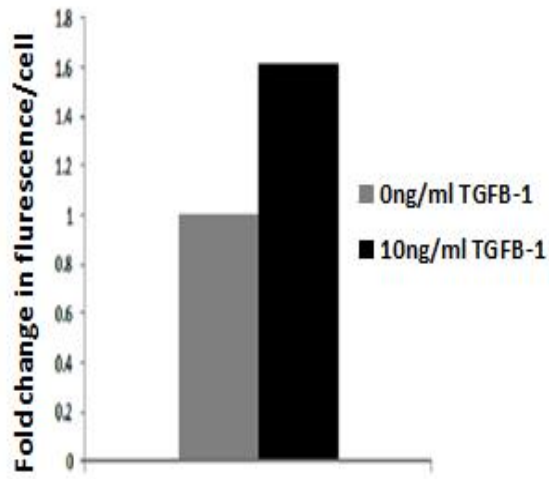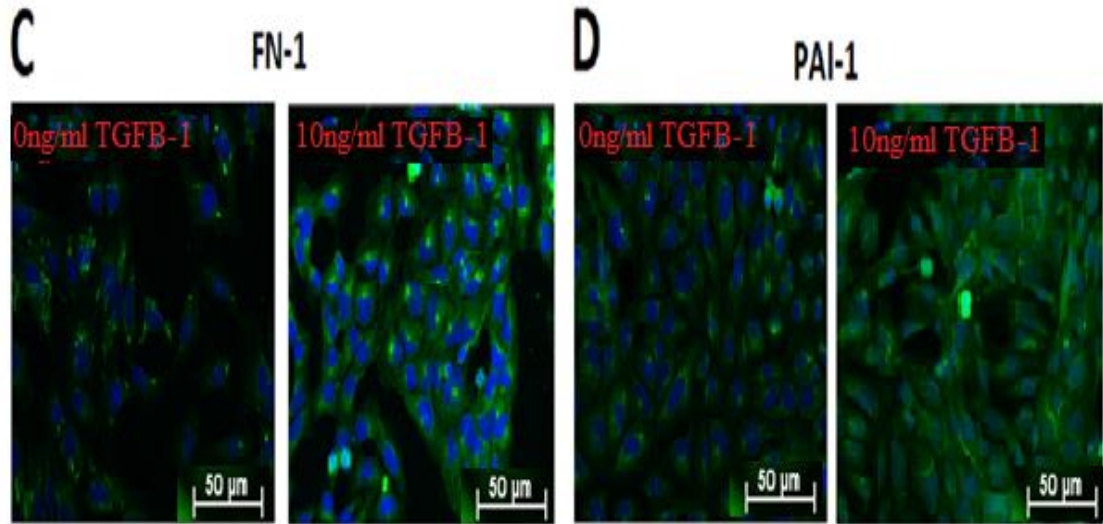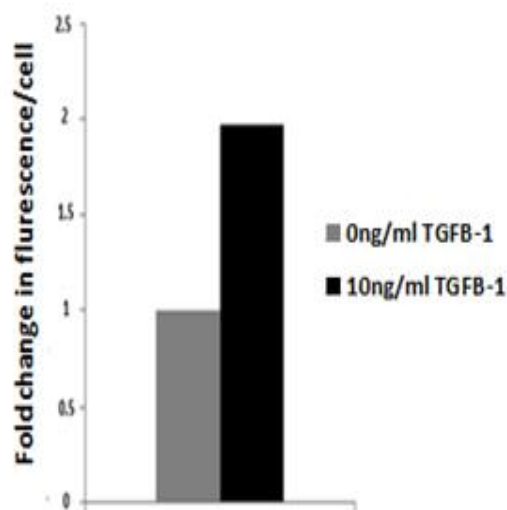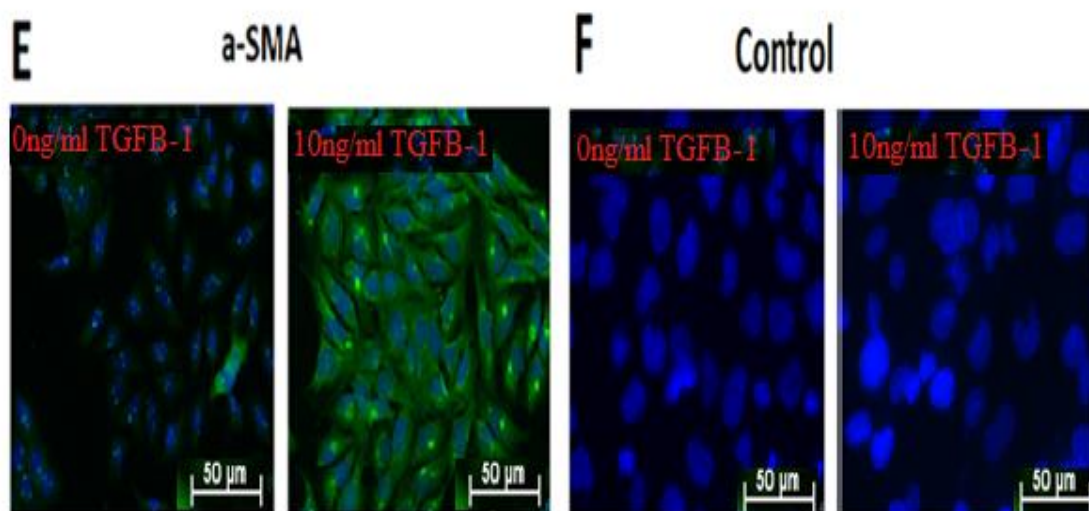
Figure 4.4. Expression of pro-fibrotic markers in THMC cell lines. *A,B,C,D,E*, F, THMCs were seeded then treated with or without TGF β-1 for 72hrs and stained for expression of the pro-fibrotic markers COLI, COLIII, FN1, PAI-1 and α-SMA or left with no primary antibody as in *F* as a control for staining. Left hand images display the expression of the pro-fibrotic markers in THMCs without TGF β-1 treatment, right images show expression of pro-fibrotic markers with TGF β-1 treatment. The images were quantitatively analysed using Image J and data expressed as fold change in fluorescence per cell. (n=1, so error bars not presented). Magnification was 40X.

4.5. SMAD3 and SET9 siRNA Treatment Results in Knockdown of Respective Proteins in Human THMCs

To determine whether the THMC cell line could be manipulated by siRNA transfection these cells were transfected with SMAD3, SET9 or SCR control siRNA for 24hrs, prior to addition of exogenous TGF β-1. Cell lysates were analysed by Western blotting which demonstrated a marked reduction in band density corresponding to the respective target gene. Gene silencing was specific, as SCR control siRNA did not affect either of the targets.
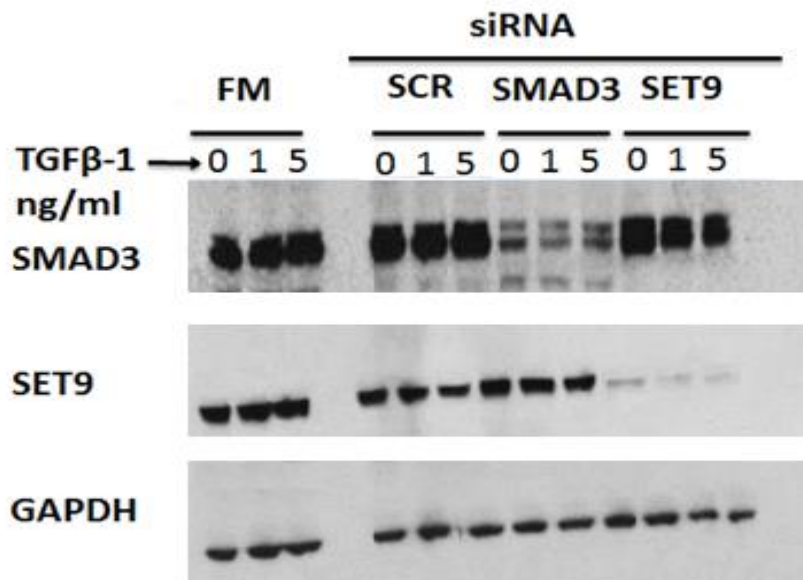
Figure 4.5. siRNA transfection efficiency. THMCs were seeded and transfected with either SCR, SMAD3 or SET9 siRNA, or in FM for 24hrs and then treated with or without TGF β-1. Cells were lysed in SDS-sample buffer, separated by SDS-PAGE, transferred to PVDF membrane, Western blotted, and probed with either anti-SMAD3 antibody or anti-SET9 antibody.GAPDH was used as a loading control.

4.6. Depletion of SMAD3/SET9 Attenuates TGF β-1–induced ECM-associated Protein Expression in THMCs

Having developed a system in which to study expression of pro-fibrotic markes, the effect of SET9 on the expression of these markers in THMCs was next examined by immunonofluorescence. THMCs were transfected with SMAD3, SET9 siRNA or control siRNA followed by staining for COLI, COLIII, FN-1, PAI-1 and α-SMA after treatment with or without TGF β-1 for 72hrs. TGF β-1–induction of COLI, COLIII (Figure 4.6A), FN-1, PAI-1 (Figure 4.6B) and α-SMA (Figure 4.6C), was attenuated by SMAD3 or SET9 siRNA compared with SCR control siRNA. This is the first evidence that a methyltransferase, SET9, can control the expression of profibrotic protein expression in response to TGF β-1. There was no significant induction of profibrotic genes in cells transfected with SET9 siRNA and not treated with TGF β-1, suggesting that SET9 may regulate the TGF-β1–SMAD3-dependent pathway.

A

COLI

COLIII

*p<0.05

**p<0.001
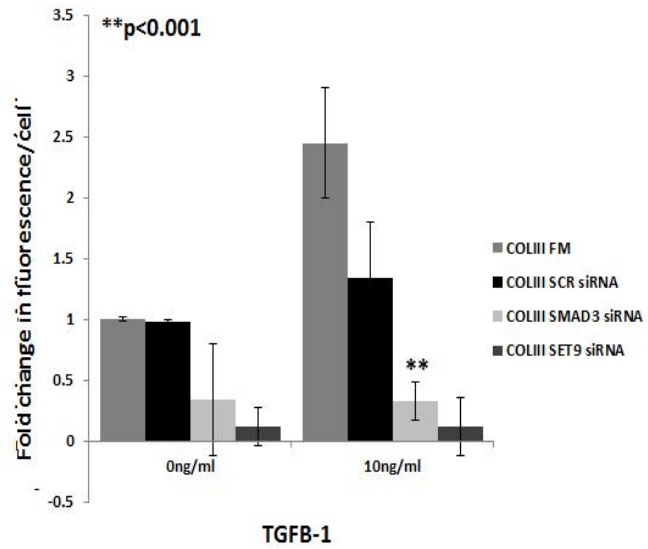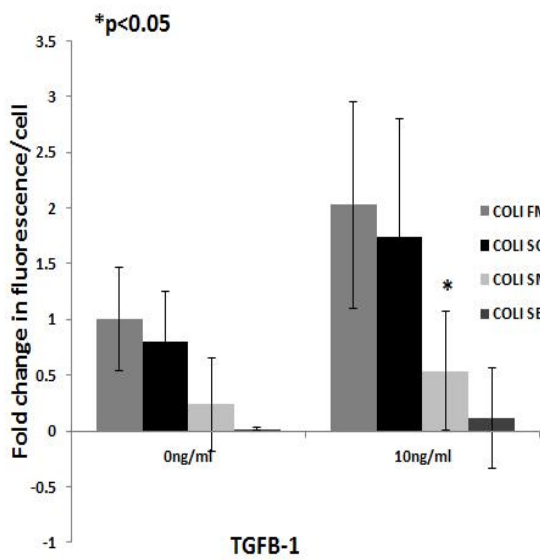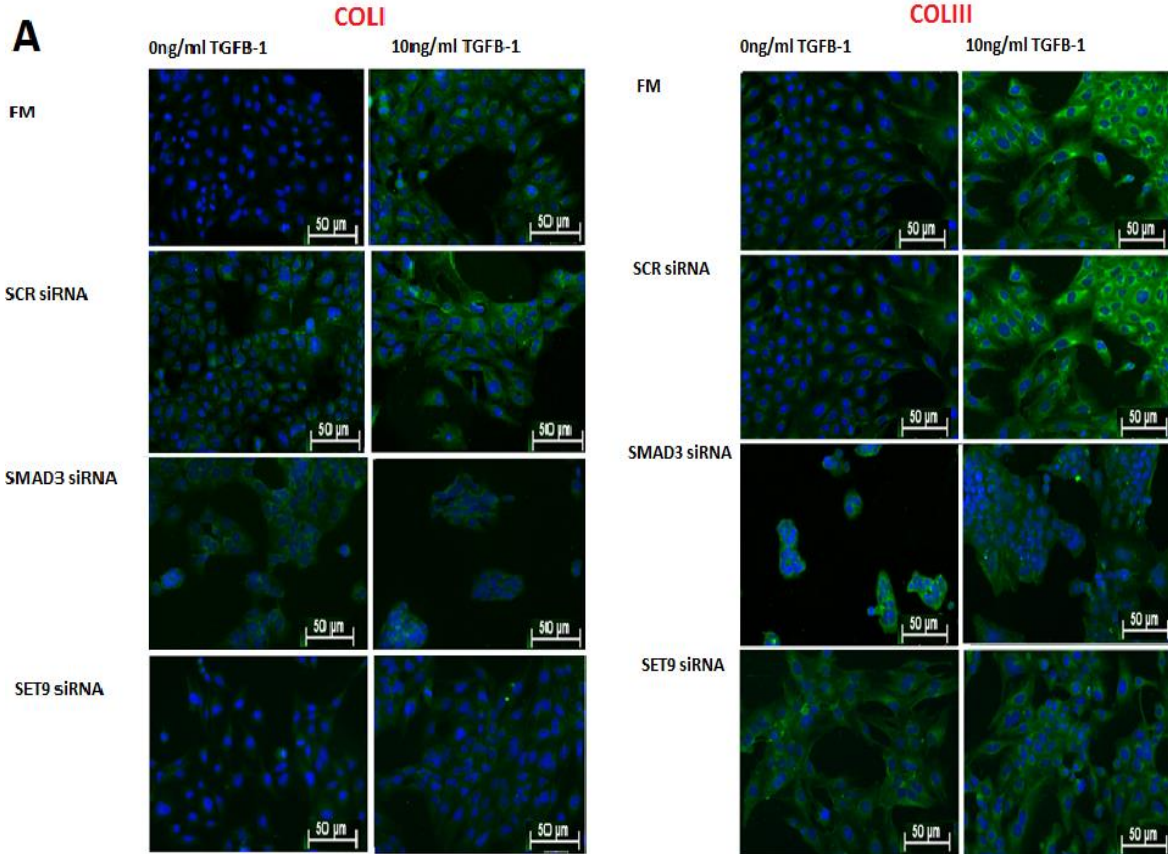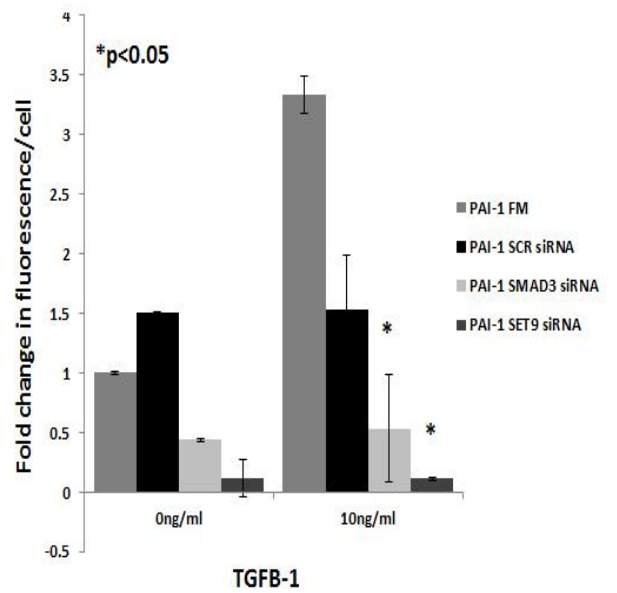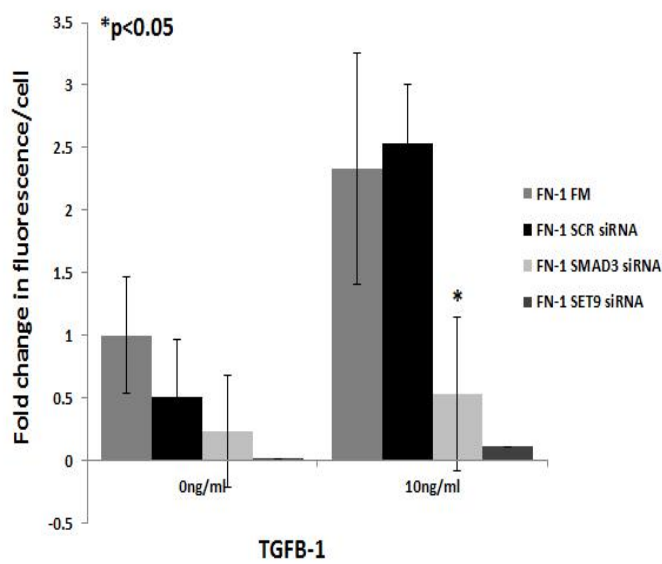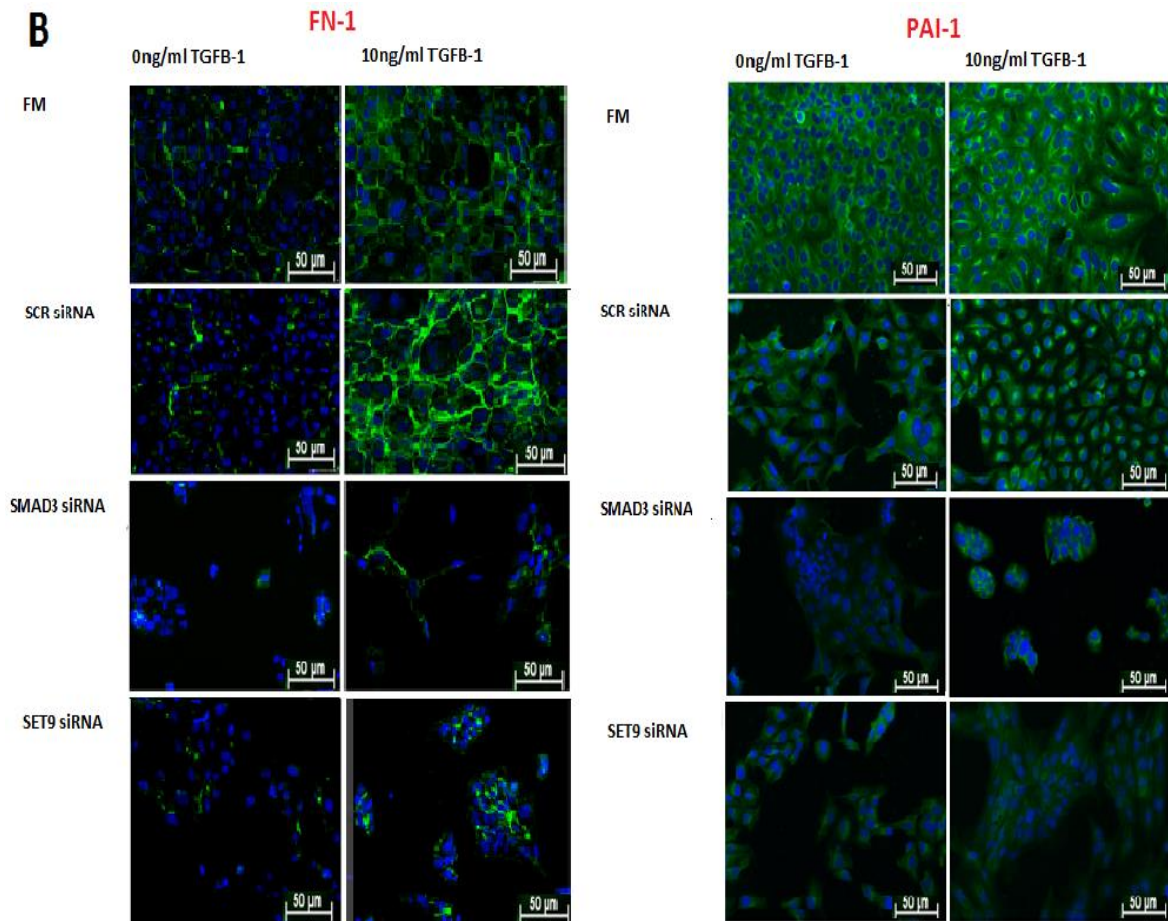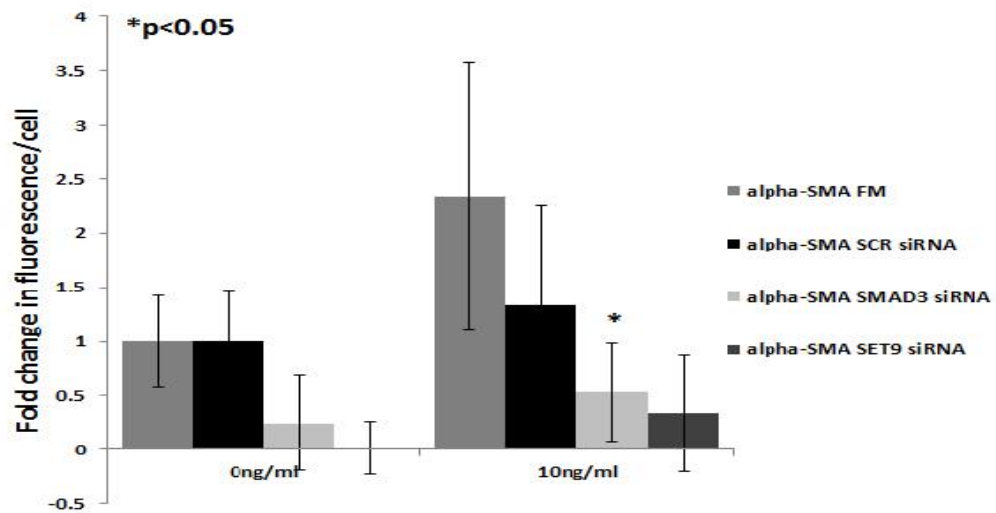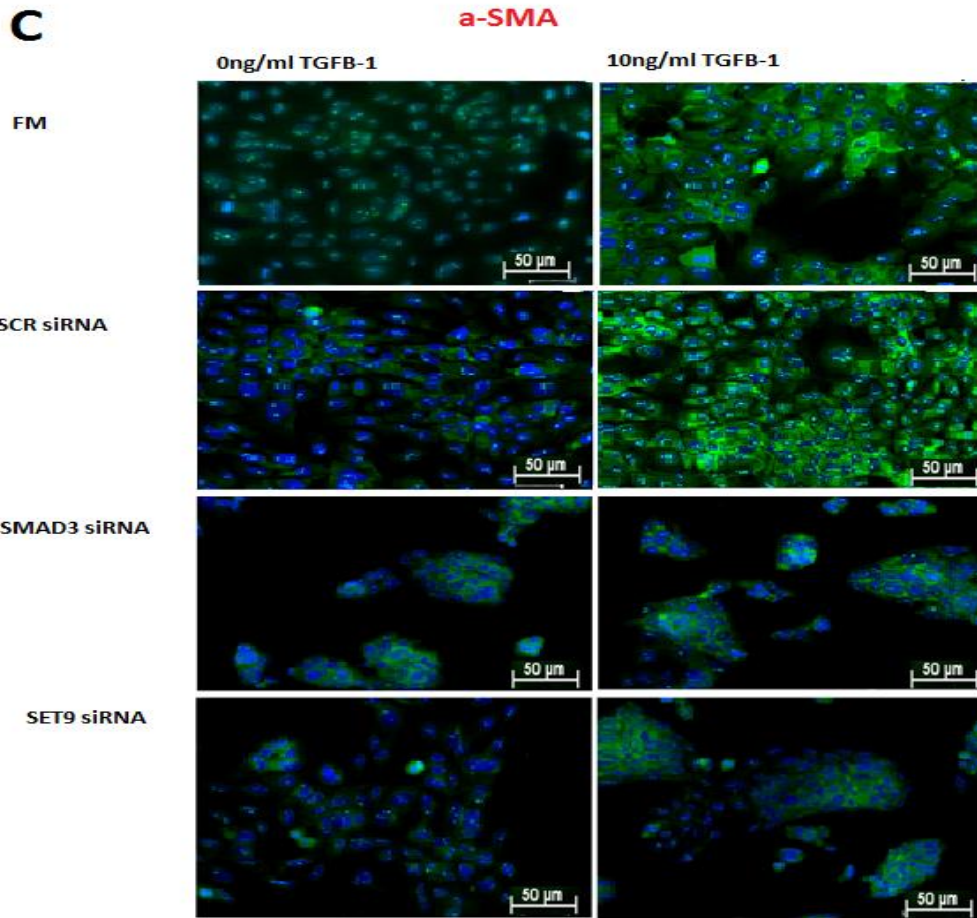
Figure 4.6. Effect of SET9 and SMAD3 siRNA on the expression of pro-fibrotic markers in THMCs. *A,B,C,* THMCs were seeded in glass cover slips, transfected with 25nM SMAD3, SET9 or SCR siRNA or left in full medium (FM). Then cells were treated with (right panels) or without (left panels) 10ng/ml TGF β-1 and stained for expression of the pro-fibrotic markers COLI (A), COLIII (A), FN1, PAI-1(B) and α-SMA(C). The images were quantitatively analysed using Image J and results presented as fold change in fluorescence per cell, combaring SMAD3 siRNA to SCR siRNA. (n=3. Mean+/-SD). Magnification was 40X.

4.7 Treatment of THMCs with a SET9 Inhibitor Significantly Reduces Expression of Pro-fibrotic Markers

Next, to further assess whether the methyltransferase activity of SET9 was responsible for the changes in fibrotic protein expression observed in THMCs, THMCs were treated with a commercially available SET9 inhibitor, R-PFI-2 as previously optimised (Barsyte-Lovejoy, Li et al. 2014). After 24hrs treatment, cells were also treated with or without TGF β-1, then stained for the pro-fibrotic proteins (Figure 4.7). Upon SET9 inhibition, expression of pro-fibrotic markers collagen I and III, fibronectin, PAI-1 and αSMA were reduced in the absence and presence of 10ng/ml TGF β-1. These results further support a key role SET9 in modulating TGF β-1 responses in THMC and suggest that the methyltransferase activity of SET9 is reposnible for these changes in fibrotic protein expression.

A

COLI

0ng/ml TGFB-1   10ng/ml TGFB-1

FM

50 µm   50 µm

SET9 Inh

50 µm   50 µm

COLIII

0ng/ml TGFB-1   10ng/ml TGFB-1

FM

50 µm   50 µm

SET9 Inh

50 µm   50 µm

B

FN-1

FM

0ng/ml TGFB-1   10ng/ml TGFB-1

SET9 Inh

PAI-1

FM

0ng/ml TGFB-1   10ng/ml TGFB-1
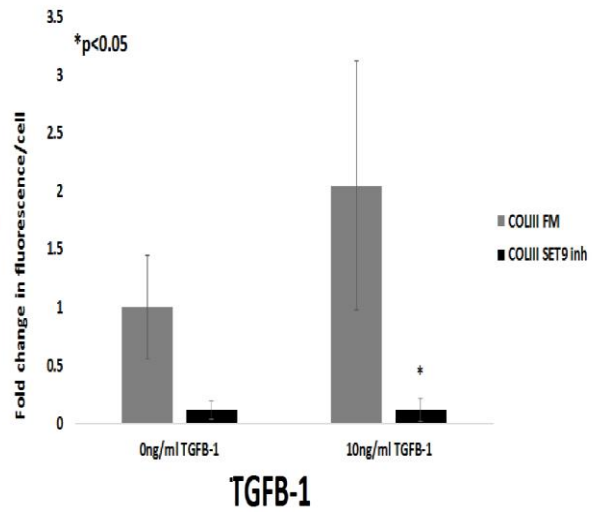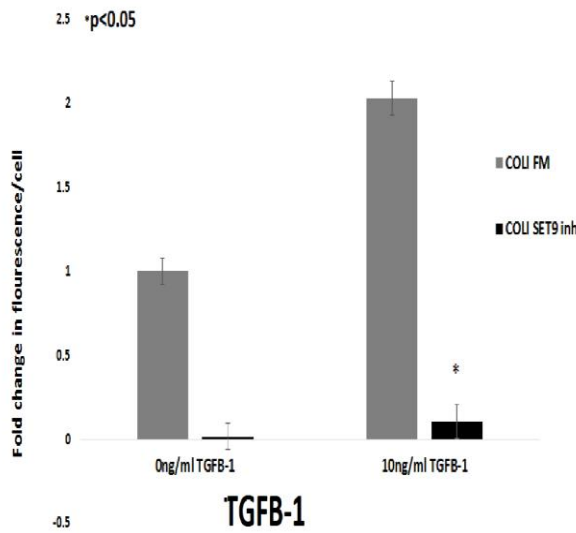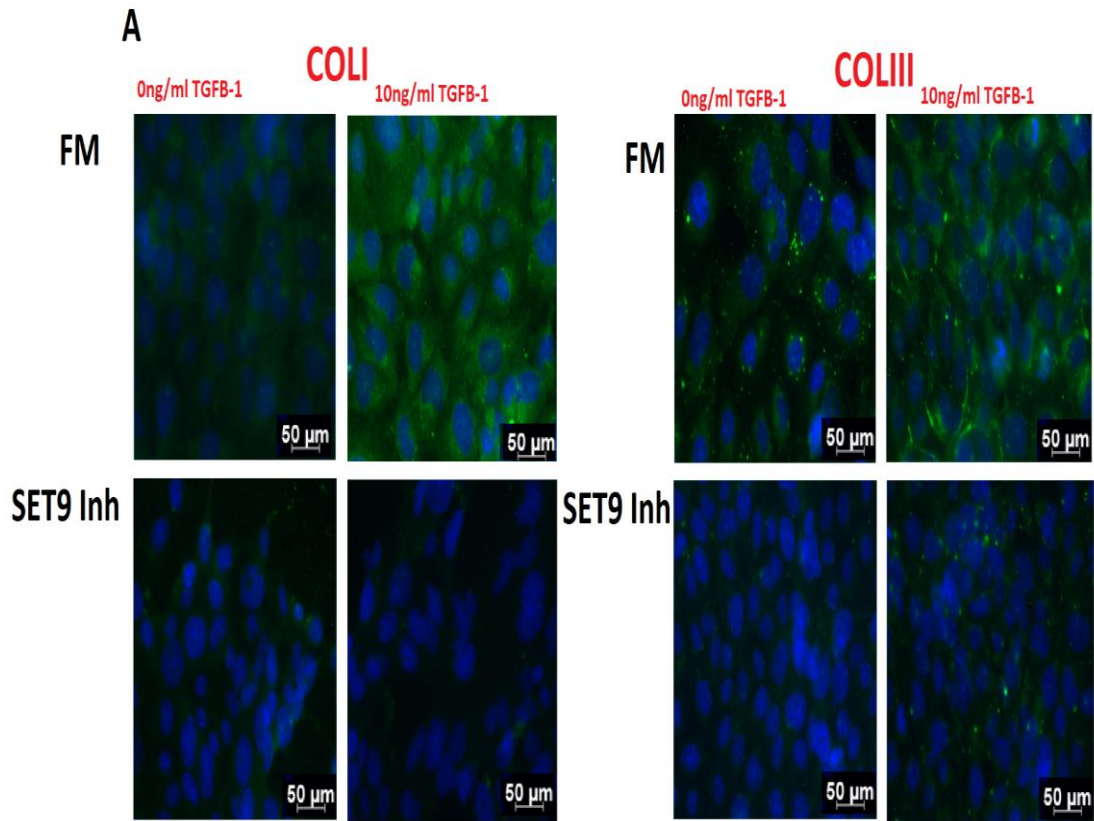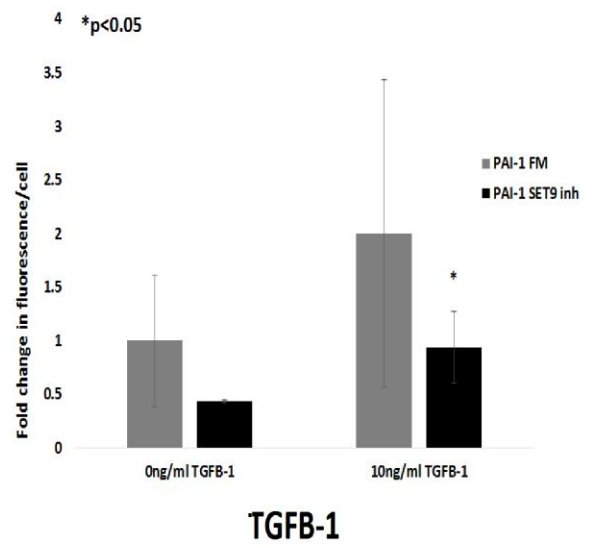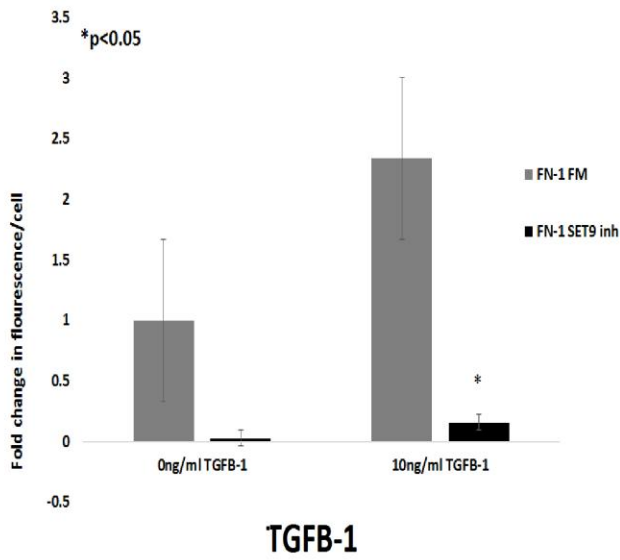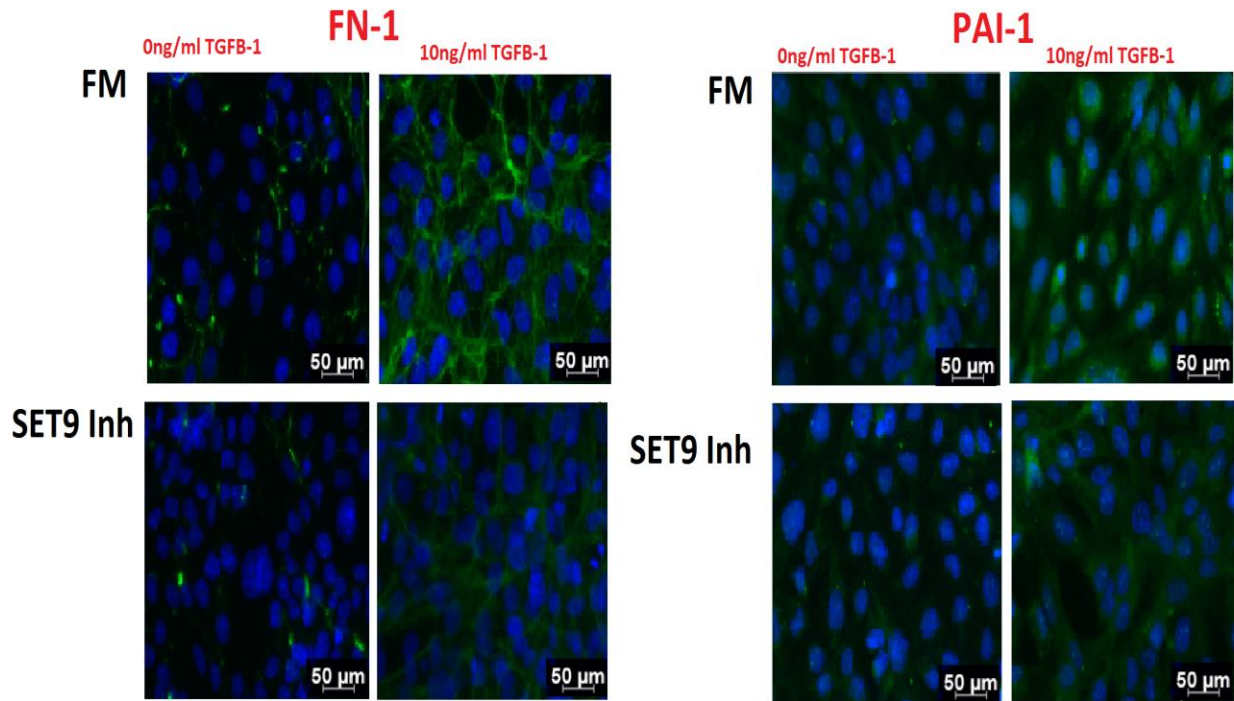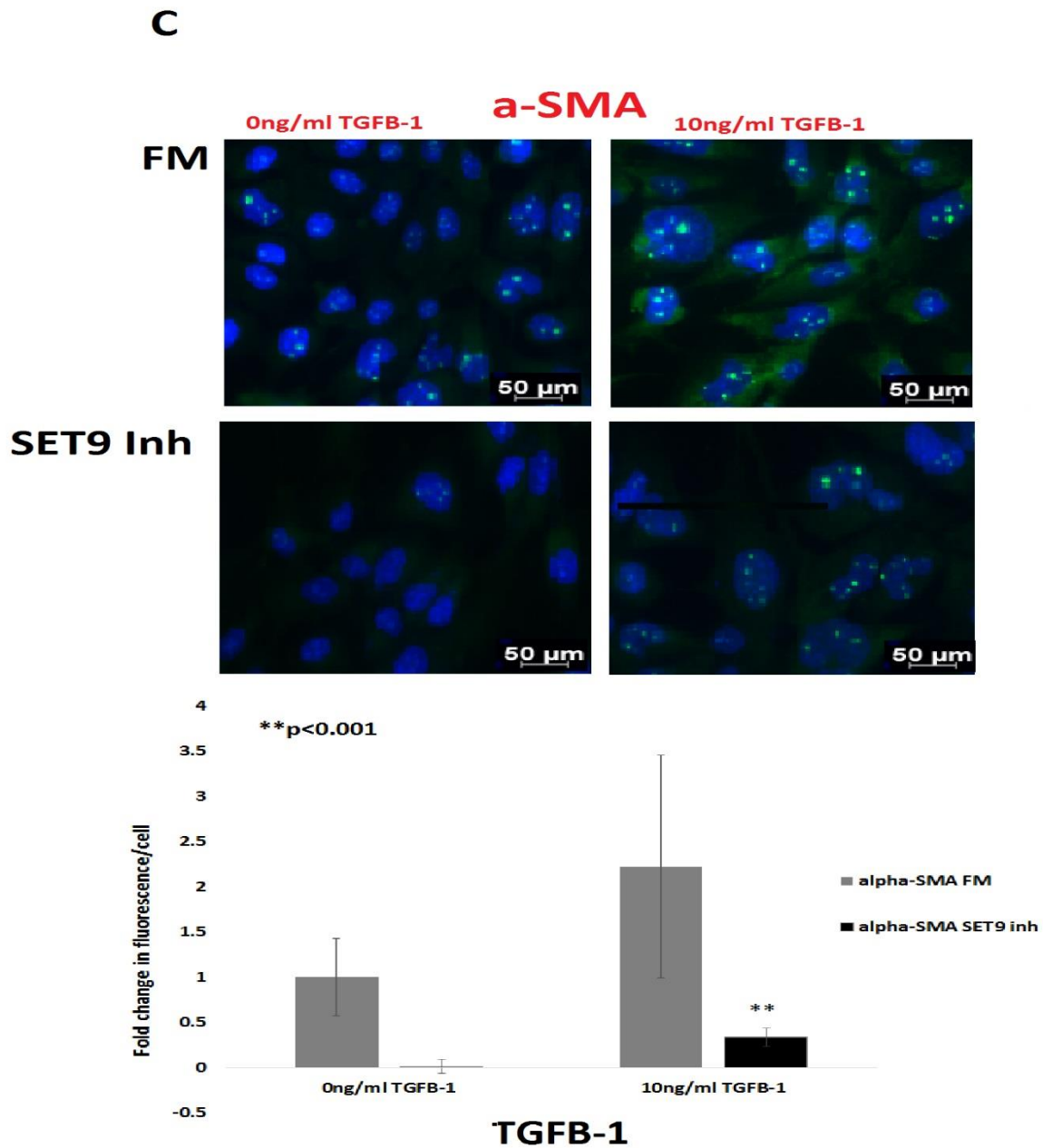
SET9 Inh

Figure 4.7. Expression of pro-fibrotic markers in THMCs upon SET9 inhibitor. *A,B,C* THMCs were seeded in glass cover slips, treated with 30uM PFI-2, SET9 inhibitor (SET9inh), or left in full medium (FM). Then cells were treated with (right panels) or without (left panels) 10ng/ml TGF β-1 and subjected to immunofluorescence for expression of the pro-fibrotic markers COLI, COLIII (A), FN1, PAI-1 (B) and α-SMA (C). The images were quantitatively analysed using Image J and are represented as bar graphs of fold change in fluorescence per cell. (n=3.Mean+/- SD). Magnification was 40X.

4.8 SET9 is Needed for Nuclear Localisation of SMAD3 in Response to TGF β-1

I next investigated the possibility that SET9 is involved in regulating SMAD3 nuclear translocation, a previously defined regulatory step in TGF β-1 signalling (Sun, Reddy et al. 2010). This was assessed by immunofluorescence to examine the localisation of SMAD3. Firstly, HK C-8 cells were transfected with SCR control siRNA, or SET9 siRNA. SMAD3 and SET9 exhibited discrete co-localisation within the nucleus, and to a lesser degree within the cytoplasm, in response to TGF β-1 (Figure 4.8A). In SET9-depleted cells SMAD3 was mostly cytoplasmic irrespective of TGF β-1 treatment (Figure 4.8B) (Logan, I. unpublished data). The single cell that does show nuclear localisation of SMAD3 was not transfected with SET9 siRNA, indicating the specificity of response. This suggests that SET9 is important in regulating SMAD3 nuclear import.
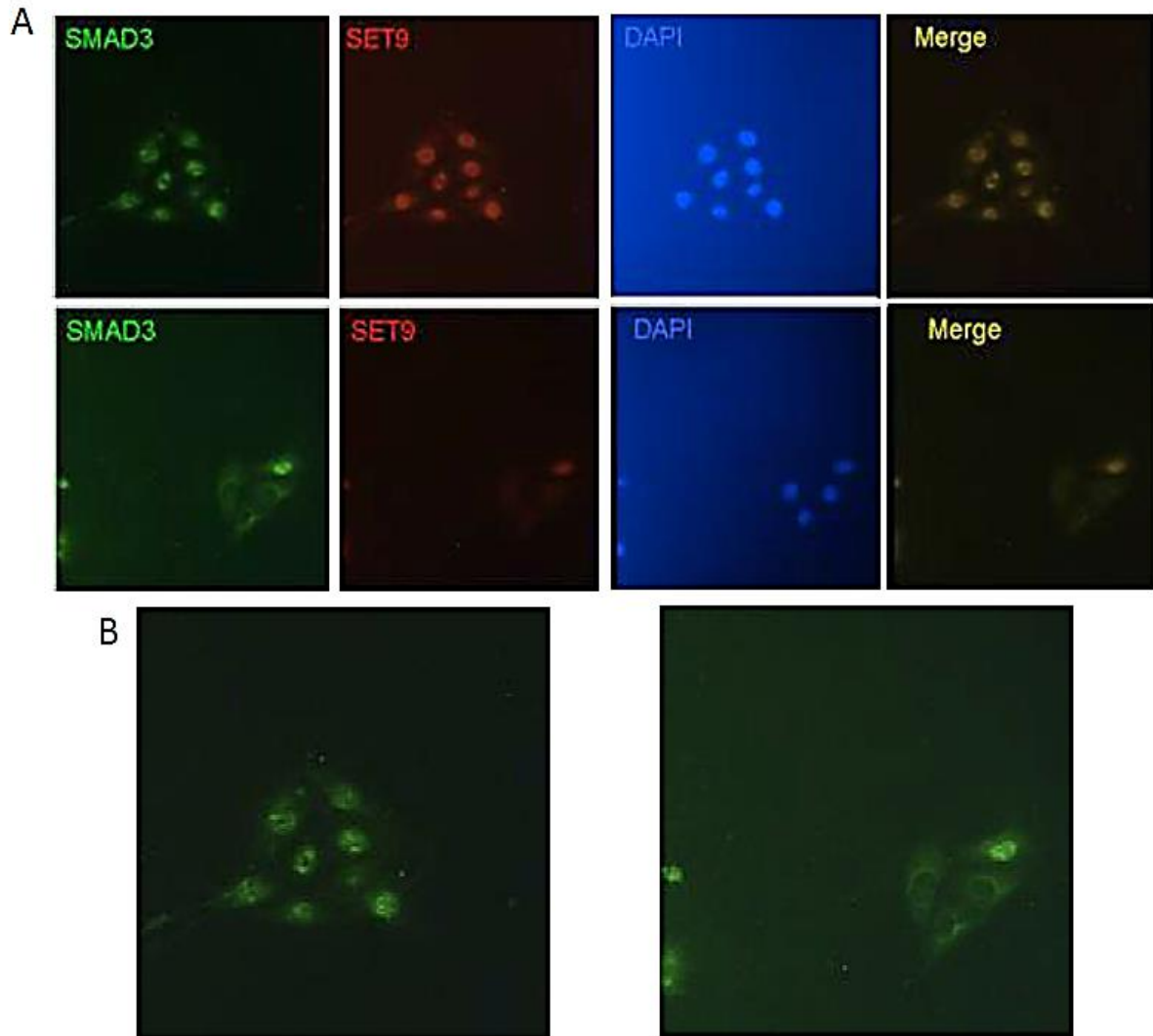
Figure 4.8. SET9 regulates SMAD3 localisation. *A*, Top row: cells transfected with non-silencing control siRNA, bottom row: cells transfected with SET9 siRNA. All cells shown were treated with TGF β-1 then subject to immunofluorescence with antibodies to endogenous SMAD3 or SET9 then stained with Alexa Fluor 488 goat anti rabbit and Alexa Fluor 594 goat anti mouse antibodies, respectively. Nuclei were counterstained with DAPI. *B*, Green image from *A* representing SMAD3 have been magnified. Note the difference in SMAD3 localisation in the un-transfected cell. Pilot data performed by Dr. Ian Logan.

4.9. Nuclear Accumulation of SMAD3 in THMCs in Response to TGF β-1

The impact of SET9 on SMAD3 nuclear import was next examined in the THMCs. Firstly, however, immunofluorescence was performed in THMCs treated with exogenous TGF β-1 to ensure that these cells respond to TGF β-1 (Figure 4.9A). Control cells demonstrated mainly cytoplasmic SMAD3 localisation. Treatment with TGF β-1 for 72hrs enhanced the SMAD3

nuclear translocation. Additionally, nuclear and cytoplasmic fractions were obtained from THMC lysates treated with or without TGF β-1 (Figure 4.9B). In cells cultured in FM, Western blotting analysis demonstrated large quantities of SMAD3 in the cytoplasm and nucleus. When treated with 10ng/ml TGF β-1, SMAD3 translocated from the cytoplasm into the nucleus. Notably, there was a considrable quantity of SMAD3 in the nucleus in cells cultured in FM. This could be as a result of the basal production of TGF β-1 on cells. These data further validate the effect of TGF β-1 on nuclear import of SMAD3 in THMCs. HDAC1 nuclear marker was used to validate nuclear extraction.
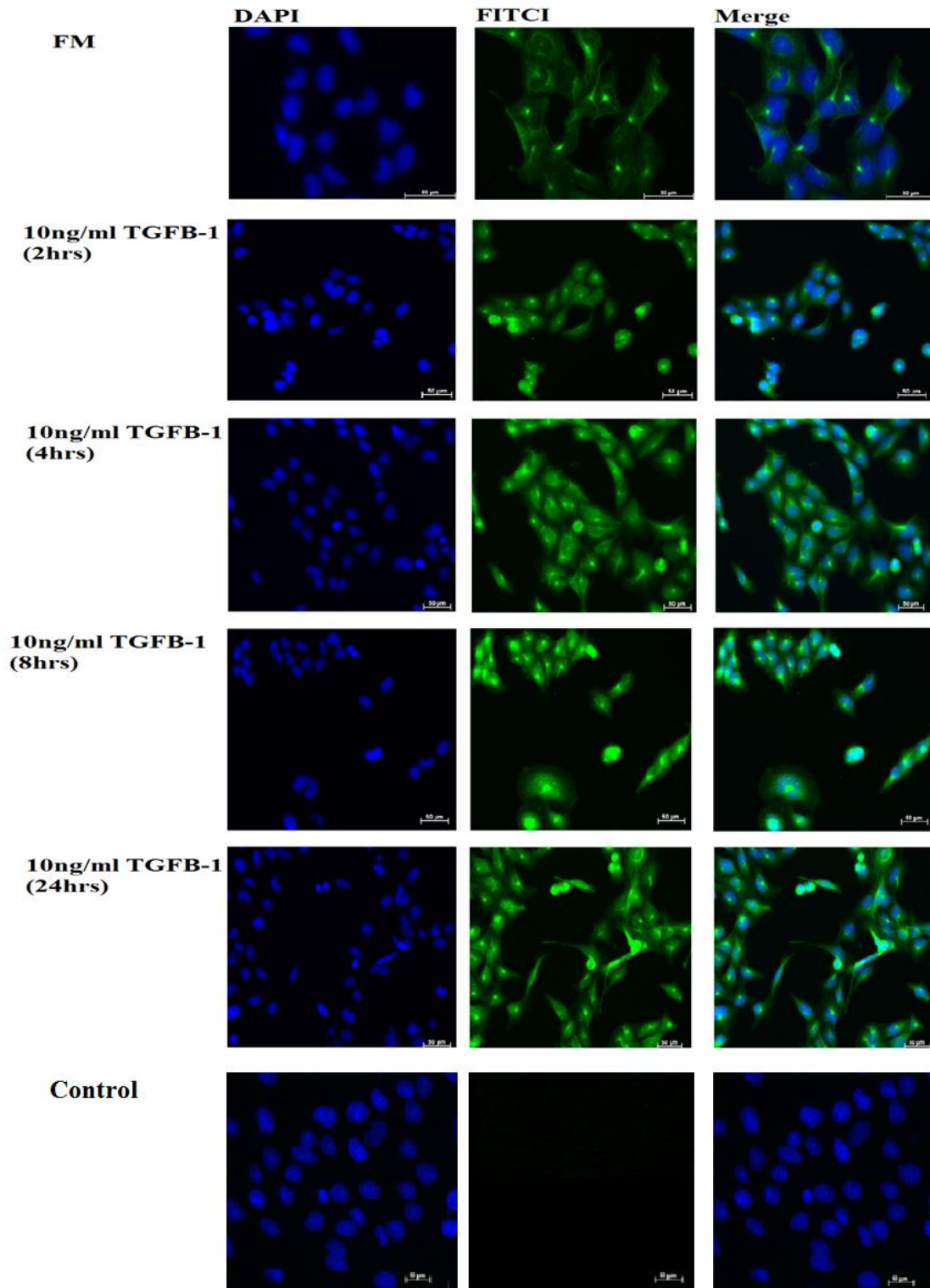
Figure 4.9A. TGF β-1 inhances SMAD3 nuclear translocation in THMCs. THMCs were seeded into glass coverslips and treated with or without TGF β-1. Cells were washed in PBS, fixed in ice cold methanol and blocked in 5% goat serum. Cells were then subjected to IF with anti-SMAD3 after incubation with TGF β-1 for the time periods shown in the figure. Cells were visualised by confocal microscopy. Magnification was 40X.
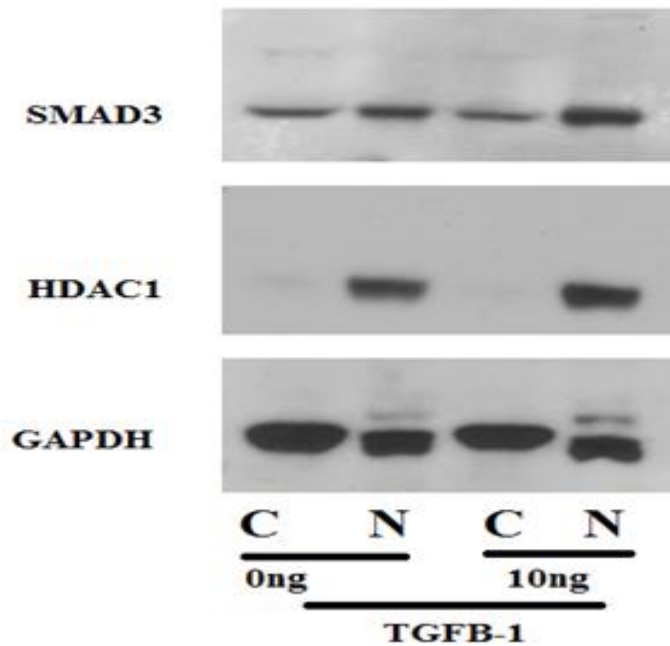
Figure 4.9B. TGF β-1 enhances SMAD3 nuclear accomulation in THMCs. THMCs were seeded, treated with or with out TGF β-1 for 24hrs. Nuclear and cytoplasmic fractions were obtained by centrifugation using a hypotonic buffer solution. Fractions were subjected to WB analysis with SMAD3, HDAC1 and GAPDH antibodies.

4.10. The Impact of SET9 on SMAD3 Nuclear Import

Having demonstrated that SET9 is important in regulating SMAD3 nuclear import in HK C-8 (Section 4.8, Ian Logan, unpublished data), it was imporant to verify this data in THMCs. Therefore, SET9 was firstly examined in this setting by immunofluorescence comparing cells transfected with SCR siRNA or SET9 siRNA. Cells were stained for SMAD3 (Figure 4.10A). This experiment was performed several times, however, the result was repeatedly the same. There was no clear evidence of SMAD3 nuclear translocation in response to TGF β-1 treatment of cells transfected with SCR siRNA. This made it diffecult to interpret the result obtained from cells transfected with SET9 siRNA, in the absence of translocation in the negative (SCR) controls (Figure 4.10A).

Nuclear accumulation of SMAD3 was further assessed using Western blotting, comparing cells treated with or without SET9 inhibitor, in presence of TGF β-1 (Figure 4.10B). Western

blotting analysis showed that cells cultured in FM, exhibited a SMAD3 cytoplasmic:nuclear of 1:1.5. This suggests that endogenous TGF β-1 is having an impact on SMAD3 nuclear localisation. When treated with exogenous TGF β-1, more SMAD3 moved from the cytoplasm into the nucleus, with the ratio between cytoplasmic SMAD3 and nuclear SMAD3 increasing to 1:3 (Figure 4.10B) .

There was a similar degree of SMAD3 nuclear translocation in cells treated with SET9 inhibitor R-PFI-2, compared to that seen without inhibitor. This suggests that SET9 seesm to have only subtle effects, as most, upon SMAD3 nuclear import in these cells (Figure 4.10B).
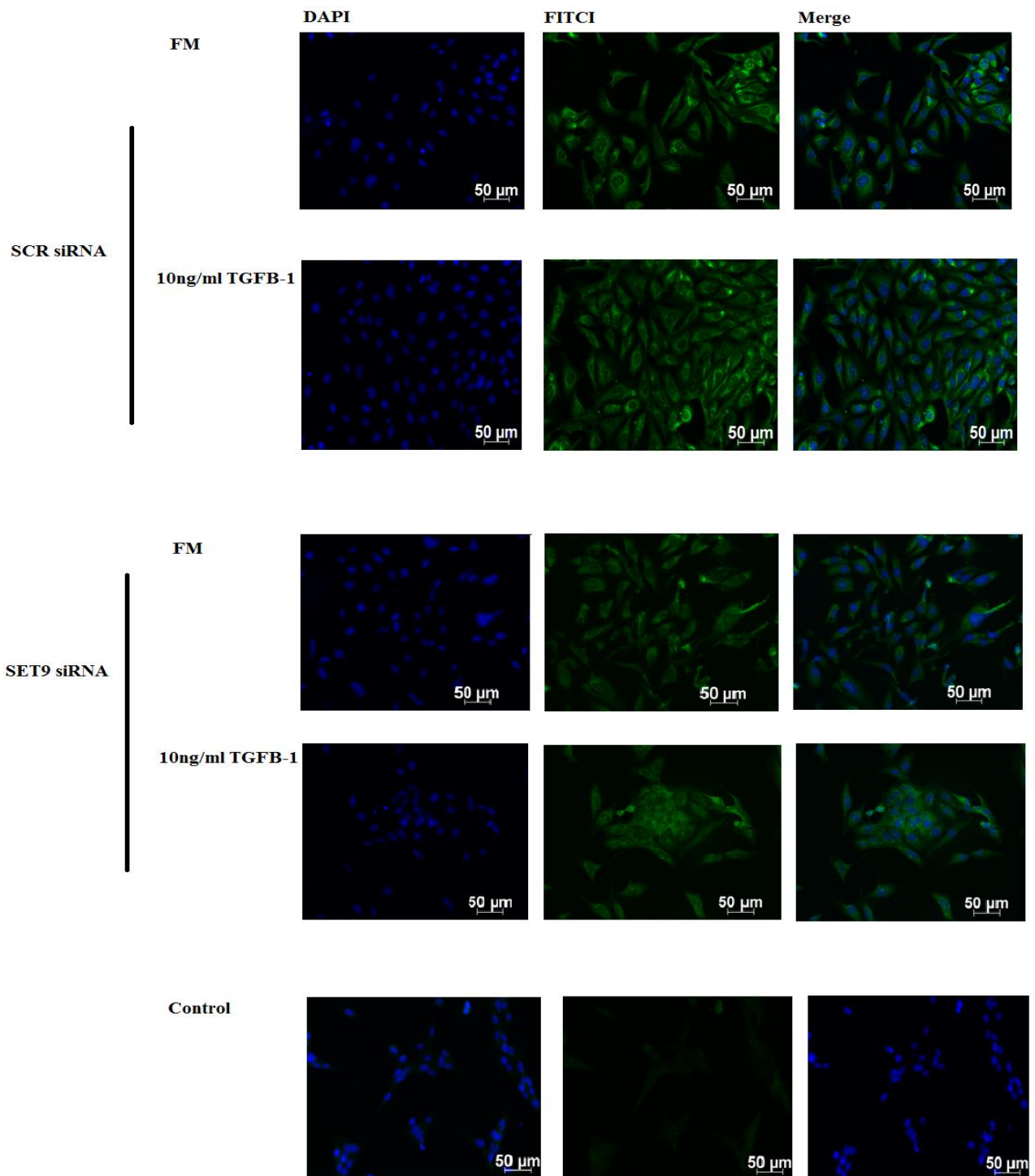
Figure 4.10A. the effect of SET9 siRNA on SMAD3 nuclear import. cells were seeded onto glass coverslips and transfected with either SCR or SET9 siRNA and treated with or without 10ng/ml TGF β-1 for 24h. Cells were washed in PBS, fixed in ice cold methanol and blocked in 5% goat serum. Cells were then stained using an anti-SMAD3 primary antibody and a secondary conjugated with Alexa Fluor 488. Control cells were left without primary antibody. Magnification was 20X.
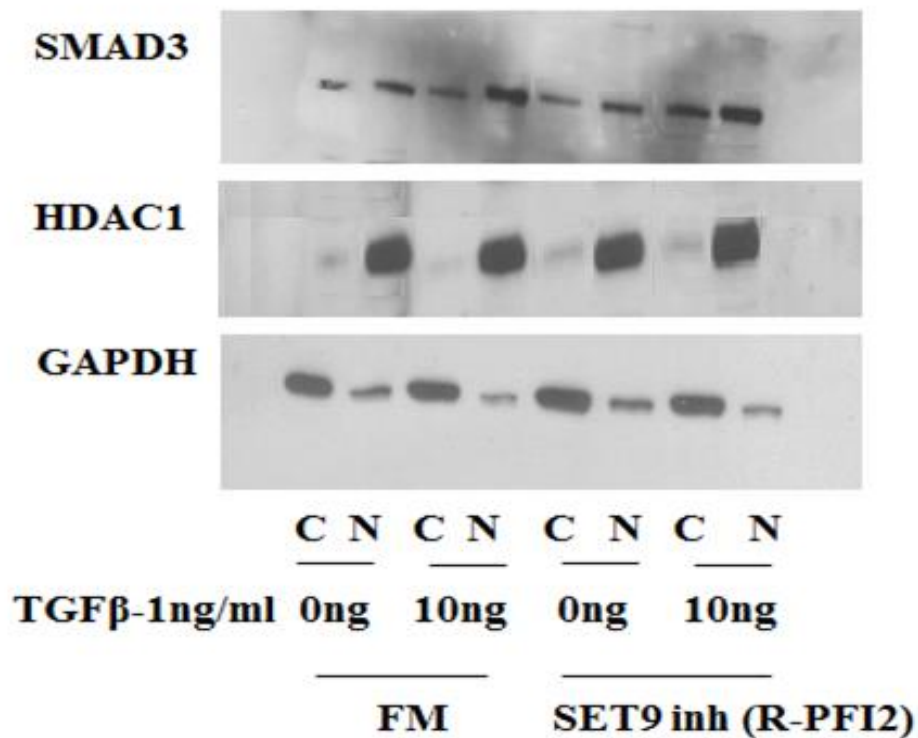
Figure 4.10B. The effect of SET9 inhibitor on SMAD3 nuclear import. THMCs were seeded, treated with SET9 inhibitor and then treated with or without TGF β-1 for 24hrs. Nuclear and cytoplasmic fractions were obtained by centrifugation using a hypotonic buffer solution. Fractions were subjected to Western blot analysis with SMAD3, HDAC1 and GAPDH antibodies. HDAC1 and GAPDH were used as nuclear marker and cytoplasmic loading conrtol, respectively. GAPDH is more cytoplasmic in this experiement as it may was affected by the cellular metabolism.

4.11. SET9 Inhibition Negatively Affects Wound Healing

Fibrosis has been described as an aberrant wound healing response (Flanders, Major et al. 2003, Wynn 2007). To examine the effect of SET9 inhibition in wound healing assays in THMC cells, cells were seeded into chambers then treated with or without SET9 inhibitor or transfected with 25nM SCR/SMAD3 siRNA and imaged at the indicated intervals until the wound was completely closed (Figure 4.11A and B). Cells in full medium exhibited wound closure within 24hrs, whereas cells treated with SET9 inhibitor only reached 70% wound closure at 24hrs (Figure 4.11B). Unexpectedly, TGF β-1 had only minimal impact on wound

healing. THMCs transfected with SMAD3 siRNA, also showed diminished wound closure compared to cells transfected with SCR control siRNA (Figure 4.11B). Overall these results indicate that the SET9 methyltransferase activity appears to be important in wound healing in an *in vitro* model of glomerulosclerosis and as such might be used as a treatment to slow down this disease process.
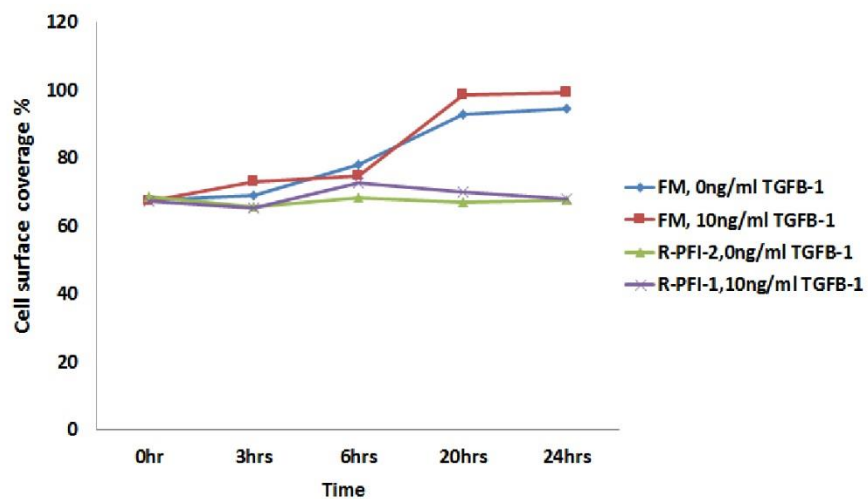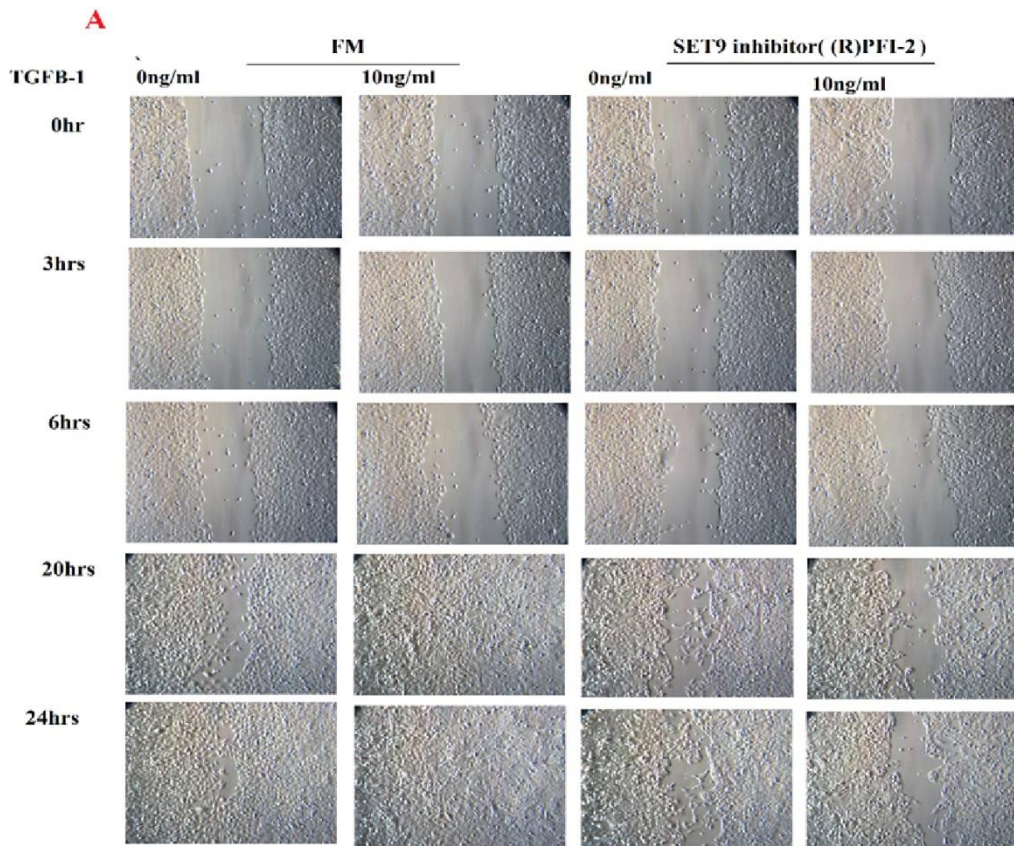
Figure 4. 11A. The effect of SET9 on wound healing in THMCs. Cells were seeded into wound healing chambers, treated with or without 30uM of SET9 inhibitor (R-PFI-2) and treated with or without 10ng/ml TGF β-1 for 24hrs. The chambers were removed and the cells imaged at different intervals to determine the differences in wound healing. N=3.
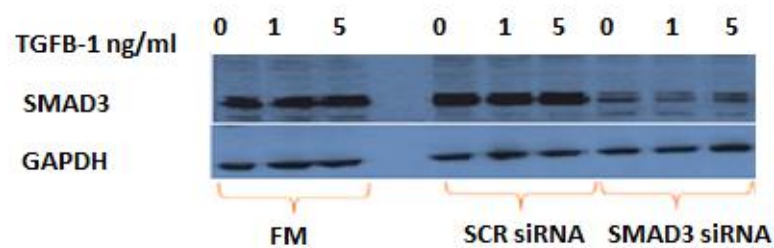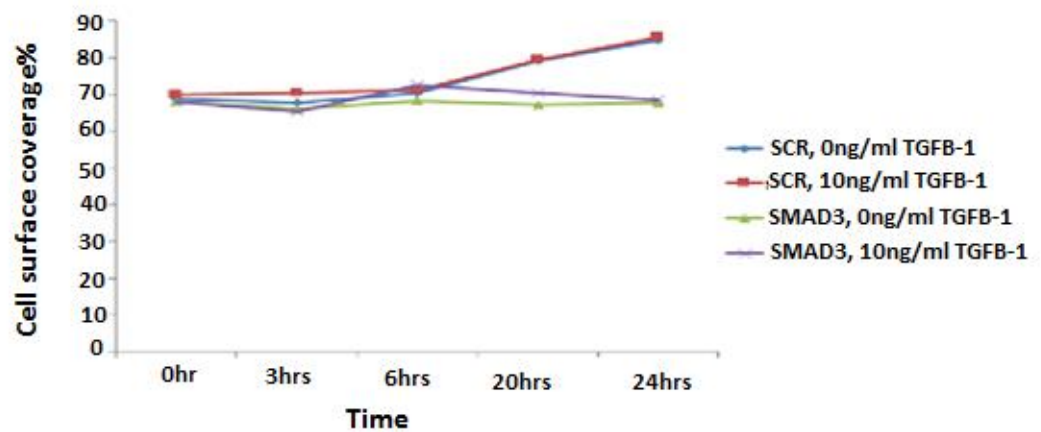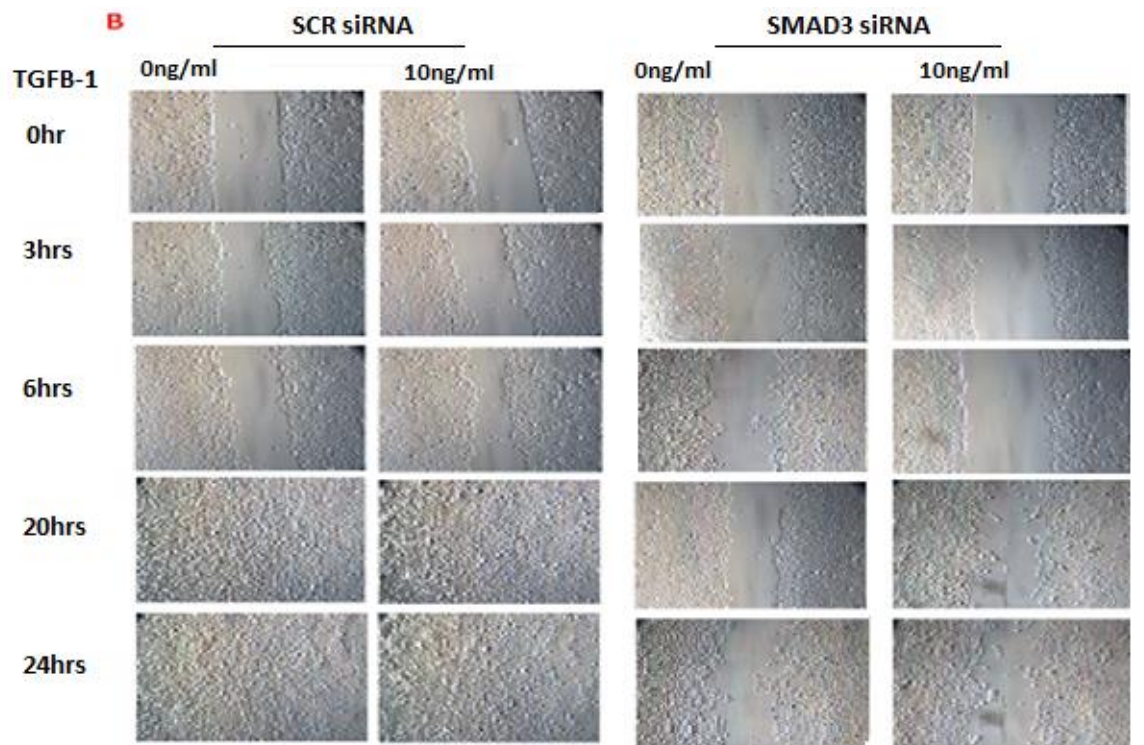
Figure 4.11B. The effect of SMAD3 on wound healing in THMCs. Cells were seeded into wound healing chambers, transfected with 25nM SCR/SMAD3 siRNA and treated with or without 10ng/ml TGF β-1 for 24hrs. The chambers around the cells were removed and the cells imaged at different intervals to determine the differences in wound healing. WB analysis shows the siRNA transfection efficiency.

4.12. Effect of SET9 Inhibition on Cell Proliferation

In order to determine whether or not the wound closure occured as a result of cell migration or cell proliferation the effect of SET9 inhibition was examined in THMCs in a colorometric proliferation assay. Cells were seeded and treated with or without SET9 inhibitor prior to exposure to TGF β-1 (Figure 4.12). Cell proliferation was assayed by assessment of cell number after 24 hours using the WST-1 assay. There was no clear trend in the effects of TGF β-1 or SET9 inhibitor on THMC proliferation after 8 and 24 hours (time points used in the wound healing assays) suggesting that the wound is more likely to heal as a result of cell migration into the wound rather than proliferation of cells into the wound.
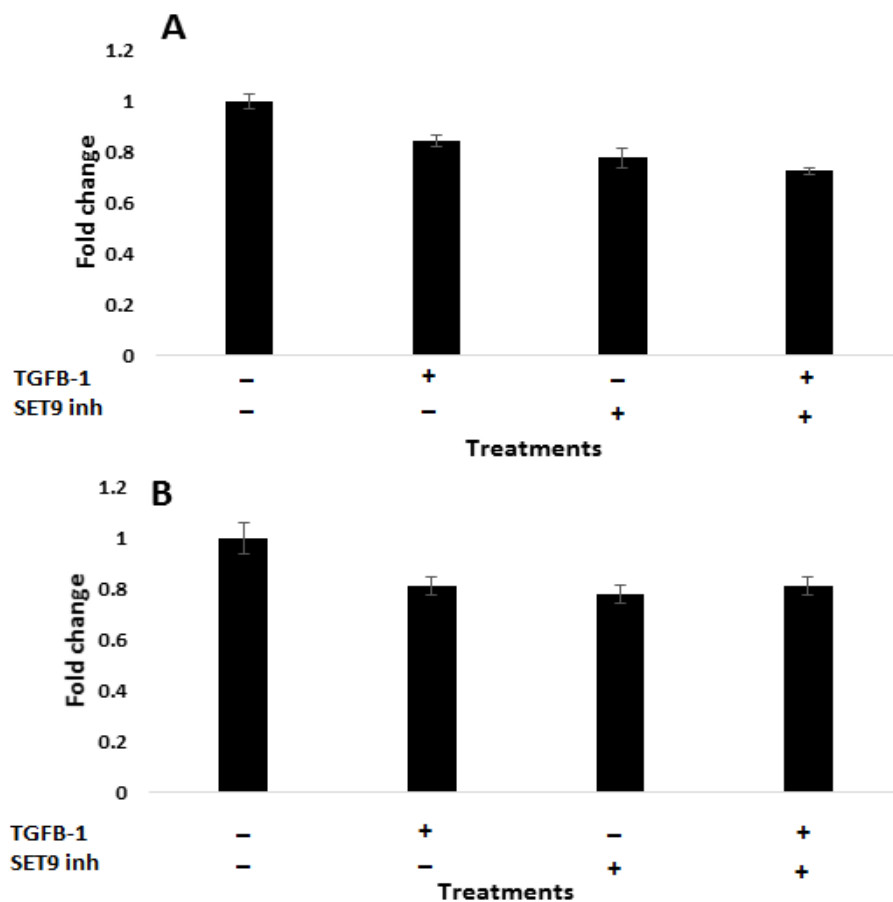
Figure 4.12. Proliferation assay for THMCs in response to TGF β-1. *A*, cells were seeded and treated with or without SET9 inhibitor prior to exposure to TGF β-1. 8 hours later, colorimetric cell proliferation was measured using WST-1 reagents. *B*, cells were treated as in *A*, however, colorimetric cell viability and proliferation were measured 24 hours after treatment with TGF β-1.(n=5.Mean+/-SD).

4.13. Discussion

The activation and translocation of SMAD3 into the nucleus is a key process in TGF β-1 signalling. Consequently, detection of factor(s) regulating SMAD3 nuclear import would be significant in developing novel treatments to down-regulate TGF β-1 signalling in renal fibrosis and other diseases (Shi and Massague 2003). Published data has identified a lysine rich KKLKK nuclear import signal in the SMAD3 protein (Xiao, Liu et al. 2000). Mutation of these lysine residues abolishes TGF β-1-induced SMAD3 nuclear import. (Yao, Chen et al. 2008).

Methylation can regulate the function of transcription factors in various ways: it can modulate interaction with other proteins, protein stability, transactivation potential, the deposition of other modifications, and nuclear retention and recruitment to genomic targets (Sarris, Nikolaou et al. 2014). It has been reported that SET9 may activate transcription by methylating nonhistone protein substrates, including SMADs (Yang, Huang et al. 2009). The identification of SET9, a lysine methyltransferase that preferentially methylates target lysine residues within sequences very similar to the KKLKK NLS within SMAD3 (Nishioka, Chuikov et al. 2002), strengthens the hypothesis of SET9 mediating nuclear import of SMAD3 via this KKLKK motif in response to TGF β-1. SET9, along with its broad substrate specificity, has distinctive features, that distinguish it from other methyltransferases. First, SET9 has neither canonical nuclear localisation signals nor nuclear translocastion signals and has been shown to be located in both the cytoplasm and the nucleus (Okabe, Orlowski et al. 2012). Therefore, it is possible that nuclear localisation of SET9 is regulated by interaction with other cellular partners. SET9, for example, is controls the transcription of target genes by interaction with other transcription factors such as NFκB (Li, Reddy et al. 2008). Furthermore, in proteins linking the membrane to the cytoskeleton, SET9 is the only methyltransferase that contains membrane occupation and recognition nexus (MORN) repeats (Garbino, van Oort et al. 2009). These observations suggest both a cytoplasmic and nuclear function for SET9.

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Data collected in this chapter indicates that there is a physical interaction between SET9 and SMAD3 raising the possibility that methylation is involved in the TGF β-1-SMAD3 signalling cascade.

SET9 interacts with SMAD3, and enhances the transcriptional activity of SMAD3-driven reporter-genes, as shown by reporter gene assays. Analysis of *CAGA*-luciferase reporter gene assay with WT SET9 and MUT SET9 (lacking methyltransferase activity) revealed that SET9 may act as a co-activator for SMAD3 in the presence of TGF β-1. The finding that SMAD3 transcriptional activity depend on SET9 was shown by the reduction of luciferase activity observed on depletion of SET9.

To further study the role of SET9 in glomerulosclerosis, a variant of renal fibrosis, THMCs were used as a disease model. Excessive production and accumulation of ECM proteins is the hallmark event of fibrotic diseases, (Wynn and Ramalingam 2012). The discovery of (R)-PFI-2 that selectively inhibits the methyltransferase activity of human SET9 via occupying the substrate peptide binding groove of SET9, including the catalytic lysine-binding channel, and by making a direct contact with the donor methyl group of the cofactor, S-adenosylmethionine (Barsyte-Lovejoy, Li et al. 2014), has made it possible to study the diverse roles of SET9 in cells and further validates protein methyltransferases as a druggable target.

Analysis showed that THMCs adopt a pro-fibrotic phenotype in response to TGF β-1. Upon depletion of SET9, IF staining showed a reduced expression of the pro-fibrotic markers collagen I, collagen III, fibronectin, PAI-1 and α-SMA, even in the presence of TGF β-1. My findings suggest that SET9 function is required for TGF-β-dependent activation of ECM genes in THMCs. The involvement of SET9 in SMAD3 nuclear accumulation was tested by immunoflourescence staining and Western blotting, wherease the inhibition of SET9 notably

reduced SMAD3 nuclear import in the presence of TGF β-1 in HK C-8 cells, it had relatively minor impact in THMC cells. The reasons for this are unclear.

Wound healing experiments indicated a significant reduction in cell migration in THMCs as a result of SMAD3 siRNA and SET9 inhibitor. In agreement, a study has shown SMAD3 null fibroblasts showed a reduced migratory activity (Dobaczewski, Bujak et al. 2010). Alternatively, in comparsion with wild-type mice, other studies document that SMAD3 null mice paradoxically show accelerated cutaneous wound healing by re-epithelialisation (Mauviel 2009). The finding that SET9 is involved in cell migration is a novel finding and suggests a possible role for SET9 in the recruitment of mesangial cells in the development of glomerulosclerosis.

The data represented in this chapter indicates that SET9 is a novel methyltransferase involved in the TGF β-1 / SMAD3 signalling pathway. SET9 was discovered to be one of the first protein lysine methyltransferases and was regarded as a monomethyltransferase of lysine 4 on histone H3 (H3K4me1) (Nishioka, Chuikov et al. 2002). Subsequently, SET9 has been shown to have a variety of targets including transcriptional regulators such as TAF10, STAT3, ER, p65, Foxo3, AR, Rb, Mypt, Tat, and p53 (Calnan, Webb et al. 2012). Here we add to this repertoire of factors by showing that SET9 also regulates SMAD3, in a manner that is dependent upon its methyltransferase activity. The novel impact of SET9 on wound healing suggests that further evaluation of SET9 as a target in the treatment of fibrotic diseases such as CKD is warranted. Given the multitude of methylation and interaction targets and lack of distinct SET9-associated phenotypes identified to date, it is likely that SET9 role may be modulatory and may be dependent on the cell type and/or physiological surroundings being considered.Other methyltransferases might also play a role in TGFβ/SMAD signalling and represent additional therapeutic targets.

5 ANALYSING THE INTERACTION BETWEEN SMAD3 AND HSPBAP-1

5.1 Introduction

Histone methylation is mediated by histone methyltransferases (HMTs) and histone demethylases (HDMs). Methylation is thought to be involved in the pathogenesis of several common diseases , including diabetic nephropathy (Sun, Cui et al. 2014) . Each of the HMTs and HDMs influence the structure of chromatin leading to different functional outcomes, based on the site and the degree of modification (Kooistra and Helin 2012).

Heat Shock Protein BAP1 (heat shock 27-kDa-associated protein 1, HSPBAP1), is a human homologue of rat PASS1 demethylase (protein associated with small stress proteins 1) (Jiang, Ma et al. 2001) that binds to one of the small heat shock proteins, specifically HSP27 (Liu, Gilmont et al. 2000). The human protein HSPBAP-1 shared 80% amino acid identity with rat PASS1, and was originally cloned from a human foetal brain cDNA library (Jiang, Ma et al. 2001). The gene encoding HSPBAP-1 is located on chromosome 3q21 and it is expressed in many tissues, but particularly highly expressed in the testis and kidney (Jiang, Ma et al. 2001). 3 isoforms of the human protein are produced by alternative mRNA splicing. HSPBAP1 chromosomal aberrations, such as loss of the short arm of chromosome 3 due to (a) deletion(s) or unbalanced translocation, have been found in a family with renal cell carcinomas. It is also involved in translocation t(2;3)(q35;q21) with the putative pseudogene *DIRC3* (an inhibitor of apoptosis), resulting in a hybrid mRNA encoding a truncated HSPBAP1 lacking the first 36 amino acids (Bodmer, Schepens et al. 2003). Human HSPBAP is not yet known to harbour demethylase activity but its sequence conservation makes it a putative demethylase enzyme. Recently, HSPBAP1 has been shown to interact with androgen receptor in the nucleus, and this interaction increases during androgen-deprivation conditions (Saeed, Ostling et al. 2015).

HSPBAP1 is speculated to promote tumour cell viability in prostate cancer by maintaining androgen receptor (AR) signalling during androgen-deprived conditions, which are relevant during androgen deprivation therapy (Saeed, Ostling et al. 2015).

5.2 HSPBAP-1 is Expressed by THMCs

Having previously demonstrated that depletion of HSPBAP-1 by siRNA knock down notably increased the activity of SMAD3-responsive *CAGA* reporter gene in the presence of TGF β-1 (Section 3.7), the HSPBAP-1 – SMAD3 interaction was studied in more detail. IF staining shows that HSPBAP-1 is expressed by THMCs, thereby offering an opportunity to study the role of HSPBAP-1 in TGF β-1/ SMAD3 signalling in this model of glomerulosclerosis (Figure 5.2). Accordingly, the specific aims of this chapter were:

7- To validate the effect of HSPBAP1 which was identified from the demethylase screen.

8- To study the interaction between SMAD3 and HSPBAP1.

9- To examine the effect of HSPBAP-1 on SMAD3 nuclear import.
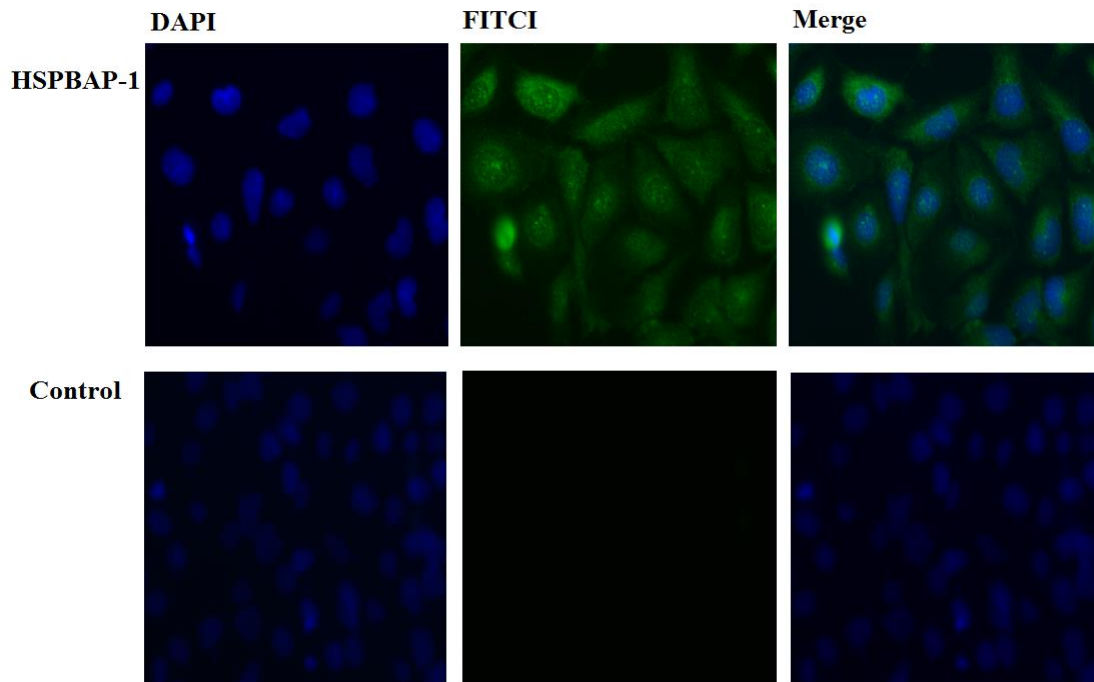
Figure 5.2. Expression of HSPBAP-1 in THMCs. THMCs were seeded onto glass coverslips and grown for 24hrs. Cells were washed in PBS, fixed in ice cold methanol and blocked in 5% goat serum. Cells were then subjected to immunofluorescence with or without HSPBAP-1 primary antibody then stained with Alexa Fluor 488 secondary antibody and visualised by confocal microscopy. Magnification was 40X.

5.3 Interaction of HSPBAP-1 with SMAD3 in Kidney Cells

Experiments were next performed to determine whether any direct interaction between HSPBAP-1 and SMAD3 proteins exists. Firstly, immunoprecipitation of HK C-8 cell lysates with SMAD3 antibody and subsequent Western blot analysis with anti-HSPBAP-1 demonstrated co-immunoprecipitation of both HSPBAP-1 and SMAD3. HSPBAP-1 could not be detected in immunoprecipitation samples with control immunoglobulin G (IgG) (Figure 5.3A). This is the first evidence of a direct interaction between the SMAD3 and HSPBAP-1 protein. There did not appear to be an enrichment of SMAD3- HSPBAP-1 co-immunoprecipitation in the presence of exogenous TGF β-1.

To determine whether the HSPBAP-1 – SMAD3 interaction could be detected in another cell type, the immunoprecipitation was repeated in THMCs (Figure 5.3B). This confirmed that SMAD3 and HSPBAP-1 interact in THMCs (Yamamoto, Nakamura et al. 1993, Alsaad and Herzenberg 2007). Again, exogenous TGF β-1 did not seem to alter the SMAD3: HSPBAP-1 interaction. Although it is unknown which protein domains might be responsible for the SMAD3:HSPBAP-1 interaction, these results raise the possibility that HSPBAP-1 might demethylate SMAD3. As before, control immunoprecipitations with rabbit IgG demonstrated specificity to the interaction between HSPBAP-1 and SMAD3.

Finally, additional experiments were performed to determine whether the HSPBAP-1 and SMAD3 interaction could be identified in a reciprocal fashion, this time using the HSPBAP-1 antibody for immunoprecipitation. In HK C-8 cells, immunoprecipitation with anti-HSPBAP-1 antibody followed by immunoblotting with anti-SMAD3 antibody demonstrated a detectable interaction between the two proteins (Figure 5.3C). Similarly, immunoprecipitation from THMCs resulted in co-immunoprecipitation of SMAD3 with HSPBAP-1 (Figure 5.3D). There was no detectable effect of TGF β-1 treatment on the interaction in either cell line.

A

HSPBAP-1

SMAD3

GAPDH

TGFB-1   −   +   −   +   −   +   −   +

Inputs   **Inputs**   **IPs**   **IPs**
(SMAD3)   **(Control IgG)** **(SMAD3)** **(Control IgG)**

B

HSPBAP-1

SMAD3

GAPDH

TGFB-1   −   +   −   +   −   +   −   +

Inputs   **Inputs**   **IPs**   **IPs**
(SMAD3)   **(Control IgG)** **(SMAD3)** **(Control IgG)**
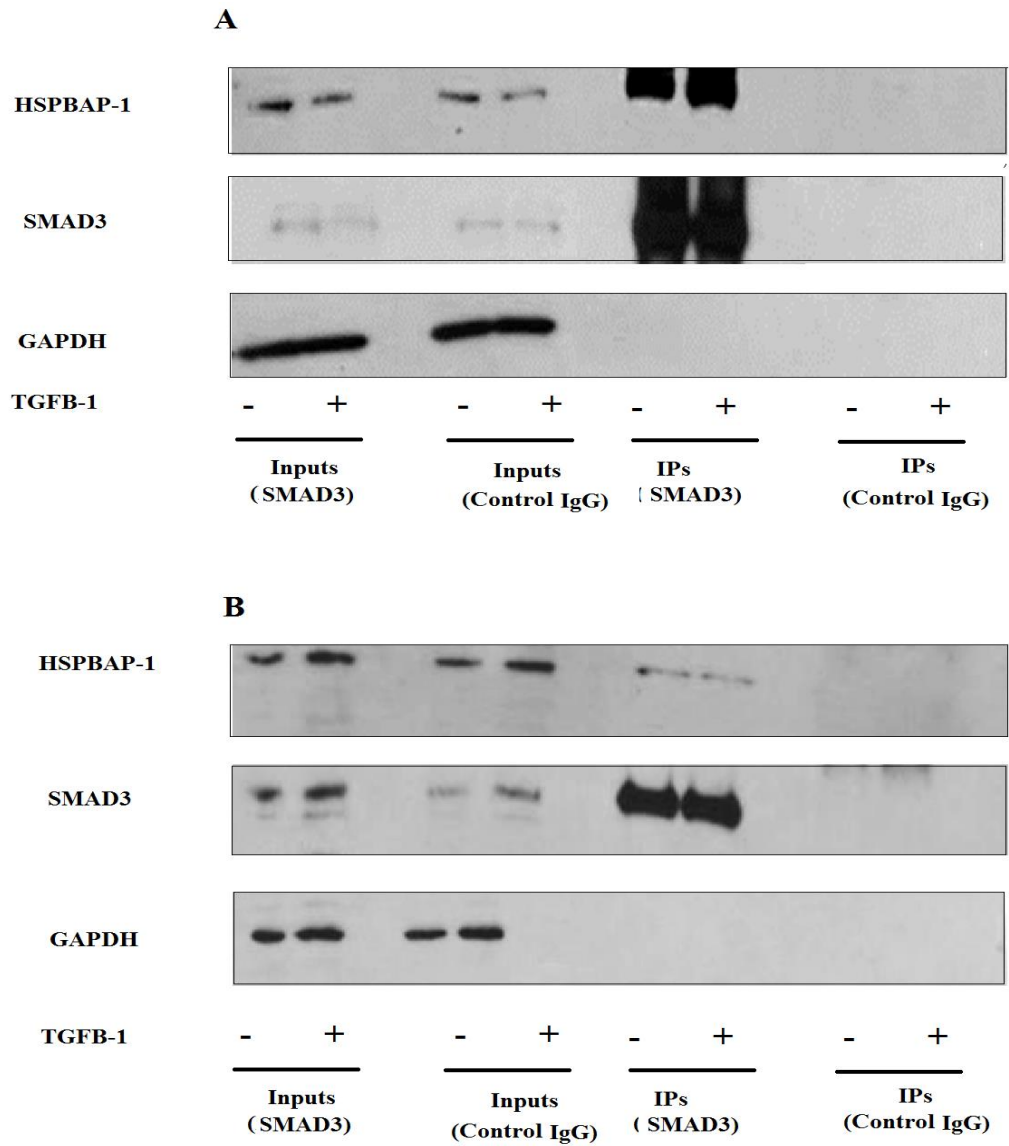
Figure 5.3 *A, B*. HSPBAP-1 interacts with SMAD3 in HK C-8 and THMCs. *A*, HK C-8 cells were starved in serum-free medium then treated with or without 10ng/ml TGF β-1 for 24hrs prior to lysis in RIPA buffer. Lysates were centrifuged to remove insoluble debris then immunoprecipitated with protein G dynabeads cross-linked to antibodies for SMAD3 or rabbit immunoglobulin G (IgG) control. *B*, THMCs were treated as in *A*. Proteins were resolved on SDS PAGE then subject to Western blotting with the indicated antibodies.
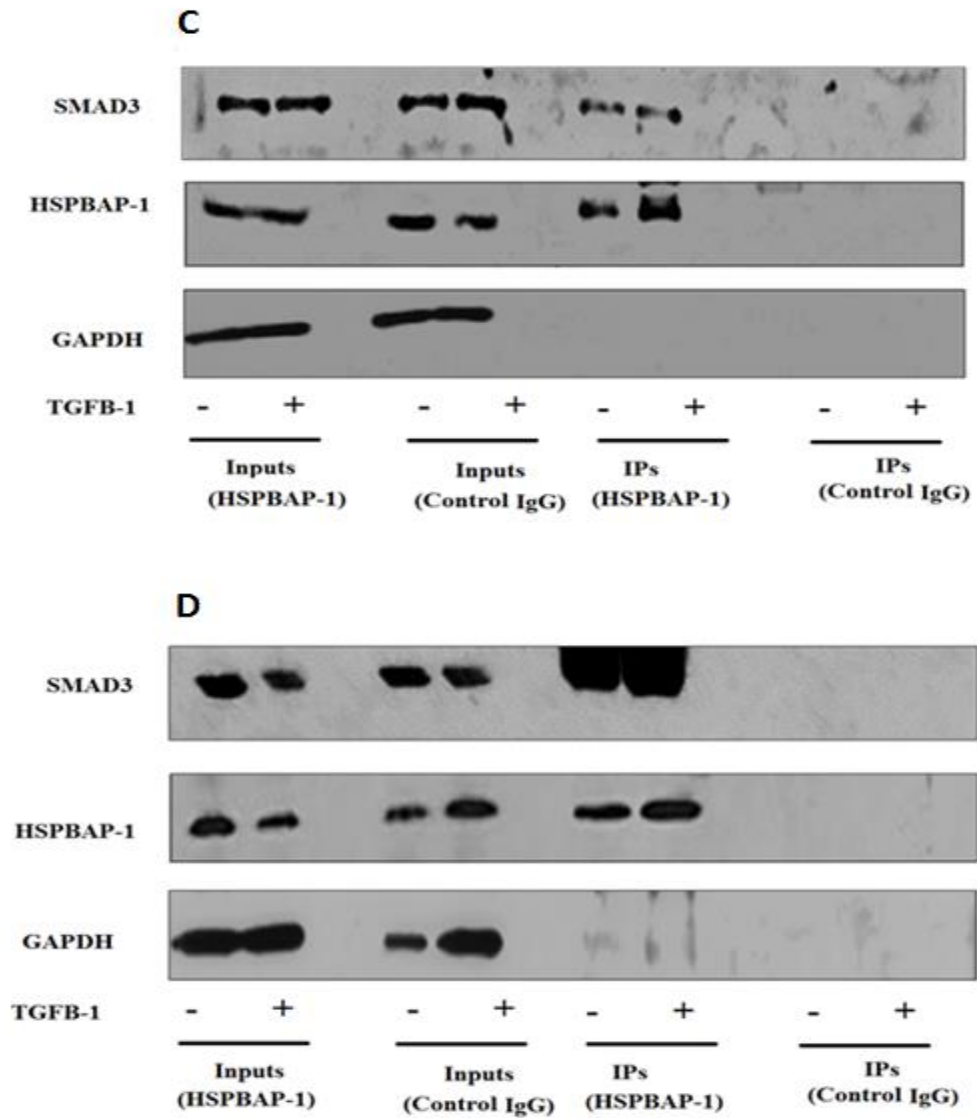
Figure 5.3 *C, D*. HSPBAP-1 interacts with SMAD3 in HK C-8 and THMC cells. *C*, HK C-8 cells were seeded then starved in serum-free medium then treated with or without 10ng/ml TGF β-1, for 24hrs prior to lysis in RIPA buffer. Lysates were centrifuged to remove insoluble debris then immunoprecipitated with protein G dynabeads cross-linked to antibodies for HSPBAP-1 or rabbit immunoglobulin G (IgG) control. *D*, THMC cells were treated as in A. Proteins were resolved on SDS PAGE then subject to Western blotting with the indicated antibodies.

5.4 PAI-1 Luciferase Response to TGF β-1

The luciferase assays used so far were performed with an artificial SMAD3-responsive promoter containing 12 repeats of the consensus *CAGA* SMAD binding element. To establish a more biologically relevant system the Plasminogen Activator Inhibitor-1 (*PAI-1*) promoter was used. Here, the luciferase reporter gene is driven by a natural, prototypic TGF β-1 / SMAD3 promoter from the *PAI-1* gene, known to be responsive to SMAD3 and TGF β-1. Cells were transiently transfected with 300ng of a *PAI-1* promoter-luciferase construct prior to transfection with SMAD3 siRNA. Cells were then treated with or without TGF β-1 and lysates collected for luciferase assay. The inhibition of SMAD3 resulted in a reduction in the PAI-1 promoter-driven luciferase signal (Figure 5.4). A response was seen from cells in the absence of exogenous TGF β-1 could be as a result of the effect of endogenous TGF β-1.
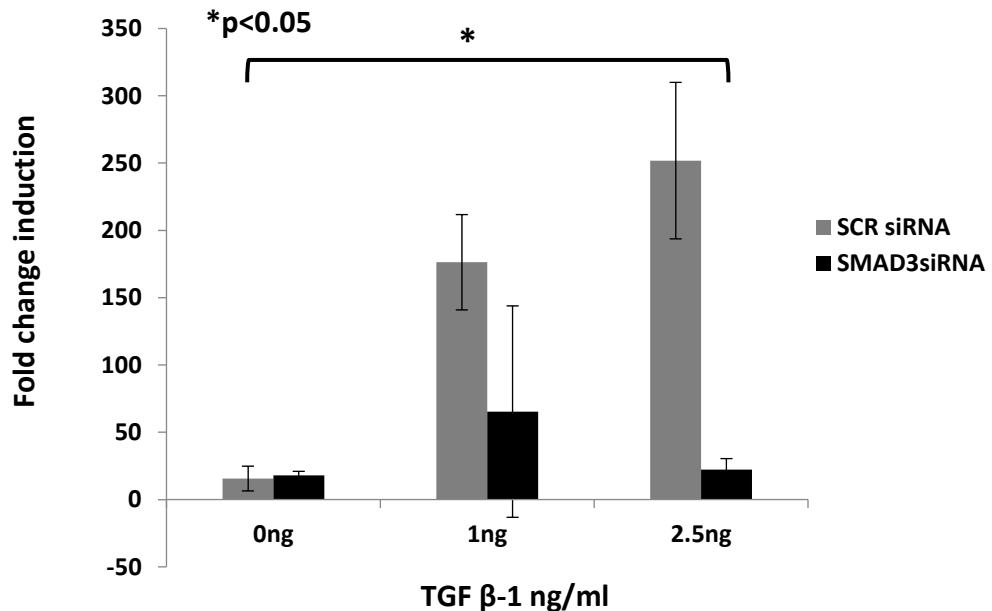
Figure 5.4. SMAD3 siRNA inhibits TGF β-1 -induced PAI-1 reporter gene expression. HK C-8 cells were seeded transfected with 25nM of SCR/SMAD3 siRNA. After 24hrs of incubation, cells were transfected with 300ng of *PAI-1* reporter gene. Cells were treated with or without TGF β-1. Cell lysates were harvested after 24hrs. Luciferase values, corrected for protein concentration, are expressed as fold change compared to that for the control SCR siRNA. The baseline of SCR siRNA untreated cells was used to calculate the fold change induction. (n=3 Mean +/- SD).

5.5. HSPBAP-1 Knockdown Increases the activity of PAI-1 Reporter Gene

TGF β-1 induces PAI-1 expression via SMAD3 in fibrosis (Li, Heaton et al. 2006). This occurs through binding of SMAD3 to regulatory sequence elements of the *PAI-1* gene (Section 5.3). Having demonstrated that HSPBAP-1 interacts with SMAD3 and influences *CAGA*-luciferase expression, the effect of HSPBAP-1 on the *PAI-1* reporter gene was examined. Cells were transiently transfected with *PAI-1* promoter-luciferase construct prior to sequential transfection with SMAD3 siRNA, HSPBAP-1 siRNA, or siRNAs against demethylases from the original screen (see Figure 5.5A). These were then treated TGF β-1 or vehicle control and lysates collected for luciferase assay. As expected, SMAD3 knockdown led to a reduction in TGF β-1-stimulated, *PAI-1* promoter-driven luciferase signal. There was an increase in the *PAI-1* promoter-driven luciferase signal as a result of HSPBAP-1 knockdown, suggesting that HSPBAP-1 is involved in the regulation of SMAD3 transcriptional activity on the *PAI-1* gene promoter. However, the change of response is much smaller than was obtained using *CAGA*-luciferase reporter gene.

HSPBAP-1 was targeted by a mixture of 3 siRNAs (referred to as A, B and C from now on), as HSPBAP-1 is exists in three isoforms. These isoforms have the same function but have differences in their sequence due to alternative splicing events and subsequently may have differential effects on their target genes. HSPBAP-1 isoform 1 has been chosen as the 'canonical' sequence. HSPBAP-1 isoform 2 sequence differs from the canonical sequence as follows:

191-225:

KRWHLFPPEDTPFLYPTRIPYEESSVFSKINVVNP → LECNGMIIAPGPQAILLPQPLK YLGLQETMASLSS

226-488: Missing.

HSPBAP-1 isoform 3 sequence differs from the canonical sequence as follows:

250-280:

FVPRHWWHYVESIDPVTVSINSWIELEEDHL → ERKWQEGTQLLLLVKRRMDFGGR

QSTRVIFI

281-488: Missing.

An additional experiment was performed to verify the effect of each individual HSPBAP-1 siRNA. HK C-8 cells were transfected with *PAI-1* luciferase after the transfection with SMAD3 siRNA or HSPBAP-1 A, B, or C siRNA individually (Figure 5.5B). Cells then treated with or without TGF β-1 and luciferase activity was determined. Luciferase activity was most noticeably increased upon introduction of the HSPBAP1 siRNA C, by producing approximately 20-fold induction, in the presence of TGF β-1.

To verify if the 3 pooled siRNA against HSPBAP-1 will behave in the same way observed form *PAI-1* reporter gene assay, HSPBAP-1 A, B, and C were separately transfected into cells transiently transfected with the TGF β-1 reporter *CAGA*-luciferase. Controls were scrambled siRNA (SCR) and SMAD3 siRNA with or without 1ng/ml TGF β-1 (Figure 5.5C).Among the 3 siRNAs for HSPBAP-1, siRNA C again produced the largest change, with approximately 11-fold induction in luciferase activity in response to TGF β-1 compared to SCR siRNA. siRNA A and B had much lesser effects, albeit with a trend towards that of siRNA C. HSPBAP-1 siRNA transfection efficiency was confirmed by Western blotting (Figure 5.5D). HK C-8 cells were transfected with HSPBAP-1(A, B, orC), SMAD3 or SCR control siRNA for 24hrs, prior to addition of exogenous TGF β-1. Cell lysates were analysed by Western blotting which demonstrated a marked reduction in band density corresponding to the respective target gene. Gene silencing was specific, as SCR control siRNA did not affect HSPBAP1 levels.

These experiments indicate that HSPBAP-1 influences TGF β-1 signalling at target promoters via interaction, likely as result of direct interaction with SMAD3, to regulate common downstream transcriptional responses.
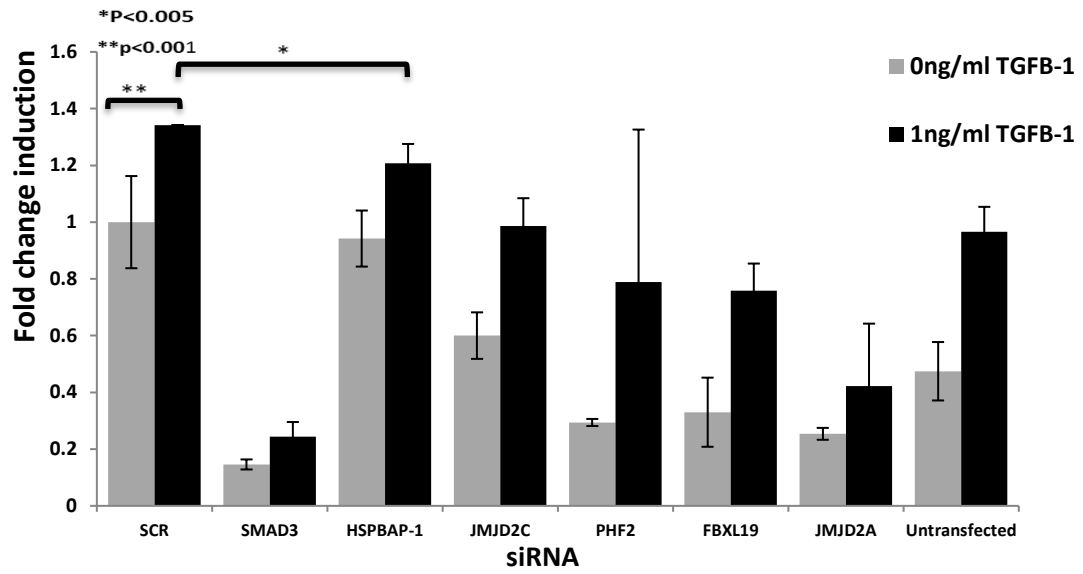
Figure 5.5A. The effect of silencing HSPBAP-1 on the expression of *CAGA* luciferase. HK C-8 Cells were seeded transfected with 25nM of SCR/SMAD3/HSPBAP-1/JMJD2C/PHF/FBXL19/JMJD2A siRNAs. 24hrs later, cells were transfected with 300ng of *PAI-1* reporter gene. Cells were treated with or without 1ng/ml TGF β-1. Cell lysates were harvested after 24hrs. Luciferase values, corrected for protein concentration, are expressed as fold change compared to that for the control SCR siRNA. The baseline of SCR siRNA untreated cells was used to calculate the fold change induction. (n=3 Mean +/- SD).
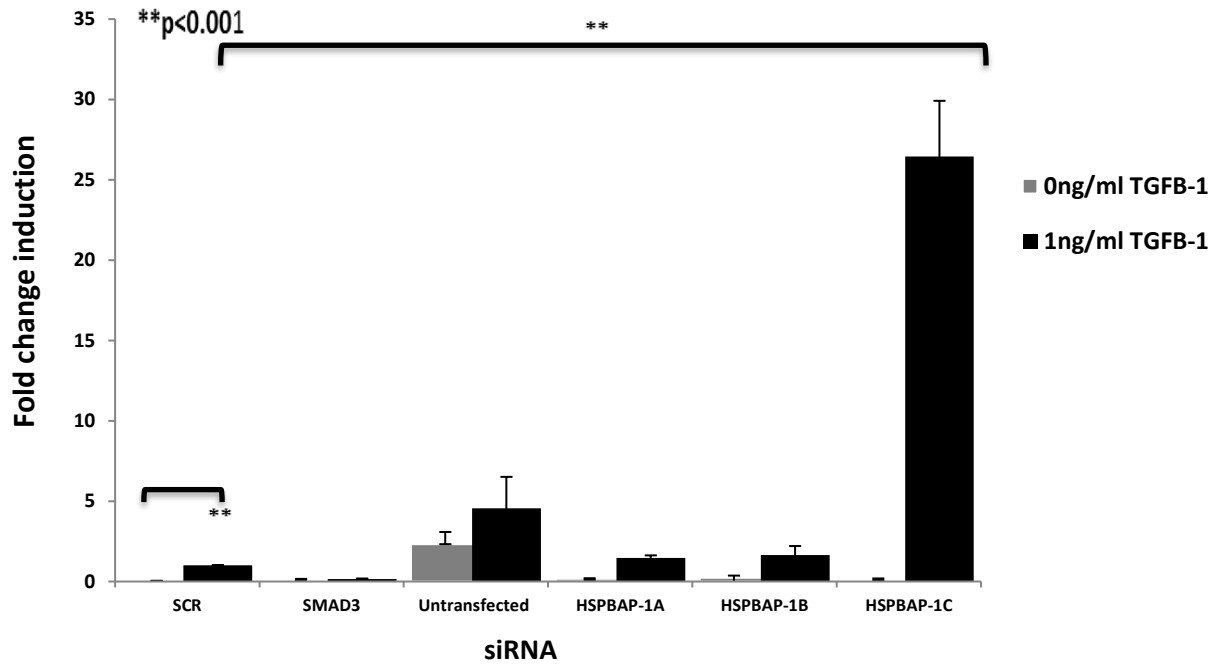
Figure 5.5B. The effect of silencing HSPBAP-1 on the expression of PAI-1. HK C-8 Cells were seeded transfected with 25nM of SCR/SMAD3/HSPBAP-1(A, B, C) siRNA. 24hrs later, cells were transfected with 300ng of *PAI-1* reporter gene. Cells were treated with or without 1ng/ml TGF β-1. Cell lysates were harvested after 24hrs. Luciferase values, corrected for protein concentration are expressed as fold change compared to that for the control SCR siRNA. The baseline of SCR siRNA treated cells was used to calculate the fold change induction. (n=3 Mean +/- SD).
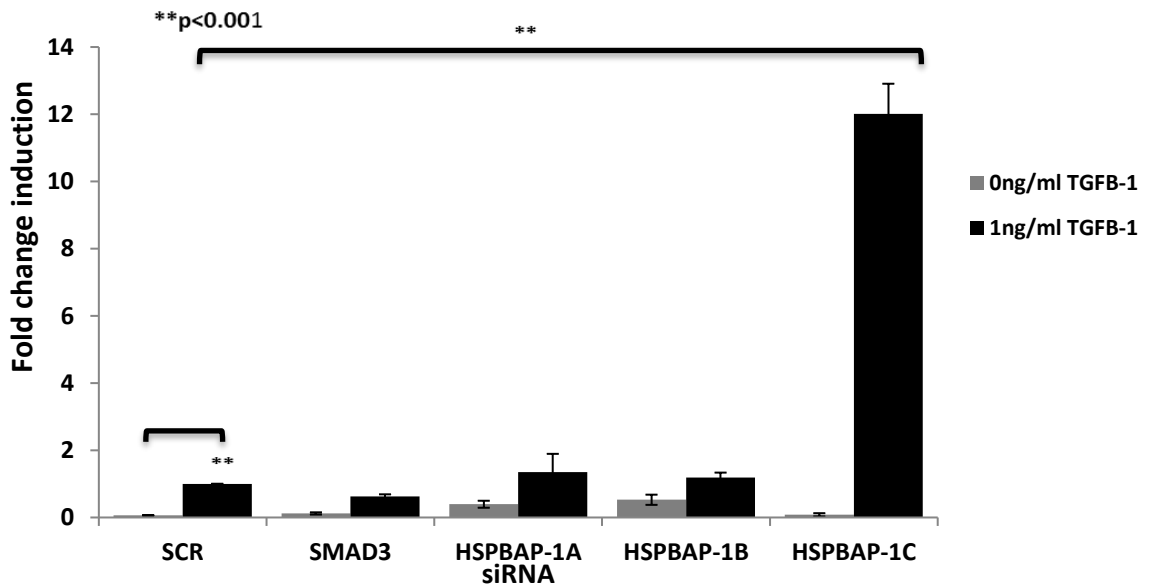
Figure 5.5C. The effect of silencing HSPBAP-1 on the expression of *CAGA* luciferase. HK C-8 were seeded transfected with 25nM of SCR/SMAD3/HSPBAP-1(A, B, C) siRNA. 24hrs later, cells were transfected with 300ng of *CAGA*-luciferase reporter gene. Cells were treated with or without 1ng/ml TGF β-1. Cell lysates were harvested next day, after 24hrs. Luciferase values, corrected for protein concentration, are expressed as fold change compared to that for the control SCR siRNA. The baseline of SCR siRNA treated cells was used to calculate the fold change induction. (n=3 Mean +/- SD).
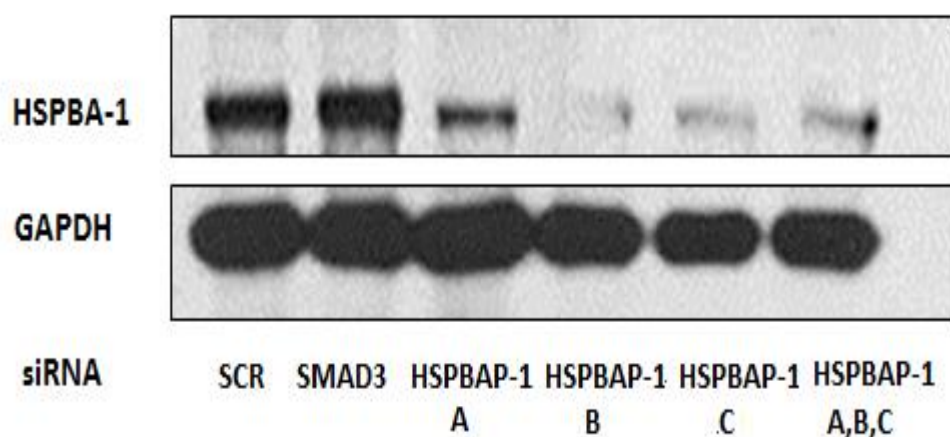
Figure 5.5D. siRNA transfection efficiency. HK C-8 were seeded and transfected with either SCR, SMAD3 or HSPBAP-1(A,B,C) siRNA for 24hrs and then treated TGF β-1. Cells were lysed in SDS-sample buffer, separated by SDS-PAGE, transferred to PVDF membrane, Western blotted, and probed with either anti-SMAD3 antibody or anti-HSPBAP-1 antibody.GAPDH was used as a loading control.

5.6. The role of HSPBAP-1 in SMAD3 Nuclear Import

Serine phosphorylation of SMAD3 and its subsequent nuclear translocation are critical regulatory steps in TGF β-1 signaling (Margarit, Sondermann et al. 2003). Unpublished work has also demonstrated that the lysine methyltransferase SET9 promotes SMAD3 nuclear import, suggesting that multiple post-translational modifications can regulate this aspect of SMAD3 signalling. We therefore examined the effect of HSPBAP-1 on SMAD3 nuclear translocation using immunofluorescence in THMCs. THMCs were transfected with SCR control siRNA or siRNA sequences A, B or C against HSPBAP-1 prior to immunofluorescence for SMAD3 (Figure 5.6). In the absence of exogenous TGF β-1, SMAD3 was distributed primarily in the cytoplasm with some weak nuclear staining (Figure 5.6A). Upon TGF β-1 stimulation, there was no detectable SMAD3 accumulation in the nucleus, which was an unexpected result given that THMCs are known to respond to TGF β-1 and that SMAD3 was

previously shown to undergo nuclear translocation in response to TGF β-1 in these cells (Chapter 4).

Although HSPBAP-1 levels were successfully reduced uupon introduction of the siRNA (Figure 5.6B), SMAD3 was again predominantly cytoplasmic with only minor staining of SMAD3 in the nucleus even in response to TGF β-1, (Figure 5.6C). This experiment was performed several times with consistent results. It is therefore difficult to conclude whether or not HSPBAP-1 affects SMAD3 nuclear import because the cells do not respond to TGF β-1. Another technique, such as nuclear extraction and Western blotting, would perhaps be informative in this case.

A

B
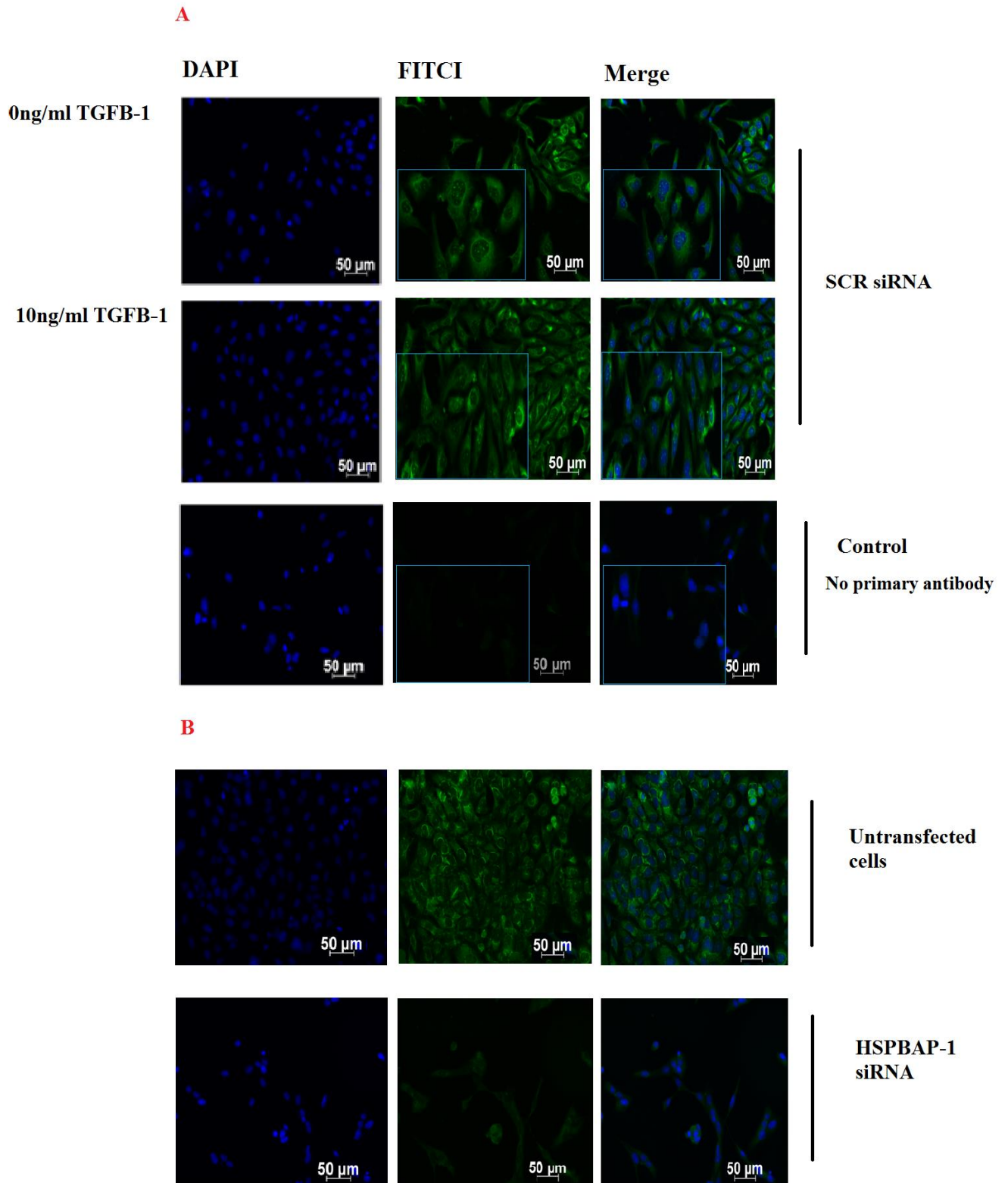
Figure 5.6A, B. HSPBAP-1 knockdown failed to alter SMAD3 nuclear translocation. *A,* THMCs were seeded onto glass coverslips and transfected with SCR siRNA and treated with 10ng/ml TGF β-1 for 24h. Cells were washed in PBS, fixed in ice cold methanol and blocked in 5% goat serum. Cells were then subjected to immunofluorescence staining for SMAD3. *B,* cells were seeded, transfected with or without HSPBAP-1, then stained for HSPBAP-1. Magnification was 20X.

C

0ng/ml TGFB-1   50 µm   50 µm   50 µm   HSPBAP-1 A
                                                                  siRNA

10ng/ml TGFB-1   50 µm   50 µm   50 µm

0ng/ml TGFB-1   50 µm   50 µm   50 µm   HSPBAP-1 B
                                                                  siRNA

10ng/ml TGFB-1   50 µm   50 µm   50 µm

0ng/ml TGFB-1   50 µm   50 µm   50 µm   HSPBAP-1 C
                                                                  siRNA

10ng/ml TGFB-1   50 µm   50 µm   50 µm
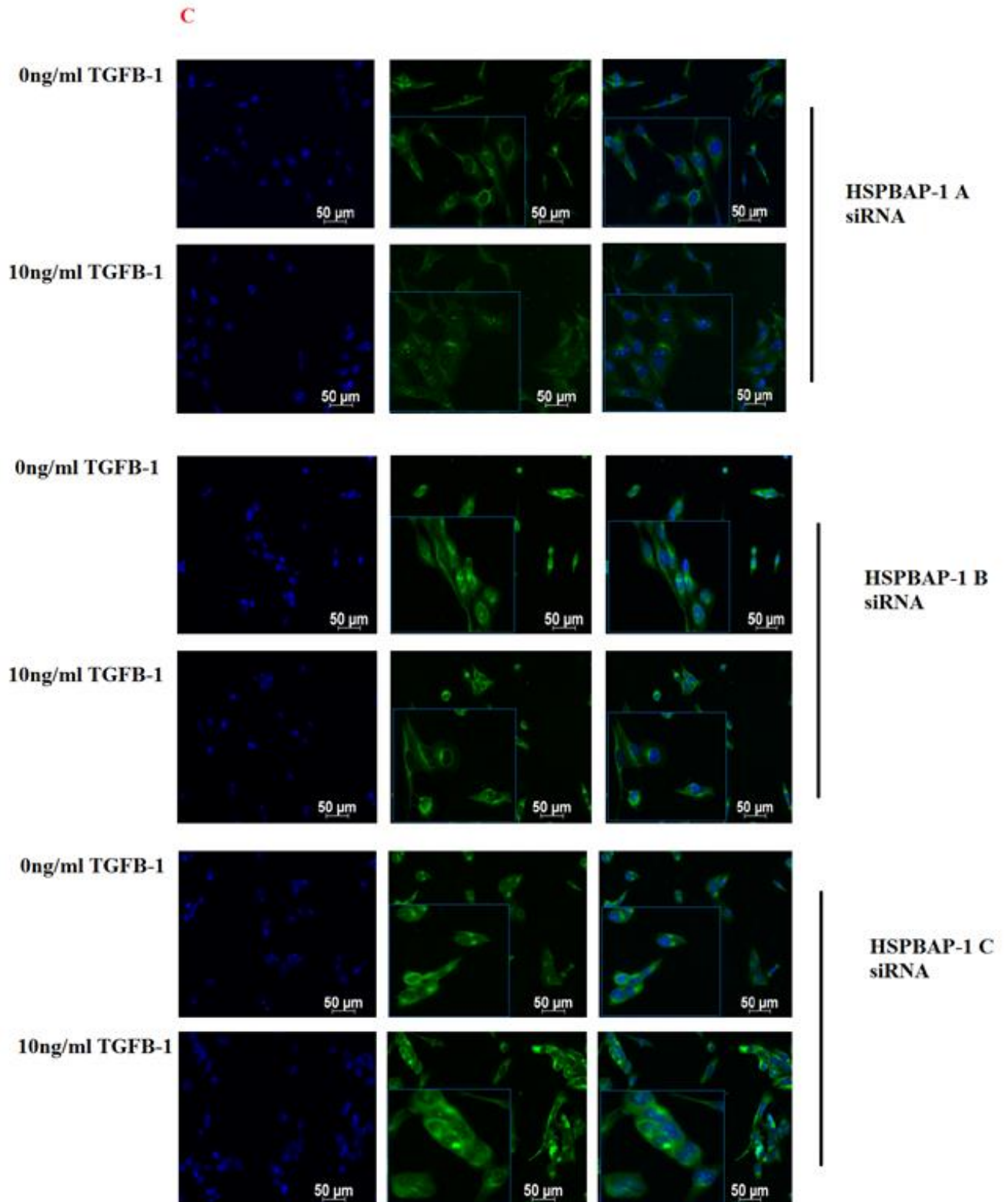
Figure 5.6C. HSPBAP-1 knockdown failed to influence SMAD3 nuclear translocation. Cells were prepared as in *A* but transfected with HSPBAP-1 siRNA stained for SMAD3 and visualised by confocal microscopy. Magnification was 20X.

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5.7 Discussion

TGF β-1 / SMAD3 signaling plays a central role in renal fibrosis (Zhang, Wang et al. 2015). Improvements in our understanding of the regulatory mechanisms governing TGF β-1 / SMAD3 signalling in CKD offers the prospect of delivering new treatment for CKD, in the hope of preventing renal failure. Disrupting the TGF β-1 / SMAD3 signalling cascade can attenuate renal fibrosis in animal models (Inazaki, Kanamaru et al. 2004). This suggests that inhibition of SMAD3 would have clinical utility in the prevention or treatment of fibrosis. Unfortunately, no SMAD3 inhibiting drugs have been developed for clinical use. Identification of factor(s) involved in the regulation of SMAD3 signalling could therefore provide a novel way to attenuate TGFβ signalling in the treatment of CKD.

HSPBAP1, heat shock protein 27 associated protein 1 (also known as PASS1), is a member of the JmjC family and was identified in a yeast two-hybrid screen using heat shock protein 27kDa (HSP27) as lure. HSPBAP1 orthologues exists from fly to humans (Liu, Gilmont et al. 2000). HSPBAP1 localises to the cytoplasm, and is expressed in many different tissues. HSPBAP1 inhibits the capacity of HSP27 to protect cells against sublethal heat shock (Bodmer, Schepens et al. 2003). It has been shown that HSPBAP1 is implicated as a fusion partner of disrupted in renal carcinoma 3 (DIRC3) in familial renal cancer (Bodmer, Schepens et al. 2003), suggesting that HSPBAP1 may also have a role in the development of cancer. HSPBAP1 orthologues contain residues that are predicted to be important for cofactor binding, demonstrating that this protein might establish functional hydroxylases with enzymatic roles in the cytoplasm (Klose, Kallin et al. 2006). It has also been suggested that HSPBAP-1 might have a role in the cellular stress response. Although it has been suggested thst HSPBAP-1 has demethylase activity its substrares have not been identified (Johansson, Tumber et al. 2014). HSPBAP1 is expressed in neuronal and glial cells in the temporal lobe of patients with intractable epilipsy (IE), at a tenfold higher level than in normal controls, indicating that HSPBAP-1 may play a role in the

development of epileptic seizures in patients with cell loss in this brain region (Xi, Wang et al. 2007). Another JMJD protein, JMJD2D, was shown to form a complex with P53 (Kim, Oh et al. 2012), but this has not been shown for HSPBAP-1.

The data I have collected in this chapter shows that the putative demethylase HSPBAP-1 is involved in TGF β-1 / SMAD3 signalling. This is the first report that HSPBAP-1 and SMAD3 interact, and the first data implicating a potential demethylation event in TGF β-1 / SMAD3 signaling.

It is currently unclear as to precisely how HSPBAP-1 is able to regulate the transcriptional activity of SMAD3, but is appaears to have a consistent repressive effect on SMAD3 transcriptional activity. One possibility is that HSPBAP-1 is able to demethylate histones within the locality of TGF β-1 target genes resulting in transcriptional repression as an *indirect* means of SMAD3 regulation. Alternatively, HSPBAP-1 might downregulate SMAD3 activity *directly*; of note HSPBAP-1 did not appear to influence the nuclear import of SMAD3, albeit within the limitations of the assay used. An alternative cell type in which to study SMAD3 translocation might be more useful, although THMCs have previously been shown to respond to TGF β-1 (Hayashida, Decaestecker et al. 2003). It is currently unclear whether or not the SMAD3 protein undergoes direct methylation and therefore the role for HSPBAP-1 demethylase activity within that context is unknown.

Interestingly, the addition of exogenous TGF β-1 to cells did not appear to alter the HSPBAP-1 – SMAD3 interaction by co-immunoprecipcitation. This is perhaps suprising given that HSPBAP-1 appears to have a regulatory role in the TGF β-1 / SMAD3 signalling, although for some reason, only the lower band of HSPBAP-1 was pulled down by SMAD3 in both cell types. One explanation for this might be that cells exhibit a constitutive interaction between HSPBAP-1 and SMAD3, perhaps in the cytoplasm, that is unaltered in response to TGF β-1 so

that whilst HSPBAP-1 can repress SMAD3 activity, it is the recruitment of additional activating factors such as p300 (Shen, Hu et al. 1998), in response to TGF β-1, that transforms SMAD3 into an active state, rather than the loss of transcriptional repressors such as HSPBAP-1. This hypothesis awaits formal testing. Of note, the HSPBAP-1 – SMAD3 interaction was detectable in both tubular cells and mesangial (THMC) cells that have differing functions within the kidney; whilst tubular cells are responsible for water and solute handling, mesangial cells provide a structural framework to support blood capillaries within the glomerulus. The extent to which the role of TGF β-1 signalling in these cells differs, and its effect upon cell fate is unknown. Whilst TGF β-1 is likely to promote pathological glomerular scarring (glomerulosclerosis) in mesangial cells, the previously documented effects of TGF β-1 on epithelial-to-mesenchymal transition in tubular cells *in vitro* has been questioned recently (Qian, Feldman et al. 2008). The role for the HSPBAP-1 – SMAD3 interaction between the two cells types therefore remains largely unknown but requires exploration in the context of glomerulosclerosis, a subtype of renal fibrosis.

It was expected that SMAD3 will undergo nuclear translocation in response to HSPBAP-1 knockdown, in the presence of TGF β-1, however immunofluorescence results showed only minimal SMAD3 nuclear translocation. This could not be explained by the level of knockdown, as the levels of HSPBAP-1 were successfully reduced upon the transfection with siRNA. One possibility that SMAD3 nuclear translocation is intrupted by another protein such as; HSP72, that is known to assist in protein folding and facilitates nuclear translocation (Zhou, Mao et al. 2010). Moreover, it has been shown that HSP72 overexpression inhibited TGF-β1-induced phosphorylation and nuclear translocation of SMAD3 and p-SMAD3 in renal epithelial-to-mesenchymal transition (Zhou, Mao et al. 2010).

Future work should focus on the mechanisms by which HSPBAP-1 can regulate SMAD3 and whether the activity and / or expression of HSPBAP-1 is altered in fibrotic diseases including CKD. Whilst pharmacological inhibition of HSPBAP-1 is theoretically undesirable, based on the data shown here, understanding the interaction between HSPBAP-1 and SMAD3 will further our understanding of the regulatory factors involved in TGF β-1 – SMAD3 signalling, in the hope of being to somehow disrupt this pathway in the future treatment of CKD.

6 GENERAL DISCUSSION AND CONCLUSION

Chronic kidney disease (CKD) is a worldwide public health problem that occurs as a result of an array of heterogeneous disorders affecting renal structure and function(Levin and Stevens 2011). At the glomerular, tubulointerstitial and vascular levels, TGFβ has been is a key player in many pathological events related to in CKD progression (Lopez-Novoa, Rodriguez-Pena et al. 2011) and TGFβ overexpression in animal models results in renal fibrosis (Mozes, Bottinger et al. 1999).Despite this recognised role of TGFβ in fibrosis, drugs that selectively target TGFβ signaling are not yet clinically available. Enzymatic modification of histones by methylation, phosphorylation, and acetylation alters chromatin structure and therefore the binding of transcriptional activators and repressors (Berger 2007). Methylation of different lysine residues in histone tails can serve as an activator or repressor by mediating topological changes in individual nucleosomes and directing chromatin dynamics (Rice, Briggs et al. 2003). SET9 was the first lysine methyltransferase (KMT) discovered to mono-methylate lysine-4 of histone 3 (H3K4), a marker for transcriptional activation (Nishioka, Chuikov et al. 2002). SET9 has been shown to be involved in many signaling and disease pathways (El-Osta, Brasacchio et al. 2008, Li, Reddy et al. 2008, Sun, Reddy et al. 2010). SET9 methyltransferase was also shown to be involved in activation of the collagenase gene, and it is recruited early after stimulation

to the collagenase promoter and that, as well as dimethylation, trimethylation of lysine 4 of histone H3 is also involved in collagenase activation (Martens, Verlaan et al. 2003).

SET9 can modify STAT signaling. It has been shown SET9 is responsible for the dimethylation of K140 of STAT3 in cells (Yang, Huang et al. 2010). Previous studies demonstrated that SET9 is required for the development of pulmonary fibrosis, and SET9 was proposed as a potential therapeutic target for treatment of the disease (Elkouris, Kontaki et al. 2016) . The methyltransferase SET9 has been indicated to potentiate TGF-β signaling by targeting SMAD7, an inhibitory SMAD. Methylation of SMAD7 promotes interaction with the E3 ligase Arkadia and, therefore, ubiquitination-dependent degradation of SMAD7. Depletion or pharmacological inhibition of SET9 results in elevated SMAD7 protein levels and inhibits TGF-β-dependent expression of genes encoding components of ECM (Elkouris, Kontaki et al. 2016) . Loss of SET9 has been shown to protect against bleomycin- or Ad-TGF-β-mediated pulmonary fibrosis in mice, with the pathological features of interstitial thickening, alveolar collapse, and the presence of cystic air spaces highly reduced in SET9-deficient mice (Elkouris, Kontaki et al. 2016). Excessive production and accumulation of ECM proteins is the hallmark of fibrotic diseases, and my findings suggest that SET9 function is required for TGF-β-dependent activation of ECM genes.

Furthermore, a role of SET9 in the regulation of reactive oxygen species (ROS) mediated signaling has been considered. The depletion of SET9 with siRNA or a SET9 small molecule inhibitor in both macrophages and a human bronchial epithelial cell line (Beas-2B) were able to counter NF-κB-induced oxidative stress and pro-inflammatory cytokine production (He, Owen et al. 2015). Methylation through SET9 has also been shown to be important for the modification of ROS signaling via its regulation of mitochondrial function, pro-inflammatory responses and the NFE2L2/ARE pathway. Furthermore, inhibition of SET9 may lead to up-

regulation of multiple antioxidant genes and improved ROS clearance, suggesting the potential advantage of targeting SET9 in treating ROS-associated diseases (He, Owen et al. 2015).

Data from this thesis has identified SET9 as a key regulator of SMAD3 in the TGF β-1 signalling pathway. This makes SET9 a potential therapeutic target for treatment of the CKD and other disorders that rely upon TGF β-1 signalling. (Levin and Stevens 2011).

I have shown here that overexpression of wild type, but not a methyltransferase-deficient form of SET9 results in increased SMAD3 transcriptional activity. These are the first showing that SET9 acts as a co-activator for SMAD3 and the first methyltransferase enzyme described as being involved in TGF β-1 / SMAD3 signalling. Moreover, depletion of SET9 by siRNA knock down reduced SMAD3 transcriptional activity in the presence of TGF β-1, supporting the hypothesis that SET9 is also required for the transcriptional activity of SMAD3.

SET9 has been implicated in various diseases (Sun, Reddy et al. 2010) althoughSET9 knockout mice do not exhibit any phenotypic changes compared to WT mice. During the course of this investigation, an independent study has shown that SET9 is be involved in renal fibrosis *in vivo* (Sasaki, Doi et al. 2016). Here I examined a human mesangial cell model of renal fibrosis (THMC). Mesangial cell proliferation and matrix overproduction, driven by TGF β-1 are predominant pathological features of glomerular sclerosis, a histological hallmark of numerous kidney diseases that result in end stage renal failure, for example diabetic nephropathy.. Immunofluorescence carried out in this project showed reduced expression of the pro-fibrotic markers α-SMA, collagen I, collagen III, fibronectin and PAI-1 in THMCs transfected with SET9 siRNA which is consistent with previous studies showing that SET9 null mice showed suppressed expression of pro-fibrotic genes including collagen (Sasaki, Doi et al. 2016). SMAD3 siRNA transfected cells have also shown a decrease in the synthesis of pro-fibrotic proteins in response to TGF β-1. These results suggest that SET9-dependent methylation and

consequent regulation of SMAD3 function plays a central role in TGF β-1-dependent activation of ECM proteins and the development of renal fibrosis in mesangial cells.

Fibrosis develops in response to persistent tissue injury, or when the normal wound healing process is uncontrolled. Cellular migration into the injured tissue accounts for the presence of activated fibroblasts or mesangial cells, the cell types responsible for fibrosis. I therefore examined whether SET9 also plays a role in cell migration. Wound healing experiments showed significantly reduced cell migration in THMCs transfected with SMAD3 siRNA or treated with SET9 inhibitor (R-PFI2) in the presence of TGF β-1. In agreement, a study has shown SMAD3 null mouse fibroblasts compared to corresponding wild type controls showed reduced migratory activity (Dobaczewski, Bujak et al. 2010). Together, the data demonstrate that SET9 and SMAD3 have similar regulatory roles in cell migration, which can be explained by their protein-protein interaction in the presence of TGF β-1. Whilst the data suggest that SET9 inhibition could be valuable in preventing fibrosis, the role of SET9 in normal wound healing should be examined.

To identify demethylases required for TGF β-1 / SMAD3 signalling, an siRNA screen was performed which identified HSPBAP1 as a repressor of SMAD3 activity. A previously published study used a similar methodology to identify other SMAD3-regulatory factors (Inui, Manfrin et al. 2011). HSPBAP1 chromosomal aberrations have been found in renal carcinoma (Bodmer, Schepens et al. 2003). The conserved sequences of human HSPBAP-1 makes it a putative demethylases enzyme, although its demethylase activity has not been confirmed yet. This study showed that HSPBAP-1 interacts with SMAD3, and suppresses the transcriptional activity of SMAD3-driven reporter-genes. This is the first report that HSPBAP-1 and SMAD3 interact, and the first data implicating a potential demethylation event in TGF β-1 / SMAD3 signalling. However, the mechanism(s) by which HSPBAP-1 regulates SMAD3 transcriptional activity requires further investigation. HSPBAP-1 may demethylate histones within the locality

of TGF β-1 target genes resulting in transcriptional repression as an indirect means of SMAD3 regulation. Another possibility is that HSPBAP-1 might directly contribute to the regulation of SMAD3 transcriptional activity; of note HSPBAP-1 did not appear to be involved in the nuclear import of SMAD3, albeit within the limitations of the assay used. I demonstrated a steady-state interaction between SMAD3 and HSPBAP1, by co-immunoprecipitation that did not seem to change in response to TGF β-1. It might therefore be possible that, rather than the loss of transcriptional repressors such as HSPBAP-1, SMAD3 is activated in response to TGF β-1 by recruitment of activating factors such as p300 (Feng, Zhang et al. 1998). Future work should be directed at determining whether HSPBAP-1 loss triggers key TGF β-1 driven processes within fibrosis such as ECM production, or fibroblast migration. Exploring the precise mechanisms by which HSPBAP-1 is involved in the regulation of SMAD3 transcriptional activities were unfortunately beyond the time constraints of the present study.

In conclusion, data from this thesis has suggested that methylation is involved in the regulation of SMAD3 transcriptional activity;

Taken together, the work in this study defines a novel role of SET9 and HSPBAP-1 in fibrosis by involvement in TGF β-1 / SMAD3 signalling. The novel impact of SET9 in the process of wound healing warrants further evaluation of SET9 as a target in the treatment of fibrotic diseases such as CKD. Other methyltransferases might also play a role in TGFβ/SMAD signalling, and represent potential therapeutic targets.

7 FUTURE WORK

Future work will be directed towards exploring SET9 expression in diseased kidneys, in order to understand the contribution of SET9 to fibrosis and CKD.

A similar assessment of HSPBAP-1expression in diseased kidneys might allow an appreciation of the importance of methyltransferase versus demethylase activity in CKD. Moreover, Future work could explore the effects of blocking HSPBAP-1 on SMAD3 localisation and wound healing assays. Using immunofluorescence for SMAD3, in fibroblasts, it is expected that SMAD3 will undergo nuclear translocation in response to HSPBAP-1 knockdown, in the presence of TGF β-1. It is also expected that HSPBAP-1 siRNA will accelerate wound healing. Using an animal model of UUO or HSPBAP-1 knock out mice would also help to identify/validate the role of HSPBAP-1.

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