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SGLT2 Dependent Novel Pro-Fibrotic Signalling in Proximal Tubule Epithelial Cells

A thesis submitted by

Xinlu Pan

In fulfilment of the requirements for the degree of Doctor of Philosophy

At

St George's, University of London

Cranmer Terrace

London

SW17 0RE

And

South West Thames Institute for Renal Research

St Helier Hospital

Surrey

SM5 1AA

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Statement of Copyright

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Declaration

This thesis is the independent work of the author under the advice and guidance of PhD supervisors Dr Mark Dockrell, Professor Deborah Baines and Dr Mysore Phanish.

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Abstract

INTRODUCTION: Diabetic kidney disease (DKD), mediated by both hyperglycaemia and transforming growth factor beta 1 (TGF- β 1), is the principal cause of end stage renal disease (ESRD). Tubulointerstitial fibrosis is the best prognostic indicator of renal function in diabetic patients. Drugs targeting the sodium glucose co-transporter 2 (SGLT2) at the proximal tubule are successful anti-diabetic therapeutics. This thesis aims to establish a cellular DKD model for studying the novel glucose/TGF- β 1 pathway and its regulation by SGLT2 inhibitors.

METHODS: Human primary proximal tubule epithelial cells (PTECs) were cultured and treated with D-glucose at either 7mM (control), 25mM (hyperglycaemia), or 7mM D-glucose+18mM L-glucose (osmotic control), with or without TGF- β 1 (0.75-5ng/ml). SGLT2 was inhibited by dapagliflozin (0.1-10nM), SGLT1 by phlorizin (500 μ M), facilitative glucose transporter 1 (GLUT1) by phloretin (1mM) and extracellular regulated signal kinase (ERK) by U0126 (10 μ M). PTECs were transfected with a Smad3 serine 204 (S204) dominant negative (DN) construct (0.5-2 μ g). Proteins were examined by western blotting, gene transcription by qPCR and dapagliflozin breakdown by high performance liquid chromatography (HPLC).

RESULTS: High D-glucose (25mM) and TGF- β 1 (0.75ng/ml) induced connective tissue growth factor (CCN2), fibronectin and phosphorylated Smad3 S204. These were all attenuated in the presence of dapagliflozin. Smad3 S204 and ERK phosphorylation was attenuated by U0126. The DN S204 construct prevented CCN2 but not fibronectin transcription. HPLC showed a time dependent decrease in dapagliflozin concentration in the medium. Urinary CCN2 and fibronectin protein were detected in the DKD patient group with high albumin to creatinine ratios (ACR) (median 336) and low estimated glomerular filtration rates (eGFR) (<60ml/min/1.73m²).

CONCLUSION: This work describes a role for SGLT2 in regulating select pro-fibrotic signalling outcomes and provides evidence for a novel disease pathway in DKD PTECs. The reduction of fibronectin and CCN2 by dapagliflozin provides strong support for the reno-protective benefits of SGLT2 inhibition in DKD.

Main Aims

The main aims of this thesis are to address the following questions:

- Are PTECs a suitable model of DKD?
- How does treatment with glucose and TGF- β 1 affect CCN2, TGF- β receptor type II, p21, K-Cadherin and fibronectin protein abundance?
- What are the effects of SGLT2 inhibition on CCN2 and fibronectin abundance?
- What are the mechanisms behind glucose-mediated TGF- β 1/Smad3 induced CCN2 signalling?
- Are there raised/measurable urinary levels of CCN2 and fibronectin protein in DKD patients?

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Published abstracts

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Pan, X., Baines, D., Phanish, M. and Dockrell, M.E., 2018. Inhibition of Glucose Transporters Ameliorates Fibrogenic Signaling in a Cellular Model of Diabetic Nephropathy: FR-PO419, 3023978.

Pan, X., Baines, D., Phanish, M. and Dockrell, M.E., 2017. Osmotic Pressure Increases TGF β 1-mediated Loss of SGLT2 Expression in Human Proximal Tubule Cells. Journal of the American Society of Nephrology: TH-PO395, 2788704.

Oral Presentations at Research Conferences

Dapagliflozin May Attenuate Renal Fibrosis by Inhibition of Novel Smad3 Linker Region Phosphorylation, UK Kidney Week 2020, Best Science Abstracts Session.

SGLT2 Mediated Fibrosis in a Cellular Model of Diabetic Kidney Disease: A Delineation of Specific Pathways Involved, European Renal Cell Study Group 2019.

Distinguishing the Metabolic and Osmotic Effects of Raised Glucose in Human Proximal Tubule Cells, UK Kidney Week 2020, Young Investigator Award Session.

Abbreviations

ATPase	Adenosine triphosphatase
AKI	Acute kidney injury
AGE	Advanced glycation end-product
Ang II	Angiotensin II
ACEi	Angiotensin converting enzyme inhibitor
ARB	Angiotensin Receptor Blocker
ANOVA	Analysis of variance
Ab	Antibody
ACR	Albumin to creatinine ratio
Asn	Asparagine
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BMP	Bone morphogenic protein
CKD	Chronic kidney disease
cDNA	Complimentary DNA
CTGF (CCN2)	Connective Tissue Growth Factor
Co-Smad	Common mediator Smad
C ^t	Threshold cycle
dNTP	Deoxynucleotide triphosphate
DAG	Diaglycerol
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulfoxide
dsRNA	Double stranded RNA
EDA-FN	Extra domain A fibronectin
EDTA	Ethylene diaminetetraacetic acid
E.coli	Escherichia coli
eGFR	Estimated glomerular filtration rate
EGF	Epidermal growth factor
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal regulated kinase
ESRD	End stage renal disease
FBS	Fetal bovine serum
FN	Fibronectin
GFR	Glomerular filtration rate
GBM	Glomerular basement membrane
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Facilitated glucose transporter

GTPase	Guanosine triphosphate
GMNA	Glutamyl-gamma-4-methoxy-napthalamide
HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cells
HK-2	Human kidney-2 cells
HKC-8	Human kidney cell-clone 8
IGF	Insulin growth factor
JNK	c-Jun N-terminal kinase
kDa	kilo-Dalton
KIM-1	Kidney injury molecule-1
LAP	Latency associated peptide
ILBP	Latency binding protein
LTBPs	Latent transforming growth factor binding proteins
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
MMP	Matrix metalloproteinase
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NGAL	Neutrophil gelatinase associated lipocalin
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PTEC	Proximal tubule epithelial cells
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PKC	Protein kinase C
PVDF	Polyvinylidene difluoride
qPCR	Quantitative real time polymerase chain reaction
RAAS	Renin-angiotensin-aldosterone system
RNA	Ribonucleic acid
R-Smad	Receptor-activated Smad
RT	Reverse transcription
ROS	Reactive oxygen species
RISC	RNA induced silencing complex
SDS	Sodium dodecyl sulphate
SD	Standard deviation
siRNA	Small interfering RNA
α -SMA	α -Smooth muscle actin
Smad	Sons of mothers against decapentaplegic
SV40	Simian virus 40

SGLT	Sodium glucose co-transporter
SLC	Small latent complex
TBS	Tris buffered saline
TGF- β 1	Transforming growth factor- β 1
THBS1	Thrombospondin-1
T β RI/T β RII	TGF- β type I/II receptor
TAK-1	TGF- β 1 activated kinase-1
TBS-T	Tris buffered saline-Tween 20
Taq	Thermus aquaticus
TNF- α	Tumour necrosis factor- α
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TmG	Tubular maximum glucose
UUO	Unilateral ureteral obstruction
VEGF	Vascular endothelial growth factor
Alpha	α
Beta	β

Chapter 1: Introduction

1.1. Diabetic Kidney Disease (DKD)

Diabetic kidney disease (DKD) is the chronic loss of renal function caused by diabetes mellitus (DM) (Lim, 2014). Not all individuals diagnosed with diabetes will progress further but approximately 20% to 30% of all DM patients will be diagnosed with DKD (Gheith *et al.*, 2016). As the worldwide prevalence of diabetes is expected to rise to ~578 million by the year 2030 (Saeedi *et al.*, 2019), the prevalence of DKD is also anticipated to increase, aggravating the current health crisis and putting further strain on public health systems. In a recent study shown, male and female patients on dialysis with end stage renal disease (ESRD) were found to have a worse unadjusted 5-year survival rate than patients with prostate and breast cancer, respectively (Naylor *et al.*, 2019).

1.1.1. Pathophysiology of DKD

In humans, DKD is typically characterised by glomerular/tubular hypertrophy, glomerulosclerosis, podocyte loss and tubulointerstitial injury. These changes are predominantly caused by hyperglycaemia (Toth-Manikowski and Atta, 2015). The clinical manifestation of these effects is the development of microalbuminuria plus a declining glomerular filtration rate (GFR). This eventually leads to overt proteinuria, progressive renal fibrosis, and ESRD. (Cravedi and Remuzzi, 2013). The pathophysiology of DKD also involves various processes like lipotoxicity, inflammation, hypoxia and altered tubuloglomerular feedback.

In a healthy kidney, albumin does not typically enter into the bowman's space/glomerular ultrafiltrate. Podocytes, located on the glomerular basement membrane, play an essential role in maintaining the integrity of the glomerular filtration barrier and preventing albumin entry into the glomerular ultrafiltrate (Coward *et al.*, 2007). In a diabetic kidney, hyperglycaemia induces podocyte depletion, apoptosis and foot process effacement, leading to excess albuminuria build up. Albuminuria is thought to drive mesangial proliferation, glomerulosclerosis, and tubular damage (DeFronzo, Reeves and Awad,

2021). Tubular damage is also thought to contribute towards albuminuria as PTECs are responsible for the reabsorption, processing and degradation of albumin. The albumin that is leaked through the glomerular capillaries is bound to the megalin/cubulin complex receptor in preparation for proximal tubule (PT) uptake. The albumin enters the PTECs via clathrin mediated endocytosis where it is targeted for lysosomal degradation. The fragments that remain are either reabsorbed or transcytosed into the tubular lumen (Dickson *et al.*, 2014; Nielsen, Christensen and Birn, 2016). Tubular damage is detected through the presence of markers like neutrophil-gelatinase associated lipocalin (NGAL) in urine (Charlton, Portilla and Okusa, 2014). The uptake of NGAL in healthy PTECs is also mediated by megalin (Mahadevappa *et al.*, 2013). The importance of the PT in DKD will be discussed in more detail in section 1.2.

During DKD, abnormal lipid metabolism leads to excessive lipid and free fatty acid accumulation in non-fat cells. This eventually leads to lipotoxicity and further renal damage by inducing inflammation, oxidative stress, and cell death (DeFronzo, Reeves and Awad, 2021).

The diabetic kidney also experiences increased glucose and sodium filtered load and thus increased reabsorption of glucose and sodium via SGLT2 in the PT. The mechanism is dependent on the consistent activity of the sodium/potassium ATPase (Na^+/K^+ ATPase) pump. Therefore, the increased reabsorption in the PT will lead to increased oxygen demand. This promotes a hypoxic environment which leads to further inflammation, fibrosis and the generation of reactive oxidative species (ROS). The increased sodium-glucose reabsorption also leads to a reduction in the delivery of sodium chloride to the macula densa. This drives glomerular hyperfiltration, over-activation of the renin-angiotensin-aldosterone system (RAAS), efferent arteriole vasoconstriction and afferent arteriole vasodilation (Carmines, 2010; Fioretto *et al.*, 2016; DeFronzo, Reeves and Awad, 2021). Figure 1.1 depicts the metabolic and hemodynamic changes that occur during DKD.

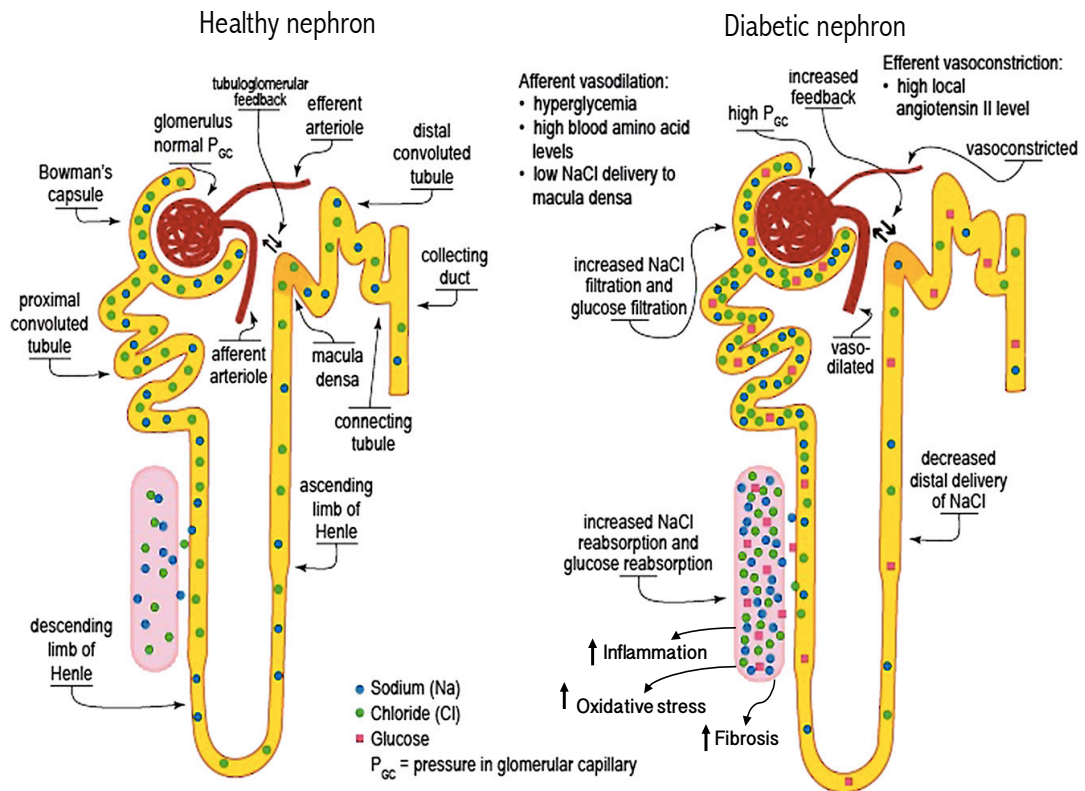


Figure 1.1: Comparison of the healthy and diabetic nephron.

Glomerular hyperfiltration causes an increase in glomerular capillary pressure which leads to an increase of NaCl and glucose filtration. This increases NaCl and glucose reabsorption by PTECs in the PT and subsequently leads to an increase in inflammation, oxidative stress and fibrosis. Furthermore, there is decreased distal delivery of NaCl to the macula densa. This altered tubuloglomerular feedback then causes efferent arteriole vasoconstriction and afferent arteriole vasodilation. Afferent vasodilation increases blood amino acid levels and efferent vasoconstriction overstimulates RAAS which leads to an overproduction of angiotensin II. Figure taken from (Tonneijck et al., 2017).

1.1.2. Natural history of DKD

DKD has traditionally been characterised by renal phenotypical and functional changes that are split into 5 progressive stages. This classical model (Figure 1.2) was primarily, although not solely, derived from epidemiologic studies focused on patients diagnosed with type 1 diabetes. Glomerular hyperfiltration was established as the first clinical stage of DKD, quickly followed by the incidence of microalbuminuria, thought to represent the first indications of renal dysfunction. Increasing albumin excretion rates eventually leads to the development of macroalbuminuria, which generally precedes GFR decline (Lin *et al.*, 2018). However, there is evidence demonstrating that patients with diabetes do not adhere to this exact sequence of events. Indeed, a cross-sectional study comparing the clinical presentation of DKD in patients with diabetes from the 1988-1994 period to the 2009-2014 period found that prevalence of albuminuria as a clinical marker of DKD progressively decreased over time (Afkarian *et al.*, 2016). This highlights the complex aetiology and dynamic nature of this disease. Thus, these stages and values are only to be observed as reference endpoints as each individual patient will differ in clinical presentation.

In the early stages (stage 1) of DKD, glomerular hyper-filtration is initiated ($\geq 90 \text{ ml/min/1.73m}^2$). Although it is probable that there is a slight increase in the albumin to creatinine ratio (ACR), it is not clinically detectable at this point and kidney function still remains normal. Structurally, one of the earliest changes that can be observed is hypertrophy of the kidney. Further along the line, stage 2 (also considered the “silent stage”) is characterised by mesangial expansion, glomerular basement membrane thickening. The GFR ranges between $60\text{--}89 \text{ ml/min/1.73m}^2$ (Marshall, 2016; Ma *et al.*, 2020; Kovesdy *et al.*, 2021). Thickening of the glomerular basement membrane is the earliest structural change that can be detected via microscopy (Fioretto and Mauer, 2007). Structural changes like mesangial expansion generally appear 5 or more years after the diagnosis of type 1 diabetes. The timeline for such changes is more variable for those diagnosed with type 2 diabetes, as the onset of the latter disease state is often unknown (Said and Nasr, 2016). The ACR is estimated to be $<30 \text{ mg/mmol}$ at this stage, and thus the patient is still considered normoalbuminuric with exception to transient episodes of microalbuminuria. While a large proportion of patients will remain in stage 2 indefinitely,

an estimated 1/3 will progress into stage 3 (Pugliese, 2014), the incipient DKD stage, approximately 10 years after diagnosis (Alicic, Rooney and Tuttle, 2017). During this phase, the ACR exceeds 30mg/mmol with a GFR ranging between 30-59ml/min/1.73m² (Alicic, Rooney and Tuttle, 2017). Patients are officially diagnosed with chronic kidney disease (CKD) when they have a GFR of <60ml/min/1.73m², ACR of >30mg/mmol and these values persist for longer than 3 months (Thomas et al., 2015). At stage 4 (overt DKD), blood pressure increases, and the ACR increases past 300mg/mmol, also known as macroalbuminuria (Pugliese, 2014). Other pathologic findings like glomerulosclerosis and inflammation are discovered in this stage. Furthermore, kidney complications like anaemia, bone and mineral metabolism dysfunction, and neuropathy also develop (Alicic, Rooney and Tuttle, 2017). Patients at stage 4 typically have a GFR of >30/min/1.73 m². This can continue for many years until the patient reaches ESRD, clinically manifesting as uraemia and a GFR of below 15ml/min/1.73m², approximately 25 years after initial diagnosis (Lim, 2014; Delanaye et al., 2016; Lin et al., 2018). The persistent increase of albuminuria is also paralleled with the development of microvascular complications like retinopathy (Pugliese, 2014). Patients who have reached stage 5 have lost almost all renal function and are put on haemodialysis (Pockros *et al.*, 2016). The prognosis of ESRD is poor and patient deaths due to complications of CVD and infection are highly prevalent (Alicic, Rooney and Tuttle, 2017). In the latter stages, excessive collagen/ECM deposition and tubular damage is also observed (Reidy *et al.*, 2014).

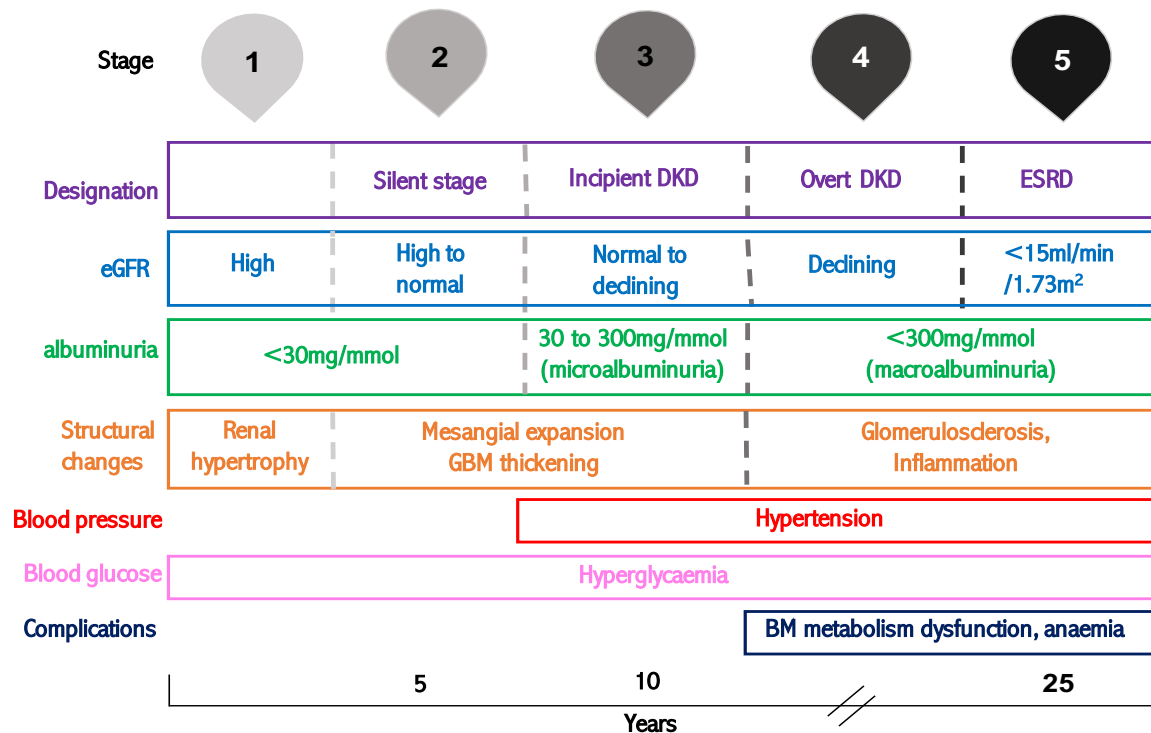


Figure 1.2. Natural history of DKD

Stage 1: High GFR (>90ml/min/1.73m²) is initiated, an increase in kidney size is observed, and albuminuria values (<30mg/mmol) are considered normal; Stage 2: the GFR ranges between high to medium and structural changes like mesangial expansion and glomerular basement membrane thickening take place; Stage 3: hypertension occurs, GFR begins to decline, and persistent increases in albuminuria leads to microalbuminuria; Stage 4: microalbuminuria develops into macroalbuminuria, the GFR declines further, and other kidney complications like anaemia and bone and mineral metabolism dysfunction develop; Stage 5 (ESRD): the GFR declines to <15ml/min/1.73m², leaving the kidneys unable to function sufficiently for the patient to survive without renal replacement therapy.

1.1.3. Treatment for DKD

Currently, there are limited clinical therapeutic interventions approved that safely ameliorate the progression of DKD. The basis of treatment for DKD is the control of common risk factors such as hyperglycaemia and hypertension (Patney, Whaley-Connell and Bakris, 2015). Glycaemic control, generally achieved with anti-diabetic therapies, has been established as a common medical intervention. In a large scale study conducted on patients diagnosed with insulin-dependent diabetes (IDDM), researchers found that there was a significant reduction in the incidence of albuminuria for patients undergoing

intensive insulin treatment and rigorous blood-glucose monitoring (Diabetes Control and Complications Trial Research Group *et al.*, 1993). A separate group carried out a clinical trial with sitagliptin, a dipeptidyl peptidase 4 (DPP-4) inhibitor commonly administered to diabetic patients, to determine whether there were any changes relating to diabetes induced complications. They found that sitagliptin significantly reduced albuminuria but there was no change in the GFR. They postulated that this was likely a result of improved glycaemic control (Mori *et al.*, 2014). More recently, Kwon *et al.* carried out a large cohort study to analyse the efficacy of metformin, a first line drug for long term diabetes, in treating DKD patients. They found that metformin significantly reduces the incidence of ESRD as well as all-cause mortality. Additionally, they found that there was no increased risk of lactic acidosis, a serious complication arising from the use of metformin (Kwon *et al.*, 2020).

RAAS inhibitors are common therapies used in DKD treatment. As mentioned above, the RAAS system is activated as a response to low blood pressure and reduced sodium chloride delivery to the macula densa. Renin released into the blood first targets angiotensinogen which is produced by the liver. Renin then converts angiotensinogen to angiotensin I. Angiotensin I is converted to angiotensin II (Ang II) by angiotensin converting enzyme (ACE) (Remuzzi *et al.*, 2005). Over-stimulation of the RAAS which results in excessive angiotensin II production, majorly contributes to the development of numerous microvascular diseases. Dysregulation and unceasing stimulation promotes oxidative stress, inflammation and an increase in glomerular capillary pressure, which all contribute towards glomerular and tubular hypertrophy (Lozano-Maneiro and Puente-García, 2015). There are 4 recognised groups of RAAS inhibitors; ACE inhibitors (ACEi), angiotensin II receptor blockers (ARBs), direct renin inhibitors and mineralocorticoid receptor antagonists.

Hsu *et al.* analysed a large cohort of individuals diagnosed with diabetic and non-diabetic advanced CKD. Their trial data concluded that the use of ACEi and ARBs, through both dual and monotherapy, significantly reduced the incidences of long-term dialysis and prevented 5.5% of pre-dialysis patients from requiring long-term dialysis (Hsu *et al.*, 2014). A retrospective study carried out by Vejakama *et al.* found that the continued treatment of RAAS blockade over the span of one year significantly reduced the number of CKD patients who reached ESRD (Vejakama *et al.*, 2017). Conversely, a clinical trial for aliskiren, a direct renin inhibitor, found no differences in the renal composite outcome

in diabetic patients when comparing the drug group to the placebo group after a follow up of ~32 months (Parving *et al.*, 2012).

ACE inhibitor ramipril significantly reduces the risk of overt nephropathy after 4.5 years of treatment even after adjustment for blood pressure changes ('Effects of ramipril on cardiovascular and microvascular outcomes in people with diabetes mellitus: results of the HOPE study and MICRO-HOPE substudy. Heart Outcomes Prevention Evaluation Study Investigators.', 2000). Additionally, a different study found that ARB Candesartan and ACEi Lisinopril dual therapy steadily reduced the protein to creatinine ratio in patients suffering from advanced proteinuric nephropathy. Meanwhile, the blood pressure was only lowered moderately after a month and then maintained consistently throughout the trial (Luño *et al.*, 2002). These results support the basis that renoprotective RAAS inhibitor benefits may be independent of their hypotensive capabilities.

Overall, there have been considerable positive advancements made in delaying the development of DKD. However, there is still a continuously increasing prevalence of patients who reach ESRD, prompting the need for more effective and selective therapeutics that target DKD.

1.2. The importance of the proximal tubule in DKD

Although the paradigm of renal injuries often focuses on the glomerulus, it is well established that tubulointerstitial fibrosis is the recurring deleterious characteristic observed in all kidney disease and the best histological predictor for DKD survival (Hewitson, 2009; Mansour *et al.*, 2017). Grgic *et al* developed a mouse model using Six2-Cre-LoxP technology to selectively cause PT insult through the administration of diphtheria toxin (DT). Multiple injections of DT to the renal tubule epithelial cells caused glomerulosclerosis, tubulointerstitial fibrosis and inflammation (Grgic *et al.*, 2012). Due to this and the growing success of anti-diabetic therapies that target the PT, there is now also a focus on the PT and its role in the pathogenesis of diabetes affecting the kidney.

As discussed before, clinical markers like ACR rise and GFR decline are widely accepted as important clinical indicators of DKD. However, this has been disputed by others who have shown that 48% of diabetic patients with normal readings of urinary albumin excretion (UAE) (Krolewski *et al.*, 2014) had reached an advanced stage of DKD ($<60\text{ml/min/1.73m}^2$) without progressing to proteinuria (Perkins *et al.*, 2010). This suggests that progression of microalbuminuria to proteinuria may not be required for diabetic cohorts to reach ESRD and challenges the monopolisation of the glomerulus in discussions concerning DKD. There is also evidence to suggest that tubular injury precedes the development of albuminuria in patients with diabetes (Fiseha and Tamir, 2016).

A group analysing localised areas of apoptosis in renal biopsy samples from DKD patients found distinct cell death in the PT but not the glomeruli (Kumar, Robertson and Burns, 2004). Similarly, they also discovered that streptozotocin (STZ) induced diabetic mice presented with apoptosis of tubular but not glomerular cells (Kumar *et al.*, 2004). These data, along with the discovery of biomarkers of CKD like kidney injury model-1 (KIM-1) and NGAL which are of PT origin, have pushed a paradigm shift from a 'glomerulocentric' perspective to a 'tubulocentric' one.

KIM-1, NGAL and cystatin C are either derived from an impairment of PT function or are a result of PT injury (Charlton, Portilla and Okusa, 2014). KIM-1 is a type 1 transmembrane glycoprotein highly upregulated in PTECs upon renal insult, thus also making it a promising urinary marker candidate for early AKI. KIM-1 is not constitutively expressed in the healthy kidney (Zhou *et al.*, 2016). Sabbasetti *et al.* examined the serum KIM-1 levels of diabetic and non-diabetic CKD patient cohorts and found that the concentration of the biomarker was directly proportional to increasing CKD stages. Pertaining to the diabetic CKD cohort, they found that only approximately 20% of patients with low levels of serum KIM-1 would eventually advance to ESRD after 12.5 years. Their results give support to KIM-1 as an effective biomarker in CKD (Sabbasetti *et al.*, 2014).

NGAL can regulate epithelial morphogenesis in healthy kidneys. It stimulates the induction of epithelial tubules from mesenchymal cells, a principal feature of the renal development, and maintains epithelial structure thereafter (Gwira *et al.*, 2005). In cell lines

derived from the kidney, NGAL sequesters iron, localises to endosomes, and regulates the activity of iron-responsive proteins like ferritin (Schmidt-Ott et al., 2007). NGAL is also filtrated by the glomerulus and endocytosed at the apical membrane of the proximal tubules where it donates iron (Mori et al., 2005). It is expressed in large quantities in injured/inflamed renal tubule cells as well as hepatocytes and immune cells, making for easy detection in urine. Bolignano et al analysed the levels of urinary and serum NGAL in normoalbuminuric, microalbuminuric and DKD groups and found that while all three sets presented with high levels of the protein in comparison to the controls, there were increased levels of urinary and serum NGAL in type 2 diabetic patients without glomerular injury (Bolignano *et al.*, 2009).

A separate group examined for both urinary and serum NGAL in a type 2 diabetic patient group and found that the levels of both were positively associated with significantly greater albumin presence. Furthermore, they also found that the normoalbuminuric diabetic group still exhibited higher concentrations of urine and serum NGAL than their control counterparts, suggesting again that PT injury potentially precedes glomerular injury (Kaul *et al.*, 2018). NGAL can be employed as a useful early predictor of early renal injury in diabetic individuals, assuming that albuminuria is the result of glomerular damage.

Cystatin C is a small non-glycosylated 13kDa protease inhibitor that is ubiquitously expressed at a constant rate in all nucleating cell types. In healthy kidneys, it potently inhibits lysosomal cysteine proteases responsible for degrading proteins taken up by cells (Fried, 2009). It is freely filtered by the glomerulus prior to being reabsorbed and fully metabolised by the PT. As serum cystatin C abundance is not affected by factors such as age, gender, race, muscle weight or inflammation, it is considered a much more precise marker of GFR than serum creatinine (Murty *et al.*, 2013). Jeon et al reported that serum cystatin C levels were also significantly higher in normoalbuminuric patients with GFR values of $<60 \text{ mL/min/1.73 m}^2$ compared to those with GFR of $>60 \text{ mL/min/1.73 m}^2$. Similar to what was discussed above, the authors conclude that this is potentially a reflection of initial tubular injury proceeding glomerular changes (Jeon *et al.*, 2011).

1.3. Diabetes mellitus

Diabetes Mellitus (DM)/Diabetes, the main driver of DKD, is defined as a group of chronic and complex metabolic diseases mainly characterised by hyperglycaemia and an impaired ability to produce insulin (American Diabetes Association, 2014). It is estimated that approximately 3.8 million individuals in the UK live with Diabetes, with cases disproportionately affecting black, Asian and minority ethnic (BAME) and 40-59yr age groups (Pham *et al.*, 2019). On a larger scale, it is estimated that ~8% of the entire global population have DM, which corresponds to approximately 424 million people, not accounting for those that may be undiagnosed (Finer *et al.*, 2018, Standl *et al.*, 2019). It is also estimated that the total global diabetes-related healthcare expenditure will rise from 1.3 trillion US dollars back in 2015 to 2.2 trillion by 2030 (Bommer *et al.*, 2018).

The human body tightly monitors its blood glucose levels under normal circumstances and maintains a slim concentration range between 4-6mM. This glycaemic control is achieved through the feedback stimulation and release of hormones insulin and glucagon. Under a state of hypoglycaemia, usually when the body is asleep or fasting, glucagon, the body's main catabolic effector, is released by pancreatic α -cells. This promotes hepatic glycogenolysis, helping the liver to stimulate glycogen breakdown and allowing the release of glucose back into the bloodstream. Additionally, it also drives gluconeogenesis to synthesise glucose from non-carbohydrate sources like amino acids and lactate. Conversely, under a state of hyperglycaemia, usually after a meal, pancreatic β -cells will release insulin, the body's main anabolic effector, which facilitates the increased uptake of glucose into muscle cells, thereby reducing the plasma concentrations of glucose. If there is an excessive decrease of glucose, the pancreas will begin producing glucagon again (Röder *et al.*, 2016; Bich, Mossio and Soto, 2020).

1.4. Insulin resistance

As insulin is a key regulator of glucose homeostasis and metabolism, disruptions to insulin signalling can lead to a host of maladaptive changes (Whaley-Connell and Sowers, 2017). Patients diagnosed with diabetes are stratified into two main categories: type 1 diabetes, characterised by an absolute deficiency of insulin secretion and type 2 diabetes,

characterised by increased insulin resistance and impaired insulin secretion (Karalliedde and Gnudi, 2016). Insulin resistance (IR) describes the pathological condition where tissues experience reduced sensitivity to insulin-mediated actions as a result of the hormone's diminished capacity to exert its usual biological effect. IR is typically characterised by defects in glycogen synthesis, glucose uptake, glucose oxidation, and lipid oxidation suppression. This disorder prompts the pancreas to increase insulin synthesis and secretion in an effort to maintain normal blood glucose levels, leading to compensatory hyperinsulinemia (Ormazabal *et al.*, 2018; Schrauben *et al.*, 2019).

From a renal disease perspective, IR triggers various hemodynamic changes within the kidney which can lead to hypertension, atherosclerosis of the renal microvasculature, and inflammation (Semenkovich, 2006; Karalliedde and Gnudi, 2016). It is also with the development of glomerular hyperfiltration, an early hallmark of renal dysfunction, suggesting that it may have a role in triggering the cascade of events that lead to DKD (De Cosmo *et al.*, 2013). Early studies established that patients in the early stages of DKD are more prone to IR; Yip *et al.* found that IR was a common feature of patients with type 1 diabetes and microalbuminuria, while Parvanova *et al.* found that IR was an independent predictor of microalbuminuria development in patients with type 2 diabetes (Yip *et al.*, 1993; Parvanova *et al.*, 2006). Furthermore, a 6-year study focused on evaluating complications in patients with type 1 diabetes found that greater insulin sensitivity at baseline independently predicted a lower probability of developing albuminuria (Bjornstad *et al.*, 2016). Similarly, hyperinsulinemia is also linked to renal deterioration in patients with diabetes (Porrini *et al.*, 2009). It can drive insulin receptor degradation in podocytes (Lay *et al.*, 2017), which exacerbates albuminuria in mouse models of DKD (Madhusudhan *et al.*, 2015).

A recent study focusing on 3 large cohorts of patients newly diagnosed with diabetes further supported the association between insulin resistance and DKD. The study split patients into 5 different clusters, stratified by clinical characteristics, disease severity, and differing risk of developing diabetic complications. Patients from cluster 3, categorised by severe-insulin resistant diabetes, were associated with a higher risk of DKD, defined as persistent albuminuria, across all 3 cohorts (Ahlqvist *et al.*, 2018). Further research to better understand the extent to which insulin contributes to kidney disease progression is required to develop novel strategies to combat DKD.

1.5. Glucotoxicity

Glucotoxicity refers to the deleterious effects of supraphysiological glucose levels on the structure and function of various cell types. In the diabetic milieu, a decline in beta cell function and insulin secretion promotes hyperglycaemia. Chronic hyperglycaemia subsequently leads to diabetic microvascular complications (Chen *et al.*, 2016). Activation of diacylglycerol (DAG)-protein kinase C (PKC) and excessive production of advanced glycated end products (AGEs) are biochemical pathways that have been implicated in the development of glucotoxicity. The continuous activation of conventional PKC isoforms is acquired through *de novo* DAG synthesis (Noh and King, 2007). The DAG-PKC pathway then facilitates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation (Atsumi *et al.*, 2011). It is established that various PKC isoforms (α , β 1, β 2) are increased in rat and mouse models of diabetes mellitus (Geraldine and King, 2010). AGEs are secondary products formed when monosaccharides like glucose attach to proteins and lipids as a response to increased glucose. Thus, whilst PKC activation is a direct pathway that facilitates glucotoxicity, the overproduction of these glycated proteins is considered an indirect pathway (Yamagishi and Matsui, 2018). In chronic diabetes, AGEs are continuously created and aggregate within the bloodstream and nearby tissues, consequently allowing microvascular complications to develop (Papachristoforou *et al.*, 2020).

One of the main underlying processes that unifies these pathways is the formation of reactive oxygen species (ROS) (Giacco and Brownlee, 2010). Under normal metabolic events, ROS is also produced but this is generally combated through upregulation of antioxidant proteins (Chang and Chuang, 2010). An increase in ROS formation is thought to be one of the main mediators of diabetic-induced complications (Fakhrudin, Alanazi and Jackson, 2017). Under diabetic conditions, an elevated level of glucose will undergo glycolysis which in turn generates more acetyl-CoA and pyruvate. This subsequently increases the production of NADH. This excess supply of NADH subjects extra electron pressure on the mitochondrial electron transport chain, leading to pronounced oxidative stress and the formation of ROS (Yan, 2014). Experiments have shown that prolonged exposure to glucose leads to pre-diabetes, beta cell dedifferentiation and an increase in ROS stress. This was prevented by the administration of glutathione, a common anti-

oxidant (Zhang *et al.*, 2019). A different group also found that high glucose induced oxidative stress and apoptosis in RSC96 cells (Jiang *et al.*, 2020).

1.6. Glucose handling in PTECs

As previously discussed, chronic and persistent hyperglycaemia and/or hypoglycaemia often leads to a wide array of pathological complications. Glucose is essential as a source of energy for cellular metabolism, and the kidney is heavily involved in preserving plasma glucose homeostasis. This homeostasis must be maintained within a relatively narrow range in order to prevent pathological complications that may arise. In the context of the kidney, this maintenance is facilitated through three main physiological processes: gluconeogenesis, glucose reabsorption and glucose excretion (Gerich, 2010).

Glucose is released into the circulation at an approximate rate of 10 μ mol/kg/min during an overnight fast. Gluconeogenesis describes the metabolic process in which organisms synthesise glucose-6-phosphate from non-carbohydrate/non-hexose precursors such as amino acids, lactate and glutamine (Triplitt, 2012). With respect to glucose handling and consumption, the kidney essentially divides into two unique roles, with the renal cortex responsible for glucose release and the renal medulla responsible for glucose utilisation. This is a result of the differing distribution and localisation of various enzymes on the nephron surface. In the kidney, the renal cortex is the site for gluconeogenesis meanwhile the medulla is the site for glucose phosphorylation and glycolysis (Mota, 2015). It is thought that the kidney is responsible for 40% of all gluconeogenesis (Mather and Pollock, 2011).

As my thesis focuses on SGLT2 glucose handling, I am most interested in glucose reabsorption. Plasma glucose is not bound by protein, and so approximately 180g of glucose is freely filtered from the plasma by the glomeruli daily (Ghezzi, Loo and Wright, 2018). This would result in a major loss of within a matter of hours if left unchecked. Therefore, the main function of the kidney is to ensure that this entire quantity is reabsorbed by the PTECs in healthy individuals. Transport of glucose across the luminal / apical cell membrane of PTECs is facilitated by Na⁺/glucose transporters SGLT1 and SGLT2. Generally, the reabsorptive capacity of these transporters continues to amplify in correlation with the increase in circulating plasma concentration or GFR (Ferrannini,

2017). However, once a certain threshold is reached, glucose begins excreting into the urine. This threshold is referred to as tubular maximum glucose (TmG) (Figure 1.2). In healthy individuals, the plasma glucose concentration threshold for urine excretion is approximately 10mmol/L. This level can fluctuate depending on the physiological circumstances. For example, the plasma glucose threshold would elevate further before glycosuria is induced as these individuals have a lower GFR. Glycosuria may also be induced at a lower plasma glucose concentration when the conditions dictate a high GFR, for example during pregnancy (Mather and Pollock, 2011).

Glucose reabsorption leads to glucose build-up in the epithelium which in turn increases the concentration gradient between the blood and cells. This change in concentration then drives the glucose to exit the cell basolaterally via facilitative glucose transporters GLUT2 and GLUT1. GLUTs passively transport molecules in either direction across the cell membrane (Navale and Paranjape, 2016). Glucose transport is also mediated by the sodium/potassium (Na^+/K^+) ATPase pump, which uses 1 mole of adenosine triphosphate (ATP) to expel 3 sodium ions from the cell for every 2 potassium ions entered. This drives glucose uptake and ensures that the electrochemical sodium gradient is also conserved (Thorsen, Drengstig and Ruoff, 2014). Figure 1.3 depicts the glucose transport pathway in PTECs.

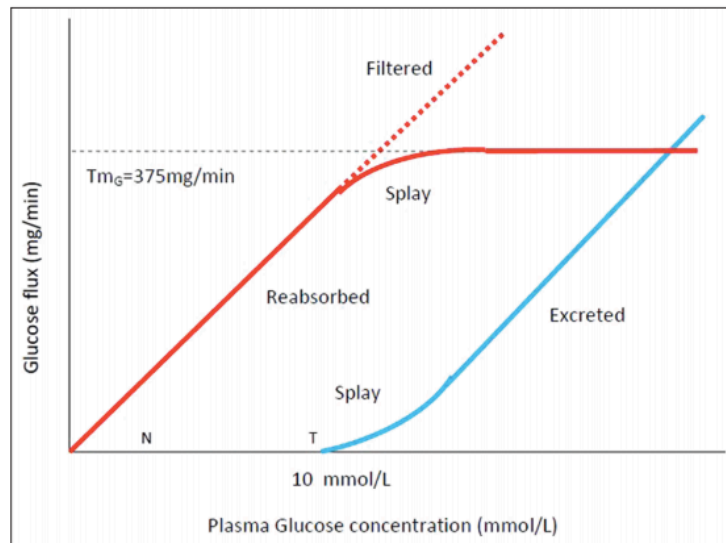


Figure 1.3: Glucose reabsorption curve.

The filtered glucose load increases in proportion to the circulating plasma glucose concentration and this is initially matched with the reabsorbed glucose load. When the plasma glucose reaches around the 10 mmol/L (200mg/dL) renal threshold, the glucose begins “spilling” into the urine. Once the T_{mG} (usually between 350 to 400 mg/min) is reached, the reabsorptive abilities of the glucose transporters become saturated. The T_{mG} is reached gradually during renal glucose handling as opposed to suddenly, accounting for the “splay” curve. Figure taken from (Poudel, 2013).

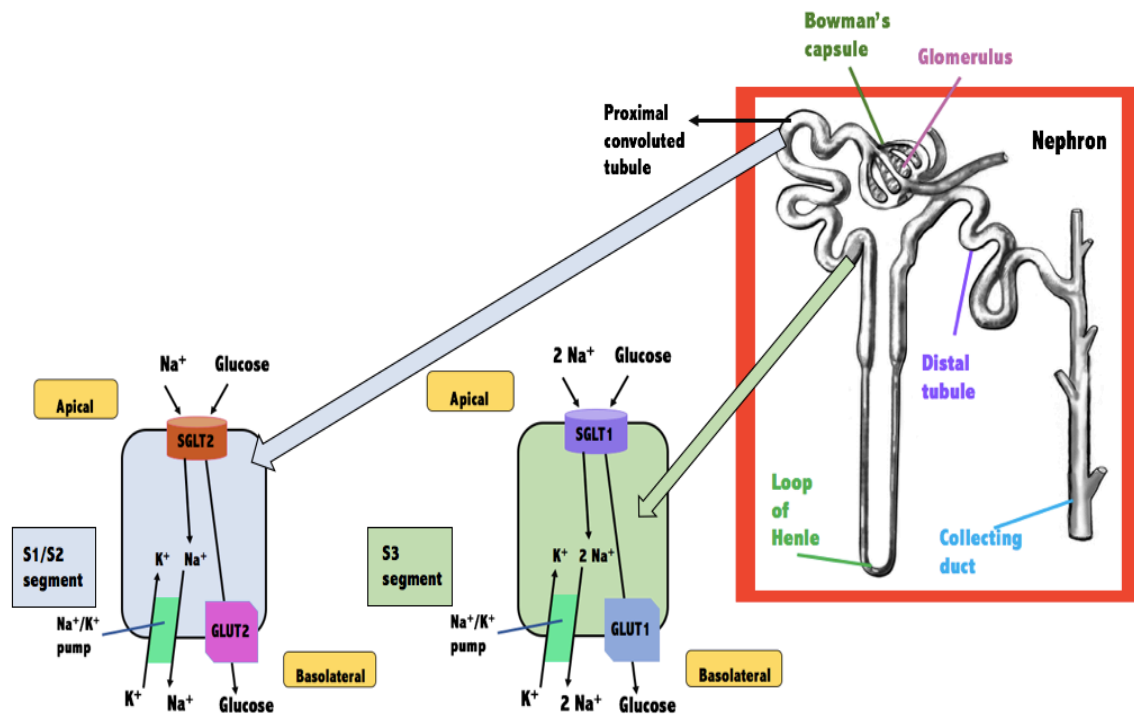


Figure 1.4: Glucose transport pathways in PTECs.

180g glucose is filtered every day and in healthy individuals, there is a negligible amount of glucose left to be secreted in the urine. The remaining glucose is reabsorbed as it flows from the glomerulus through to the proximal convoluted tubule, passing from the S1 to S3 segment. The concentration of glucose decreases rapidly during the transport through each segment. SGLT1 and SGLT2 first catalyse the active transport of the glucose by coupling it for downhill transport with sodium. Upon glucose entry, GLUT1 and GLUT2 then prepare to mediate the exit of the glucose basolaterally and back into the blood.

1.7. Sodium glucose transporters (SGLTs)

1.7.1. SGLT properties

SGLTs are encoded from the *SLC5* human gene family. The low affinity, high capacity SGLT2 is known as the principal renal glucose transporter as it is responsible for reabsorption of approximately 90% of the filtered glucose in the kidney (Lu *et al.*, 2014). It is primarily expressed in the apical brush border membrane of the S1 and S2 segments of the renal proximal convoluted tubules, although low levels of the protein have also been identified and located in the brain and liver. SGLT2 has a molecular weight of approximately 73kDa. It has a K_m of 6mM, maintains a 1:1 ratio of sodium to glucose coupling, and is highly selective for D-glucose, the most abundant monosaccharide (Hummel *et al.*, 2011). Contrarily, the outstanding glucose not reabsorbed by SGLT2 is taken up by its high affinity, low capacity counterpart SGLT1, primarily located on the S3 segment of the PT. Although SGLT1 shares approximately 60% structural homology and weighs the same as the former (Harada and Inagaki, 2012), it has a comparatively lower K_m of ~1mM and a 2:1 sodium to glucose stoichiometry ratio. Additionally, whilst SGLT2 is found almost exclusively in the kidney, SGLT1 can also be found in the liver and small intestine (Koepsell, 2020).

The secondary physical structure of this type of transporter has been studied although parts of its architecture are still yet to be fully clarified. Nevertheless, the basic core structure of SGLTs has been elucidated from the crystal structure of vSGLT2, a bacterial homolog of SGLT from the *Vibrio parahaemolyticus* organism. It is proposed to consist of 14 transmembrane (TM) domains (Figure 1.4), consisting of approximately 660-70 amino acid residues, with amino (NH₂) and carboxyl (COOH) termini groups opposing the extracellular side of the plasma membrane (Sabino-Silva *et al.*, 2010). The structure also contains an inverted topology motif where TMs 2-6 and 7-11 are conformed by an approximate 153-degree rotation angle. This “5 plus 5” inverted repeat TM is also highly conserved in the leucine transporter (Deng and Yan, 2016; Paz *et al.*, 2018). N-linked glycosylation is anticipated to take place between TMs 6 and 7 (Wright, Hirayama and Loo, 2007), specifically at asparagine (Asn) 248. A further diagram depicting the specific residues that facilitate sodium and glucose binding is included below in Figure 1.5.

Improving glycaemic control has long been considered the optimal approach to managing diabetes and its complications. However, the accumulating body of evidence over the years from clinical trials evaluating the effect of glycaemic control tell a seemingly different story. While guidelines and statements from medical journals overwhelmingly endorse intensive glycaemic control as a beneficial therapeutic strategy, data from various studies found no significant improvement in various microvascular complications like reduced risk of renal-related deaths or ESRD development following tight glycaemic control. Additionally, intensive glycaemic control did not assuage any macrovascular complications, with exception to non-fatal myocardial infarctions. Instead, the clinical intervention increased the risk of developing severe hypoglycaemia (Rodríguez-Gutiérrez and Montori, 2016). Therefore, the ideal treatment strategy must effectively reduce blood glucose levels and maintain a tolerable safety profile.

Mutations in the SGLT2 gene result in a condition called familial renal glycosuria. Individuals diagnosed report differing degrees of glucose excretion into the urine, but most are able to maintain a normal level of blood glucose with no serious health complications. In a molecular study, a 19-year-old female patient suffering from glycosuria and no prior medical history of any severe illnesses or surgeries was found to have two heterozygous SGLT2 gene missense mutations in exons 6 and 14. A full laboratory evaluation found no abnormalities in any major clinical parameters except HbA1c and insulin levels, which were slightly below the reference range (Kleta *et al.*, 2004). Furthermore, Jurczak *et al* conducted a study where SGLT2 knockout mice on a regular diet/high fat diet (HFD) were compared to controls in order to analyse resulting glucose homeostasis and insulin insensitivity. They found that the knockout mice on HFD showed markedly improved glucose intolerance and reduced HFD-associated hyperglycaemia (Jurczak *et al.*, 2011). These data suggest that renal glycosuria may alleviate symptoms associated with diabetes.

Furthermore, Quaggin *et al* also reported findings from their *Sweet Pea* mutant, a mouse model carrying a nonsense SGLT2 mutation in the *slc5a2* gene, suppressing SGLT2 protein function. Compared to their wild-type littermates, the sweet pea mutants had significantly lower peak blood glucose values that returned to baseline more promptly after injection with dextrose. Additionally, the sweet pea mutants did not exhibit increased levels of KIM-1 or NGAL (markers of tubular injury) at baseline compared to the WT

mice. After induction of diabetes (STZ injection), SP mutants exhibited the best glycaemic control but had the highest mortality rate (Ly et al., 2011). This model indicates that targeting SGLT for the management of diabetes is promising although future clinical studies must produce comprehensive safety data.

Loss of function mutations in the SGLT1 gene can lead to glucose-galactose malabsorption, an autosomal recessive disease caused by a flawed glucose-galactose transport system in the enterocyte. Diagnosed neonatal patients often present with symptoms of life threatening dehydration and watery diarrhoea (Bothinah *et al.*, 2014). The medical consequences of the adult population who also have these genetic mutations have been studied to a lesser extent. Seidlemann et al conducted a clinical trial with over 8000 participants from across 4 US communities and found that approximately 14% of these individuals carried at least one SGLT1 mutation. They found that individuals with these haplotypes had a significantly lower risk of acquiring impaired glucose intolerance and lower incidence of developing diabetes mellitus or heart failure (Seidlemann *et al.*, 2018).

Results from trials analysing both SGLT2 and 1 mutations indicate these genes as attractive targets for therapeutics aimed at treating metabolic disorders.

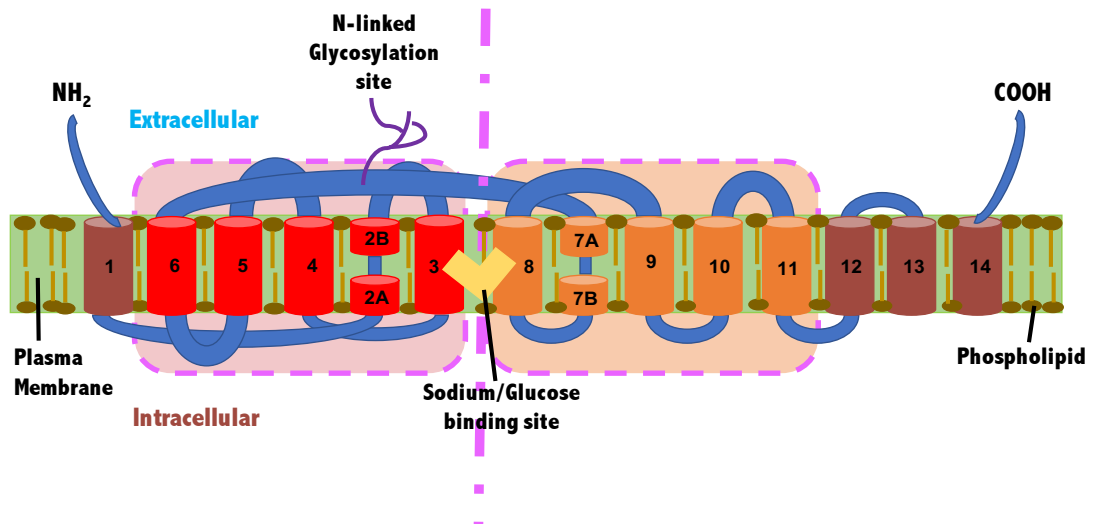


Figure 1.5: Proposed secondary structure of SGLT.

A transmembrane protein comprising of 14 helices, with both the NH₂ and COOH terminal groups facing the extracellular side of the plasma membrane. The light orange and pink boxes bordered by a dashed line represent the 5+5 inverted topology motif repeats, of which the sodium/glucose binding sites are located at the centre. The remaining transmembrane domains are coloured in maroon. N-linked glycosylation occurs between transmembrane domains 6 and 7a at Asn 248.

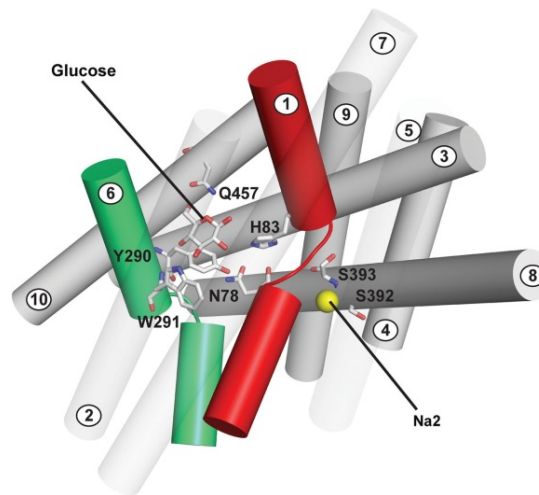


Figure 1.6: SGLT binding sites.

A more detailed look at the intracellular cavity where sodium and glucose binding take place in the inward facing vSGLT2. The transmembrane domains surrounding the cavity as well as the main amino acid residues that help form these binding sites for both are highlighted and numbered. Figure taken from (Loo *et al.*, 2013).

1.7.2. SGLT2 as a therapeutic target

The push on providing new treatment options to combat diabetes by targeting the SGLTs within the kidney has been building momentum. The rationale behind these SGLT inhibitors is that blocking glucose entry at the PTEC induces glycosuria, which decreases plasma glucose and glycated haemoglobin (HbA1c) concentrations. These inhibitors, when given at the full dose, facilitate the excretion of between 50 to 100g of glucose into urine daily, which corresponds to weight loss (Rosenstock and Ferrannini, 2015). Their mode of action is independent of insulin mediated metabolic processes and so avoids issues associated with insulin resistance, indicating that SGLT2 inhibitors can be given in combination with other drugs that control glycaemia (Cefalu and Riddle, 2015).

The first glucose transporter inhibitor discovered was phlorizin, a glucoside derived naturally from the bark of apple trees. Early experiments indicated that phlorizin was able to inhibit the transport of glucose in the small intestine, kidney and erythrocytes. It is a non-specific competitive inhibitor for SGLT1 and SGLT2, and exerts its mode of action by competing with D-glucose to bind to the transporter (Chao and Henry, 2010). Development of clinical trials for phlorizin proved difficult as the drug also inhibited the functions of SGLT1 and GLUT1.

Consequently, further medicinal research was carried out to produce an inhibitor with stronger SGLT2 selectivity. Presently, canagliflozin, dapagliflozin, and empagliflozin are all currently in clinical use. All three inhibitors are C-glucosides, as opposed to its O-glucoside predecessor phlorizin. O-glucosides are easily degraded by β -glucosidase whereas C-glucoside possess a higher metabolic stability. Canagliflozin has a 250 fold selectivity of SGLT2 compared to SGLT1 and maintains a half-life of approximately 10h with an oral bioavailability of 65% (Devineni *et al.*, 2015). A phase 3 trial revealed a decrease in fasting plasma glucose (FPG) in a dose dependent manner for patients who received 100mg and 300mg of canagliflozin. There was also a lower incidence of patients progressing to albuminuria in both groups (Yale *et al.*, 2013).

Dapagliflozin has also yielded successful results and is highly selective for SGLT2 when compared to canagliflozin. It has an oral bioavailability of approximately 78%, a half-life of 12.9h, and an approximate 1200 fold selectivity for SGLT2 (Kasichayanula *et al.*,

2014). A 24-week randomised international trial found that patients with type 2 diabetes and inadequate glycaemic control treated with 5mg and 10mg of dapagliflozin per day had significantly reduced HbA1c levels relative to the placebo group (Ferrannini et al., 2010).

Empagliflozin has a 2500 fold selectivity of SGLT2 over SGLT1 with a half-life between 13-18h and similar oral bioavailability to dapagliflozin. The guideline recommends that the maximum daily dose remain at 25mg. A study that was carried out in 100 patients with T2DM who were administered with different doses of empagliflozin found a dose dependent increase of urinary glucose excretion levels (Kanada *et al.*, 2013).

Aside from improving glycaemic control, these anti-diabetic agents have gone on to surpass expectations and also offer both renal and cardiovascular benefits (Rabizadeh, Nakhjavani and Esteghamati, 2019). The CANVAS program combined patients who were both diagnosed with type 2 diabetes and at risk of major adverse cardiac events. This merged group were administered with canagliflozin or placebo. The results after a follow up of ~3.5 years showed that participants who received canagliflozin treatment were at a lower risk of cardiovascular events in comparison to those who received placebo (Neal *et al.*, 2017).

1.8. Facilitative glucose transporters (GLUTs)

GLUT2, encoded by the *SLC2* human gene family, is ubiquitously expressed on the plasma membrane of the kidney, liver, intestine and pancreas. A bidirectional transporter with a K_m spanning approximately 15 to 20mM, it has a particularly low affinity for D-glucose. However, it is also capable of utilising other monosaccharides such as galactose, fructose and mannose as low affinity substrates (Mueckler and Thorens, 2013). It is a high affinity transporter for glucosamine ($K_m = \sim 0.8\text{mM}$). The protein ($\sim 55\text{kDa}$) consists of 12 membrane spanning helices, is proposed to consist of around 524 amino acid residues (Powers, 1998) with one N-linked oligosaccharide between TM domains 1 and 2 (Barron *et al.*, 2016) and a large cytoplasmic loop between TM domains 6 and 7 (Mueckler and Thorens, 2013). GLUT2 levels are elevated in the diabetic kidney (Rahmoune *et al.*, 2005; Jiang *et al.*, 2019). There is also evidence showing that GLUT2

ablated neonatal mice were hyperglycaemic and unable to survive past 3 weeks (Guillam *et al.*, 1997).

GLUT1 is also widely expressed on the basolateral membrane of a wide variety of mammalian cells, including the S3 segment of the PTEC, but they are expressed at the highest level in erythrocytes (Montel-Hagen *et al.*, 2008; Mather and Pollock, 2011). It is the only glucose transporter responsible for transporting glucose across the blood brain barrier. The brain rapidly breaks down this glucose for energy, indicating a crucial role for GLUT1 in maintaining neurological function (Veggiotti and De Giorgis, 2014; Patching, 2017). Structurally similar to isoform GLUT2, it also has a molecular weight of approximately 58kDa and comprises of 12 transmembrane spanning domains. Both GLUTs form a large pore that is situated in the plasma membrane in order to facilitate the transportation of glucose down the concentration gradient (Holman, 2020).

1.9. Renal fibrosis

Renal fibrosis is characterised by the excessive pathological accumulation of extracellular matrix (ECM) protein. The ECM is an extensive non-cellular structural support network consisting of diverse proteins that provide vital scaffolding for cellular components and regulates a variety of essential cellular processes like adhesion, differentiation and proliferation (Frantz, Stewart and Weaver, 2010). There is a fine balance between ECM synthesis, deposition and degradation in healthy tissue wound healing, but this equilibrium is compromised during renal fibrosis and becomes uncontrolled (Genovese *et al.*, 2014). The process is described as the continuous replacement of functional renal parenchymal tissue with connective tissue (ECM proteins like fibronectin, laminin, and collagen) and eventually leaves the kidney unable to repair itself (Mutsaers *et al.*, 2015). Although the initial development of CKD is dependent on various underlying factors like environmental stressors or predisposed genetics, renal fibrosis is the final common pathway in ESRD (Obrador *et al.*, 2017; Sakuma *et al.*, 2020). Uncontrolled ECM deposition and disrupted wound healing leads to oxidative stress, apoptosis, inflammation and cell senescence (Portilla, 2014).

Various cytokines and growth factors can mediate renal fibrosis. These include tumour necrosis factor alpha (TNF- α), interleukin-6 (IL-6) and transforming growth factor beta (TGF- β). Neutralisation of TNF- α reduces renal inflammation, fibrosis and albuminuria in rats with renal failure (Therrien *et al.*, 2012). Chen *et al* investigated the importance of IL-6 as a mediator of fibrosis in unilateral ureteral obstruction (UUO) mice. UUO mice are widely used as an animal model of renal fibrosis. These mice exhibited elevated levels of IL-6 accompanied with tubular injury and increased levels of fibronectin, collagen I and vimentin protein. Blockade of IL-6 signalling by recombinant mouse chimera protein subsequently reduced the ECM proteins and improved tubular injury (Chen *et al.*, 2019).

TGF- β , an important immunoregulatory polypeptide responsible for modulating essential biological responses like cell differentiation, apoptosis and angiogenesis (Abnaof *et al.*, 2014), is the major driver in renal fibrosis. It is the main inducer of pro-fibrotic marker connective tissue growth factor (CCN2/CTGF) (Mason, 2009) and contributes to ECM accumulation through the upregulation of collagens (Isaka, 2018). It can suppress enzymes that degrade the ECM like matrix metalloproteinases (MMPs) and can exert this effect through the induction of MMP inhibitors like Plasminogen Activator Inhibitor-1 (PAI-1) (Rerolle *et al.*, 2000) (Figure 1.6). It is also an effector of podocyte injury and death, the main hallmark of glomerulosclerosis, through the Smad ¹(mothers against decapentaplegic) pathway (Ilatovskaya *et al.*, 2015; Hao, Baker and Dijke, 2019).

The significance of epithelial to mesenchymal transition (EMT) in mediating renal fibrosis has been heavily debated within the scientific community. EMT describes the process where injured epithelial cells undergo phenotypical changes and transform into myofibroblasts. Although the important role that myofibroblasts play in producing ECM proteins is broadly accepted, the origins of these activated fibroblasts is a contentious topic (Fragiadaki and Mason, 2011). It is generally agreed that the majority of the myofibroblasts are recruited from local resident fibroblasts meanwhile the contribution of EMT to the myofibroblastic group is still unclear (Meran and Steadman, 2011; Micallef *et al.*, 2012). Interestingly, Grande *et al* found that the transcription factor Snail-1, another mediator of renal fibrosis, caused damage to renal tubular cells. They reported that these

¹ Smad proteins are homologues of the *C. elegans* protein SMA and the drosophila protein “mothers against decapentaplegic”, hence the combinatory name. Decapentaplegic is a key morphogen that plays an essential role drosophila embryo development. A female fly will stop encoding for the decapentaplegic gene if there is a fault. The name “Mothers Against Decapentaplegic” was inspired by the non-profit “Mothers Against Drunk Driving” campaigns.

damaged tubular cells displayed a loss of epithelial phenotypic markers and polarity/differentiation. They also promoted interstitial fibroblastic proliferation but remained integrated to the tubules, suggesting that it is more likely that epithelial cells may only undergo “partial EMT” during renal fibrosis, as opposed to full EMT (Grande *et al.*, 2015).

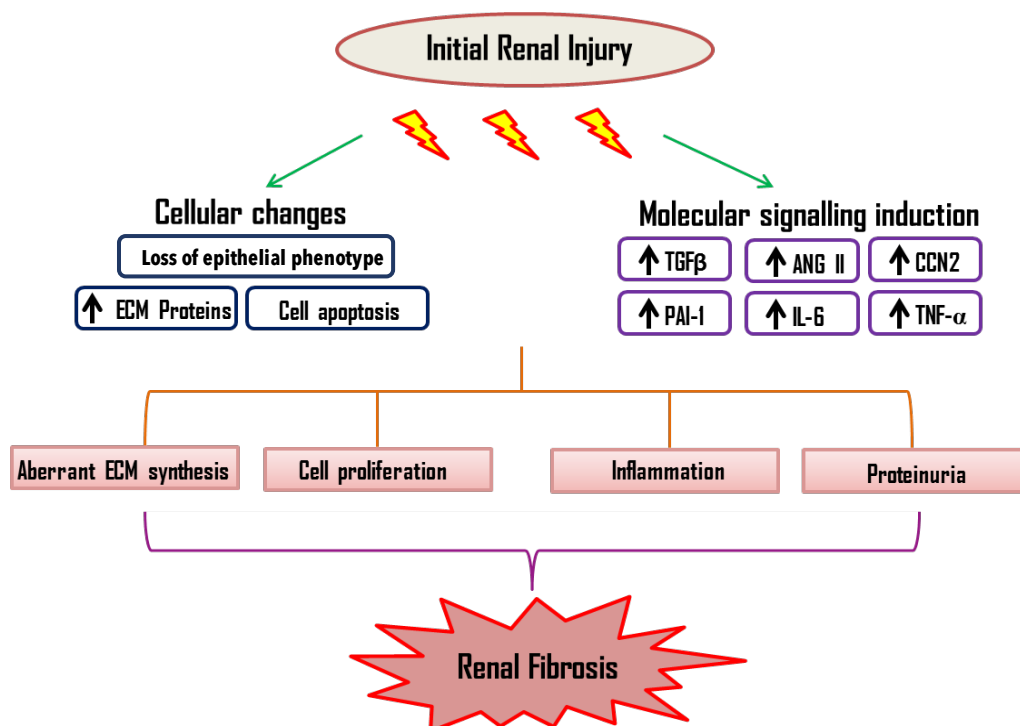


Figure 1.7: Mechanisms leading up to renal fibrosis.

Repeated insults to the renal parenchyma can trigger both cellular and molecular changes within the structure. This leads to the activation of inflammatory and pro-fibrotic markers, the aberrant synthesis of ECM proteins and inflammation. These processes all contribute towards renal fibrosis.

1.9. TGF- β in renal fibrosis

The TGF- β superfamily, consisting of isoforms, TGF- β 1, TGF- β 2, and TGF- β 3, are responsible for exerting a plethora of essential functions in the human body including but not limited to cell differentiation, proliferation, migration, angiogenesis and wound healing. Of the three, TGF- β 1 is the most abundant isoform (Zhao, Zou and Liu, 2020). The process of its synthesis involves an inactivated precursor protein monomer consisting of an N-terminal latency associated peptide (LAP) and a C-terminal mature domain. In the golgi, this complex is cleaved by furin. After cleavage, the mature domain still maintains its non-covalent association with LAP, thereby forming a small latent complex (SLC). As the majority of cell types are not able to secrete this directly, it is bound to latent TGF- β binding proteins (LTBPs), forming the large latent complex (LLC) before secretion into the ECM. It binds onto ECM proteins like fibronectin which will store TGF- β in the latent form. As it is unable to bind to surface receptors in this inactive form, it is then proteolytically cleaved from LAP by serine proteases (Biernacka, Dobaczewski and Frangogiannis, 2011; Nüchel *et al.*, 2018; Brown and Marshall, 2019).

There has been extensive evidence over the years to show that TGF- β 1 is an important mediator of renal fibrosis and that inhibition of this pathway can reduce pro-fibrogenic damage. Early on, Murakami *et al* found significant correlation between the rising urinary TGF- β 1 levels and degree of interstitial fibrosis and proteinuria (Murakami *et al.*, 1997). Ma *et al* used a UUO model in β 6-integrin-null mice to examine the role of TGF- β 1 activation as integrin α v β 6 is a matrix receptor which activates TGF- β 1. These null mice showed considerably lower collagen I/II, PAI-1 levels as well as a reduced degree of tubulointerstitial fibrosis when compared to the UUO wild type mice, suggesting that inhibition of the latter growth factor alleviates markers of renal fibrosis (Ma *et al.*, 2003). Additionally, overexpression of TGF- β 1 in the renal tubules of a tetracycline controlled transgenic mouse model induced interstitial proliferation, fibrosis and tubular autophagy (Koesters *et al.*, 2010).

TGF- β mainly exerts its pro-fibrotic effects in a canonical smad-dependent fashion. Work from our own group found that TGF- β 1 induced pro-fibrotic markers alpha SMA and CCN2 whilst decreasing epithelial marker E-cadherin. These outcomes were all found to

be Smad dependent (Phanish *et al.*, 2006). Gu *et al* found significantly reduced levels of Smad protein after 3'-deoxyadenosine decreased TGF- β 1 induced collagen protein. This suggests that the suppression of pro-fibrotic markers induced by TGF- β 1 may be achieved by targeting Smad (Gu *et al.*, 2013) .

TGF- β can also exert its effects in a non-canonical/Smad-independent fashion by activating pathways such as extracellular regulated signal kinase (ERK) or p38 mitogen activated protein kinase (MAPK) (Chung and Lan, 2013). This has also been demonstrated many times by our own group. Niculescu-Duvaz *et al* discovered that TGF- β 1 induced a significant increase in fibronectin, an important ECM glycoprotein, in transformed proximal tubule cells. Attenuation of fibronectin was achieved by p38 MAPK inhibition but not by knockdown of Smad through siRNA (Niculescu-Duvaz *et al.*, 2007). In the same transformed cells, Browne *et al* discovered that TGF- β 1 activation of ERK5 was also dependent on p38 MAP kinase (Browne *et al.*, 2008). Interestingly, Badshah *et al* found that the TGF- β induced loss of podocyte function and altered podocyte phenotype was regulated by ERK5 (Badshah, Baines and Dockrell, 2014). Furthermore, Phanish *et al* found that TGF- β 1 induced extra domain A fibronectin (EDA-fibronectin), a key component of the ECM during renal fibrosis, was regulated by phosphatidylinositol/protein kinase B signalling (Phanish *et al.*, 2015).

As TGF- β is accountable for regulating a multitude of essential cellular processes it does not make for an attractive target to treat kidney disease as it is unclear what other functions it may inhibit. Additionally, anti-TGF- β therapy clinical trials have not produced promising results. Voelker *et al* found that their TGF- β neutralising antibody LY2382770 failed to improve serum creatinine or proteinuria levels in patients with DKD (Voelker *et al.*, 2017). Similarly, Vincenti *et al* found that their monoclonal anti- TGF- β antibody did not change GFR or proteinuria levels in patients with focal segmental glomerulosclerosis (Vincenti *et al.*, 2017). Subsequently, I propose to investigate its parallel or downstream mediators with the goal of identifying and developing more selective anti-fibrotic drugs.

1.10. Glucose, TGF- β 1 and renal fibrosis

The standard criteria for a diagnosis of diabetic kidney disease is established diabetes/hyperglycaemia and albuminuria. When PTECs are exposed to serum albumin, they stimulate TGF- β 1, the main driver of renal fibrosis (Diwakar, Alex L Pearson, *et al.*, 2007; Zhao, Tramontano and Makker, 2007). There is evidence in literature detailing the interactions between glucose and TGF- β 1, as well the effects of glucose on fibrotic outcomes. High glucose increases ECM protein assembly in mesangial cells (Miller *et al.*, 2014). An increase in TGF- β 1 expression was found in both the glomerular and tubular compartments of the diabetic db/db mouse compared to its non-diabetic counterpart (Hong *et al.*, 2001), and high glucose increases the expression of TGF- β 1 in vascular smooth muscle cells (Lindschau *et al.*, 2003). Hayashida et al also discovered that high glucose increased receptor-Smad (R-Smad) phosphorylation and its association with common-Smad4 (co-Smad4), indicating that high glucose increases the activity of TGF- β 1 in mesangial cells (Hayashida and H William Schnaper, 2004). The combination of elevated blood glucose levels and TGF- β 1 caused an increase in glomerular volume and renal collagen production in STZ diabetic mice (Krag, Nyengaard and Wogensen, 2007). Li et al also found that high glucose induced collagen production in renal epithelial and mesangial cells was TGF- β dependent through the use of a neutralising TGF- β antibody. Furthermore, they found that high glucose induced an increase of TGF- β 1 in the medium (Li *et al.*, 2003). The results chapters of this thesis will address the effects of high D-glucose in combination with TGF- β 1 on PTECs.

Chapter 2: Materials and Methods

2.1. Materials

Table 2.1 details supplier and catalogue numbers for materials, reagents, and inhibitors used in this project. Suppliers and catalogue numbers for antibodies are detailed in table 2.4.

Materials	Catalogue number	Supplier
0.5ml Microcentrifuge Tubes	KC123	Appleton Woods
1.5ml microcentrifuge Tubes	KC124	
7.5% Mini-PROTEAN TGX [®] stain free gels	4568023	Bio-Rad Laboratories
10% Mini-PROTEAN TGX [®] stain free gels	4568033	
4-15% Mini-PROTEAN TGX [®] stain free gels	4568083	
Precision Protein Plus WesternC Standards	1610376	
Trans-Blot Turbo Mini 0.45um PVDF Transfer Kit	1704274	
Clarity Max Western ECL Reagent	1705062	
2x Laemmli sample buffer	1610737	
Trypan Blue Cell Counting Kit	1450003	
Dapagliflozin	11574	Cayman Chemical
50ml Centrifuge Tubes	430828	Corning
15ml Centrifuge Tubes	430790	
T75 Tissue Culture Flasks	353156	
35mm Tissue Culture Dishes	430165	
10ml Stripette [™] serological pipets	4488	
24 Well Plates	3524	
100mm Agar Plates	353003	Falcon
REGM [™] Renal Epithelial Growth Medium	CC-3190	Lonza
Primary Human Renal Proximal Tubule Epithelial Cells	CC-2553	
Trypsin/EDTA solution	CC-5012	
Glucose Uptake Glo [™] Assay	J1341	Promega
FuGENE [®] Transfection Reagent	E2691	
RNeasy Mini Kit (250)	74106	Qiagen
EASYpack Protease Inhibitor	4693116001	Roche
PhosSTOP Phosphatase Inhibitor	4906837001	
Phloretin	S2342	Selleck Chem
Phlorizin	S2343	
Heparin-Agarose Bead Suspension	H6508	Sigma-Aldrich
Ampicillin	A9393	
Luria Broth	L3522	
Luria Broth with Agar	L2897	
Fetal Bovine Serum	F2442	

Table 2.1: Suppliers and catalogue numbers for materials and reagents used.

Materials	Catalogue number	Supplier
Acetonitrile	271004	Sigma-Aldrich
TBS 10x	T5912	
Collagen IV	CC-076	
Dimethyl Sulfoxide	C6164	
GMNA	G0141	
Recombinant TGF- β 1	T7039	
Bovine Serum Albumin	A7030	
U0126 MAPK/ERK Inhibitor	662005	
D(+) Glucose	G8769	
L(-) Glucose	G5500	
Glutamyl-gamma-(4-methoxy- β - naphthylamide)	G0141	
Fast blue BB salt	F3378	Thermofisher
Silencer® select SGLT2 siRNA	4392420	
Silencer® select SGLT1 siRNA	AM16708	
Silencer® select negative control siRNA	4390843	
DMEM/F-12 Medium	11320033	
4% Formaldehyde Fixation Solution	FB002	
Gold Antifade Mountant with DAPI	S36938	
Opti-MEM™ Reduced Serum Medium	31985062	
BCA Protein Assay	23225	
High Capacity cDNA Reverse Transcription kit	4374966	VWR
Tween® 20	8.22184.0500	
99% Industrial Methylated Spirits	23684.329	

Table 2.1 Continued: Suppliers and catalogue numbers for materials and reagents used.

2.2. Cell maintenance

Ethics statement: The use of all human tissue was carried out with the approval of the South London and Surrey Research Ethics Committee, REC reference number 07/Q0806/24.

2.2.1. Cell culture

All cell culture work was carried within a vertical and unidirectional airflow safety hood which was sterilised once a week with a 20 min ultraviolet light session. All materials utilised in the hood were sprayed with 70% industrial methylated spirits (IMS) (VWR) before use.

Human primary proximal tubule epithelial cells (PTECs) are responsible for the majority of glucose handling/reabsorption in the kidneys and heavily implicated in development of tubulointerstitial fibrosis. For this project, both PTECs and immortalised proximal tubule epithelial cells (Human kidney cell-clone 8, HKC-8) transfected with temperature-sensitive simian virus 40 (SV40) were utilised (Figure 2.1&2.2). Human dermal fibroblasts (Cell Biologics), which do not mediate glucose handling, will be used a negative control cell type. In-house primary PTECs were previously isolated and prepared in our laboratory from human nephrectomy samples using percoll density gradient centrifugation (Sharpe and Dockrell, 2012). To ensure reliability, primary PTECs isolated from other donors were purchased from Lonza, USA and used for experimentation at passage number 3. The HKC-8 cell line was generously provided by Dr Lorraine Racusen of Johns Hopkins University, Baltimore and used for experimentation between passages 10 to 20. PTECs were cultured in Renal Epithelial Growth Medium (REGM™) and supplements consisting of human epidermal growth factor (EGF), insulin, hydrocortisone, gentamicin-amphotericin, foetal bovine serum (FBS), transferrin, triiodothyronine, and epinephrine (Lonza). The HKC-8 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium with D-Glucose (7mM) supplemented with insulin (5µg/ml), sodium selenite (5µg/ml), transferrin (5ug/ml), triiodothyronine (4pg/ml), hydrocortisone (36ng/ml) and FBS (5% w/v, Sigma-Aldrich).

Human primary dermal fibroblasts were purchased from Cell Biologics and cultured in Complete Fibroblast Medium (Cell Biologics). All medium was sterile filtered using a 0.2µM Puradisc™ (Whatman). All cell lines were grown at 37°C in 5% CO₂ humidity on T75 tissue culture flasks (Corning). Medium was replaced on alternating days. When cells reached ~80% confluence and were ready to be seeded on to dishes, they were dissociated and detached from the surface using 5ml of trypsin-ethylene diaminetetraacetic acid (EDTA) (Lonza) per T75 flask. PTECs, fibroblasts and HKC-8 cells were subsequently seeded onto 35mm collagen IV coated dishes at densities of 2500, 5000 and 10,000 cells/cm² respectively.

2.2.2. Collagen IV coating

Collagen IV is the main extracellular matrix component in the basement membranes of the human kidney (Butkowski *et al.*, 1989), so it is proposed that cells grown on collagen IV coated culture dishes are more physiologically relevant than its cells grown on plastic.

Collagen IV in powder form (Sigma-Aldrich) was reconstituted in 0.25% acetic acid. T75 Tissue culture flasks and dishes (Corning) were coated with 5µg/cm² of collagen IV, exposed to UV radiation for 20 min, and incubated overnight at room temperature. Flasks and dishes were then rinsed once with sterile phosphate buffered saline (PBS), air dried, and stored at 4°C before use.

2.2.3 Optimisation of SGLT2 western blots

There is currently a shortage of high-quality commercial antibodies for SGLT2 which increases the difficulty of detecting protein expression. Thus, for the optimisation of SGLT2 expression in primary PTECs, two SGLT2 antibodies were tested. Firstly, an anti-SGLT2 rabbit polyclonal antibody (ab58298, Abcam) which exhibited strong target protein expression in renal cells (Kuang *et al.*, 2017) was examined. This membrane was blocked in 5% BSA non-fat milk. PTECs and HKC8 cells were run on the same gel to ensure a more reliable comparison. For each lane, approximately 25µg of protein was loaded. SGLT2 (~73kDa) was detected in PTECs cells under these specific conditions but

was not detected in the HKC-8 cells (Figure 2.1). Distributors of the second mouse monoclonal SGLT2 antibody (sc-393350, Santa Cruz) provided the immunogen sequence (amino acids 226 to 268 of human SGLT2) used to produce the peptide. This sequence was analysed using <https://blast.ncbi.nlm.nih.gov> to determine shared SGLT2 sequence homology with other proteins. The results indicated only an approximate 28% homology with the SGLT3 sequence. Tissue lysate obtained from human kidney (see section 2.9 for tissue lysate extraction protocol) was used as a positive control and run alongside the cell lysate. Using the Santa Cruz antibody produced a relatively cleaner blot with a band at ~73kDa, thought to be equivalent to the glycosylated form of SGLT2. The Santa Cruz SGLT2 antibody was used on all SGLT2 western blots in subsequent experiments. There was a fainter band at ~64kDa, thought to be equivalent to the non-glycosylated form of the protein (Figure 2.2). The latter form was not always reproducible. The human kidney tissue sample run alongside the PTECs also expressed the same ~73kDa glycosylated protein. As figure 2.2 was produced for visualisation purposes only, it was not possible to make a direct comparison of quantity between *in vitro* and *in vivo* models. Human dermal fibroblasts, which do not express SGLT2, were also probed with the SGLT2 antibody from Santa Cruz. This was to confirm that the ~73kDa protein band observed in my PTECs was indeed SGLT2 and not due to non-specific binding. There was no detectable SGLT2 protein in the fibroblasts (Figure 2.3).

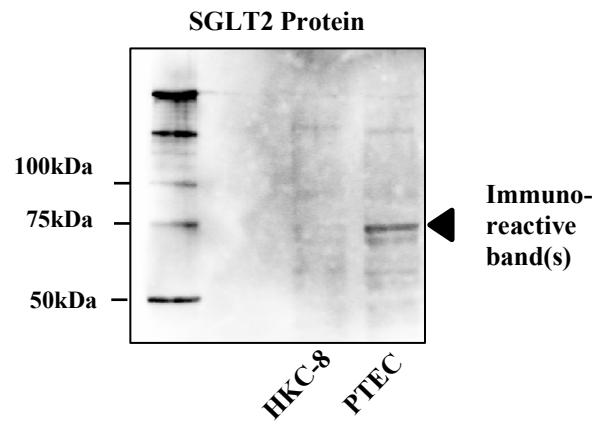


Figure 2.1: Western blot immuno-stained for SGLT2 protein in HKC-8 cells and PTECs using Abcam antibody.

There was no detectable SGLT2 protein in HKC-8 cells, while PTECs expressed SGLT2 protein at approximately 73kDa.

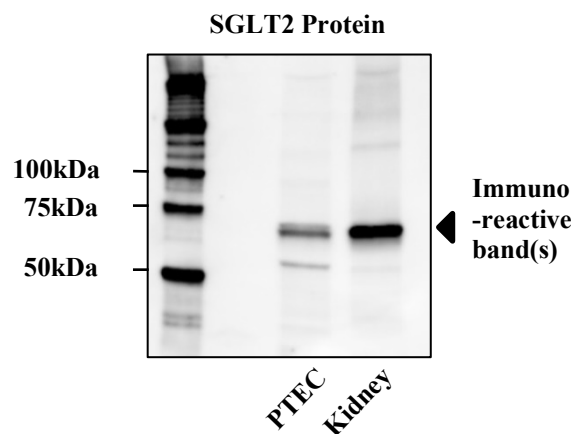


Figure 2.2: Western blot immuno-stained for SGLT2 protein in PTECs and kidney tissue using Santa Cruz antibody.

Primary PTECs expressed both ~73kDa and ~60kDa sized SGLT2 protein, while human kidney tissue expressed the ~73kDa sized SGLT2 protein.

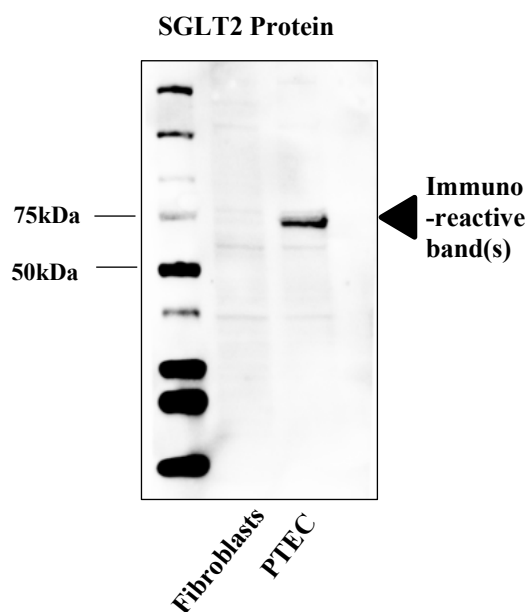


Figure 2.3: Western blot immuno-stained for SGLT2 protein in fibroblasts.

Fibroblasts (negative control) did not detect SGLT2 protein, while PTECs expressed SGLT2 protein at approximately 73kDa.

2.2.4. Cell cryopreservation

Cells were detached from the flask surface using trypsin/EDTA and centrifuged (210g, Hettich Mikro) for 6 min. Supernatant was removed and cells were re-suspended in DMEM/F-12 medium with FBS (5/10% w/v, Sigma-Aldrich) on ice in a micro-centrifuge tube. Dimethyl sulfoxide (DMSO) (10% w/v, Sigma-Aldrich) was slowly added while mixing the tube. Diluted cells were then aliquoted into cryovials at a concentration of 0.5×10^5 cells/ml. Cryovials were frozen for 2h at -20°C and stored at -80°C overnight before being transferred into liquid nitrogen for long term storage. For recovery of the cells, these cryovials were retrieved from liquid nitrogen and quickly defrosted in a 37°C water bath. Cells were then diluted in complete medium also warmed to the same temperature. This mixture was then aspirated into a T75 flask and kept at 37°C ; 5% CO_2 .

2.2.5. Staining for gamma glutamyl transferase (GGT) activity in PTECs

GGT is constitutively expressed on the cell surface of absorptive tissues in the kidney as well as the liver, pancreas, bile ducts, and lungs (Hanigan, 2014; Bulusu and Sharma, 2015). In the kidneys, GGT is a brush border membrane enzyme whose confirmed activity is an indicator of differentiated and healthy PTECs. The bi-substrate enzyme induces the cleavage and transfer of gamma-glutamyl moiety from glutathione, its natural donor substrate, to either water or peptides/amino acids, both acceptor substrates (Verma, Gupta and Goel, 2015).

The PTECs I have used for my experiments were characterised by confirming activity of this enzyme via enzyme histochemistry. PTECs with 80% confluence were washed in 1xPBS twice before being stained with a solution comprising of 3.8mM glycyl-glycine, 0.6mM Fast Blue B Salt (diatozed 4'-amino-2',5'-diethoxybenzanilide), 100mM NaCl, 25mM Tris-hydrochloride (Tris-HCL), and 6mM glutamyl gamma-(4-methoxy- β -naphthylamide) (GMNA) (Sigma Aldrich) at a pH of 7.4 in deionised water for 45 min at room temperature. GMNA is the synthetic substrate of GGT and glycyl-glycine serves as the glutamyl acceptor (Dahboul et al., 2012). Cells were rinsed with 1xPBS again before examination under the microscope. The same solution was prepared again without GMNA to incubate for the same time in the negative control dishes (Figure 3.2 E&F). The GGT enzyme is responsible for the cleavage of GMNA (similar to glutathione), which in turn

liberates 4-methoxy- β - naphthylamide (Rutenburg *et al.*, 1969). This is then coupled with the Fast Blue B salt to form an insoluble azo dye which is distinctively orange-red in colour, accounting for the orange-red staining I observed (Figure 2.4).

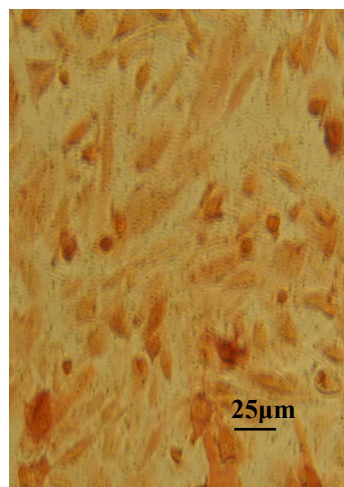


Figure 2.4: GGT enzyme histochemistry.

Primary PTECs showed distinctive orange-red staining, confirming the presence of GGT enzyme.
40 x 10 magnification.

2.3. Treatment and Inhibitors

2.3.1. Glucose and growth factor treatment

PTECs that had reached approximately 80% confluence were serum starved for 24h prior to treatment. PTECs were exposed to three different conditions: (i) control conditions (7mM D-glucose alone), (ii) high D-glucose/"hyperglycaemic" conditions (7mM D-glucose plus 18mM D-glucose) and (iii) osmotic control/"normoglycaemic" conditions (7mM D-glucose plus 18mM of non-transportable, metabolically inert L-glucose). I used L-glucose to account for the osmotic stress the PTECs are subjected to upon >7mM D-glucose treatment. All glucose was purchased from Sigma-Aldrich. Sterilisation of the different solutions was carried out by passing the glucose treated media through a 0.2µm sterile filter (Whatman). PTECs were also treated with recombinant TGF-β1 (Sigma-Aldrich) at a concentration 0.75-5ng/ml. All conditions were supplemented with bovine serum albumin (BSA) (0.1% w/v, Sigma-Aldrich). For both protein and mRNA analysis, the treatment period for all conditions ranged from 5 min to 72h.

2.3.2. Chemical inhibitors

The following chemical inhibitors were given in combination with the glucose and TGF-β1 treatment. SGLT2 inhibitor dapagliflozin (Cayman Chemical) was administered at 0.1, 1 and 10nM. These doses were chosen for my experiments as the half maximal inhibitory concentration (IC50) of dapagliflozin for human SGLT2 (hSGLT2) is ~1nM (Mudaliar *et al.*, 2015). This indicates that an estimated dapagliflozin concentration of 1nM is required to inhibit 50% of biological activity. A lower and higher dose (0.1nM and 10nM) of dapagliflozin were also added to check for a dose response. MAPK/ERK inhibitor U0126 (Sigma-Aldrich) was administered at 10µM, the same concentration used in previous MEK inhibitor experiments carried out by our group (Browne, 2009). GLUT1 inhibitor Phloretin and SGLT inhibitor Phlorizin (Selleck Chem) were administered at 1mM and 500µM, respectively, in accordance with concentrations used by our collaborators in their airway epithelial cells (Kalsi *et al.*, 2007, 2009). Knockdown of phospho-ERK protein knockdown by U0126 was first confirmed (Figure 2.5). All inhibitors were reconstituted in DMSO (0.1% w/v, Sigma-Aldrich). Treatment times ranged from 5 min to 72h.

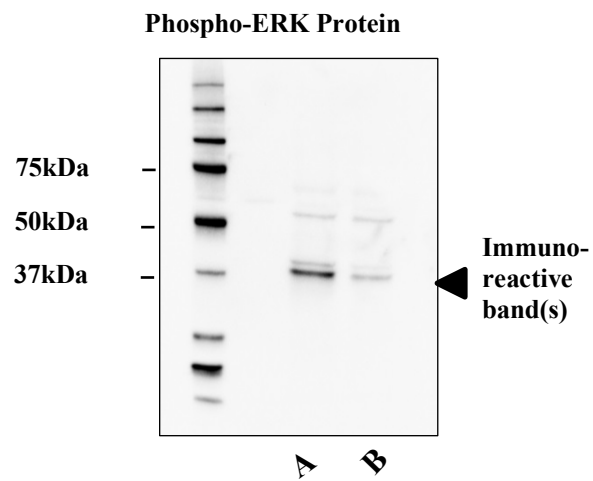


Figure 2.5: Western blot immuno-stained for phospho-ERK protein in PTECs.

- (A) (Without U0126 inhibitor) Primary PTECs expressed an approximate 40kDa phospho-ERK protein band;
- (B) (With U0126 inhibitor) U0126 MAPK/ERK inhibitor at 10 μ M reduced phospho-ERK protein.

2.3.3. Cell lysis

The process of lysis involves the destruction of the cell membrane through enzymatic or osmotic means without denaturing DNA. The purpose of this is to allow us to extract, purify and analyse intracellular proteins of interest released by the cell.

Culture dishes were washed with ice cold 1x PBS after removal of spent medium. Cells were then lysed on ice with lysis buffer comprised of Tris-HCl (20mM), NaCl (150mM), EDTA 1mM, triton X (1%), sodium deoxycholate (0.5%), and sodium dodecyl sulphate (SDS) (0.1%) at pH 7.2. EASYpack protease inhibitor and PHOSstop phosphatase inhibitor cocktails (10ml/L, Roche) were added just prior to application onto dishes. Cells were scraped off, transferred to microcentrifuge tubes and kept on ice for 20 min. To separate cell debris, the lysis mixture was then centrifuged (6270g, Henderson Biomedical) for 10 min. The supernatant was aspirated into new microcentrifuge tubes. Both the spent medium and supernatant lysate samples were then stored at -80°C until further use.

2.4. Bicinchoninic (BCA) protein assay

Protein concentration was determined using a bicinchoninic acid (BCA) protein assay (ThermoFisher). The BCA protein assay is used for quantitation of total protein in a sample. The basis of this analytical method is centred on a temperature dependent Biuret reaction where peptide bonds reduce Cu^{2+} to Cu^{1+} in an alkaline solution. BCA then chelates with each Cu^{+} ion, which produces a purple-coloured substance which absorbs light at a wavelength of 562nm. The intensity of the purple complex is directly proportional to the amount of protein present in the sample. Samples were diluted at a ratio of 1:5 using 1x PBS and plated into a 96 well plate (Corning) along with the constructed standard curve of known concentrations of BSA also dissolved in 1xPBS (Figure 2.6). Both the standard curve and samples were plated (10µl per well) in duplicate. 200µl of working reagent- prepared by mixing 50 parts of reagent A with 1 part of reagent B- was then added into each well. The plate was then incubated at 37°C for 30 min. Protein absorbance was measured using the Sunrise microplate absorbance reader (Tecan,

wavelength range of 340nm to 720nm) and concentration subsequently calculated using the standard curve.

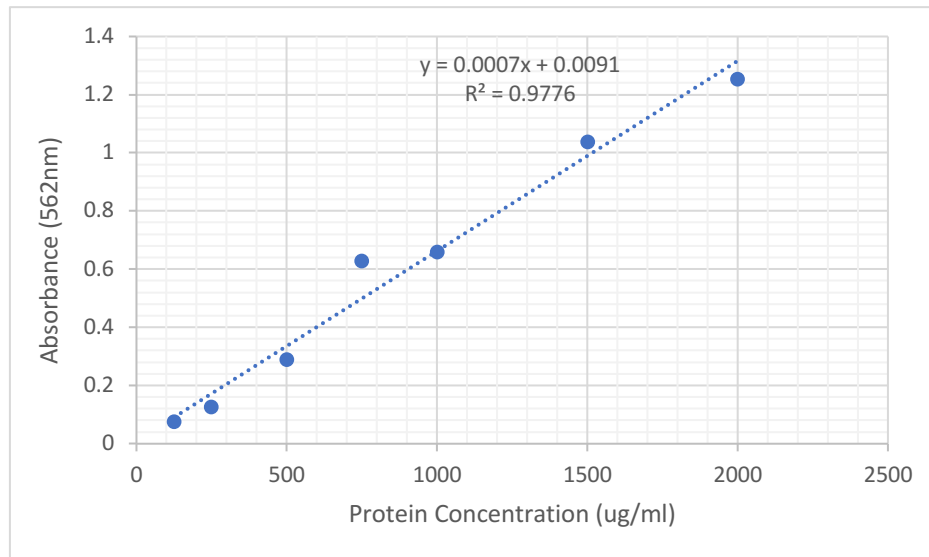


Figure 2.6: Standard curve graph generated from BCA protein assay.

2.5. Polyacrylamide gel electrophoresis (PAGE)

PAGE is a technique used to analyse and separate proteins on the basis of molecular mass and charge. Specific antibodies are then used to assess the presence and relative quantification of target proteins.

Equal amounts of protein (25µg per well) were boiled in 2x Laemmli buffer (Bio-Rad), heated at 95°C for 5 min in a thermal cycler (Fisher Scientific) and then loaded onto either 4-15%, 7.5% or 10% “mini-PROTEAN” stain free TGX gels (Bio-Rad) for 30 min at 220V. Precision Protein Plus WesternC standards were used for visual confirmation of band weight (Bio-Rad). Total protein stain free gel images were captured on the ChemiDoc™ Touch system (Bio-Rad) prior to the transfer onto polyvinylidene (PVDF) membranes for total protein normalisation and loading adjustments later.

2.6. Western blotting

After the transfer, PVDF membranes were blocked in 5% milk or bovine serum albumin (BSA) buffer for 1h at room temperature and then given 3 x 15 min washes in 1x tris buffered saline- tween (TBST) (Sigma-Aldrich, VWR). Membranes were incubated in primary antibody overnight at 4°C and washed another 3 times in TBST the following day. Table 2.2 and 2.3 provide further details on gel electrophoresis settings and western blotting reagents used. Secondary antibody conjugated with horseradish peroxidase (HRP, Cell Signaling Technology) was then applied to the membrane and incubated on a rocker at room temperature for 1h followed by a 5 min incubation in clarity max enhanced chemiluminescent (ECL) substrate system (Bio-Rad) in preparation for band visualisation. This detection system is dependent on the combination of HRP and the oxidation of substrate luminol contained in reagent A to generate a chemiluminescent signal. As luminol is only able to emit light at a maximum wavelength of 428nm, an enhancer found in reagent B is used to increase the luminol based signal. Reagents A and B are mixed together at a ratio of 1:1. Protein density analysis was carried out using ImageLab™ software (Bio-Rad) where target protein density was expressed relative to the amount of total protein. Table 2.4 has a detailed list of antibodies used.

	Current (mA)	Voltage (V, Constant)	Time
Gel electrophoresis	~120-160	220	30 min
Blot transfer	~100-120	15	10 min

Table 2.2: Gel electrophoresis and transfer settings.

Type of reagent/buffer	Components
TGS (Tris glycine SDS) buffer	25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3
Transfer buffer	Proprietary (Bio-rad)
Laemmli sample buffer	Proprietary (Bio-rad)
Tris-buffered saline (TBS)	20mM Tris base, 137mM NaCl, pH 7.4
TBS-T	1x TBS buffer, 0.1% w/v tween
Monoclonal antibody diluent	5% non-fat milk in TBS-T
Polyclonal antibody diluent	5% BSA in TBS-T

Table 2.3: Components of reagents used for gel electrophoresis and western blotting.

Antibody	Dilution	Cat no	Supplier
Anti-SGLT2 antibody, rabbit polyclonal	1:500 for WB 1:200 for IF	58298	Abcam
Phospho Smad3 serine 204, rabbit polyclonal	1:500 for WB	63042	Abcam
p21 antibody, rabbit polyclonal	1:1000 for WB	2947	Cell Signaling Technology
Phospho ERK, rabbit polyclonal	1:1000 for WB	4370	Cell Signaling Technology
Phospho Smad3, rabbit polyclonal (SS ^p XS ^p)	1:1000 for WB	25A9	Cell Signaling Technology
TGF- β receptor type 2 (T β RII) antibody	1:1000 for WB	2518	Cell Signaling Technology
Alexa Fluor goat anti-mouse antibody	1:100 for IF	11029	Invitrogen
CCN2 antibody, rabbit polyclonal	1:500 for WB	32193	Invitrogen
Anti-rabbit IgG HRP conjugated antibody	1:2000 for WB	7074	New England Biolabs
Anti-SGLT2, mouse monoclonal	1:500 for WB	393350	Santa-Cruz
CCN2 antibody L-20, goat polyclonal	1:2000 for WB	14939	Santa-Cruz
Fibronectin IST-9 antibody, mouse monoclonal	1:1000 for WB	59826	Santa-Cruz
Rabbit anti-mouse IgG HRP conjugated antibody	1:30000 for WB	9044	Sigma-Aldrich
K-cadherin antibody, mouse monoclonal	1:1000 for WB	06305	Thermofisher

Table 2.4: Antibodies list.

2.7. Preparation of urine samples for western blotting

EASYpack protease inhibitor and PHOSstop phosphatase inhibitor cocktails ([10ml/l], Roche) were added to each 1ml urine sample aliquot. The mixtures are then transferred to new microcentrifuge tubes and placed in the rotary evaporator (CHRIST) at 45°C for approximately 18h to increase the concentration 5 fold. After evaporation, the urine samples were boiled with 2x laemmli buffer (Bio-Rad) at a 1:1 ratio for 5 min at 95°C on the thermal cycler (Fisher Scientific). The rest of the urine sample analysis follows the western blotting protocol in section 2.6.

2.8. Heparin bead purification

The harvested supernatant samples were mixed with heparin beads to carry out affinity purification of secreted CCN2. This heparin bead purification process concentrates heparin binding proteins like CCN2 (Mason, 2009) to levels which can be analysed by western blot. Harvested supernatant samples and heparin-agarose bead suspension (Sigma-Aldrich) were mixed together at a ratio of 10:1 and placed on a roller-mixer overnight at room temperature. Medium and bead suspension were mixed on a roller for a further 6h in order to create precipitation of all heparin binding proteins secreted from PTECs. Tubes were centrifuged at (6270g, Henderson Biomedical) for 10 min at 4°C with the supernatant aspirated carefully, ensuring no dislodgment of the beads. Ice cold 1x PBS (100µl) was then added to each tube, re-suspended by gentle pipetting, and centrifuged again at 10000rpm for 1 min at 4°C before removing the supernatant. This step was repeated a further two times. Equal amounts of bound proteins were then extracted in 2x laemmli sample buffer (Bio-Rad) with 5% mercaptoethanol, boiled for 5 min at 95°C on the thermal cycler (Fisher Scientific) and run on the “mini-PROTEAN” TGX gels (Bio-Rad) in accordance with the settings detailed in table 2.2.

2.9. Tissue lysate extraction

Human kidney tissue was dissected into approximately 5mg pieces using a sterile scalpel and placed in 2ml round bottom microcentrifuge tubes (Appleton Woods). Ice cold lysis

buffer (total volume 300ul) containing NaCl (150mM), NP-40 (1% w/v), sodium deoxycholate (0.5% w/v), SDS (0.1% w/v), and Trizma base (50mM) at a pH of 8.0 was then mixed to the same tube and homogenised (Sonics Vibra-Cell). This step was repeated a further two times, ensuring that the blade was rinsed in between each time. The tubes were then kept on a roller-mixer at 4°C for 2h before centrifugation (15142g, Henderson Biomedical) for 20 min at 4°C. The supernatant was then aspirated and kept while the pellet was discarded. Tissue samples were stored at -80°C until ready for use. Protein concentration was determined using the same method BCA protein assay as discussed above in section 2.4.

2.10. RNA extraction

In order to identify and assess the relative RNA abundance in the target genes of my PTECs, RNA was first extracted from PTEC lysates. RNA was harvested from PTEC lysates using the RNeasy kit (Qiagen). RNA lysates were prepared on ice by aspirating the medium and then rinsing the PTECs with ice cold 1x PBS. PTECs were then immersed in 350ul of buffer RLT plus 2-mercaptoethanol in order to release the RNA and scraped off the dishes. The lysates were then transferred into QIA-shredder spin columns (Qiagen) for homogenisation and centrifuged (6720g, Henderson Biomedical) for 2 min at room temperature. To this flow through, a further 350ul of 70% ethanol was added, mixed gently, and dispensed into new RNeasy spin columns with a 2ml collection tube. This was centrifuged (6270g, Henderson Biomedical) for 15 sec also at room temperature. Next, 700ul of buffer RW1 was added into the spin column and spun (6270g, Henderson Biomedical) for 15 sec again. The flow through was discarded and 500ul of buffer RPE was transferred into the column and spun (6270g, Henderson Biomedical) for a further 15 sec. This step was repeated once more, this time centrifuging (6270g, Henderson Biomedical) for 2 min. The columns were then dry spun for (6270g, Henderson Biomedical) 1 min before being placed into a new 1.5ml microcentrifuge tube. Finally, 30ul of RNase free water was passed through the column, incubated for 2 min, and centrifuged for 1 min (6270g, Henderson Biomedical) to elute the RNA. Columns were discarded and RNA stored at -80°C before the subsequent steps of reverse transcription and qPCR.

2.11. Reverse transcription

Reverse transcription is the biological process where the reverse transcriptase enzyme generates a copy of single stranded complementary DNA (cDNA) from total RNA. As total RNA is too unstable, the cDNA synthesised is used as a template for the polymerase chain reaction (PCR).

RNA concentration was measured and quantified on a Nanodrop® 2000C spectrophotometer (Thermofisher). The 260/280 absorbance ratio value is an indicator of RNA purity and level of contamination with DNA. Samples were considered to possess low DNA contamination if the absorbance ratio was greater than 1.8. The volume required for 1µg of RNA was calculated and made up to 10µl using nuclease free water. Complementary DNA (cDNA) was produced via a reverse transcription polymerase chain reaction (RT-PCR) using the High Capacity cDNA Reverse Transcription kit (Thermofisher). This kit was a master mix recipe comprising of 10x RT buffer, 10x RT random primers, multiscribe reverse transcriptase, 25x deoxynucleoside triphosphate (dNTP) mix, RNase inhibitor, and nuclease free water to create a total volume of 20µl per reaction (Table 2.5). The samples were briefly centrifuged to eliminate air bubbles before being loaded into the thermal cycler with the following settings: 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C.

Component	Volume per reaction (μl)
10 X RT Buffer	2
25 X dNTP Mix (100mM)	0.8
10x RT random primers	2
Reverse Transcriptase	1
RNase Inhibitor	1
RNase free water	3.2
RNA sample (1ug)	10

Table 2.5: Required volumes of each reverse transcription reagent per reaction.

2.12. Quantitative real time polymerase chain reaction (qPCR)

Relative gene expression was quantified using qPCR. This process involves the continuous measurement of amplified mRNA targets using fluorescence, allowing us to obtain data in real time. I have used the TaqMan® primer probes (Thermofisher) to analyse my specific genes of interest. This probe was designed to bind to a specific DNA sequence between the forward and reverse primers and are joined with a 5' reporter dye and 3' quencher molecule. When the reporter and quencher are bound together in close proximity, the reporter dye's fluorescence is absorbed by the quencher. When the probe is cleaved during the extension phase, the reporter is released and its fluorescent emissions begins to increase. An increase in the fluorescent signal of the reporter is directly proportional to the abundance of PCR product. The point in time at which the fluorescent signal becomes greater than the background fluorescence is referred to as cycle threshold (Ct), and is relative to the level of gene expression. Therefore, a sample that contains a greater initial quantity of the target gene will require a lower number of amplification cycles to pass the threshold in comparison to a sample that has a lower quantity of the target gene.

To begin, the cDNA obtained from reverse transcription was diluted 1 in 10 in nuclease-free water. For every 1µl of diluted cDNA, 1µl of a housekeeping primer probe (GAPDH [glyceraldehyde 3-phosphate dehydrogenase], VIC® labelled, Thermofisher) was combined with 1µl of Taqman target gene expression assay (FAM™ labelled, Thermofisher) and 10µl of 1x Taqman gene expression master mix (Thermofisher) on a 96-well PCR plate (Bio-Rad) (Table 2.6). This was covered with adhesive seal to prevent spillage and cross contamination. Quantification of gene targets was carried out by the CFX96 Real Time system thermocycler (Bio-Rad) using their pre-optimised qPCR thermal cycler program (Table 2.7). This was accompanied with the CFX manager software which performed the subsequent data analysis. The fold changes in cDNA expression were presented as values normalised to the housekeeping gene using the $\Delta\Delta C_t$ method (Equation 2.1). GAPDH was selected as the housekeeping gene as it was ranked high in stability during subjection to hypoxic and hyperglycaemic conditions after comprehensive genome wide array and qPCR analysis (Bakhashab *et al.*, 2014). A negative control was also included on the 96 well plate, where the cDNA sample was

replaced with nuclease free water to confirm that no signal occurs in the absence of genetic material.

Gene	Assay ID	Reference sequence
GAPDH	Hs99999905_m1	NM_002046.3
SGLT2/SLC5A2	Hs00894642_m1	NM_003041.3
SGLT1/SLC5A1	Hs01573793_m1	NM_000343.3
CCN2/CTGF	Hs00170014_m1	NM_001901.2
FN (Fibronectin)	Hs01549976_m1	NM_001306129.1

Table 2.6: Taqman Gene Expression Assays.

Cycle Step	Temperature	Time	Cycles
Initial denaturation	95°C	10 min	1
Denaturation	95°C	15s	1x
Annealing	55°C	1 min	1
Extension	95°C	15 sec	40 x
Melting curve	65°C	5 sec	1
Melting curve	95°C	50 sec	1

Table 2.7: PCR thermal cycler program.

$$2^{-\Delta\Delta C_t}(\text{sample}) = [C_t(\text{target gene}) - C_t(\text{housekeeping gene}) \text{ treatment sample} \\ - C_t(\text{target gene}) - C_t(\text{housekeeping gene}) \text{ control sample}]$$

Equation 2.1: Relative Ct method for calculating fold change

2.13. Plasmid production and purification

2.13.1 Ampicillin preparation

Ampicillin stock solution (1000x, Sigma-Aldrich) was prepared by dissolving 100mg Ampicillin in 1 ml of deionised water, followed by filtration using 0.2µm filter (Whatman). The stock solution was aliquoted and stored at -20°C until required.

2.13.2 Luria broth (LB) preparation

LB broth was prepared by dissolving 10g LB Broth (Sigma-Aldrich) powder in 500ml of deionised water to give a 10g/L tryptone (pancreatic digest of casein), 5g/L yeast extract and 5g/L NaCl composition. This was sterilised by autoclaving. After cooling down the broth, ampicillin stock solution was added to give a final working concentration of 100µg/ml.

2.13.3 LB agar preparation

LB agar was prepared by dissolving 17.5g LB agar powder (Sigma-Aldrich) in 500ml of deionised water to give 10g/L tryptone (pancreatic digest of casein), 5g/L yeast extract, 5g/L NaCl and 15g/L agar. This was sterilised by autoclaving. After cooling down, ampicillin stock solution was added to the molten agar to give a final concentration of 100µg/ml. The agar was then poured into 100mm sterile Petri dishes (BD Falcon) and left to set for 20 min.

2.13.4 Plasmid processing

The pCS2 Flag Smad3 S204A dominant negative mutant plasmid (Addgene plasmid # 27114), generated by encoding a serine to alanine mutation at the serine 204 phosphorylation site of the Smad3 linker region (LR), was kindly provided by Dr Joan Massague of the Sloan Kettering Cancer Centre (Gao *et al.*, 2009). The empty vector

pCS2+ plasmid (Addgene plasmid # 34931) was kindly provided by Dr Amro Hamdoun of the Scripps Institution of Oceanography (Gökirmak *et al.*, 2012). Both plasmids were delivered as bacterial *Escherichia coli* (E. coli) stabs and processed for long term storage within two weeks. Cell spreaders (Scientific Laboratory Supplies) were used to streak E. coli onto the agar plates. These plates were then incubated overnight at 37°C in order to obtain isolated colonies in an orbital shaker (Labnet). Agar plates (BD Falcon) were inspected for isolated bacterial growth the next day with identifiable single colonies indicating confirmation of successful transformation. Singular ampicillin resistant colonies from different plates were selected and cultured in 5ml of LB broth containing ampicillin (100µg/ml) overnight at 37°C in the orbital shaker at 200rpm. From the 5ml culture, 3ml was stored at -80°C in 20% glycerol for future stock, and 2ml was used to inoculate 200ml of LB broth with ampicillin (100µg/ml). The latter was then incubated overnight at 37°C in the orbital shaker at 200rpm.

2.13.5 Plasmid purification

Purification of the plasmids were carried out in accordance to the QIAprep® “maxi prep” protocol (Qiagen). The overnight bacterial growth cultures were left to rotate in the orbital shaker until the solution became turbid. A 50ml aliquot for maxi prep was centrifuged at 4°C for 5 min (10000g, Henderson Biomedical). The supernatant was quickly discarded and the pellet kept for the following stage. The QIAprep miniprep technique is centred on the alkaline lysis of various bacterial cells. The lysate is neutralized and adjusted to high salt binding conditions. After the lysate is cleared via centrifugation, the DNA from the lysate is bound by QIAprep tips containing a silica membrane by anion-exchange whilst the cellular proteins, metabolites and RNA are cleared through the column by gravity flow. Decanting buffer QC through the silica membrane washed and then removed endonucleases and salts. The plasmid DNA is then eluted via the QIAprep spin column using approximately 50µl of nuclease free water. DNA concentration was quantified using UV spectrophotometry on the Nanodrop® 2000C (Thermoscientific). The 260/280 absorbance ratio value is an indicator of DNA purity and level of contamination with protein. Samples were considered to possess low protein contamination if the absorbance ratio was greater than 1.8. The DNA was then subsequently stored at 20°C until further use.

2.13.6. Plasmid transfection

Eukaryotes do not typically maintain plasmids in nature, but transient insertion/transfection of plasmid DNA into cultured adherent mammalian cells has enabled the genetic code that is encoded within the plasmid to also be expressed in the host cell. Transfection was performed using the cationic liposome-mediated transfection reagent Fugene 6 (Promega), a non-liposomal agent that transfects with high efficiency and minimal toxicity. The Fugene 6 to plasmid DNA ratio used for transfection experiments was 3(ml):1(μ g), so that each 35mm culture dish receives 0.5 μ g/1 μ g/2 μ g of plasmid DNA and 1.5ml/3ml/6ml Fugene 6 respectively. The manufacturer's recommendations stated that cells between 50-80% confluent could be transfected in Lonza medium with supplements (see section 2.2.1) and 0.5% FBS (Lonza). PTECs were grown to ~70% confluence. Once the Fugene 6 was incubated with the medium, it was vortexed briefly and left to sit for 15 min at room temperature. Plasmid DNA was then added to the mixture, vortexed briefly and left to sit out for 5 min. 100 μ l of this plasmid DNA mixture was then added dropwise to each dish to ensure equal distribution. These dishes were incubated at 37°C for 24h and recovered in normal medium for 4 or 8h. PTECs were then lysed using the same method in section 2.3.3.

2.14. Small interference RNA (siRNA) transfection

RNA interference is the biological mechanism by which the introduction of double-stranded RNA (dsRNA) into cells can trigger gene silencing by causing cleavage and degradation of complementary mRNA. These small interfering RNAs (siRNAs) are between 20-23 nucleotides long. Once this siRNA is incorporated with other protein components like ribonucleoprotein, it forms an RNA induced silencing complex (RISC). As the RISC complex becomes activated, the double stranded siRNA strands are unwound. The anti-sense single stranded siRNA that is no longer integrated to the RISC now guides the complex while searching for a target mRNA. Once found, the mRNA is cleaved and degraded by catalytic RISC protein. This disrupts the translation process and effectively reduces the abundance of the protein of interest.

Transfections were performed when PTECs were at approximately 70% confluence. The spent medium was replaced by Opti-MEM™ reduced serum medium (Thermofisher) in order to accommodate the addition of Lipofectamine™ 2000 transfection reagent (Thermofisher). The transfection mixture of Opti-MEM and Lipofectamine (diluted 1/50 from the original stock) was combined at a 900µl:100µl volume ratio in each 35mm dish. To this mixture, either silencer® select SGLT2 siRNA, silencer® select SGLT1 siRNA, or non targeting silencer® select negative control siRNA was added. All three siRNAs (Thermofisher) were used at concentrations of 15nM and 30nM. Transfection was performed for 24h before the mixture was changed back to Lonza medium for a further period of 24h to allow for recovery from transfection. PTECs were then lysed and analysed via qPCR as detailed above in section 2.12 above.

2.15. Immunofluorescence (IF) microscopy

Immunofluorescence microscopy is an effective technique widely employed by scientists that utilises fluorescently labelled antibodies to detect the location and abundance levels of a specific target protein. The primary antibody is used to probe the antigen within the protein of interest and is bound to a secondary antibody of a different origin labelled with a fluorescent dye. This specific fluorophore conjugated secondary antibody will then fluoresce at a distinct wavelength when excited. Visualisation of multiple antigens at the same time is possible as long as the primary antibodies used are raised from different species, thus allowing the secondary antibodies conjugated with different fluorophores to emit at differing wavelengths.

PTECs were cultured on collagen IV coated coverslips kept in 24 well plates (Corning) with an approximate growth area of 2cm². The spent medium was removed and the cells were gently washed with 1x PBS twice. Formaldehyde (4%) solution (Thermofisher) was then added to fix and permeabilise the PTECs and incubated at room temperature for 15 min in a fume cupboard. Upon fixation, the solution was removed and the PTECs were washed gently 3 times with 1x PBS. At this point, the cells can be stored in PBS at 4°C for up to one month. The PTECs were then incubated with glycine (0.1M) in 1xPBS for 5 min at room temperature before gently washing with 1xPBS 3 times. Each well was then

incubated with 300µl blocking buffer (10% w/v FBS, 0.1% w/v Triton X-100, Sigma-Aldrich) for 45 min at room temperature. The purpose of this stage was to block non-specific antigens from binding to the primary antibody. After blocking, the PTECs were once again gently washed with 1xPBS before being incubated in 200µl of primary antibody diluted in blocking buffer and left at 4°C overnight. The PTECs were gently washed in 1xPBS 3 times before incubation in 200µl of secondary antibody diluted in blocking buffer for 1h at room temperature in the dark. The PTECs were rinsed in 1xPBS for a final time before the coverslips are mounted with a few drops of 4',6-diamidino-2-phenylindole (DAPI) solution (1mg/ml, Thermofisher) on a slide and left to dry for 20 min. Nail varnish was then used to seal the edges of the coverslip. Images were captured on a fluorescent microscope (Nikon) (Figure 2.7).

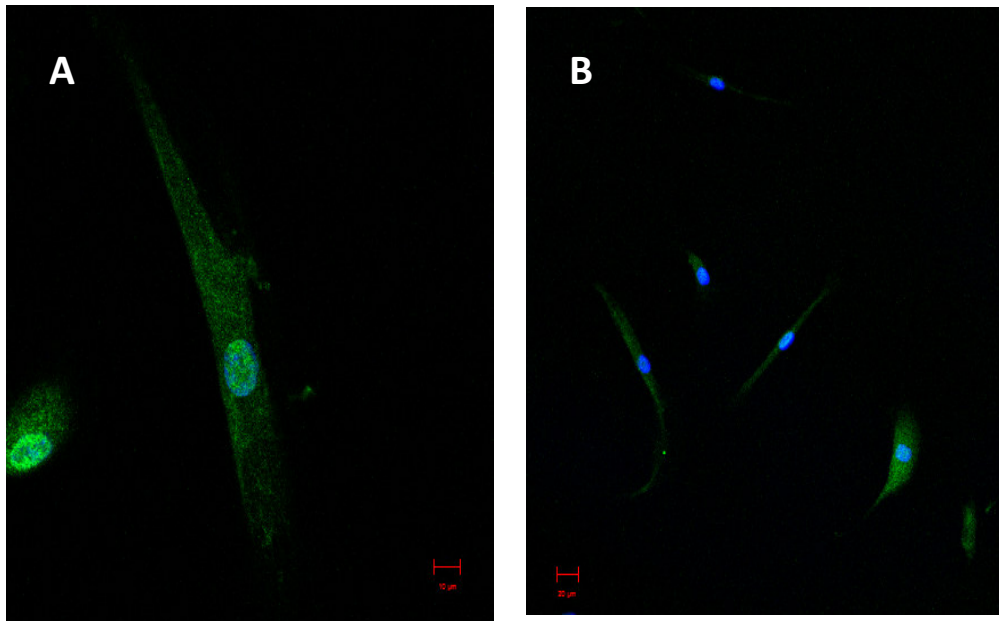


Figure 2.7: Immunofluorescent staining of SGLT2 protein in PTECs.

Immunofluorescent visualisation of SGLT2 in (A) a singular PTEC and (B) a group of PTECs. SGLT2=green stain, Nucleus=blue stain.

2.16. Glucose Uptake Assay

I have used the Glucose Uptake-GloTM assay (Promega) to analyse the rate of phosphorylated glucose take up by PTECs. This assay is a non-radioactive, end point plate-based bioluminescent method centred around the detection of 2-deoxyglucose-6-phosphate (2DG6P). When 2-deoxyglucose (2DG) is administered to target cells, it is transported across the membrane and efficiently phosphorylated to 2DG6P by hexokinase in the same fashion as D-glucose. The differentiation is that the enzyme responsible for metabolising glucose-6-phosphate during glycolysis (phosphoglucose isomerase) cannot modify 2DG6P specifically, therefore the latter will begin accumulating in the cell. After treatment with 1nM dapagliflozin, (Cayman chemical), phloretin, and phlorizin (100µM and 200µM, Selleck Chem), PTECs were washed by 1xPBS 3 times before 2DG (50µl, 1mM) was added to each well and left for 20 min at room temperature. After this incubation period, stop buffer (25µl), an acidic detergent solution, was then added to lyse PTECs, stop uptake and eliminate cellular NADPH. In order to neutralise the acidic mixture, a highly alkaline buffer solution (25µl) was then added. Finally, a 2DG6P detection reagent solution comprised of NADP⁶⁺, reductase, proluciferin substrate, Ultra-GloTM recombinant luciferase, and glucose-6-phosphate dehydrogenase (G6PDH) was added to each well. Reductase aids the NADPH- mediated conversion of proluciferin to luciferin which was subsequently used by the Ultra-GloTM recombinant luciferase to produce luminescence. This light signal was measured on the FLUOstar Omega microplate reader (BMG Labtech). The strength of the luminescent signal emitted from the plate is directly proportional to 2DG6P concentration (Figure 2.8).

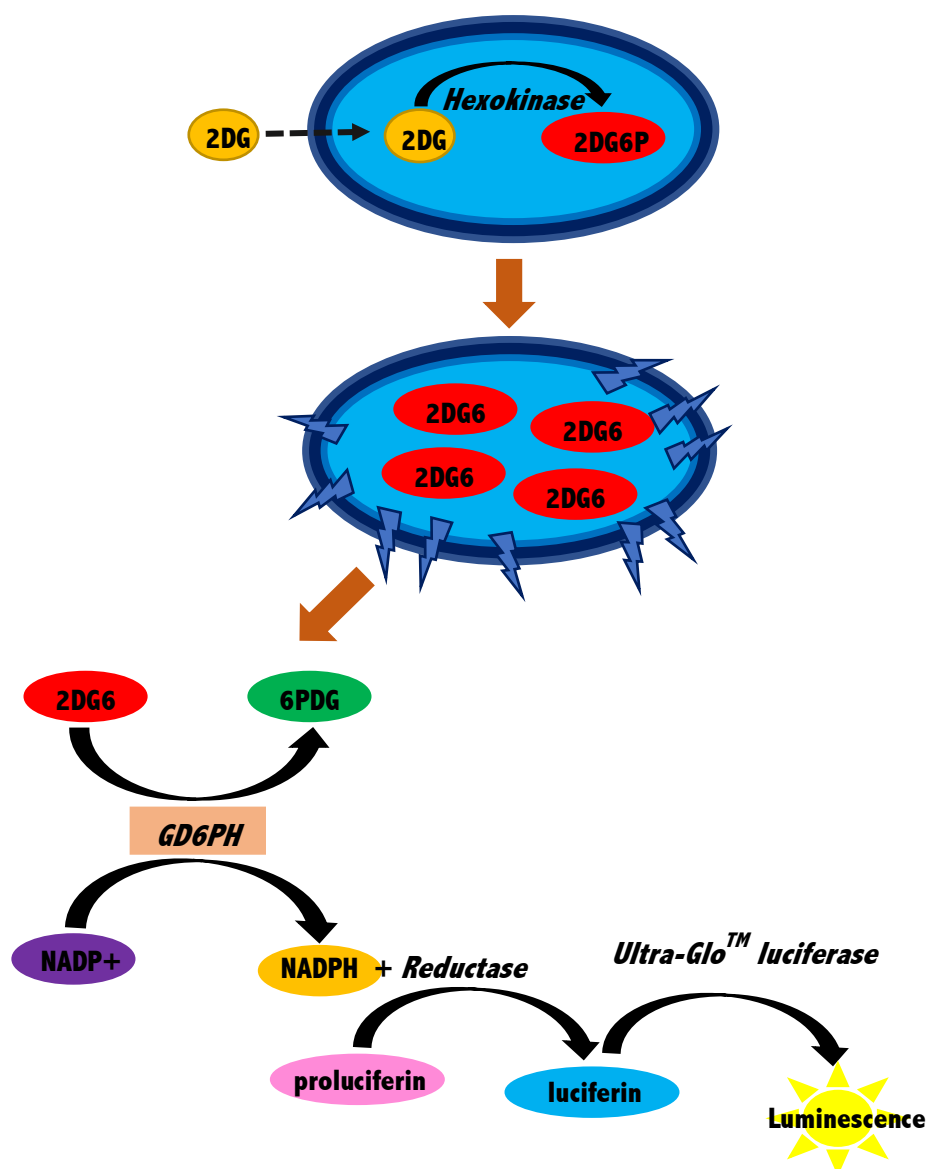


Figure 2.8: Schematic diagram of the Promega Glucose Uptake Glo-™ Process.

2-deoxyglucose (2DG) enters the PTEC and is phosphorylated to 2-deoxyglucose-6-phosphate (2DG6P) by hexokinase. 2DG6P cannot be phosphorylated by the enzymes responsible for glycolysis so it begins accumulating in the cell. The addition of stop buffer halts 2DG transport, lyses cells and stops 2DG uptake. This is then neutralized by using neutralization buffer which is followed by the addition of 2DG6P detection reagent. The glucose-6-phosphate dehydrogenase (G6PDH) found in the 2DG6P Detection Reagent mixture simultaneously mediates both the oxidation of 2DG6P to 6-phosphodeoxygluconate (6PDG) and reduction of NADP⁺ to NADPH. Reductase then facilitates the NADPH mediated conversion of the proluciferin to luciferin, which the Ultra-Glo™ Recombinant Luciferase then uses to produce luminescence in the final stage. Figure adapted from Promega Glucose Uptake-Glo™ Assay Technical Manual TM467.

2.17. Deproteinisation of medium

As proteins can interfere with the sensitivity of the high performance liquid chromatography (HPLC) assay and degrade the HPLC column, a prior deproteinisation step is required. Spent medium was diluted 1 in 20 with 1xPBS in preparation for the micro-BCA assay (Thermo scientific). The remainder of this spent medium was then mixed with ice-cold acetonitrile, an organic solvent used to pull apart and separate proteins, at a ratio of 40:60 in microcentrifuge tubes. This mixture was then left on ice for 15 min before centrifugation (6270g, Henderson Biomedical) for 10 min at 4°C. The supernatant was removed and retained in new tubes, with the volume retrieved noted down. This mixture was then diluted 1 in 8 (after deproteinisation sample) and plated along with the original 1 in 20 spent medium dilution (before deproteinisation sample) into a 96 well plate (Corning). This was plated along with the constructed standard curve of known concentrations of BSA (Figure 2.9) also dissolved in 1xPBS. The plate was then incubated at 37°C for 2h. The difference in dilution factors was to account for the 2.4x dilution of medium upon addition of acetonitrile. Measuring the protein before and after deproteinisation using the BCA assay allows assessment of deproteinisation efficiency. The deproteinisation validation data was calculated using the generated standard curve equation (Table 2.8). The deproteinised mixture is then placed in the rotary evaporator (CHRIST) at 60°C for 24h to increase the concentration approximately 10x before noting down the new volume. The evaporated samples were kept in the fridge until further use. Protein absorbance was measured using the Sunrise microplate absorbance reader (Tecan, wavelength range of 340nm to 720nm) and concentration subsequently calculated using the standard curve.

Standard curve equation

$$Y = 0.0128x + 0.0282$$

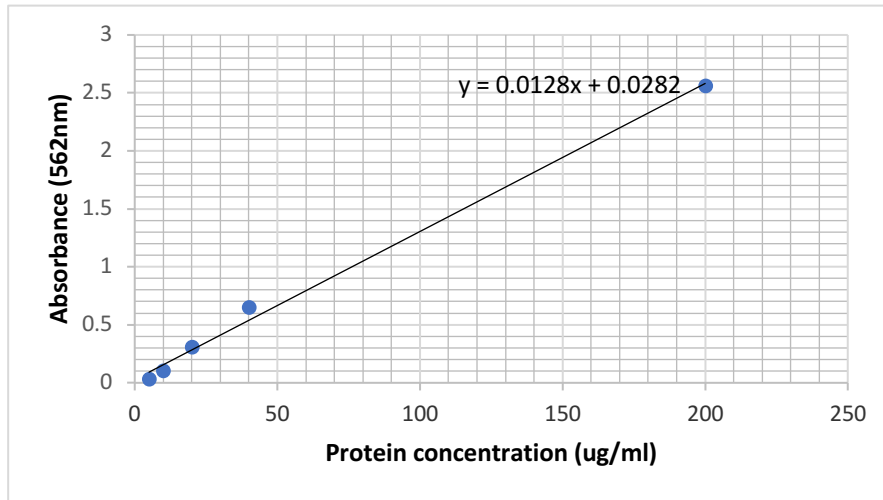


Figure 2.9: Standard curve graph generated from micro-BCA assay.

Y= average absorbance value, x= concentration, m=0.0128, c=0.0282

	Average Absorbance (avg A) (n=3)	Concentration (conc) (x) ($\mu\text{g/ml}$) $x=(y-c)/m$	Final conc ($\mu\text{g/ml}$)	Deproteinisation efficiency (%) [final conc(DB)-final conc(AD)]/final conc(BD)]*100
Before deproteinisation (BD) (1 in 20 dilution)	0.8483	64.07	$x*20$ = 1281.46	~86% deproteinisation efficiency
After deproteinisation (AD) (1 in 8 dilution)	0.2983	21.1	$x*8$ =168.83	

Table 2.8: Deproteinisation validation data.

2.18. High performance liquid chromatography (HPLC)

HPLC is a chromatographic technique employed to separate a mixture of compounds in order to identify and quantify the individual components. The separation principle is primarily based on the interaction between the analyte mixture (sample), mobile phase (eluent), and stationary phase (porous material packed into the column). The samples injected by an auto-sampler in the mobile phase are pushed through to the separation column (stationary phase) by a pump. If a constituent of the sample has more affinity with the mobile phase compared to the stationary phase, then that specific constituent will elute through the column at a quicker rate. The quicker the constituent elutes from the column, the shorter the retention time. The retention time is important as different constituents of each sample will have different retention times. A detection unit will recognise the constituents as they elute from the column. The signals are then converted and recorded by a software system and displayed as chromatographic peaks. Each constituent can be identified by comparing each peak's retention time with the retention times of injected working standards from the same mobile and stationary phase. The area under an identified peak (peak area) measures the concentration of the constituent it represents. Thus, the greater the peak area, the more concentrated the constituent is. Similarly, the greater the peak height is, the more concentrated the constituent is. The peak area and height are automatically calculated from the standard curve by the software system.

The HPLC experiment parameters used in this thesis were based on a previously established HPLC-UV method used to detect dapagliflozin (Donepudi and Achanta, 2019). Chromatography was performed with the Agilent 1100 HPLC system consisting of a binary pump, auto-sampler and UV detector. Signals were converted into peaks and analysed by Agilent Chemstation software. The mobile phase consisted of 0.1% orthophosphoric acid and acetonitrile (40:60) and separation was achieved using a Prodigy C18 column (150x5 μ mX4.6mm) at a 1ml/min flow rate. Stock solutions of dapagliflozin were diluted to obtain standards of known concentrations of the inhibitor (0.05 μ M, 0.1 μ M, 0.2 μ M, 0.5 μ M and 1 μ M) and run under the same conditions as my samples to generate the standard curve.

2.19. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 and Bio-Rad CFX 1000 manager software where P values < 0.05 were considered statistically significant. Quantitative data are presented as means $\pm$ standard deviation (SD). Comparison between two means was carried out using the unpaired t-test. One-way analysis of variance (ANOVA) with Bonferroni's post-hoc comparison test was used to compare the means of more than two treatment groups. The P values of the qPCR data carried out using the CFX 1000 manager software was calculated as follows (Equation 2.2):

$$p\text{-value} = 1 - A$$

Where:

•

$$A = \int_{x=-t}^t = \frac{\Gamma\left(\frac{v+1}{2}\right)}{\sqrt{v\pi}\Gamma\left(\frac{v}{2}\right)} \left(1 + \frac{x^2}{v}\right)^{-\frac{v+1}{2}}$$

Where:

$$v = \text{Count (NE}_{\text{sample (Experimental)}}) + \text{Count (NE}_{\text{sample (Control)}}) - 2$$

- Γ = gamma function
- t = t-statistic

$$t = \frac{\left| \text{Mean}(\text{NE}_{\text{sample(exp.)}}) - \text{Mean}(\text{NE}_{\text{sample(control.)}}) \right|}{\sqrt{\frac{\left(\text{Count}(\text{NE}_{\text{sample(exp.)}}) - 1 \right)^2 \text{SD}(\text{NE}_{\text{sample(exp.)}})^2 + \left(\text{Count}(\text{NE}_{\text{sample(control.)}}) - 1 \right)^2 \text{SD}(\text{NE}_{\text{sample(control.)}})^2}{\text{Count}(\text{NE}_{\text{sample(exp.)}}) + \text{Count}(\text{NE}_{\text{sample(control.)}}) - 2}} * \sqrt{\left(\frac{1}{\text{Count}(\text{NE}_{\text{sample(exp.)}})} + \frac{1}{\text{Count}(\text{NE}_{\text{sample(control.)}})} \right)}}$$

Where:

- Mean = Arithmetic mean
- NE = Normalized expression
- Count(x) = Size of list x
- SD = Sample standard deviation

Equation 2.2: Mathematical formula used by the Bio-Rad CFX Manager Software to calculate P value.

Chapter 3:

Characterisation of the Diabetic Tubule Epithelial Cell Model

3.1. Introduction

Albumin that has gone through the glomerular ultrafiltrate travels to the PT where it is taken up by large glycoproteins megalin and cubulin via clathrin-mediated endocytosis through vesicles produced from the cell membrane. They are then targeted for degradation in lysosomes (Gianesello *et al.*, 2017). The presence of increased urinary albumin excretion (UAE), an important characteristic of diabetic kidney disease, was originally only attributed to an increase in glomerular filtration as a result of persistent glomerular damage or hyperfiltration (Tojo *et al.*, 2001).

Reports in the following years however, also highlighted the importance of the PT in urinary albumin production. Russo *et al* reported that while there was a substantial increase in UAE and albumin-derived peptide excretion in their streptozotocin (STZ) induced diabetic rats, there was no change in both the glomerular sieving coefficient (GSC) or glomerular filtration rate (GFR) when compared to the control group. Interestingly, they did find that there was a dramatically reduced uptake of albumin in the PT of diabetic rats, indicating the significance of PT albumin handling in generating albuminuria seen in DKD (Russo *et al.*, 2009). In cultured LLC-PK1 proximal tubule cells, a different group found a substantial decrease in colloidal albumin endocytosis mediated by Rab38 mRNA knockdown without causing any changes to glomerular permeability. Rab38 knockdown in their transgenic mouse model produced a proteinuric phenotype (Rangel-Filho *et al.*, 2013). Furthermore, Wagner *et al* examined both the GSC and PT albumin uptake in their albumin overload Munich Wistar Fomter (MWF) rat model. These rats showed positive correlation between increases in urinary albumin values and decreases in PT associated albumin uptake but no correlation with GSC (Wagner *et al.*, 2016).

These findings, along with the identification of various PT markers of kidney disease (Fassett *et al.*, 2011) and the fact that tubulointerstitial fibrosis remains the best prognostic determinant of renal function, have highlighted the importance of the PT in DKD. Experiments described in this first results chapter will focus on characterising and examining the function of my DKD primary PTEC model. This *in vitro* model is the cell line used for all subsequent experiments.

3.2. Aims and Objectives

1. To investigate the regulation of SGLT2 and SGLT1 protein and mRNA abundance in PTECs following treatment with glucose and TGF- β 1.
2. To confirm PTEC phenotype through gamma glutamyl-transferase (GGT) staining.
3. To confirm successful knockdown of SGLT2 protein and mRNA in PTECs through the use of SGLT2 targeted small interference RNA (siRNA).
4. To examine the function of SGLT2, SGLT1 and GLUT in PTECs through the use of inhibitors that selectively target these transporters.

3.3. Results

3.3.1. SGLT2 and SGLT1 protein and mRNA abundance in PTECs treated with glucose and TGF- β 1

SGLT2 western blots revealed a mature glycosylated (~73kDa) and immature non-glycosylated (~60kDa) protein band in PTECs, although the latter was not always reproducible. The results revealed no change of SGLT2 protein abundance in the PTECs grown under control conditions (7mM D-glucose alone) compared to the osmotic control (7mM D-glucose+18mM L-glucose). Likewise, 0.75ng/ml TGF- β 1 treatment on its own did not change SGLT2 protein abundance. However, there was a significant decrease of SGLT2 protein abundance in TGF- β 1 treated PTECs under both high D-glucose (7mM D-glucose+18mM D-glucose) and osmotic control conditions (Figure 3.1A). This result was only observed in the PTECs grown on collagen IV (Figure 3.1A&B). Therefore, experiments from here onwards focused exclusively on PTECs grown on collagen IV.

There was no change in SGLT2 mRNA abundance under control, osmotic control, or high D-glucose conditions, with or without TGF- β 1 (Figure 3.1 C).

SGLT1 western blots also revealed a mature glycosylated 73kDa and immature non-glycosylated 60kDa protein band in PTECs. There was no change in SGLT1 protein and mRNA abundance in PTECs exposed to the conditions detailed above (Figure 3.1 D&E).

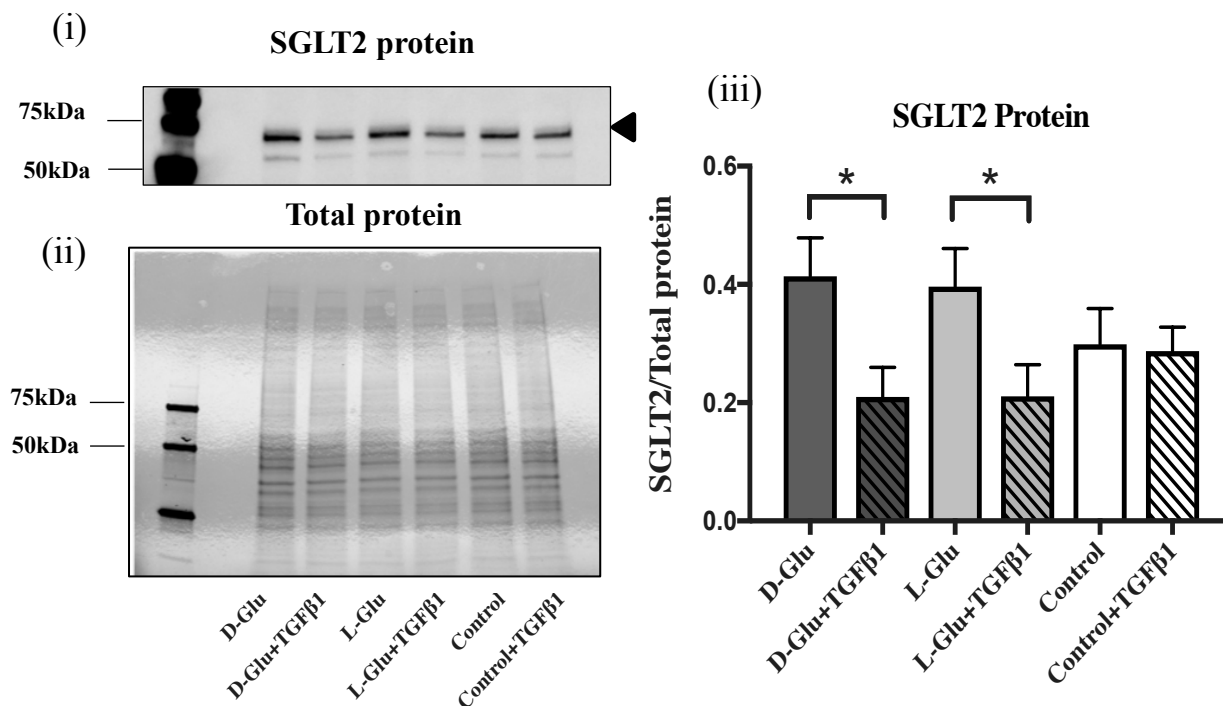


Figure 3.1A: SGLT2 protein abundance in PTECs cultured on collagen IV and treated with glucose and TGF-β1.

Key: **Control**= 7mM D-glucose, **Control+ TGF-β1**= 7mM D-glucose+ 0.75ng/ml TGF-β1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGF-β1**=7mM D-glucose +18mM D-glucose+ 0.75ng/ml TGF-β1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGF-β1**= 7mM D-glucose+18mM L-glucose+ 0.75ng/ml TGF-β1. (i) Western blot showing SGLT2 protein (~73kDa) in PTECs subjected to different treatments for 24h. The black arrowhead indicates the target immuno-reactive band, (ii) corresponding total protein stain free gel which is used as the loading control, (iii) plotted data points after quantification of SGLT2 protein (SGLT2 protein abundance/total protein abundance) presented in graph format. There was a significant decrease of SGLT2 protein in PTECs under both L-Glu and D-Glu conditions after treatment with TGF-β1. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test, *P<0.05, all n=3. Data are shown as mean +/- SD.

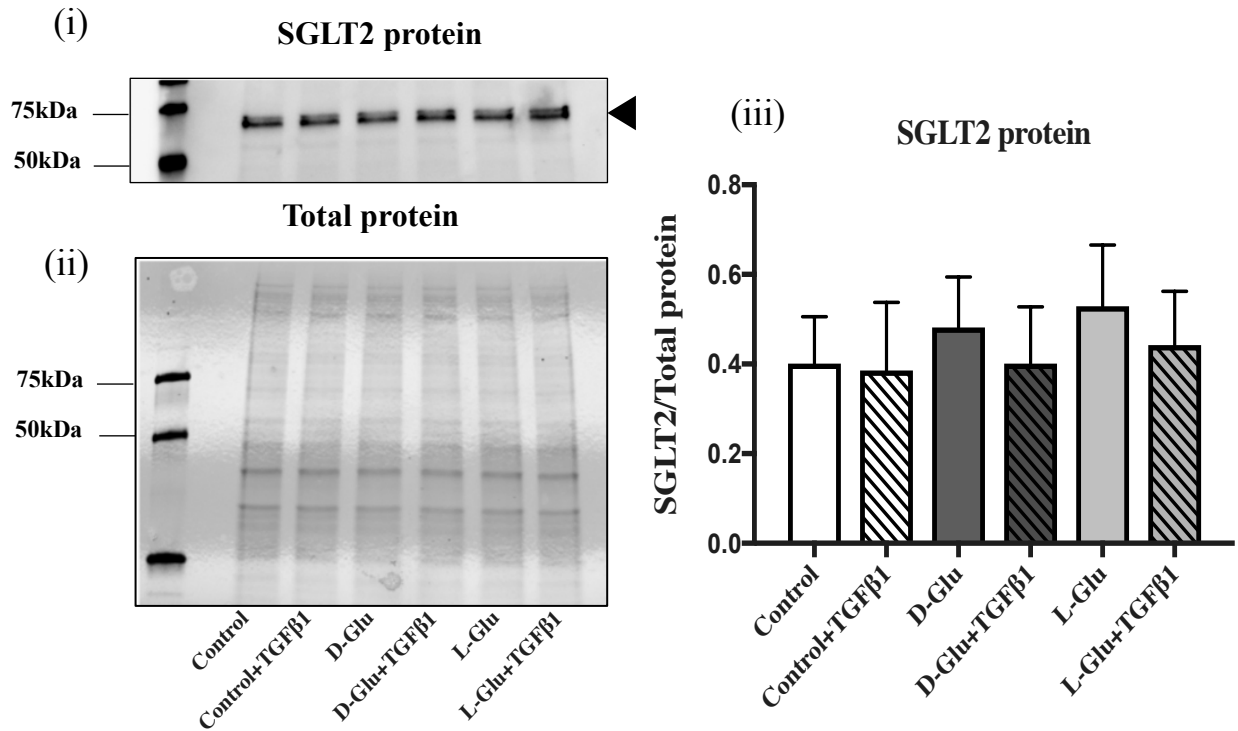


Figure 3.1B: SGLT2 protein abundance in PTECS cultured on plastic and treated with glucose and TGF-β1.

Key: **Control**= 7mM D-glucose, **Control+ TGF-β1**= 7mM D-glucose+ 0.75ng/ml TGF-β1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGF-β1**=7mM D-glucose +18mM D-glucose+ 0.75ng/ml TGF-β1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGF-β1**= 7mM D-glucose+18mM L-glucose+ 0.75ng/ml TGF-β1. (i) Western blot showing SGLT2 protein (~73kDa) in PTECs subjected to different treatments for 24h. The black arrowhead indicates the target immuno-reactive band, (ii) corresponding total protein stain free gel which is used as the loading control, (iii) plotted data points after quantification of SGLT2 protein (SGLT2 protein abundance/total protein abundance) presented in graph format. There was no change in SGLT2 protein under any of the conditions. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test, all n=3. Data are shown as mean +/- SD.

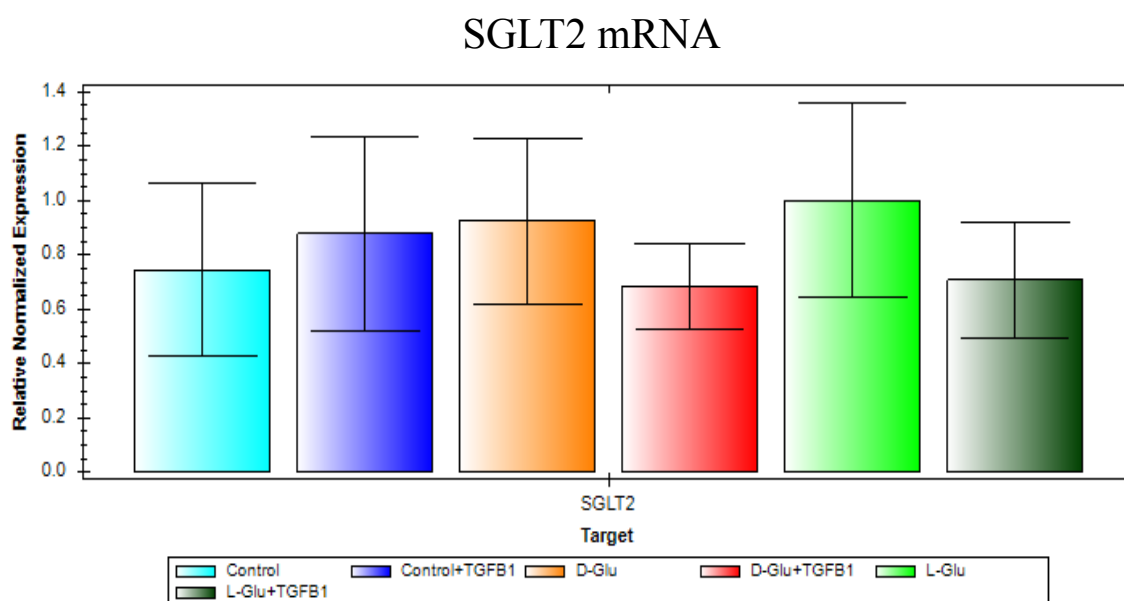


Figure 3.1C: SGLT2 mRNA abundance in PTECS cultured on collagen IV and treated with glucose and TGF-β1.

Key: **Control**= 7mM D-glucose, **Control+ TGF-β1**= 7mM D-glucose+ 0.75ng/ml TGF-β1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGF-β1**=7mM D-glucose+18mM D-glucose+ 0.75ng/ml TGF-β1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGF-β1**= 7mM D-glucose+18mM L-glucose+ 0.75ng/ml TGF-β1. All treatment times lasted 24h. The figure shows plotted data points after quantification of SGLT2 mRNA (SGLT2 mRNA abundance/GAPDH mRNA abundance) presented in graph form. There was no change in SGLT2 mRNA abundance under any of the treatments. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. All n=4. Data are shown as mean +/- SD.

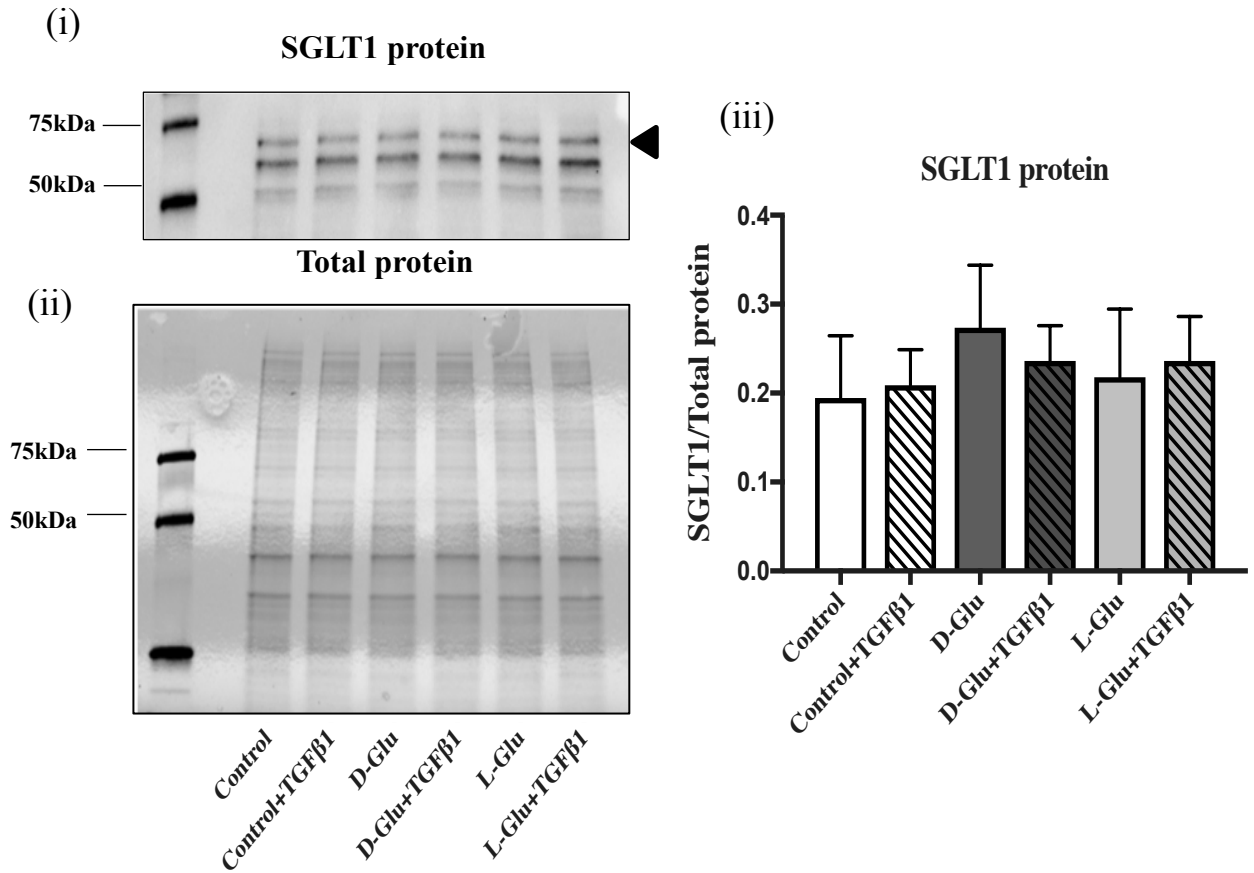


Figure 3.1D: SGLT1 protein abundance in PTECS treated with glucose and TGF-β1.

Key: **Control**= 7mM D-glucose, **Control+ TGF-β1**= 7mM D-glucose+ 0.75ng/ml TGF-β1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGF-β1**=7mM D-glucose+18mM D-glucose+ 0.75ng/ml TGF-β1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGF-β1**= 7mM D-glucose+18mM L-glucose+ 0.75ng/ml TGF-β1. (i) Western blot showing SGLT1 protein (~73kDa) from PTECs subjected to different treatments for 24h. The black arrowhead indicates the target immuno-reactive band, (ii) corresponding total protein stain free gel which is used as the loading control, (iii) plotted data points after quantification of SGLT1 protein (SGLT1 protein abundance/total protein abundance) presented in graph format. There was no change in SGLT1 protein in PTECs under any of the treatments. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test, all n=3. Data are shown as mean +/- SD.

SGLT1 mRNA

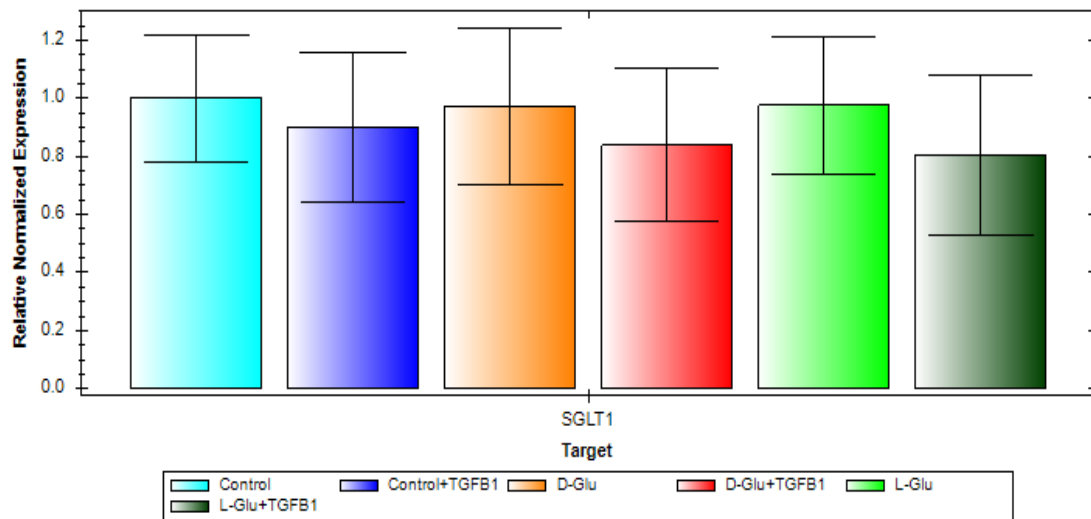


Figure 3.1E: SGLT1 mRNA abundance in PTECS treated with glucose and TGF- β 1.

Key: **Control**= 7mM D-glucose, **Control+ TGF- β 1**= 7mM D-glucose+ 0.75ng/ml TGF- β 1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGF- β 1**=7mM D-glucose+18mM D-glucose+ 0.75ng/ml TGF- β 1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGF- β 1**= 7mM D-glucose+18mM L-glucose+ 0.75ng/ml TGF- β 1. All treatment times lasted 24h. The figure shows plotted data points after quantification of SGLT1 mRNA (SGLT1 mRNA abundance/GAPDH mRNA abundance) presented in graph form. There was no change in SGLT1 mRNA abundance under any of the treatments. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. All n=4. Data are shown as mean $\pm$ SD.

3.3.2. Confirmation of PTEC phenotype

To check for any changes in phenotype or structure following high D-glucose+0.75ng/ml TGF- β 1 treatment, microscopic images of PTECs were taken. Microscopic image results indicate that osmotic control PTECs form a compact epithelial structure, creating a cobblestone-like pattern. This did not change under high D-glucose+0.75ng/ml TGF- β 1 treatment (Figure 3.2A&B).

Distinct orange-red staining indicated the presence of GGT in healthy PTECs with a functioning brush border membrane. The presence of this enzyme did not change upon treatment of PTECs with high D-glucose and 0.75ng/ml TGF- β 1 treatment (Figure 3.2C&D).

A negative control solution (containing the same buffer solution without GMNA) was also applied on PTECs under the same treatments. These PTECs showed no presence of the distinct bright orange-red staining (Figure 3.2E&F). Comparison of BCA protein assay results across all treatments revealed no noticeable patterns or changes in total cellular content.

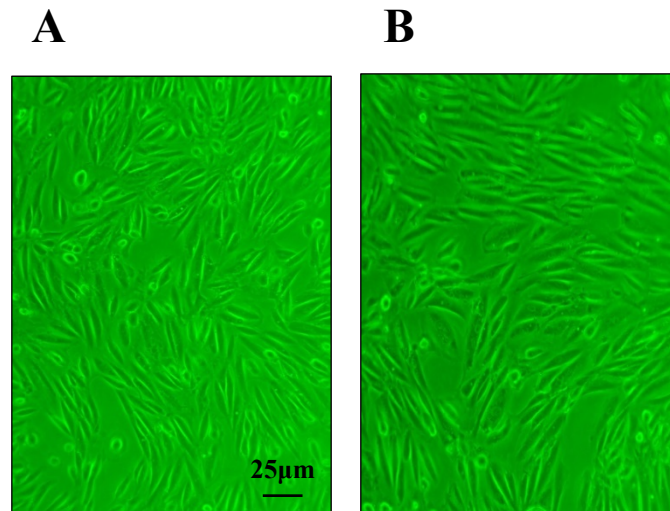


Figure 3.2A-B: Phase contrast microscopic images of PTECs.

Light microscopic images of **(A)** L-Glu (7mM D-glucose+18mM L-glucose) and **(B)** D-Glu+TGF- β 1 (7mM D-glucose+18mM D-glucose+ 0.75ng/ml TGF- β 1) treated PTECs were captured under the green filter to observe any structural changes between the two conditions after 24h.

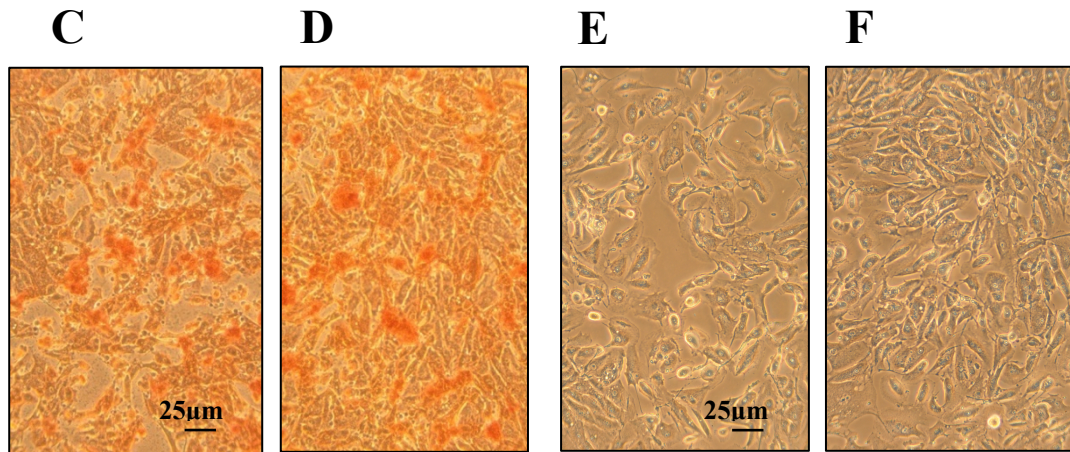


Figure 3.2C-F: GGT enzyme presence in PTECs

Light microscopic images (green filter removed) of 24h **(C)** L-Glu (7mM D-glucose+18mM L-glucose) and **(D)** D-Glu+TGF-β1 (7mM D-glucose+18mM D-glucose and 0.75ng/ml TGF-β1) treated PTECs stained with buffer solution and GMNA to confirm presence of GGT. There was distinct orange-red staining. **(E)** L-Glu and **(F)** D-Glu+TGF-β1 treated PTECs were also stained with the same buffer solution lacking GMNA. There was no distinct orange-red staining in comparison to images C&D.

3.3.3. SGLT2 knockdown in PTECs

In order to show that the 73kDa immunoreactive band I have identified as SGLT2 is indeed the correct target band, I knocked down SGLT2 in PTECs through the use of selective siRNA transfection targeted at SGLT2. After a 24h transfection, qPCR results confirmed that there was significant knockdown of SGLT2 mRNA (Figure 3.3A) in the selective SGLT2 target siRNA (15nM and 30nM) transfected PTECs. To establish that the selective SGLT2 target siRNA did not change SGLT1 mRNA abundance, an SGLT1 qPCR assay was also run. The SGLT2 targeted siRNA transfection did not change SGLT1 mRNA at 15nM or 30nM (Figure 3.3B). Similar to the mRNA results, the identified SGLT2 protein (~73kDa) band was significantly reduced after SGLT2 targeted siRNA treatment (30nM) (Figure 3.3C).

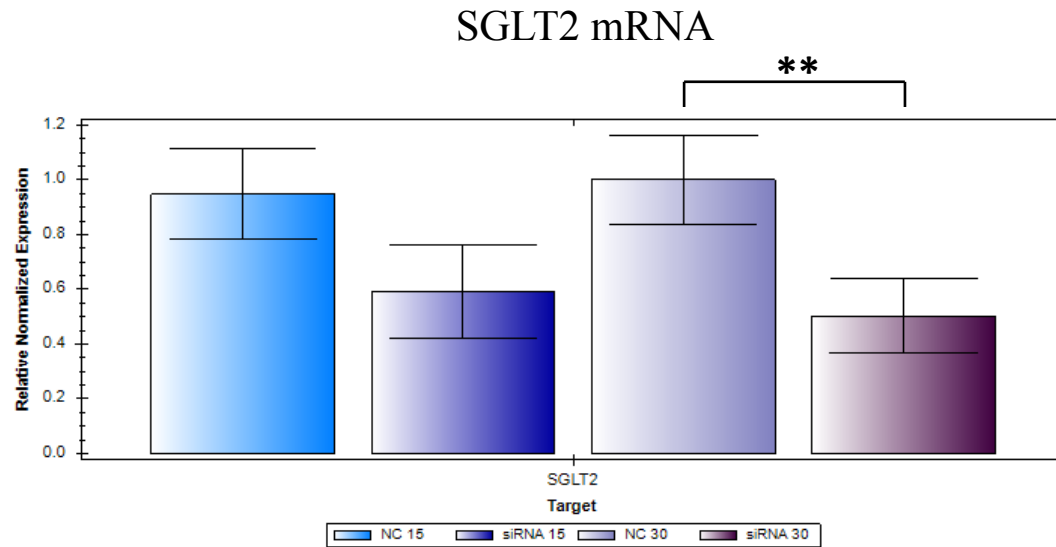


Figure 3.3A: SGLT2 mRNA knockdown in PTECs.

Key: **NC 15**= 15nM non-targeting negative control siRNA, **siRNA 15**= 15nM SGLT2 targeting siRNA, **NC30**= 30nM non-targeting negative control siRNA, **siRNA 30**= 30nM SGLT2 targeting siRNA. The figure shows plotted data points after quantification of SGLT2 mRNA (SGLT2 mRNA abundance/GAPDH mRNA abundance) presented in graph form. There was significant reduction of SGLT2 mRNA by SGLT2 targeted siRNA at 30nM after 24h transfection. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. $P^{**} < 0.01$, all $n=3$. Data are shown as mean $\pm$ SD.

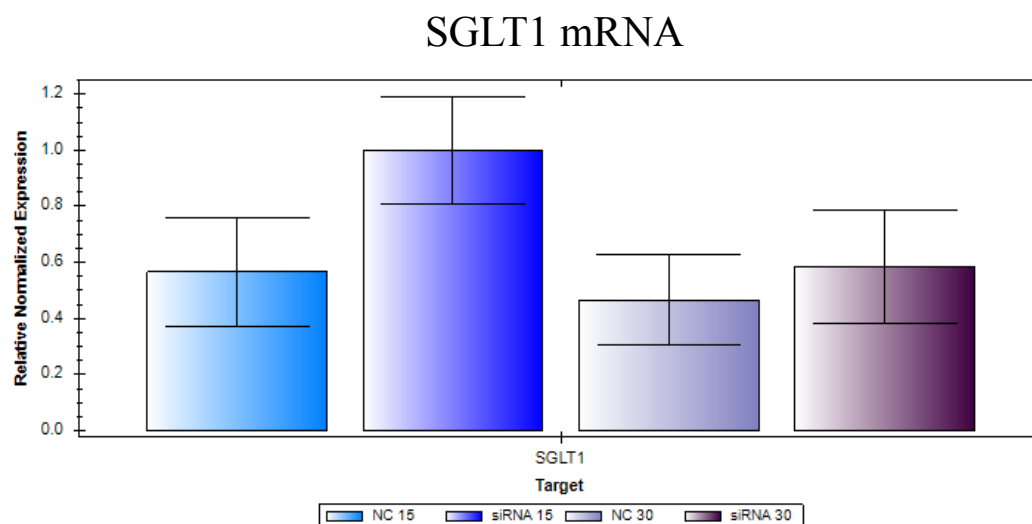


Figure 3.3B: SGLT1 mRNA levels following SGLT2 knockdown in PTECs.

Key: **NC 15**= 15nM non-targeting negative control siRNA, **siRNA 15**= 15nM SGLT2 targeting siRNA, **NC30**= 30nM non-targeting negative control siRNA, **siRNA 30**= 30nM SGLT2 targeting siRNA. The figure shows plotted data points after quantification of SGLT1 mRNA (SGLT1 mRNA abundance/GAPDH mRNA abundance) presented in graph form. There was no change in SGLT1 mRNA by SGLT2 siRNA at 15nM or 30nM after 24h transfection. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. All n=3. Data are shown as mean +/- SD.

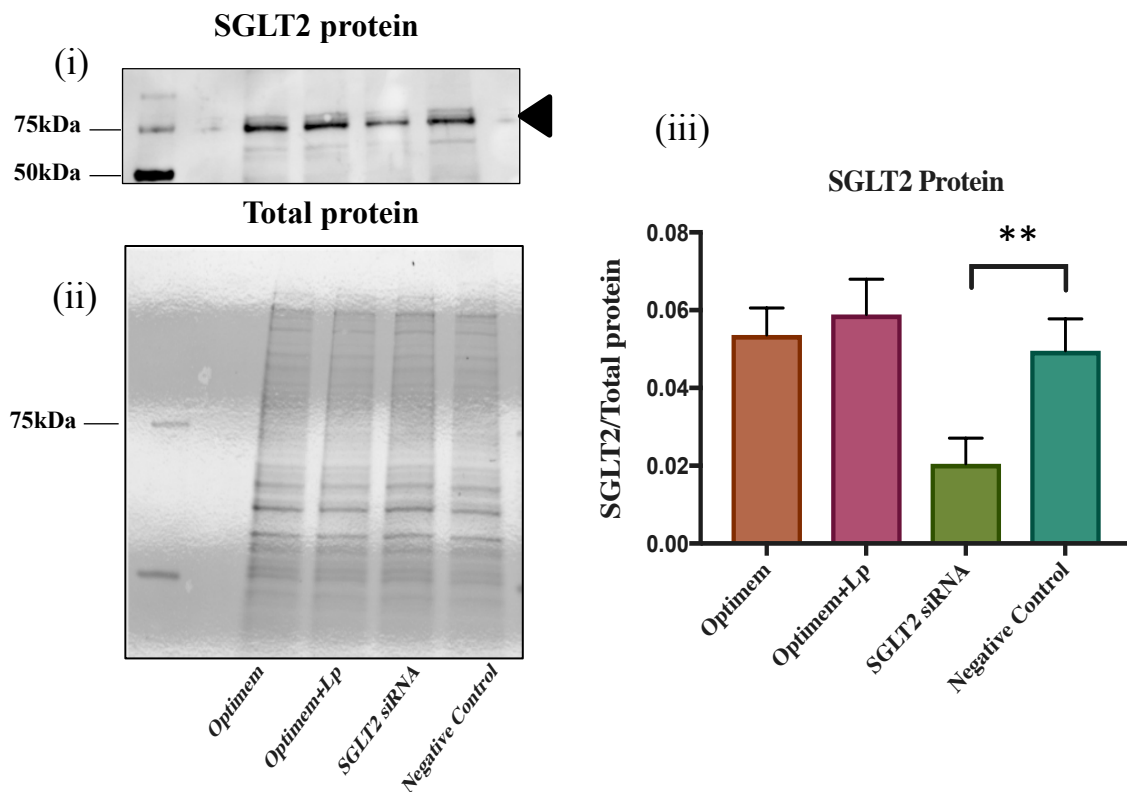


Figure 3.3C: SGLT2 protein knockdown in PTECs.

Key: **Optimem**= Optimem reduced serum medium with no additional reagents, **Optimem+Lp**= Optimem+ lipofectamine, **SGLT2 siRNA**= 30nM SGLT2 target siRNA, **negative control**= optimum+lipofectamine+non-targeting negative control siRNA at 30nM. (i) Western blot showing SGLT2 protein (~73kDa) in PTECs subjected to different conditions. The black arrowhead indicates the target immunoreactive band, (ii) corresponding total protein stain free gel which is used as the loading control, (iii) plotted data points after quantification of SGLT2 protein (SGLT2 protein abundance/total protein abundance) presented in graph format. SGLT2 protein was significantly reduced upon SGLT2 siRNA treatment after 24h transfection. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test, **P<0.01, all n=3. Data are shown as mean +/- SD.

3.3.4. Examining SGLT2, SGLT1 and GLUT function in PTECs

As the structure, phenotype and expression of SGLT2 and SGLT1 in PTECs under various treatments has been examined, the next steps will focus on examining the glucose uptake by these transporters when subjected to different concentrations of glucose. I used the non-radioactive, end-point plate-based, bioluminescent Glucose Uptake-Glo™ assay (Promega) kit based on the quantification of 2-deoxyglucose-6-phosphate (2DG6P) which amasses in the cell after administration of 2-deoxyglucose (2DG).

The results showed that under osmotic control conditions, phlorizin alone and in combination with dapagliflozin significantly reduced 2DG6P uptake, whilst dapagliflozin alone did not (Figure 3.4A). The combination of phlorizin, dapagliflozin and phloretin also significantly reduced the 2DG6P uptake (Figure 3.4A). Conversely, under high D-glucose conditions, dapagliflozin alone and in combination with phlorizin significantly reduced glucose uptake whilst phlorizin alone had no effect (Figure 3.4B). The combination of phlorizin, dapagliflozin and phloretin treatment significantly reduced glucose uptake under both conditions (Figure 3.4A&B). There was a greater rate of glucose uptake under high D-glucose conditions without inhibitor compared to the osmotic control conditions without inhibitor (Figure 3.4A&B). However, it should be noted that the glucose uptake rates of these two conditions were not directly compared for statistical significance. Glucose uptake rate was calculated and expressed in femtomole/cell/min (fmol/cell/min).

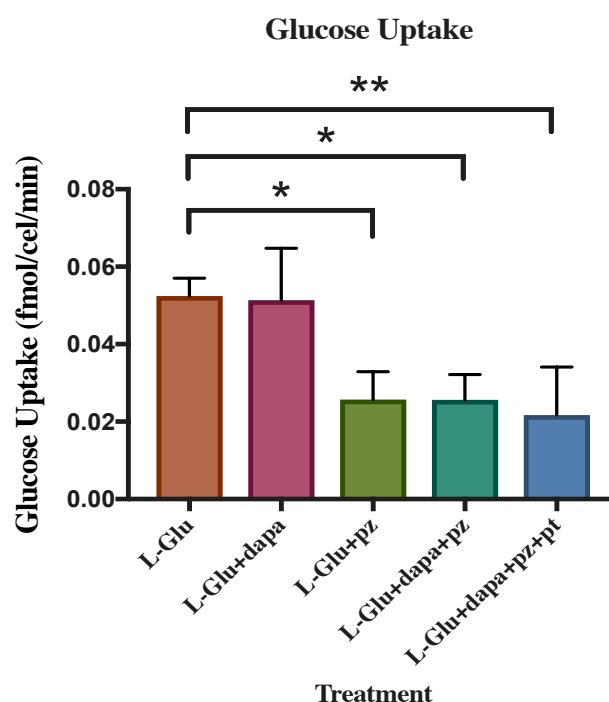


Figure 3.4A: Glucose uptake rate in PTECs under osmotic control conditions.

Key: **L-Glu**= 7mM Dglucose+18mM L-glucose (osmotic control), **L-Glu+dapa**= 7mM D-glucose+18mM L-glucose+ 1nM dapagliflozin, **L-Glu+pz**= 7mM D-glucose+18mM L-glucose+500μM phlorizin, **L-Glu+dapa+pz**= 7mM D-glucose+18mM L-glucose+ 1nM dapagliflozin +500μM phlorizin, **L-Glu+dapa+pz+pt**= 7mM D-glucose+18mM L-glucose+1nM dapagliflozin +500μM phlorizin +1mM phloretin. Plotted data points after quantification of glucose uptake (fmol/cell/min) in PTECs presented in graph format. Phlorizin significantly reduced the glucose uptake rate whilst dapagliflozin did not. The combination of the two inhibitors significantly reduced the glucose uptake rate. Phloretin administered in combination with phlorizin and dapagliflozin also reduced the glucose uptake rate. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test, $P^* < 0.05$, $P^{**} < 0.01$, $P^{***} < 0.001$, all $n=4$. Data are shown as mean $\pm$ SD.

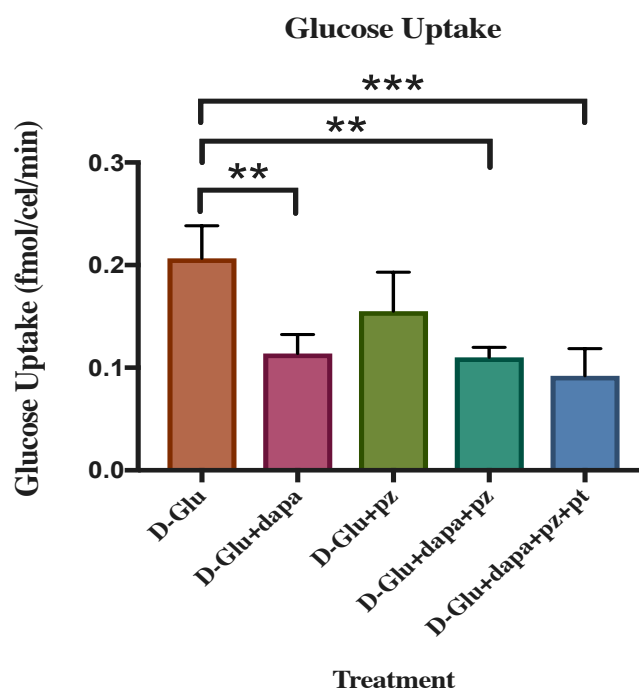


Figure 3.4B: Glucose uptake rate in PTECs under high D-glucose conditions:

Key: **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+dapa**= 7mM D-glucose+18mM D-glucose+ 1nM dapagliflozin, **D-Glu+pz**= 7mM D-glucose+18mM D-glucose+500µM phlorizin, **D-Glu+dapa+pz**= 7mM D-glucose+18mM D-glucose+1nM dapagliflozin +500µM phlorizin, **L-Glu+dapa+pz+pt**= 7mM D-glucose+18mM D-glucose+1nM dapagliflozin +500µM phlorizin +1mM phloretin. Dapagliflozin significantly reduced the glucose uptake rate whilst phlorizin did not. The combination of the two inhibitors significantly reduced the glucose uptake rate. The addition of phloretin administered in combination with phlorizin and dapagliflozin reduced the glucose uptake rate. Plotted data points after quantification of glucose uptake (fmol/cell/min) in PTECs presented in graph format. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test, $P^{**}<0.01$, $P^{***}<0.001$, all $n=4$. Data are shown as mean $\pm$ SD.

3.4. Discussion

This chapter demonstrates the first identification of SGLT2 protein in primary human proximal tubule epithelial cells extracted from human nephrectomy samples and cultured on collagen IV. I was previously unable to detect SGLT2 protein in the HKC-8 cells (Figure 2.1). This may be due to the fact that these are a transformed clonal cell line derived from an exclusive part of the proximal tubule such the S3 segment that do not express enough SGLT2.

PTEC phenotype and functionality were confirmed through positive SGLT2 protein expression and GGT activity. Although primary cell lines like PTECs have considerable proliferative capacity, they begin to lose their specialised phenotypic and genotypic characteristics after a finite number of passages/population doublings. Gradual loss of phenotype/genotype may lead to other issues like altered functional properties, greater susceptibility to infection, and eventual senescence (Geraghty et al., 2014). Due to these risks, it was important to confirm the expression/activity of these PT markers in my PTECs prior to any experimentation. PTECs were also passaged a maximum of 3 times.

Other PTEC markers include megalin, a transmembrane protein responsible for mediating receptor mediated endocytosis in the PT; aquaporin-1 (AQP1), a selective channel protein responsible for the majority of water reabsorption in the PT; and K-cadherin, a cell-adhesion molecule specifically expressed on the basolateral membrane of PTECs (Maeda et al., 2008; Yates et al., 2015; Sun et al., 2017).

Although it has been long established that SGLT2 is predominantly localised to the early proximal tubule (Wright, 2001; Vallon *et al.*, 2011), there is limited SGLT2 western blotting data in literature. This can be attributed to the current lack of well-characterized antibodies available for purchase commercially. This has in turn hindered comprehensive analyses of SGLT2 protein expression in the human kidney. I hypothesise that a reason behind the difficulty in obtaining clear SGLT2 bands is the conformation and localisation of the protein as it is intertwined within the plasma membrane (Figure 1.4). This increases the difficulty for the non-membrane specific lysis buffer used to extract the protein. However, section 2.2.3 in the materials and methods section describes the troubleshooting

steps taken to obtain and optimise protein expression of the transporter in PTECs (Figure 2.1&2.2).

I chose to normalise the density of all target protein bands against the “total protein” visualised in the electrophoresis gel. This patented stain-free imaging technology was engineered by Biorad. Briefly, the imaging application photo-activates a proprietary trihalocompound upon exposure to UV light. Following this, the compound covalently binds to tryptophan amino acids which intensifies the fluorescent signal. This allows visualisation of gels without the need for laborious staining and de-staining stages.

This method was adopted in place of traditional housekeeping protein (e.g. tubulin, β -actin) normalisation which is unreliable due to variables like blotting and differing antibody affinities. Total protein normalisation indexes target band densities to the total protein in the entire lane, resulting in more accurate data. Additionally, the traditional method requires a membrane to be reprobed with the selected housekeeping protein. In order for a membrane to be reprobed, it must be completely stripped of the primary and secondary antibodies used to detect the first target. Although this practice is straightforward in theory, the resulting blots are often not clear enough for valid data extraction.

I report two SGLT2 proteins; a mature glycosylated ~73kDa form and an immature non-glycosylated ~60kDa form using an antibody that was raised against amino acids 226 to 268 corresponding to an extracellular loop of human SGLT2. These weights were predicted according to the Bio-Rad Precision Plus Protein™ WesternC protein ladder, although values can vary slightly depending on the percentage gel that has been run. Recently, western blot analysis by a different group found a ~15kDa reduction of SGLT2 protein from human kidneys upon removal of glycosylation residues by PNGaseF, a deglycosylation enzyme (Chae *et al.*, 2020). This shift in size, which was similar to what I observed in my blots, supports my conclusion that the lighter band is a result of non-glycosylation.

Although the mature ~73kDa protein has been observed in human PTECs as well as in murine kidneys (Rahmoune *et al.*, 2005; Shepard *et al.*, 2016; Di Franco *et al.*, 2017),

there has not been a report of the non-glycosylated form to the best of my knowledge. However, as I have cultured primary cells on collagen IV, a major component of human proximal tubule basement membranes (Miner, 1999), I propose that my PTEC model is more physiologically relevant in regards to its cell-matrix interactions than cells that have not been cultured on collagen IV. Takesue et al found positive SGLT2 protein expression in human kidney (HK-2) cells, but they only observed one protein band of which the weight was not indicated. HK-2s are immortalised proximal tubule cells initially isolated from primary cells, but it is a clonal expansion of a single cell which was transformed by the human papilloma virus (Ryan *et al.*, 1994). They therefore represent a single cell selected by its ability to be transformed. Interestingly, they did find a greater abundance of both SGLT2 protein and mRNA levels in the human kidney than in HK-2 cells (Takesue *et al.*, 2018), much like my observation (Figure 2.2), suggesting that SGLT2 is highly glycosylated *in vivo*.

SGLT2 siRNA at 30nM significantly reduced SGLT2 mRNA by approximately 50% whilst 15nM concentration did not, suggesting a dose dependent decrease of SGLT2 mRNA. The siRNA concentrations used in my experiments were within a similar range to the concentrations used in studies on HK-2 cells (Takesue *et al.*, 2018), although there are others that have reported using concentrations as high as 100nM on the same cell line (Li *et al.*, 2020).

My results showed a significant decrease in SGLT2 protein for PTECs treated with TGF- β 1 grown on collagen IV. This indicates that the response to treatments differ depending on whether cells were grown on collagen IV or plastic. I propose that this may be due to different signalling pathways being activated as a response to cells interacting with collagen IV. Interestingly, previous work from our group revealed a dose dependent reduction of cellular ERK protein abundance only in collagen IV cultured PTECs (unpublished data). A similar pattern is observed in other work, where human Caco-2 intestinal epithelial cells exhibited upregulation of ERK signalling after collagen IV adhesion for 10 min (Sanders and Basson, 2000).

The significant decrease in SGLT2 protein that I observe only occurs in TGF- β 1 treated PTECs under high D-glucose or osmotic control conditions. I utilised L-glucose in my

experiments to account for the hyperosmotic stress that are placed upon the PTECs after 24h treatment of $>7\text{mM}$ D-glucose. Osmotic stress induces cell cycle arrest in epithelial cells (Arsenijevic *et al.*, 2013). Although D-glucose can enter the PTECs, the glucose that is not metabolised by them will exit through the GLUTs and remain in the surrounding medium, maintaining osmotic pressure. As there is no structural distinction between the two glucose isomers, I conclude that TGF- β 1 appears to block the osmotic upregulation of SGLT2. It is unclear what drives this osmotic effect, but I suggest that it could be partly due to SGLT2 association with Caveolin-1 (Cav-1), a membrane protein and important component of caveolae, which forms invaginations on the plasma membrane (de Almeida, 2017). Osmotic pressure has been shown to disrupt caveolae pockets and trigger the dispersion of Cav-1 (Tachikawa *et al.*, 2017). TGF- β 1, at 2ng/ml , significantly downregulates Cav-1 mRNA abundance (Sanders *et al.*, 2015). There is also evidence showing the co-localisation of Caveolin-1 and SGLT2 in renal proximal tubule cells, further indicating a positive association between both membrane proteins (Lee *et al.*, 2012). Thus, factors that prevent Cav-1 upregulation at the membrane under hyperosmotic conditions may also reduce SGLT2 protein.

My SGLT2 results contradict a previous study carried out by Panchapakesan *et al.*, who reported that 0.5ng/ml treatment with TGF- β 1 for 72h significantly increased SGLT2 protein abundance, although they utilised the transformed HK-2 kidney cell line to carry out their experiments (Panchapakesan *et al.*, 2013). A separate group that did use primary PTECs found a significant decrease in SGLT2 protein after TGF- β 1 treatment (Lee and Han, 2010). Notably, they used a 5ng/ml concentration of the growth factor and subjected their cells to a treatment time of 72h. This perhaps offers a rationale as to why I did not observe a significant decrease in SGLT2 protein after exclusive TGF- β 1 treatment at 0.75ng/ml for 24h.

The significant reduction of SGLT2 protein was not reproduced at mRNA level, suggesting that this effect is translationally mediated. However, the non-glycosylated $\sim 60\text{kDa}$ protein was not always present and therefore it was not possible to carry out semi-quantitative western blot analysis of the band. It is not necessarily uncommon for differing results to be seen between protein and mRNA abundance. There are many complex post-transcriptional mechanisms like adenylation, nucleotide base methylation, or 5' capping

which are key for successful protein translation and subsequent native protein synthesis but they are not well understood (Greenbaum *et al.*, 2003). Therefore it should not be assumed that there is a positive correlation between the two. Additionally, I applied the same experimental parameters to my qPCR experiments as I did to my western blotting experiments, i.e. 24h treatment time. Whilst protein is made and then accumulated, mRNA follows a transient cycle of mRNA production and degradation. Hence, the 24h timepoint may not have been the most optimal time to analyse mRNA transcription levels.

Under osmotic control conditions, the dual administration of phlorizin and dapagliflozin did not reduce the glucose uptake rate any more significantly than phlorizin did by itself when compared to the osmotic control ($P < 0.05$). This leads to the conclusion that the observed reduction in glucose uptake is due to the inhibition of the SGLT1 transporter and not SGLT2. This would make sense as SGLT1 is a high affinity/low capacity transporter, meaning that under relatively normal levels of D-glucose, it remains relatively active and is responsible for the majority of glucose transport and uptake. Likewise, SGLT2 is a low affinity/high capacity transporter and is therefore not substantially active in relatively low or normal levels of circulating D-glucose. The administration of phloretin in combined treatment with dapagliflozin and phlorizin also further significantly reduced glucose uptake when compared to the osmotic control alone ($P < 0.01$). Phloretin is a non-specific GLUT inhibitor and so this further reduction is likely to be due to the additional restriction of glucose uptake via GLUT.

Conversely, under high D-glucose conditions, dapagliflozin significantly reduced glucose uptake ($P < 0.01$ vs high D-glucose treatment without inhibitor), whilst phlorizin did not change the uptake. This is likely due to SGLT2 activation in response to an increasing concentration of D-glucose. SGLT1's function at high levels of D-glucose is potentially insignificant due to its low capacity for D-glucose.

This glucose uptake assay was likely not sensitive enough to detect a small but significant further glucose uptake reduction induced by phlorizin on top of dapagliflozin in the high D-glucose experiment. The standard deviation of the mean of my glucose uptake values (in the PTECs subjected to high D-glucose alone) is approximately 0.03, which would mean that at $n=4$, my experiments were not powered to detect a difference of less than approximately 0.09 fmol/cell/min. Thus, my experiment was not powered to determine

whether the glucose uptake difference between the PTECs subjected to high D-glucose (mean of ~ 0.2 fmol/cell/min) and the PTECs subjected to high D-glucose plus phlorizin (mean of approximately ~ 0.15 fmol/cell/min) was significant.

Although my glucose uptake studies examined SGLT1, SGLT2 and GLUT function in my *in vitro* PTEC model, I was not able to track the pattern of glucose transport and uptake over time. The specific kit used was an end-point assay that only measures the accumulation of 2DG6P. Other methods that would allow for the tracking of glucose uptake and glucose transport inhibitor efficacy as a function of time include the use of radioactive labelled glucose analogues. These include [^{14}C]methyl- α -D-glucopyranoside (AMG) (Choi *et al.*, 2020), a non-metabolised specific substrate of SGLT labelled with radioactive-carbon 14 or radiolabelled glucose isomers like [^3H]-D-glucose (Kalsi *et al.*, 2009). In general, samples of target cells are incubated together with the radiolabelled glucose/glucose analogues (Castaneda and Kinne, 2005). The amount of radioactive substance in these samples can then be counted/measured at different timepoints. Due to time constraints, the non-radioactive plate-based assay was the most appropriate choice of glucose measurement tool available. Additionally, there are data that have previously demonstrated the feasibility of using non-radioactive methods to measure glucose transport mediated by SGLT in cultured human kidney cells (Lu *et al.*, 2019). Further methods of tracking glucose signalling *in vitro* will be discussed in chapter 7.

Chapter 4:

The Effects of Glucose and TGF- β 1 on Connective Tissue Growth Factor, T β RII, p21, and K-Cadherin.

4.1. Introduction

Connective tissue growth factor (CCN2/CTGF) is a secreted 36/38kDa matricellular protein and major downstream mediator of TGF- β 1 signalling that regulates various important biological functions (Mason, 2009). It is significantly upregulated during fibrosis, cancer and as a response to diabetic cardiovascular complications (Leask and Abraham, 2006; Ha *et al.*, 2013). CCN2 belongs to the CCN (Cyr61/CTGF/Nov) family, a class of secreted cysteine rich regulatory proteins which serve as multi-domain adaptor molecules that help link together the ECM and cell surface. It was first identified three decades ago upon isolation from human umbilical vein endothelial cells (HUVECs) (Bradham *et al.*, 1991). Structurally, CCN2 consists of 4 domains/modules, connected together through highly conserved disulphide bonds, which share homology to various other proteins: (1) insulin-like growth factor (IGF) binding module, (2) Von Willebrand (VWF) factor type C repeats homologous module, (3) thrombospondin type 1 repeat homologous module and (4) carboxyl terminal cysteine knot motif module (Lipson *et al.*, 2012; Mendes *et al.*, 2015). Modules 1 and 2 comprise the N-terminus signal peptide (SP) whilst the latter 2 comprise the C-terminus (Figure 4.1). These domains are each responsible for interacting with various ligand-binding partners like integrins, vascular endothelial growth factor (VEGF) and fibronectin in order to exert its effects and modulate various biological process like fibrosis, cell adhesion/migration, ECM remodelling, angiogenesis and morphogenesis (Malik, Liszewska and Jaworski, 2015). CCN2, in association with both high D-glucose (James *et al.*, 2013) and TGF- β 1 (Tsai *et al.*, 2018; Yanagihara *et al.*, 2020), plays a pivotal role in the promotion of fibrosis.

Consequently, the experiments described in this second results chapter will mainly focus on the effects of high D-glucose and 0.75ng/ml TGF- β 1 on CCN2 and whether SGLT2 plays a role in regulating pro-fibrotic signalling. Additionally, this chapter will also

address whether high D-glucose/TGF- β 1 treatment affects other proteins such as K-Cadherin, a marker of PTEC phenotype (Paul *et al.*, 1997), cyclin-dependent kinase (CDK) complex inhibitor p21, which prevents cell cycle progression (Shtutman *et al.*, 2017) and TGF- β 1 receptor type II (T β RII).

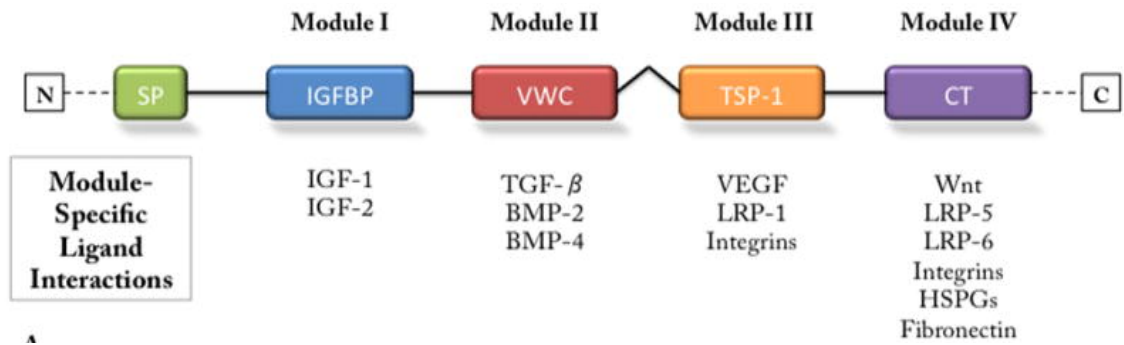


Figure 4.1: Structure of CCN2.

CCN2 has 4 modules, with modules 1-2 and modules 3-4 linked together by a hinge region susceptible to cleavage by matrix metalloproteinases (MMPs). Module 1 shares homology with IGF-1 binding proteins, module 2 shares homology with VWF type C repeat, module 3 shares homology with thrombospondin type 1 repeat and module 4 is a carboxyl terminal cysteine knot motif. Figure taken from (Arnott et al., 2011).

4.2. Aims and Objectives

1. To investigate the changes in T β RII, p21, and K-Cadherin protein abundance in PTECs following treatment with glucose and TGF- β 1.
2. To investigate the changes in CCN2 protein and mRNA abundance in PTECs following treatment with glucose and TGF- β 1 treatment.
3. To investigate the effect of SGLT2 inhibition on CCN2 protein and mRNA abundance using dapagliflozin, a drug that selectively inhibits the transporter.
4. To investigate the presence of urinary CCN2 protein in diabetic kidney disease patient samples.

4.3. Results

4.3.1. TGF- β receptor 2 (T β RII), p21 and K-Cadherin protein abundance in PTECs treated with glucose and TGF- β 1

T β RII (~75kDa), p21 (~21kDa), and K-Cadherin (~120kDa) protein were detected under control (7mM D-glucose alone), osmotic control (7mM D-glucose+18mM L-glucose) and high D-glucose (7mM D-glucose+18mM D-glucose) conditions. Data analysis revealed no change in T β RII protein in response to the different treatments (Figure 4.2A). Although neither exclusive high D-glucose nor 0.75ng/ml TGF- β 1 treatment changed p21 protein abundance, there was a significant increase of p21 protein abundance in the combined high D-glucose and 0.75ng/ml TGF- β 1 treatment compared to all other treatments (Figure 4.2B). Similar to T β RII, K-Cadherin protein abundance did not change under any of the treatments (Figure 4.2C).

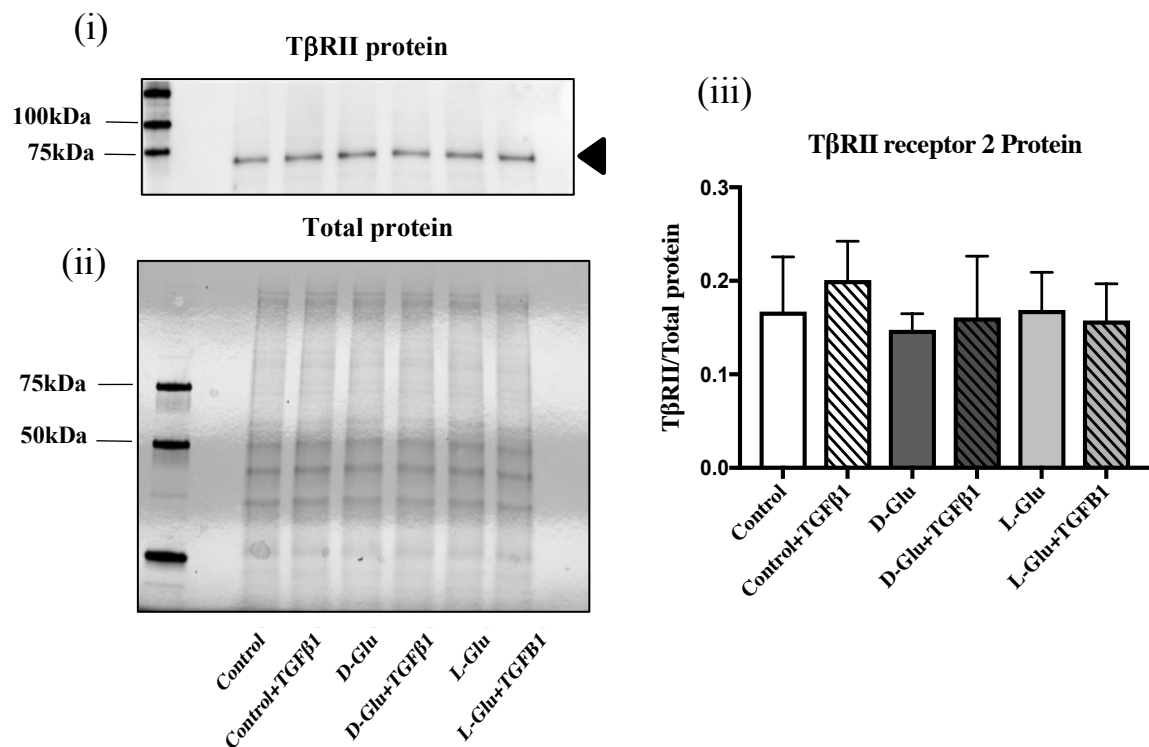


Figure 4.2A: TβRII protein abundance in PTECS treated with glucose and TGF-β1.

Key: **Control**= 7mM D-glucose, **Control+ TGF-β1**= 7mM D-glucose+ 0.75ng/ml TGF-β1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGF-β1**=7mM D-glucose+18mM D-glucose+ 0.75ng/ml TGF-β1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGF-β1**= 7mM D-glucose+18mM L-glucose+ 0.75ng/ml TGF-β1. (i) Western blot showing TβRII protein (~75kDa) in PTECs subjected to different treatments at 24h. The black arrowhead indicates the target immuno-reactive band, (ii) corresponding total protein stain free gel which is used as the loading control, (iii) plotted data points after quantification of TβRII protein (TβRII protein abundance/total protein abundance) presented in graph format. There were no changes in TβRII protein abundance under any of the treatments after 24h. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. All n=3. Data are shown as mean +/- SD.

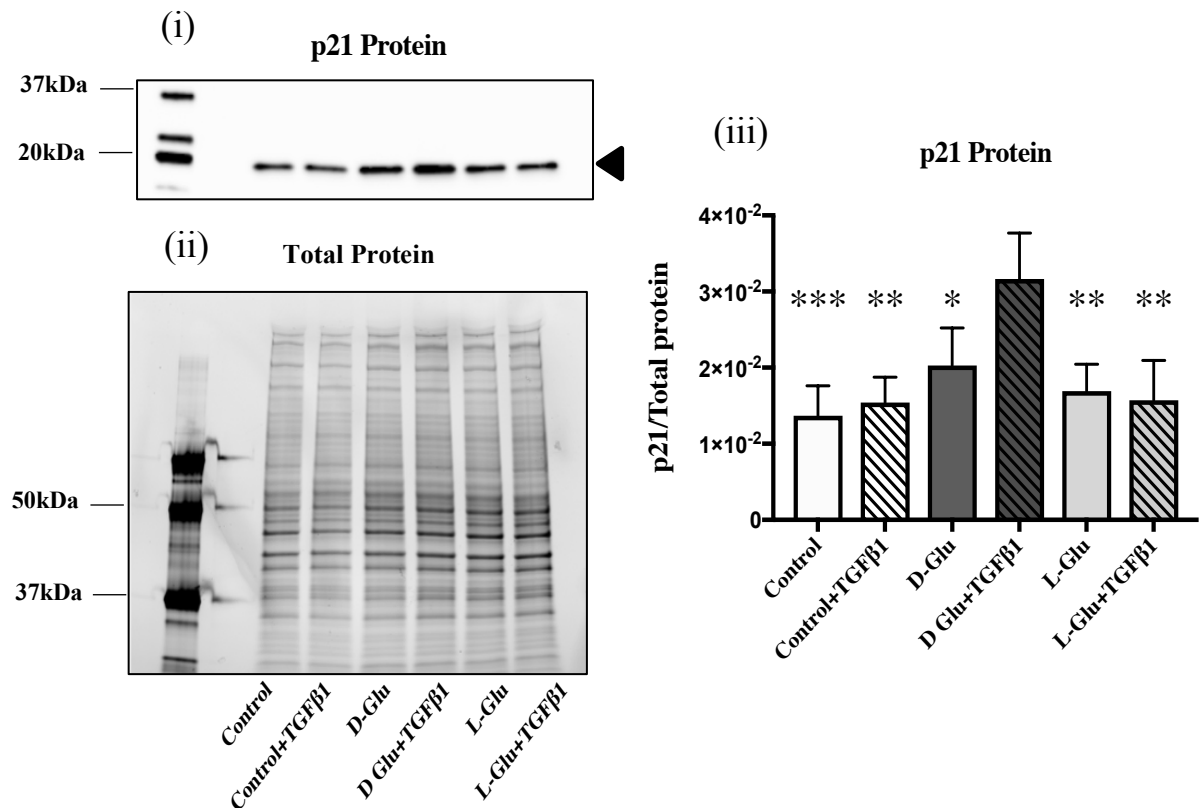


Figure 4.2B: p21 protein abundance in PTECS treated with glucose and TGF-β1.

Key: **Control**= 7mM D-glucose, **Control+ TGF-β1**= 7mM D-glucose+ 0.75ng/ml TGF-β1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGF-β1**=7mM D-glucose+18mM D-glucose+ 0.75ng/ml TGF-β1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGF-β1**= 7mM D-glucose+18mM L-glucose+ 0.75ng/ml TGF-β1. (i) Western blot showing p21 protein (~21kDa) in PTECs subjected to different treatments for 24h. The black arrowhead indicates the target immuno-reactive band, (ii) corresponding total protein stain free gel which is used as the loading control. (iii) Plotted data points after quantification of p21 protein (p21 protein abundance/total protein abundance) presented in graph format. D-Glu+TGF-β1 treatment significantly increased p21 protein abundance in comparison to all other treatments. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. ***P<0.001, **P<0.01 and *P<0.05 compared to D-Glu+TGF-β1. All n=3. Data are shown as mean +/- SD.

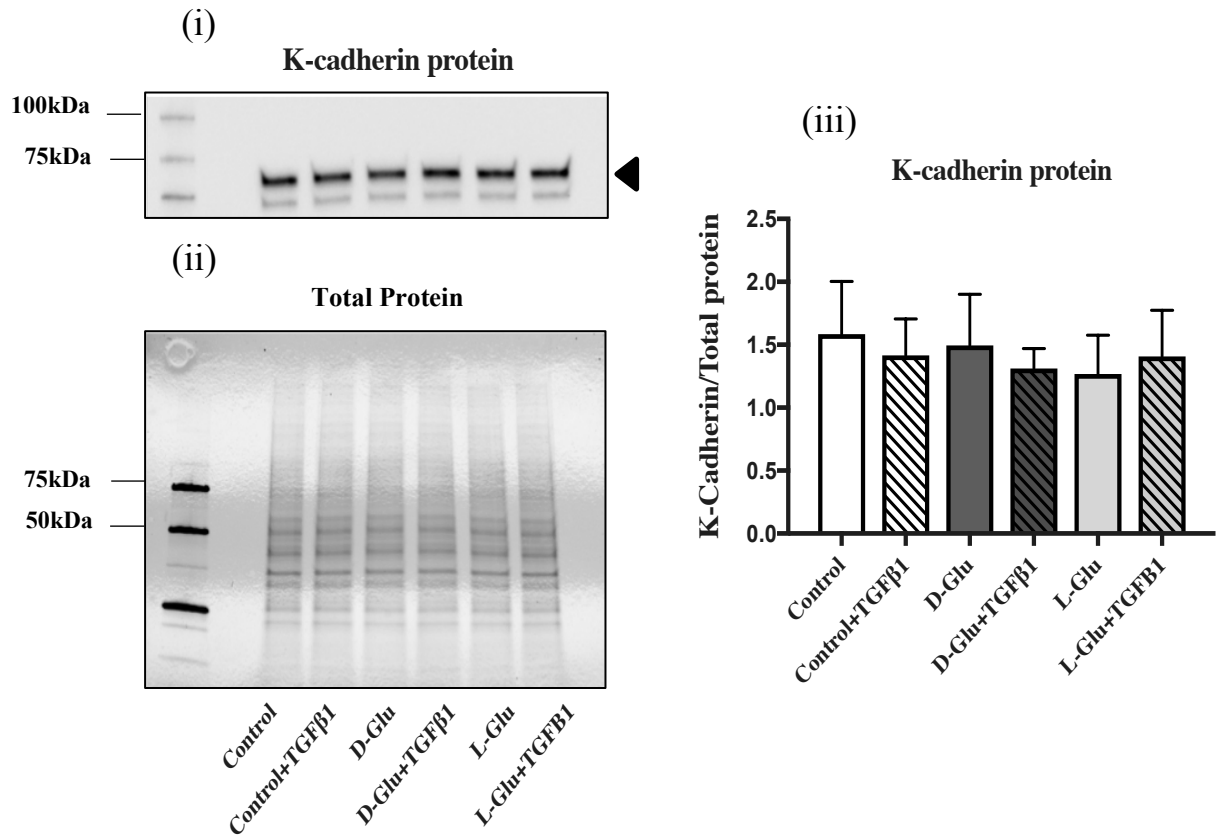


Figure 4.2C: K-Cadherin protein abundance in PTECS treated with glucose and TGF-β1.

Key: **Control**= 7mM D-glucose, **Control+ TGF-β1**= 7mM D-glucose+ 0.75ng/ml TGF-β1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGF-β1**=7mM D-glucose+18mM D-glucose+ 0.75ng/ml TGF-β1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGF-β1**= 7mM D-glucose+18mM L-glucose+ 0.75ng/ml TGF-β1. (i) Western blot showing K-Cadherin protein (~120kDa) in PTECs subjected to different treatments for 24h. The black arrowhead indicates the target immuno-reactive band, (ii) corresponding total protein stain free gel which is used as the loading control, (iii) plotted data points after quantification of K-Cadherin protein (K-Cadherin abundance/total protein abundance) presented in graph format. There were no changes in K-Cadherin protein abundance under any of the treatments. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. All n=3. Data are presented as mean +/- SD.

4.3.2. CCN2 protein and mRNA abundance in PTECs treated with glucose, TGF- β 1 and dapagliflozin

CCN2 protein (~36-38kDa) and mRNA were detected under all conditions. Protein and qPCR data analysis revealed that neither high D-glucose nor 0.75ng/ml TGF- β 1 alone changed CCN2. However, the combined treatment of the two did significantly increase CCN2 protein and mRNA abundance compared to all other treatments (Figure 4.3A&B). The increase of CCN2 protein was significantly attenuated by 0.1, 1 and 10nM dapagliflozin (Figure 4.3C). Dapagliflozin also reduced CCN2 mRNA at the two higher concentrations, 1 and 10nM (Figure 4.3D).

To investigate whether CCN2 mRNA was upregulated with TGF- β 1 treatment alone under osmotic control conditions, PTECs were treated with additional higher concentrations of TGF- β 1 (0.75, 2.5, and 5ng/ml). CCN2 mRNA abundance was significantly increased with 5ng/ml TGF- β 1 (Figure 4.3E).

Dapagliflozin (1nM) was administered to PTECs treated with 5ng/ml TGF- β 1 under osmotic control conditions in order to investigate whether SGLT2 inhibition could affect TGF- β 1 signalling, independent of elevated D-glucose exposure. Dapagliflozin did not change the TGF- β 1-increased CCN2 mRNA abundance (Figure 4.3F).

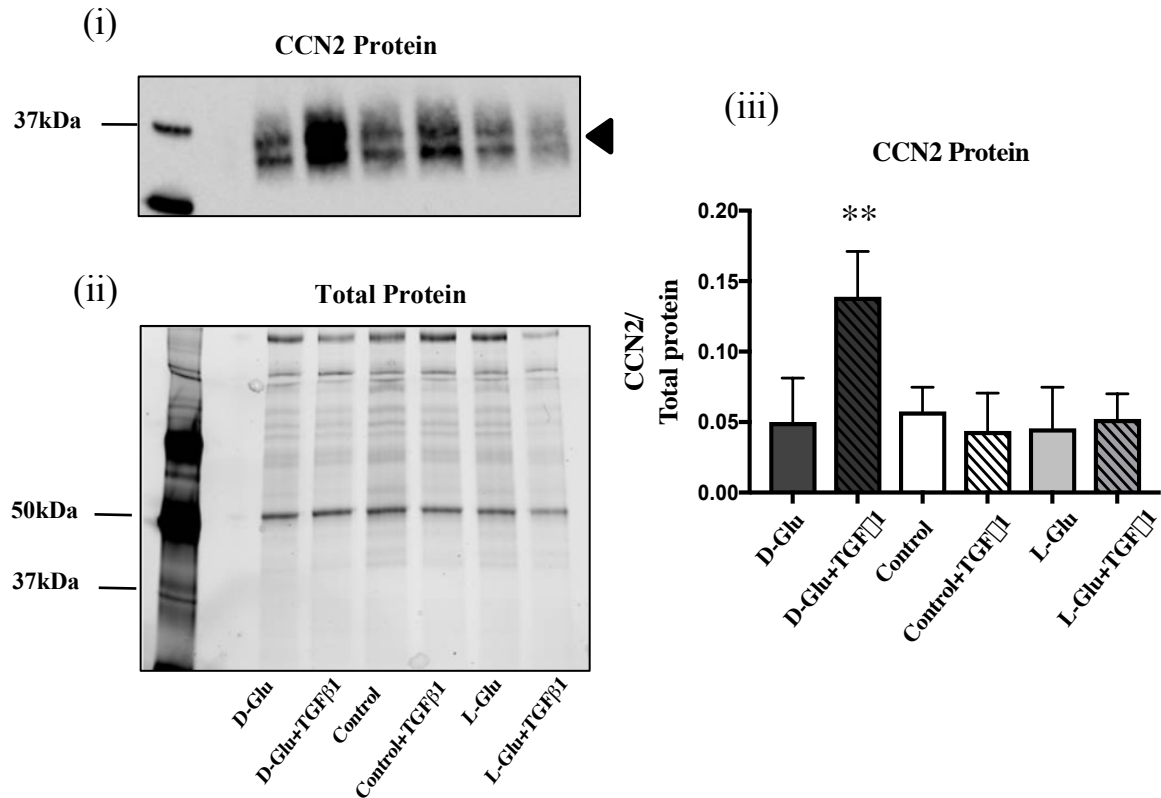


Figure 4.3A: CCN2 protein abundance in PTECs treated with glucose and TGF-β1.

Key: **Control**= 7mM D-glucose, **Control+ TGF-β1**= 7mM D-glucose+ 0.75ng/ml TGF-β1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGF-β1**=7mM D-glucose+18mM D-glucose+ 0.75ng/ml TGF-β1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGF-β1**= 7mM D-glucose+18mM L-glucose+ 0.75ng/ml TGF-β1. (i) Western blot showing CCN2 protein (~36-38kDa) in PTECs subjected to different treatments for 24h. The black arrowhead indicates the target immuno-reactive band, (ii) corresponding total protein stain free gel which is used as the loading control, (iii) plotted data points after quantification of CCN2 protein (CCN2 abundance/total protein abundance) presented in graph format. D-Glu+ TGF-β1 treatment significantly increased CCN2 protein abundance in comparison to all other treatments. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. **P<0.01 compared to all other treatments, all n=3. Data are shown as mean +/- SD.

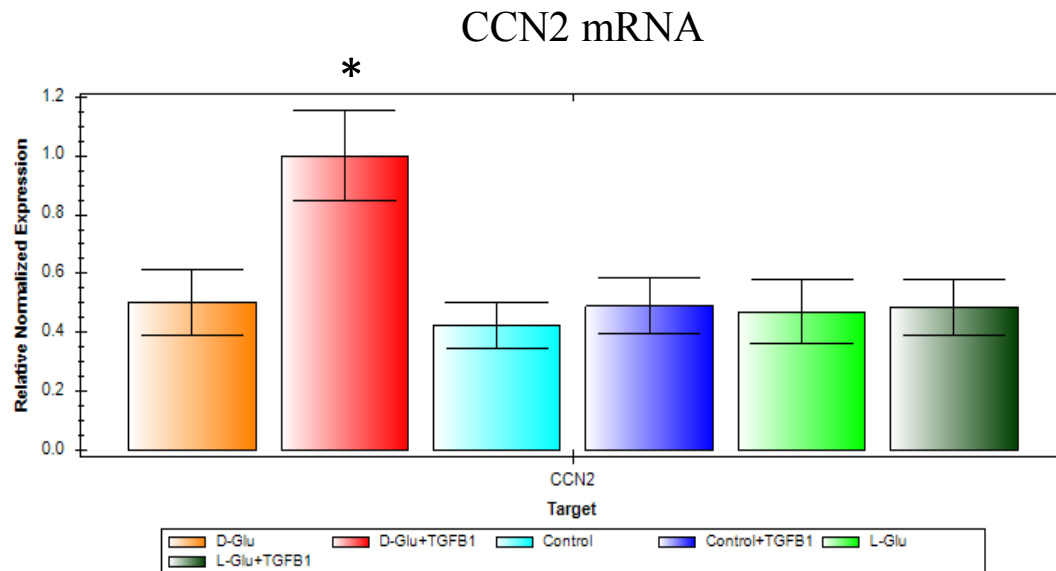


Figure 4.3B: CCN2 mRNA abundance in PTECS treated with glucose and TGF- β 1.

Key: **Control**= 7mM D-glucose, **Control+ TGF- β 1**= 7mM D-glucose+ 0.75ng/ml TGF- β 1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose) **D-Glu+TGF- β 1**=7mM D-glucose+18mM D-glucose+ 0.75ng/ml TGF- β 1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGF- β 1**= 7mM D-glucose+18mM L-glucose+ 0.75ng/ml TGF- β 1. All treatment times lasted 24h. The figure shows plotted data points after quantification of CCN2 mRNA (CCN2 mRNA abundance/GAPDH mRNA abundance) presented in graph form. qPCR analysis showed that TGF- β 1 did not change CCN2 mRNA and neither did D-Glu on its own. D-Glu+TGF- β 1 treatment significantly increased CCN2 mRNA abundance in comparison to all other treatments. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. *P<0.05 compared to all other treatments, all n=4. Data are shown as mean +/- SD.

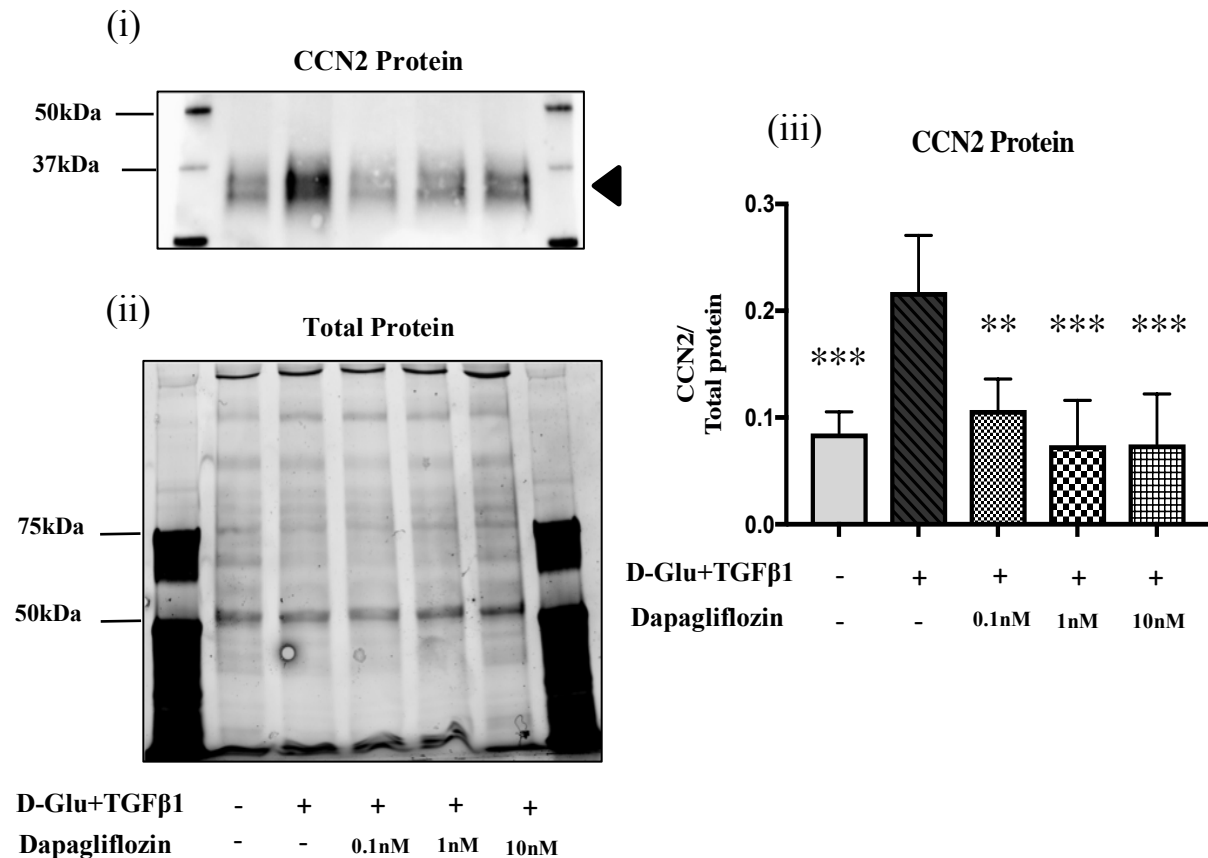


Figure 4.3C: CCN2 protein abundance in PTECS treated with glucose, TGF-β1 and dapagliflozin.

Key: **D-Glu+TGF-β1**= 7mM D-glucose+18mM D-glucose+ 0.75ng/ml TGF-β1. (i) Western blot showing CCN2 protein (~36-38kDa) in PTECs subjected to different treatments for 24h. The black arrowhead indicates the target immuno-reactive band, (ii) corresponding total protein stain free gel which is used as the loading control, (iii) plotted data points after quantification of CCN2 protein (CCN2 protein abundance/total protein abundance) presented in graph format. D-Glu+TGF-β1 treatment significantly increased CCN2 protein abundance. Dapagliflozin significantly reduced this increased CCN2 protein. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. ***P<0.001 compared to D-Glu+TGF-β1, **P<0.01 compared to D-Glu+TGF-β1, all n=5. Data are shown as mean +/- SD.

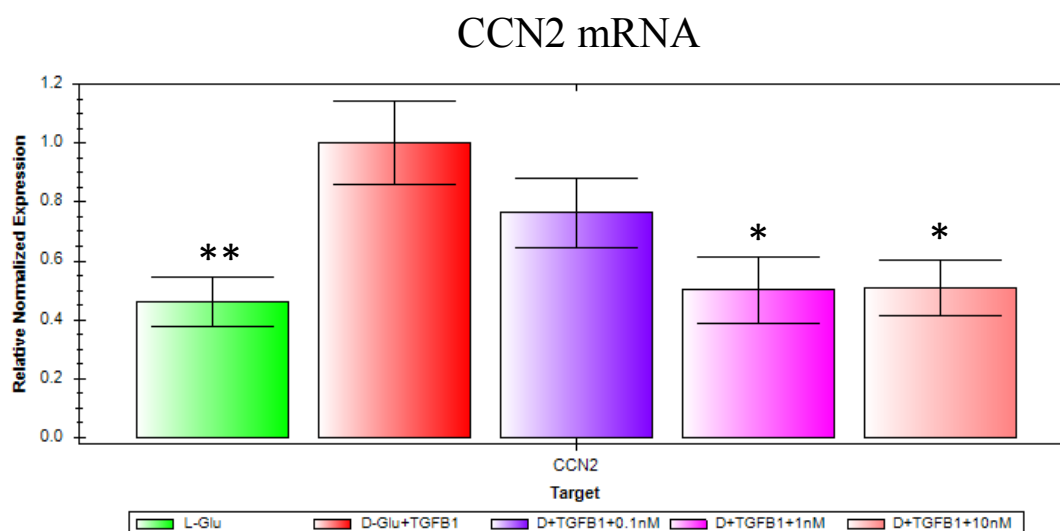


Figure 4.3D: CCN2 mRNA abundance in PTECS treated with glucose, TGF- β 1 and dapagliflozin.

Key: **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **D-Glu+TGF- β 1**=7mM D-glucose+18mM D-glucose+ 0.75ng/ml TGF- β 1, **D+TGF- β 1+0.1nM**=7mM D-glucose+18mM D-glucose+0.75ng/ml TGF- β 1+0.1nM dapagliflozin, **D+TGF- β 1+10nM**=7mM D-glucose+18mM D-glucose+0.75ng/ml TGF- β 1+10nM dapagliflozin. All treatment times lasted 24h. The figure shows plotted data points after quantification of CCN2 mRNA (CCN2 mRNA abundance/GAPDH mRNA abundance) presented in graph form. qPCR analysis showed that D-Glu+TGF- β 1 significantly increased CCN2 mRNA abundance. Dapagliflozin at 1 and 10nM significantly reduced this increased CCN2 mRNA abundance. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. **P<0.01 compared to D-Glu+TGF- β 1, *P<0.05 compared to D-Glu+TGF- β , all n=4. Data are shown as mean +/- SD.

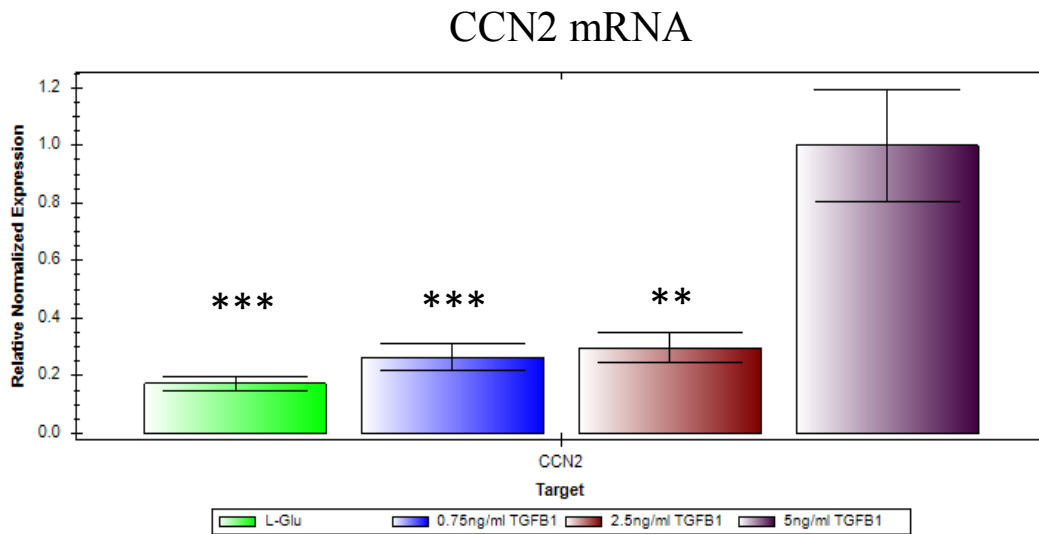


Figure 4.3E: CCN2 mRNA abundance in PTECS treated with TGF-β1.

Key: **L-Glu**= 7mM D-glucose+18mM L-glucose, **0.75ng/ml TGF-β1**=7mM D-glucose+18mM L-glucose+0.75ng/ml, **2.5ng/ml TGF-β1**= 7mM D-glucose+18mM L-glucose+2.5ng/ml TGF-β1, **5ng/ml TGF-β1**= 7mM D-glucose+18mM L-glucose+5ng/ml TGF-β1. All treatment times lasted 24h. The figure shows plotted data points after quantification of CCN2 mRNA (CCN2 mRNA abundance/GAPDH mRNA abundance) presented in graph form. qPCR analysis showed that treatment with 0.75 and 2.5ng/ml TGF-β1 did not change CCN2 mRNA abundance, but stimulation of TGF-β1 at a higher concentration of 5ng/ml did significantly induce its abundance. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. ***P<0.001 compared to 5ng/ml TGF-β1, **P<0.01 compared to 5ng/ml TGF-β1, all n=4. Data are shown as mean +/- SD.

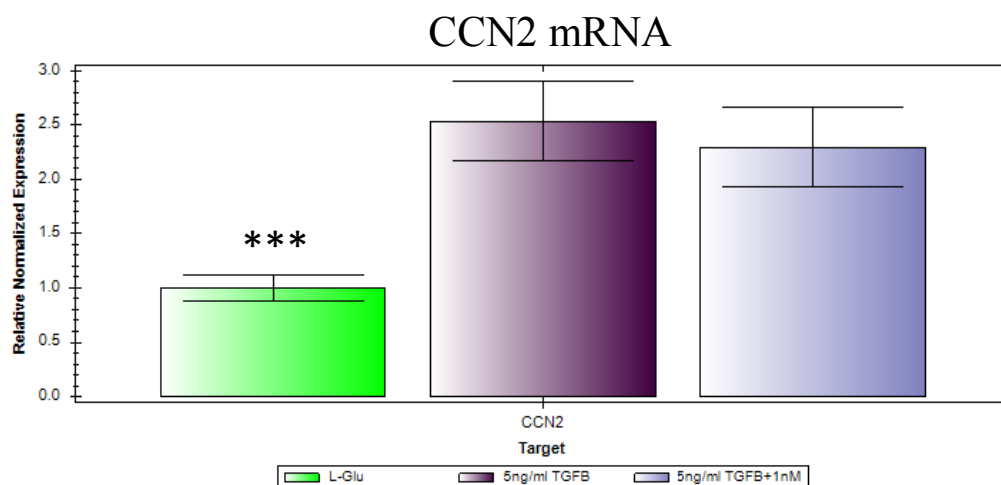


Figure 4.3F: CCN2 mRNA abundance in PTECS treated with TGF- β 1 and dapagliflozin

Key: **L-Glu**= 7mM D-glucose+18mM L-glucose, **5ng/ml TGF- β 1**= 7mM D-glucose+18mM L-glucose+5ng/ml TGF- β 1, **5ng/ml TGF- β 1+1nM**= 7mM D-glucose+18mM L-glucose+5ng/ml TGF- β 1+1nM dapagliflozin. All treatment times lasted 24h. The figure shows plotted data points after quantification of CCN2 mRNA (CCN2 mRNA abundance/GAPDH mRNA abundance) presented in graph form. qPCR analysis showed that treatment with 5ng/ml TGF- β 1 significantly increased CCN2 mRNA abundance. Dapagliflozin, at 1nM, did not change the increased CCN2 mRNA abundance. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. ***P<0.001 compared to 5ng/ml TGF- β 1 and 5ng/ml TGF- β 1+1nM, all n=4. Data are shown as mean $\pm$ SD.

4.3.3. Presence of urinary CCN2 protein in samples from patients with diabetic kidney disease

As CCN2 protein abundance was increased by high D-glucose and 0.75ng/ml TGF- β , a pilot clinical study was carried out to gain more insight into whether CCN2 would be an appropriate and effective clinical biomarker of DKD. Urine samples from a pool of eight DKD patients were used, split equally into group A and B. Group A, in comparison to group B, had better DKD outcomes consisting lower HbA1C levels, lower albumin to creatinine ratios (ACR) and eGFR values of $>60\text{ml/min/1.73m}^2$ (Table 4.1). The western blotting results showed that all 4 of the DKD patients in group B had detectable CCN2 protein in their urine, whilst none of the DKD patients in group A had any detectable urinary CCN2 protein (Figure 4.4). Samples were previously segregated by DKD outcomes, but the two groups were blinded during this study.

	HbA1c median (%)	ACR Median	eGFR (ml/min/1.73 m ²) >60:<60	Ethnicity (self- identified) Black:White:Asian	Gender (self- identified) Male:Female
Group A (4 samples)	6.7	0.42	4:0	1:3:0	2:2
Group B (4 samples)	12.2	336	1:3	1:2:1	2:2

Table 4.1: Diabetic kidney disease patient information for pilot clinical study.

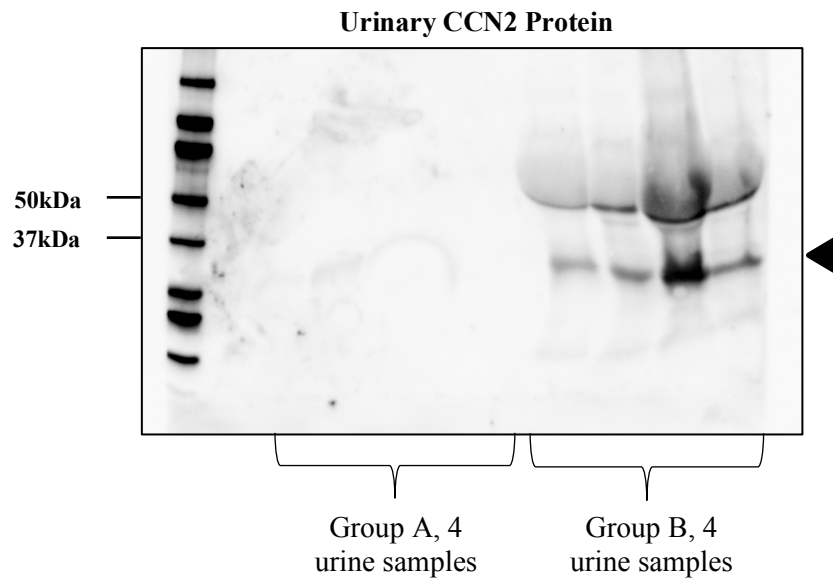


Figure 4.4: Western blot immuno-stained for urinary CCN2 protein.

Western blot probed for CCN2 protein (~36-38kDa) in DKD urine samples. The black arrowhead indicates the target immuno-reactive band. There was no observable CCN2 protein in samples from group A, but all 4 urine samples from group B contained urinary CCN2 protein.

4.4. Discussion

My results demonstrate that in this DKD PTEC model, 0.75ng/ml TGF- β 1 significantly induce CCN2 protein abundance in the presence of high D-glucose. Zorena *et al* determined that serum TGF- β 1 levels was the most superior power compared to other markers like HbA1c and ACR, in regards to predicting diabetes induced outcomes. The group established the threshold concentration for TGF- β 1 where it became strongly associated with diabetes induced complications was approximately 0.4ng/ml (Zorena *et al.*, 2013). Thus, I have included a threshold concentration (0.75ng/ml) of TGF- β 1 in my DKD model.

Much higher concentrations of TGF- β 1 (5ng/ml) were required to elevate CCN2 mRNA in osmotic control conditions. CCN2 promotes renal fibrosis and plays a pivotal role in the development of DKD. CCN2 protein has been found in the glomeruli and podocytes of DKD mice and human kidneys (Wahab *et al.*, 2001; Roestenberg *et al.*, 2006). Chronic exposure to elevated levels of D-glucose has significantly increased CCN2 protein abundance in renal fibroblasts (Lam, van der Geest, *et al.*, 2003). There has also been research supporting CCN2 as a potential target for anti-DKD therapy. CCN2 antisense treatment administered to streptozotocin (STZ) induced diabetic mice showed a significant reduction in albuminuria and proteinuria (Guha *et al.*, 2007). Furthermore, a phase 1 clinical trial treating microalbuminuric diabetic patients with FG-3019, an anti-CCN2 monoclonal antibody, significantly decreased their ACR with minimal adverse events (Adler *et al.*, 2010).

Increasing TGF- β 1 to 5ng/ml induced CCN2 in osmotic control media, indicating that higher concentrations of TGF- β 1 do not require high concentrations of D-glucose to activate this pathway in PTECs. Similarly, other work in literature has also revealed significant CCN2 induction after $\geq$ 5ng/ml TGF- β 1 treatment alone in various cell types (Phanish *et al.*, 2005; Liu *et al.*, 2013; Cheng *et al.*, 2015).

A concentration of TGF- β 1 ranging from 1 to 5ng/ml activates epithelial mesenchymal transition (EMT), a process that contributes towards renal fibrosis, in breast, kidney and

alveolar epithelial cells (Kasai *et al.*, 2005; Tian and Schiemann, 2017; He *et al.*, 2018). There is also compelling evidence of a TGF- β 1 independent CTGF induced EMT in rat kidney cells (Burns *et al.*, 2006). There is no evidence of EMT in human DKD.

Although there has been a selection of different strategies attached with varying levels of success, there is currently no approved therapeutic that selectively targets renal fibrosis with high efficacy. Administration of dapagliflozin, a selective SGLT2 inhibitor (Kasichayanula *et al.*, 2014), significantly reduced CCN2 protein and mRNA in PTECs treated with high D-glucose and 0.75ng/ml TGF- β 1. This indicates a crucial role for SGLT2 in the pro-fibrotic regulation of CCN2 in DKD. Whilst dapagliflozin at all three concentrations (0.1, 1, 10nM) significantly attenuated this rise in CCN2 protein, only the two higher concentrations significantly decreased CCN2 mRNA. This inconsistency could be accounted by the fact that CCN2 is an early immediate gene with unique properties including transitory nucleic acid expression. The lowest concentration of dapagliflozin administered may only be sufficient enough to “flatten the curve” as opposed to significantly reducing mRNA abundance at the 24h timepoint.

The CCN2 mRNA induced by 5ng/ml TGF- β 1 in osmotic control conditions was not attenuated by dapagliflozin, indicating that SGLT2 is not involved in TGF- β 1 signalling or CCN2 regulation under normal levels of D-glucose. There have been a couple of pre-clinical trials which have investigated the role of SGLT2 inhibition in non-diabetic kidney disease (Zhang *et al.*, 2016; Ma, Steiger and Anders, 2017). Although these animal studies demonstrated no significant renoprotective benefits, the more recent long-term DAPA-CKD clinical study evidenced promising data. Researchers found that CKD patients administered with dapagliflozin had significantly reduced risk of progressing to ESRD or death, irrespective of the presence/absence of diabetes (Heerspink *et al.*, 2020).

Despite this, future long-term studies are required to properly evaluate whether gliflozins can improve renal outcomes in non-diabetic patients, with minimal adverse side effects.

My pilot clinical study showed a clear presence of urinary CCN2 protein in all of the DKD patients with high HbA1c levels (median 12.2%), high albumin to creatinine ratios (ACR) (median 336), and eGFR values of <60ml/min/1.73m², thus supporting CCN2 as a clinical

biomarker candidate for DKD. A separate clinical trial showed that diabetic patients with micro and macro-albuminuria had a 10 and 100-fold increase of urinary CCN2-N terminal fragments respectively compared to their normoalbuminuric counterparts (Gilbert *et al.*, 2003). This signifies a positive correlation between CCN2 abundance and severity of renal damage. Additionally, Gerritsen *et al.* also found an independent association between urinary CCN2 and various markers of tubular dysfunction including KIM-1 and NGAL (Gerritsen *et al.*, 2015).

Aside from pro- fibrotic outcomes, I also examined the abundance of other proteins such as T β RII, p21 and K-Cadherin to observe whether elevated glucose and 0.75ng/ml TGF- β 1 stimulation changed receptor level proteins, cell cycle regulation and PTEC morphology, respectively.

There was no change to T β RII protein under any of the treatments, demonstrating that the induction of CCN2 is not dependent on receptor abundance. Interestingly, the upregulation of p21 protein by high D-glucose and 0.75ng/ml TGF- β 1 treatment indicated that the combination promotes cell senescence. Cell senescence is typically elicited in response to an internal or external stressor (Yosef *et al.*, 2017). Other groups have found an overexpression of p21 protein in STZ rats and epithelial cells cultured in high D-glucose without the addition of TGF- β 1 (Satriano *et al.*, 2010; Kitada *et al.*, 2014). However, it should be noted that their treatments lasted between 10 to 28 days as opposed to the 24h condition my PTECs were subjected to. Clements *et al.* also found that an increase of p21 protein helps to promote the progression of renal fibrosis from acute kidney injury (AKI) (Clements *et al.*, 2013).

K-cadherin is a cell adhesion protein localised to the basolateral membranes of PTECs (Thedieck *et al.*, 2005). Prior work from our group found that a loss of full-length urinary K-cadherin protein was considered a potential biomarker in diabetic patients with deteriorating kidney function (Yates *et al.*, 2015). K-cadherin protein did not change in my experimental model, indicating that the PTECs maintained their cellular integrity and that the induction of pro-fibrotic CCN2 was mediated by a specific and selective mechanism. This will be investigated in the next results chapter.

Chapter 5:

The Mechanisms Behind CCN2 Induction

5.1. Introduction

As briefly mentioned in the introduction, TGF- β exerts its effects through Smad dependent and independent signalling pathways. The Smad family is made up of 8 members. These comprise of two R-Smads (Smad2 and 3) that are activated in response to TGF- β , one common-Smad (co-Smad4), 3 R-Smads (Smad1, 5, 8) that are activated in response to bone morphogenetic proteins (BMPs) and two inhibitory Smads (Smad 6 and 7) (Heldin and Moustakas, 2012; Hata and Chen, 2016).

The active TGF- β homodimer engages the TGF- β type 2 (T β RII) receptor, which then activates and forms a heteromeric complex with the TGF- β type 1 (T β RI) receptor. The T β RI then phosphorylates the R-Smads, important downstream mediators for the cytokine. This phosphorylated homologous/heterologous Smad complex is then bound to co-Smad4 (Hata and Chen, 2016). This final complex is then translocated to the nucleus to regulate transcription and encode for genes like collagen, fibronectin, CCN2 and α -SMA. Inhibitory Smad7, which prevents TGF- β induced signalling, can disrupt the phosphorylation of R-Smads (Zhang *et al.*, 2014). It carries this out with the help of ubiquitin ligases like Smurf1 and 2 and competing with the other R-Smads to bind to T β RI (Yan *et al.*, 2016).

Whilst Smad3 is known to be a pro-fibrotic agent (Zarjou *et al.*, 2011, Feng *et al.*, 2018)), Smad2 has anti-fibrotic properties (Meng *et al.*, 2010, Loeffler *et al.*, 2018). The role of Smad4 is less well defined due to the limited availability of Smad4 knockout mice models. Interestingly, Meng *et al* found that whilst the deletion of Smad4 in their UUO mouse model increased markers of renal inflammation, the Smad4 knockout model also presented with attenuated levels of renal fibrosis. Additionally, they found that Smad4 was able to inhibit the transcription of Smad7 but did not change the levels of phosphorylated Smad2

and 3. Their results indicate that whilst Smad4 can ameliorate inflammation through the regulation of Smad7 transcriptional activity, it does not affect pro-fibrotic TGF- β signalling (Meng *et al.*, 2012). Studies have also supported the notion that Smad3 also plays a vital role in hyperglycaemia-associated injury and fibrosis in podocytes and tubule cells (Jiang, Cui and Ding, 2020; Wang *et al.*, 2020). TGF- β phosphorylates serines 423 and 425 on the mad homology 2 (MH2) domain of Smad3 (Matsuzaki, 2006).

TGF- β can activate other non-canonical signal transduction pathways that also play important roles in the development of renal diseases. Amongst these pathways include Rho and Ras family guanine triphosphate enzymes (GTPases), TGF- β activated kinase 1 (TAK1), and MAPK/ERK. In renal cells, the Rho pathway is heavily implicated in driving the loss of epithelial phenotype through the downregulation of membrane proteins like E-cadherin (Hutchison, Hendry and Sharpe, 2009) and breakdown of tight junctions (Zhang *et al.*, 2013). TAK1 is a key player in renal fibrosis. A dominant-negative TAK1 construct, responsible for disrupting the TGF- β pathway, also attenuated levels of collagen and fibronectin in renal mesangial cells (Ono *et al.*, 2003). Although the majority of the focus has been given to the TGF- β /Smad3 pathway in regards to renal fibrosis, a recent study found that the attenuation of TGF- β 1 induced renal fibrosis in HK-2 cells was achieved through the inhibition of ERK (Wu *et al.*, 2017). This indicates the potential pro-fibrotic properties of ERK. A schematic diagram of both Smad dependent and independent TGF- β 1 signalling pathways is detailed in figure 5.1.

Whilst it is established that ERK and Smad3 serines 423 and 425 are downstream mediators of pro-fibrotic TGF- β signalling, there is also evidence that phosphorylation of this Smad3 serine 204, found on the LR of Smad3, can also potentiate fibrotic effects (Browne *et al.*, 2013). Consequently, this chapter will address the putative signalling mechanisms that are involved in the upregulation of pro-fibrotic marker CCN2, in particular focusing on the Smad and MAPK/ERK pathways.

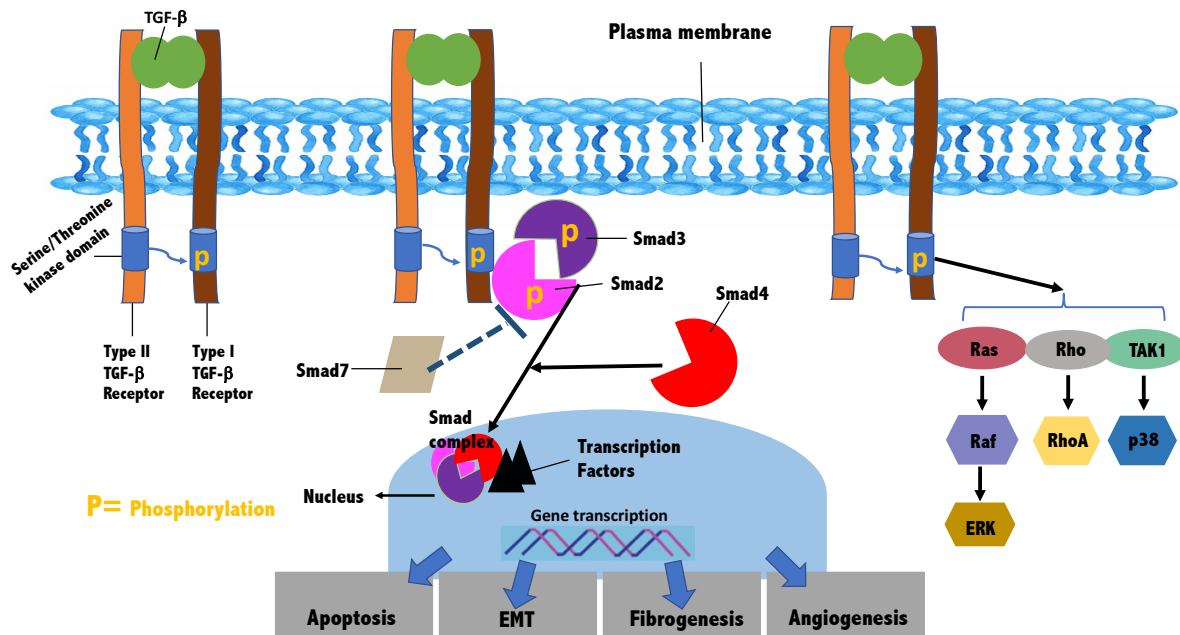


Figure 5.1: Canonical (Smad dependent) and non-canonical (Smad independent) TGF-β signalling.

Canonical signalling: Active TGF-β first binds to serine/threonine kinase TGF-β type 2 receptor (TβRII), which in turn phosphorylates and forms a heteromeric complex with TGF-β1 type 1 receptor (TβRI). The TβRI then phosphorylates R-Smads, which form a complex with co-Smad4. This complex then translocates to the nucleus to control gene transcription. In addition, the presence of Smad3 triggers the expression of smad7 which is inhibitory and suppresses phosphorylation of Smad 2/3. Non-canonical signalling: TGF-β1 also activates various Smad-independent pathways like Ras, Rho and TAK1. These subsequently activate Raf/ERK, RhoA and p38 respectively. These pathways can act separately or in combination with the Smad pathway to mediate various cellular processes.

5.2. Aims and Objectives

1. To investigate phosphorylated (phospho) ERK, phospho-Smad3 serine 423/425 and phospho-Smad3 serine 204 protein abundance in following treatment with glucose and TGF- β 1.
2. To investigate whether SGLT2 and ERK inhibition can prevent Smad3 serine 204 phosphorylation.
3. To evidence that phospho-Smad3 serine 204 protein is the point of convergence between high D-glucose and TGF- β 1 treatment using a dominant negative mutant construct which prevents Smad3 serine 204 phosphorylation.

5.3. Results

5.3.1. Phospho-ERK, phospho-Smad3 serine 423/425 and phospho-Smad3 serine 204 protein abundance in PTECs treated with glucose and TGF- β 1

There was no change in phospho-ERK protein levels over 60 min under osmotic control (7mM D-glucose+18mM L-glucose) conditions. TGF- β 1 treatment under osmotic control conditions increased the abundance of phospho-ERK protein from 5 to 60 min whilst high D-glucose (7mM D-glucose+18mM D-glucose) alone significantly increased phospho-ERK protein at 30 min before dropping to osmotic control levels by 60 min (Figure 5.2A).

Smad3 was also phosphorylated at the serines 423 & 425, after 0.75ng/ml TGF- β 1 treatment. This phosphorylation became statistically significant at 30 min and remained at 60 min (Figure 5.2B).

Finally, phospho-Smad3 serine 204 protein was detected in the western blot of PTECs with 3 bands weighing at approximately 25, 50, and 75kDa. There was a significant increase in phospho-Smad3 serine 204 protein abundance at 30 min after the combined treatment of high D-glucose and 0.75ng/ml TGF- β 1 compared to all other treatments (Figure 5.2C). This significant rise was only observed at the ~25kDa weight.

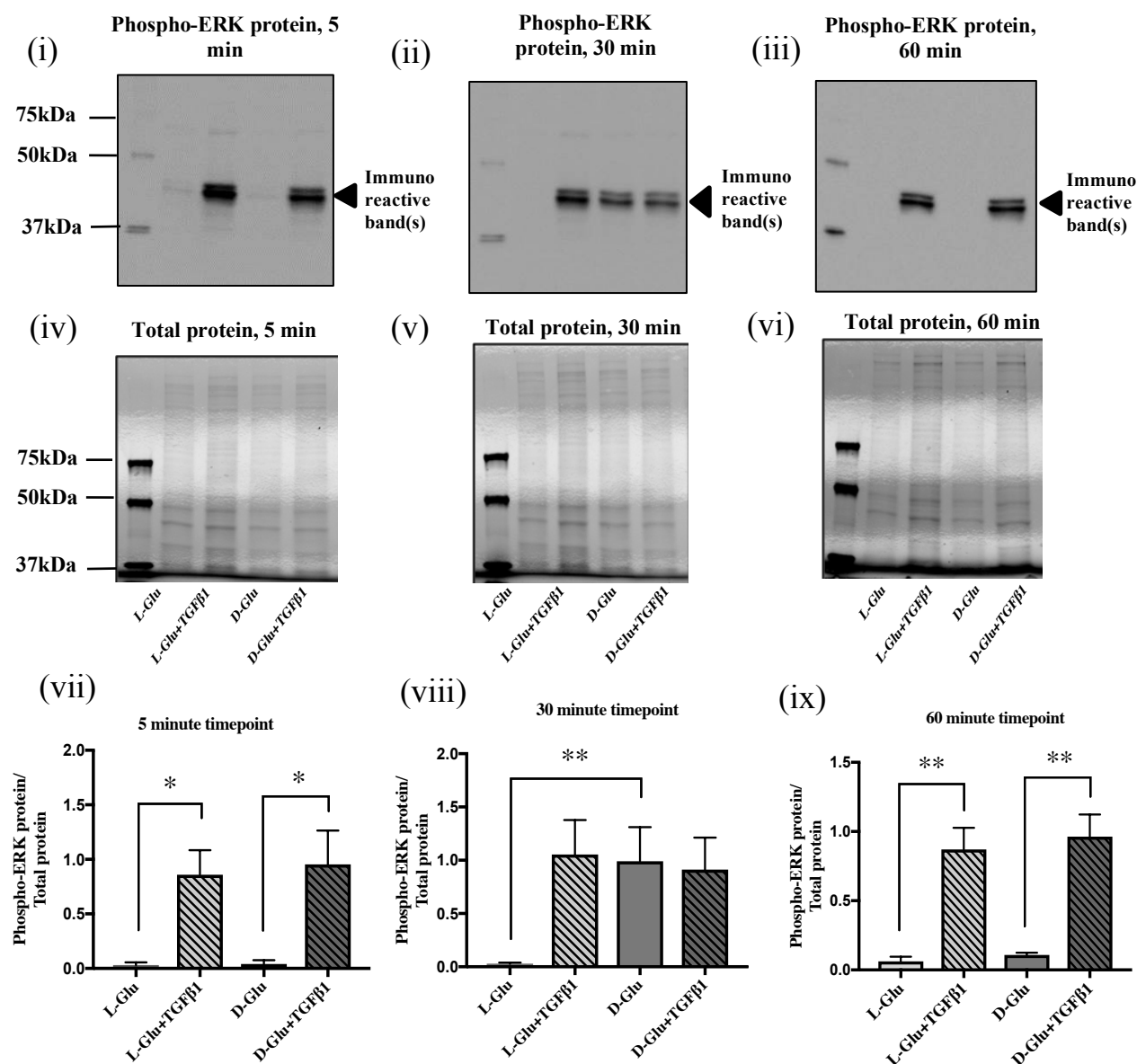


Figure 5.2A: Phospho-ERK protein abundance in PTECs after glucose and TGF- β 1 treatment.

Key: **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGF β 1**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGF β 1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGF β 1**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGF β 1. (i-iii) Western blot showing immunostained phospho-ERK protein (~40kDa) in PTECs under various treatments and timepoints. (iv-vi) Corresponding total protein stain free gel which is used as the loading control. (vii-ix) Plotted data points after quantification of phospho-ERK protein (phospho-ERK abundance/total protein abundance) presented in graph format. TGF- β 1 significantly induced phospho-ERK protein for 60 min, while high D-glucose alone induced phospho-ERK protein starting at 30 min. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. * $P < 0.05$ and ** $P < 0.01$, all $n = 4$. Data are shown as mean $\pm$ SD.

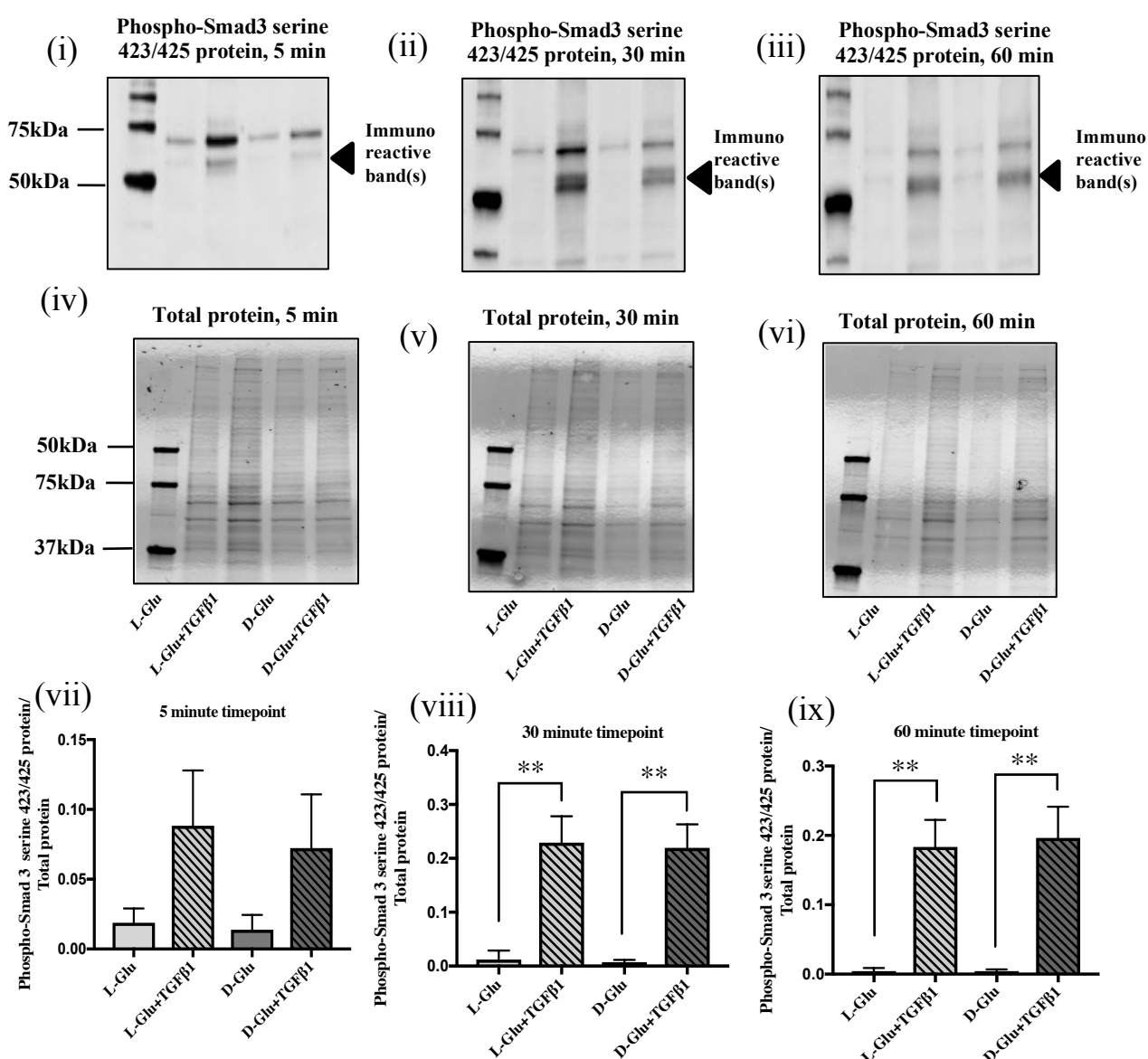


Figure 5.2B: Phospho-Smad3 serine 423/425 protein abundance in PTECs after glucose and TGF-β1 treatment.

Key: **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGFβ1**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGFβ1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGFβ1**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGFβ1. (i-iii) Western blot showing immunostained phospho-Smad3 serine 423/425 protein (~53kDa) in PTECs under various treatments and time points. (iv-vi) Corresponding total protein stain free gel which is used as the loading control. (vii-ix) Plotted data points after quantification of phospho-Smad3 serine 423/425 protein (phospho-Smad3 serine 423/425 abundance/total protein abundance) presented in graph format. TGF-β1 significantly induced phospho-Smad3 serine 423 and 425 protein from 30 min onwards while D-Glu alone did not induce any changes. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. **P<0.01, all n=3. Data are shown as mean +/- SD.

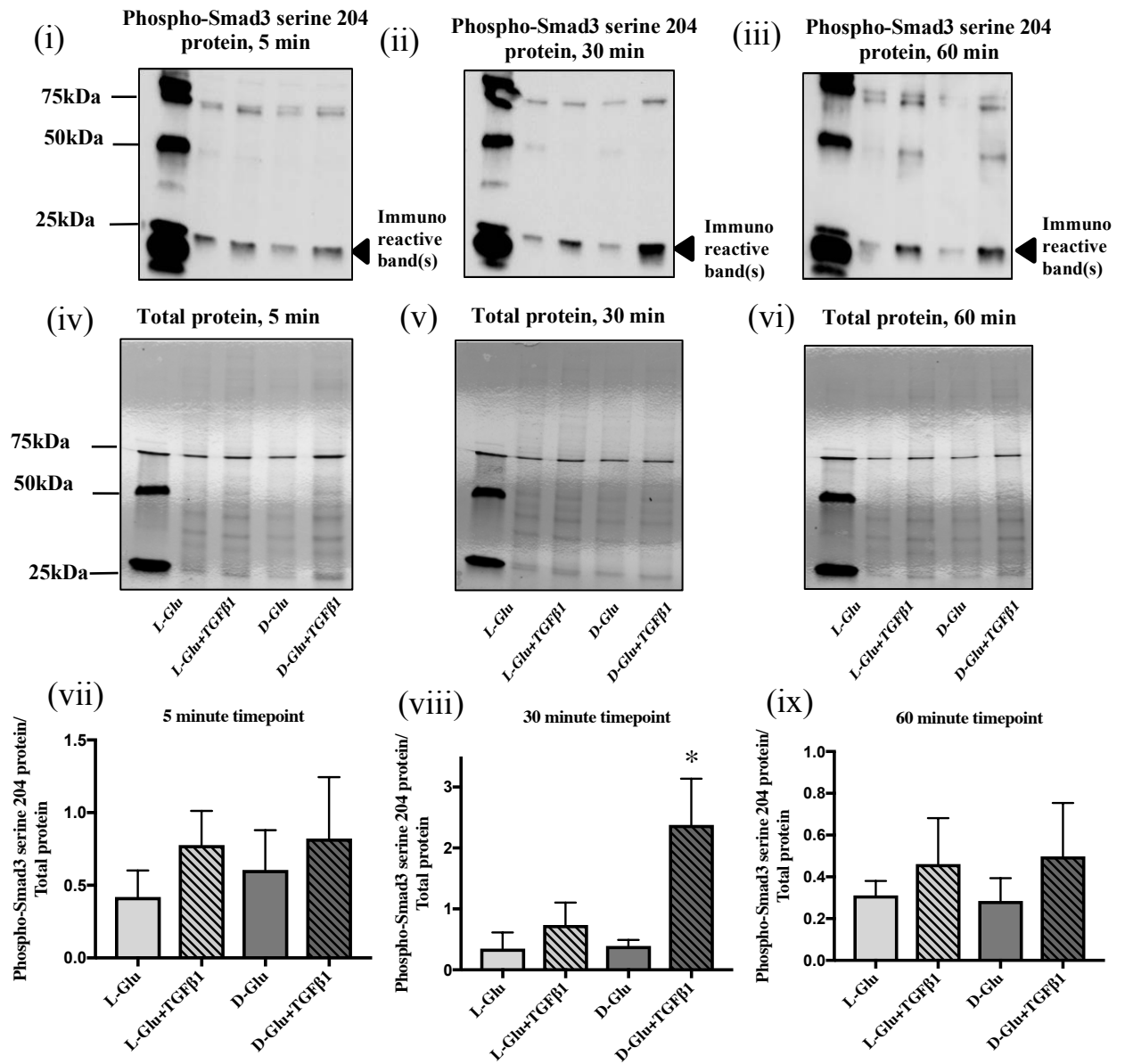


Figure 5.2C: Phospho-Smad3 serine 204 protein abundance in PTECs after glucose and TGF-β1 treatment.

Key: **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGFβ1**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGFβ1, **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGFβ1**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGFβ1. (i-iii) Western blot showing immunostained phospho-Smad3 serine 204 protein (~25kDa) in PTECs under various treatments and time points. (iv-vi) Corresponding total protein stain free gel which is used as the loading control. (vii-ix) Plotted data points after quantification of phospho-Smad3 serine 204 protein (phospho-Smad3 serine 204 abundance/total protein abundance) presented in graph format. Neither TGF-β1 nor high D-Glu alone significantly induced phospho-Smad3 serine 204 protein, but the combined treatment of the two significantly induce phospho-Smad3 serine 204 protein at 30 min. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. *P<0.05 vs L-Glu, L-Glu+TGFβ1, and D-Glu, all n=3. Data are shown as mean +/- SD.

5.3.2. Phospho-Smad3 serine 204 protein abundance in PTECs after ERK and SGLT2 inhibition

As observed from 5.3.1, high D-glucose treatment alone significantly increased cellular phospho-ERK, beginning at 30 min and reducing down to osmotic control levels by 60 min. Furthermore, the co- incubation of high D-glucose and 0.75ng/ml TGF- β 1 treatment significantly increased abundance of phospho-Smad3 serine 204 protein as well as abundance of CCN2 protein and mRNA as shown in the chapter 4.

The next steps carried out were to determine whether prevention of ERK phosphorylation could result in a decrease in phospho-Smad3 serine 204 protein using U0126, a MAPK/ERK inhibitor. Additionally, as SGLT2 inhibition significantly reduced CCN2 abundance after high D-glucose and 0.75ng/ml TGF- β 1 treatment, PTECs were also administered with dapagliflozin and probed for phospho-Smad3 serine 204 protein to check for changes in phospho-Smad3 serine 204 protein abundance after SGLT2 inhibition.

Phospho-Smad3 serine 204 protein was significantly attenuated after high D-glucose and 0.75ng/ml TGF- β 1 treated PTECs were administered with 10 μ M U0126. Similarly, high D-glucose and 0.75ng/ml TGF- β 1 treated PTECs co-incubated with 1nM dapagliflozin also significantly reduced phospho-Smad3 serine 204 protein abundance (Figure 5.3A&B).

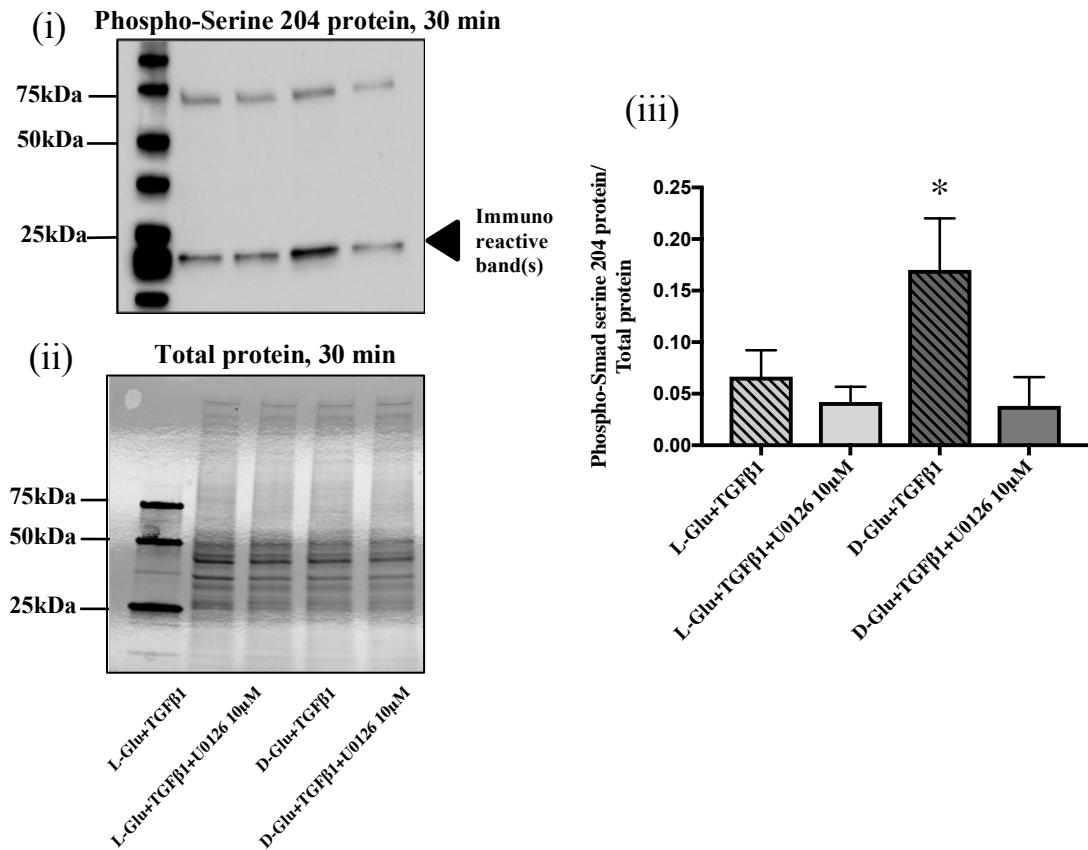


Figure 5.3A: Phospho-Smad3 serine 204 protein abundance in PTECs after glucose, TGF-β1 and U0126 treatment.

Key: **L-Glu+TGFβ1**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGFβ1, **L-Glu+TGFβ1+U0126 10μM**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGFβ1+10μM U0126 ERK inhibitor, **D-Glu+TGFβ1**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGFβ1, **D-Glu+TGFβ1+U0126 10μM**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGFβ1+10μM U0126. (i) Western blot showing immune-stained phospho-Smad3 serine 204 protein (~25kDa) in PTECs after 30 min treatments, (ii) corresponding total protein stain free gel which is used as the loading control, (iii) plotted data points after quantification of phospho-Smad3 serine 204 protein (phospho-Smad3 serine 204 abundance/total protein abundance) presented in graph format. U0126 significantly decreased phospho-Smad3 serine 204. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. *P<0.05 vs L-Glu+TGFβ1, L-Glu+TGFβ1+ U0126 10μM, and D-Glu+TGFβ1+ U0126 10μM, all n=3. Data are shown as mean +/- SD.

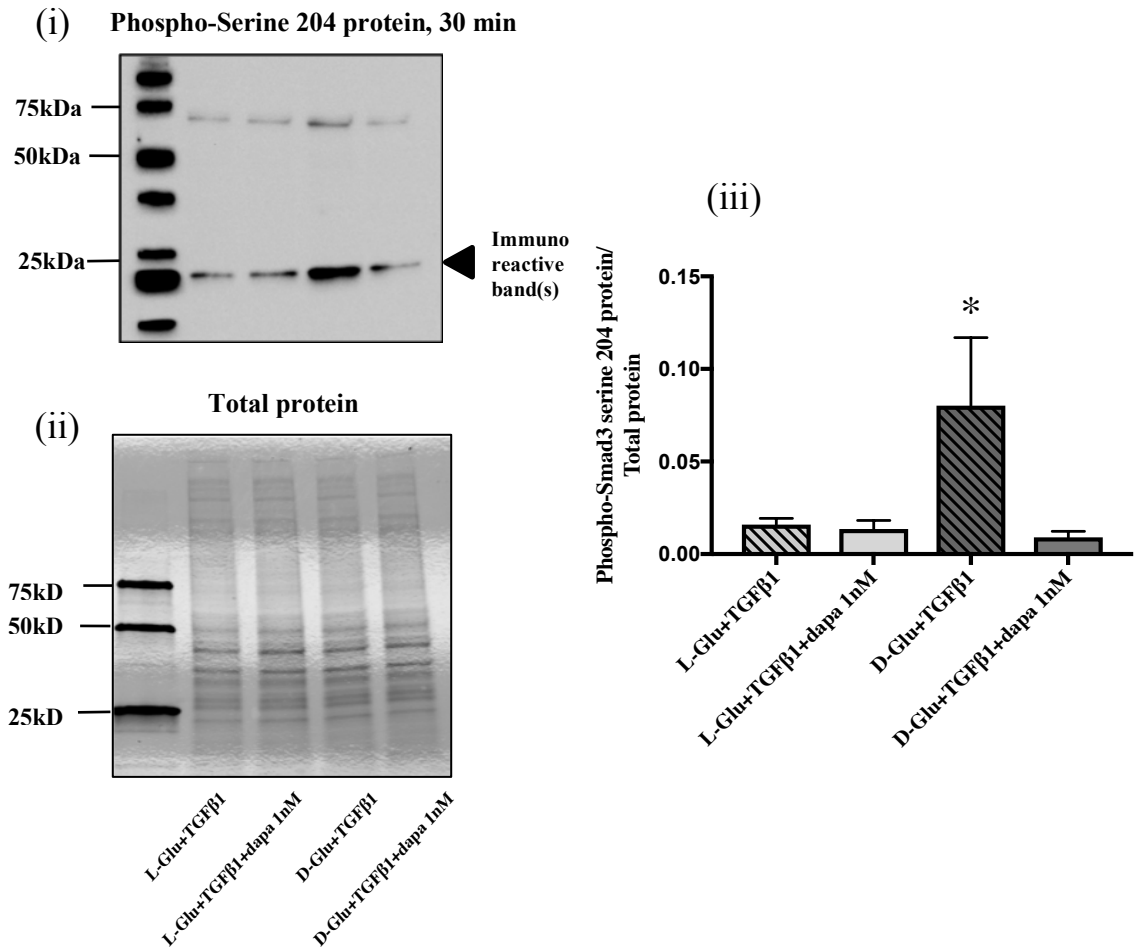


Figure 5.3B: Phospho-Smad3 serine 204 protein abundance in PTECs after glucose, TGF- β 1 and dapagliflozin treatment.

L-Glu+TGF β 1= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGF β 1, **L-Glu+TGF β 1+dapa 1nM**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGF β 1+1nM dapagliflozin, **D-Glu+TGF β 1**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGF β 1, **D-Glu+TGF β 1+dapa 1nM**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGF β 1+1nM dapagliflozin. (i) Western blot showing immuno-stained phospho-Smad3 serine 204 protein (~25kDa) in PTECs after 30min treatments, (ii) corresponding total protein stain free gel which is used as the loading control, (iii) plotted data points after quantification of phospho-Smad3 serine 204 protein (phospho-Smad3 serine 204 abundance/total protein abundance) presented in graph format. Dapagliflozin (1nM) significantly decreased phospho-Smad3 serine 204 protein. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. * $P < 0.05$ vs L-Glu+TGF β 1, L-Glu+TGF β 1+ dapa 1nM, and D-Glu+TGF β 1+dapa 1nM, all $n=3$. Data are shown as mean $\pm$ SD.

5.3.3. Phospho-ERK, total Smad3, phospho-Smad3 serine 423/425, phospho-Smad3 serine 204 and CCN2 protein abundance in PTECs transfected with mutant plasmid DNA transfection

The results depicted above strongly support an important role for phospho-Smad3 serine 204 in regulating the SGLT2-mediated induction of profibrotic marker CCN2. This next part will provide evidence of direct involvement of phospho-Smad3 serine 204 in CCN2 induction under high D-glucose and 0.75ng/ml TGF- β 1 conditions. PTECs were transfected with a dominant negative pCS2 Smad3 serine 204A plasmid construct (mutant plasmid DNA) that has substituted the wild-type serine into an alanine, thus preventing serine 204 phosphorylation.

PTECs grown under osmotic control conditions were transfected with the mutant plasmid (0.5, 1 and 2 μ g) and pCS2 empty vector (EV) control plasmid using Fugene 6 transfection reagent for 24h. The spent medium was then removed from the dishes and replaced with normal medium to recover for 4 and 8h. To confirm successful transfection and to determine optimal parameters, PTECs were lysed and protein harvested for western blot analysis. For both 4 and 8h, the amount of total Smad3 protein increased with increasing amounts of mutant plasmid DNA transfected into PTECs. There was also visibly less total Smad3 protein in the EV plasmid transfected PTECs compared to the 1 μ g/2 μ g mutant plasmid transfected PTECs (Figure 5.4A). There was no statistical analysis performed at this point as the aim of the experiment was only to confirm total Smad3 protein overexpression and that only one recovery timepoint was required. The transfection and recovery time for all subsequent transfection experiments was 24h and 8h, respectively.

The next step was to investigate the abundance of total Smad3 and phospho-Smad3 serine 204 protein in my DKD model after mutant plasmid/EV plasmid DNA transfection. After 30 min of high D-glucose and 0.75ng/ml TGF- β 1 treatment, total Smad3 protein was significantly increased in mutant plasmid transfected PTECs (Figure 5.4B), while phospho-Smad3 serine 204 protein was significantly reduced (Figure 5.4C).

In order to confirm that overexpression of this mutant plasmid did not alter the levels of my other proteins of interest, I analysed the protein abundance of phospho-ERK and

phospho-Smad3 serine 423/425 protein under osmotic control, osmotic control+0.75ng/ml TGF- β 1 and high D-glucose+0.75ng/ml TGF- β 1 treatments, with transfected mutant and EV plasmid. There was significant induction of phospho-ERK and phospho-Smad3 serine 423/425 protein after the osmotic control+0.75ng/ml TGF- β 1 and high D-glucose+0.75ng/ml TGF- β 1 treatments compared to osmotic control alone at 30 min. There were no differences between the mutant and EV plasmid DNA interventions (Figure 5.4D&E).

Similar to the results above, phospho-Smad3 serine 204 protein was only significantly induced after high D-glucose+0.75ng/ml TGF- β 1 treatment compared to osmotic control and osmotic control+TGF- β 1 after 30 min. This rise was significantly reduced upon mutant plasmid DNA transfection, 2 μ g. With the exception of the high D-glucose+0.75ng/ml TGF- β 1 treatment, there were no differences between mutant and empty vector plasmid DNA interventions (Figure 5.4F).

The last experiment of this chapter is to prove that significant reduction of phospho-Smad3 serine 204 protein prevents CCN2 transcription. I analysed CCN2 mRNA levels after mutant and empty vector plasmid transfection in osmotic control, osmotic control+0.75ng/ml TGF- β 1 and high D-glucose+0.75ng/ml TGF- β 1 treated PTECs. After 30 min treatment, there were no detectable changes in CCN2 mRNA in osmotic control and osmotic control+0.75ng/ml TGF- β 1 treated PTECs after both mutant and empty vector plasmid DNA transfection. CCN2 mRNA was only significantly increased after combined high D-glucose+0.75ng/ml TGF- β 1 treatment. After mutant plasmid DNA transfection, CCN2 mRNA abundance was significantly attenuated back down to osmotic control levels (Figure 5.4G).

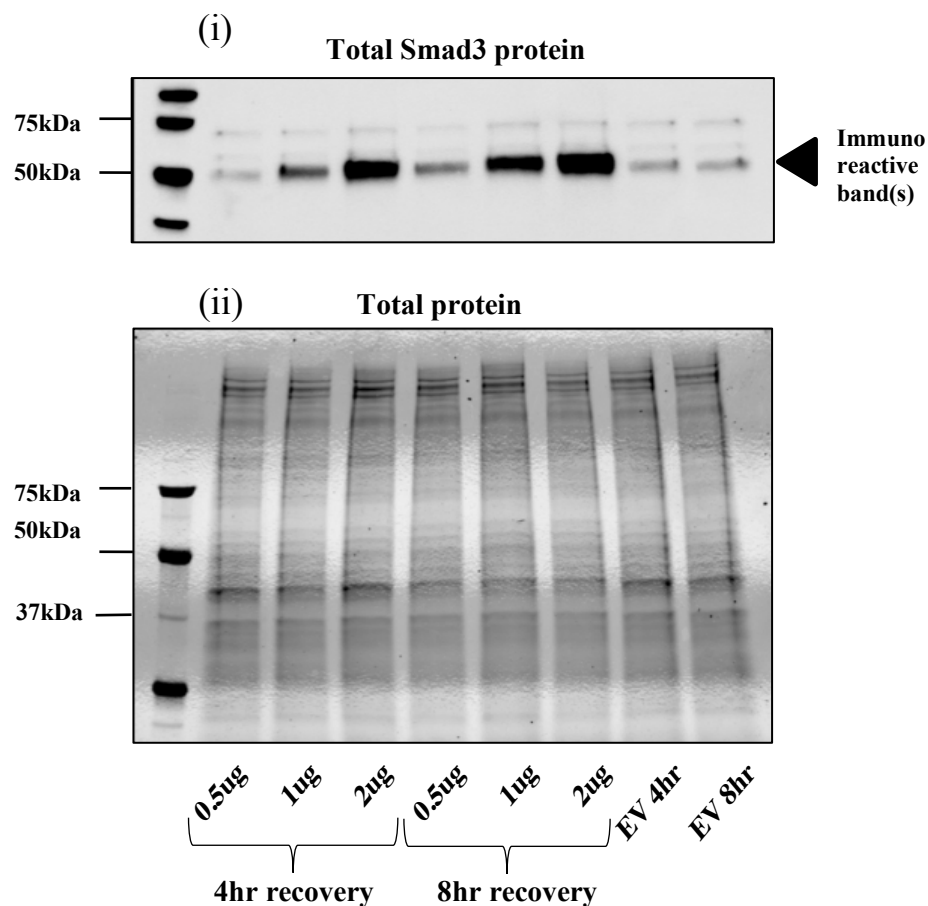


Figure 5.4A: Example western blot and total protein gel of total smad3 protein abundance in PTECs after mutant plasmid DNA transfection.

Key: **0.5 μ g**=7mM D-glucose+18mM L-glucose+0.5 μ g mutant plasmid DNA, **1 μ g**= 7mM D-glucose+18mM L-glucose+1 μ g mutant plasmid DNA, **2 μ g**=7mM D-glucose+18mM L-glucose+2 μ g mutant plasmid DNA, **EV 4h**= 7mM D-glucose+18mM L-glucose+2 μ g empty vector plasmid DNA (4h recovery), **EV 8h**= 7mM D-glucose+18mM L-glucose+2 μ g empty vector plasmid DNA (8hr recovery). (i) Western blot showing immunostained total Smad3 protein (~53kDa) in PTECs under various interventions and (ii) corresponding total protein stain free gel separated by electrophoresis.

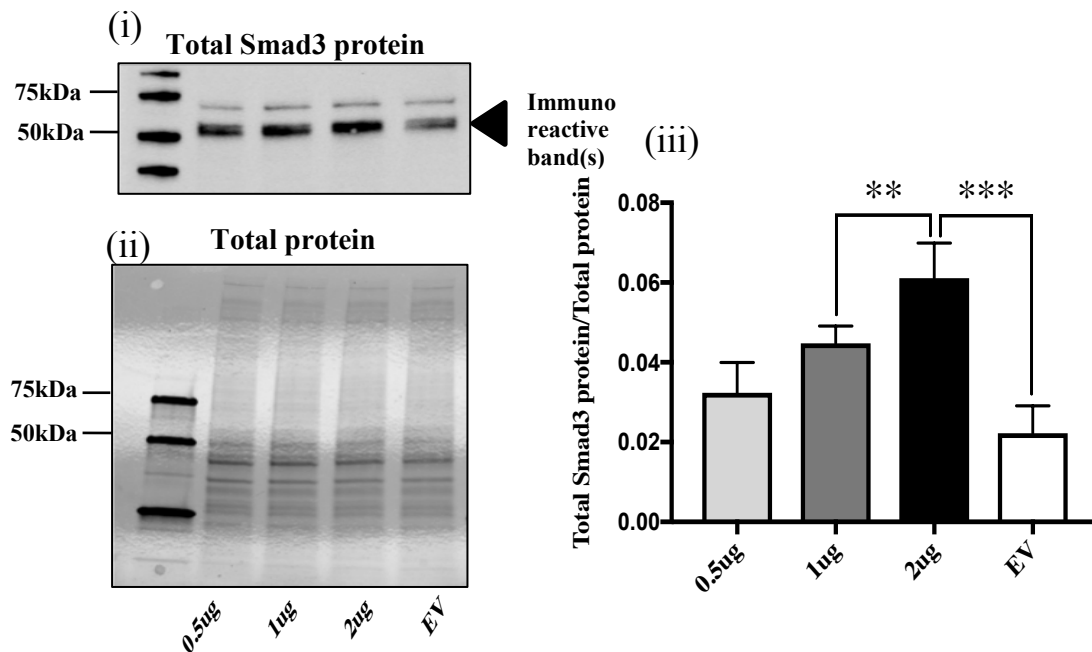


Figure 5.4B: Total Smad3 protein abundance in PTECs after mutant plasmid DNA transfection.

Key: **0.5µg**=7mM D-glucose+18mM D-glucose+0.75ng/ml TGFβ1+0.5µg mutant plasmid DNA, **1µg**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGFβ1+1µg mutant plasmid DNA, **2µg**=7mM D-glucose+18mM D-glucose+2µg mutant plasmid DNA, **EV**= 7mM D-glucose+18mM D-glucose+TGFβ1+2µg empty vector plasmid DNA. (i) Western blot showing immunostained total Smad3 protein (~53kDa) in PTECs under various interventions and 30 min treatment, (ii) corresponding total protein stain free gel which is used as a loading control, (iii) plotted data points after quantification of total Smad3 protein (total Smad3 protein abundance/total protein abundance) presented in graph format. There was significantly more total Smad3 protein in the 1 and 2µg mutant plasmid DNA transfected PTECs compared to the EV plasmid DNA transfected PTECs. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. **P<0.01, and P***<0.001, all n=3. Data are shown as mean +/- SD.

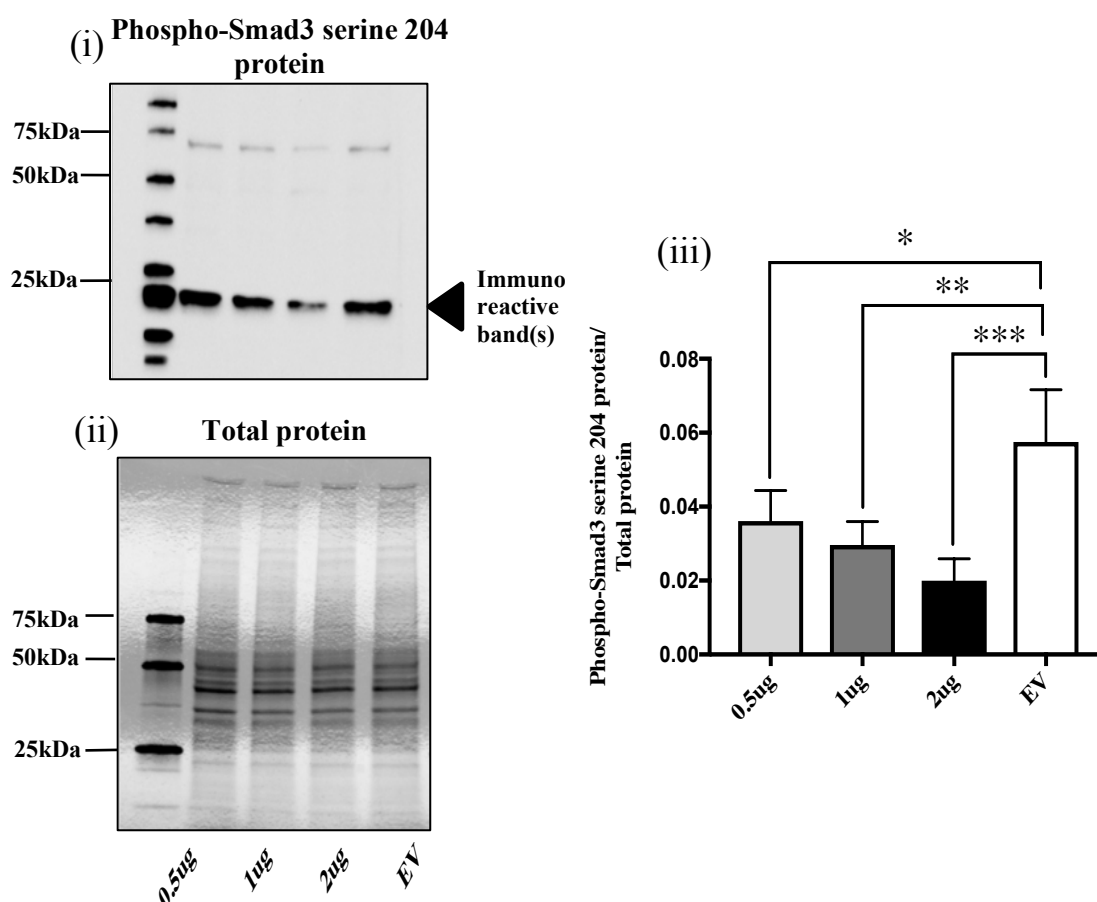


Figure 5.4C: Phospho-Smad3 serine 204 protein abundance in PTECs after mutant plasmid DNA transfection.

Key: **0.5 μ g**=7mM D-glucose+18mM D-glucose+0.75ng/ml TGF β 1+0.5 μ g mutant plasmid DNA, **1 μ g**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGF β 1+1 μ g mutant plasmid DNA, **2 μ g**=7mM D-glucose+18mM D-glucose+2 μ g mutant plasmid DNA, **EV**= 7mM D-glucose+18mM D-glucose+TGF β 1+2 μ g empty vector plasmid DNA. (i) Western blot showing immunostained phospho-Smad3 serine 204 protein (~25kDa) in PTECs under various interventions and 30 min treatment, (ii) corresponding total protein stain free gel which is used as a loading control, (iii) plotted data points after quantification of phospho-Smad3 serine 204 protein (phospho-Smad3 serine 204 protein abundance/total protein abundance) presented in graph format. There was a significant decrease in phospho-Smad3 serine 204 protein in the mutant plasmid DNA transfected PTECs compared to the empty vector plasmid DNA transfected PTECs. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. *P<0.05, **P<0.01, and P***<0.001, all n=4. Data are shown as mean +/- SD.

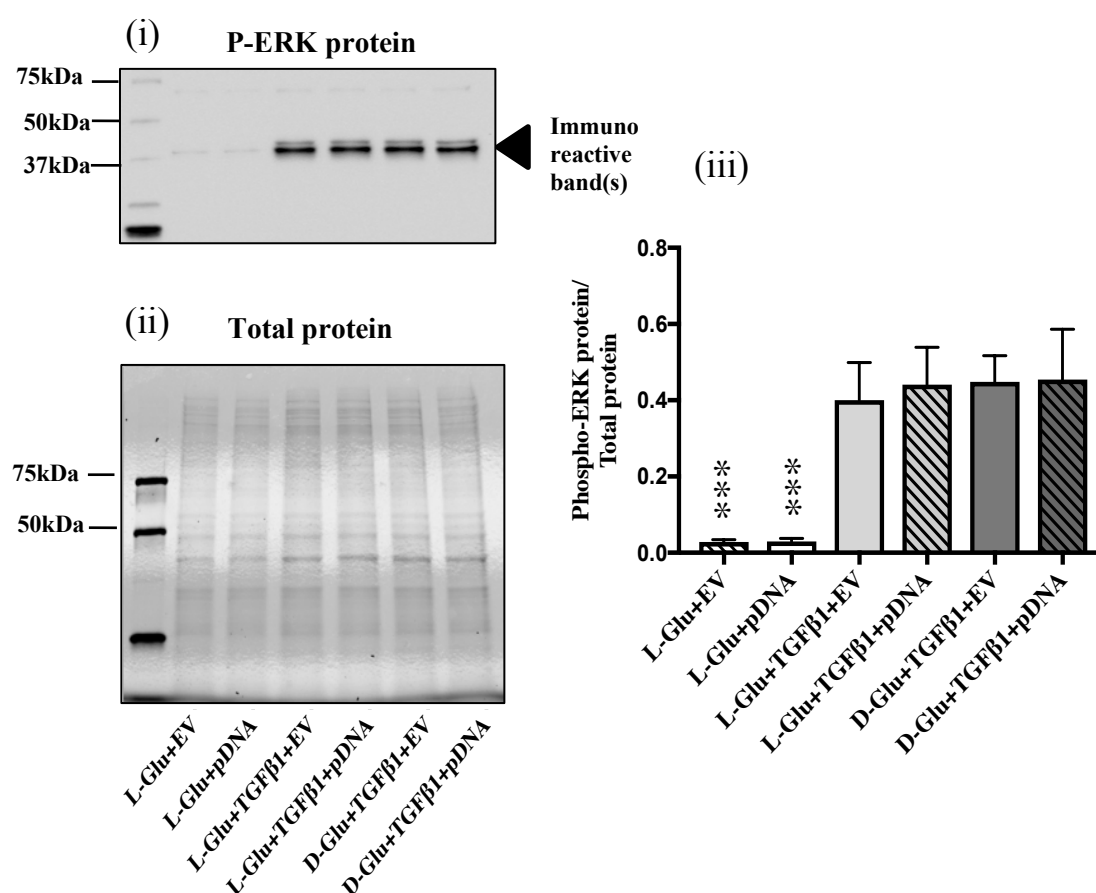


Figure 5.4D: Phospho-ERK protein abundance in PTECs treated with glucose and TGF-β1 after mutant plasmid DNA transfection.

Key: **L-Glu+EV**= 7mM D-glucose+18mM L-glucose+2μg empty vector plasmid DNA, **L-Glu+pDNA**= 7mM D-glucose+18mM L-glucose+2μg mutant plasmid DNA, **L-Glu+TGFβ1+EV**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGFβ1+2μg empty vector plasmid DNA, **L-Glu+TGFβ1+pDNA**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGFβ1+2μg mutant plasmid DNA, **D-Glu+TGFβ1+EV**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGFβ1+2μg empty vector plasmid DNA, **D-Glu+TGFβ1+pDNA**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGFβ1+2μg mutant plasmid DNA. (i) Western blot showing immunostained phospho-ERK protein (~40kDa) in PTECs under various interventions and 30 min treatment, (ii) corresponding total protein stain free gel which is used as a loading control, (iii) plotted data points after quantification of phospho-ERK protein (phospho-ERK protein abundance/total protein abundance) presented in graph format. There were no differences in phospho-ERK protein between mutant and empty vector plasmid DNA transfected PTECs. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. $P^{***}<0.001$ vs all other treatments, all $n=3$. Data are shown as mean +/- SD.

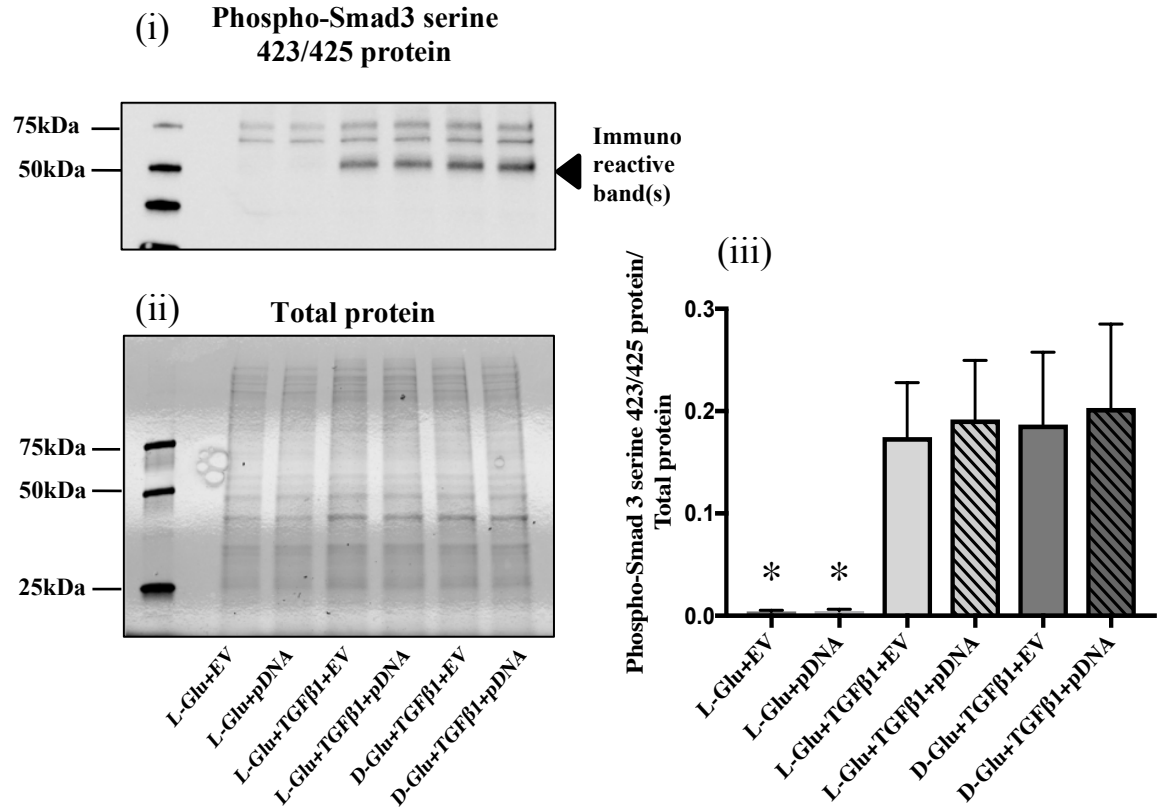


Figure 5.4E: Phospho-Smad3 serine 423/435 protein abundance in PTECs treated with glucose and TGF-β1 after mutant plasmid DNA transfection.

Key: **L-Glu+EV**= 7mM D-glucose+18mM L-glucose+2μg empty vector plasmid DNA, **L-Glu+pDNA**= 7mM D-glucose+18mM L-glucose+2μg mutant plasmid DNA, **L-Glu+TGFβ1+EV**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGFβ1+2μg empty vector plasmid DNA, **L-Glu+TGFβ1+pDNA**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGFβ1+2μg mutant plasmid DNA, **D-Glu+TGFβ1+EV**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGFβ1+2μg empty vector plasmid DNA, **D-Glu+TGFβ1+pDNA**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGFβ1+2μg mutant plasmid DNA. (i) Western blot showing immunostained phospho-Smad3 serine 423/425 protein (~53kDa) in PTECs under various interventions and 30 min treatment, (ii) corresponding total protein stain free gel which is used as a loading control, (iii) plotted data points after quantification of phospho-Smad3 serine 423/425 protein (phospho-Smad3 serine 423/425 protein abundance/total protein abundance) presented in graph format. There were no differences in phospho-Smad3 serine 423/425 protein between mutant and empty vector plasmid DNA transfected PTECs. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. $P^* < 0.05$ vs all other treatments, all $n=3$. Data are shown as mean $\pm$ SD.

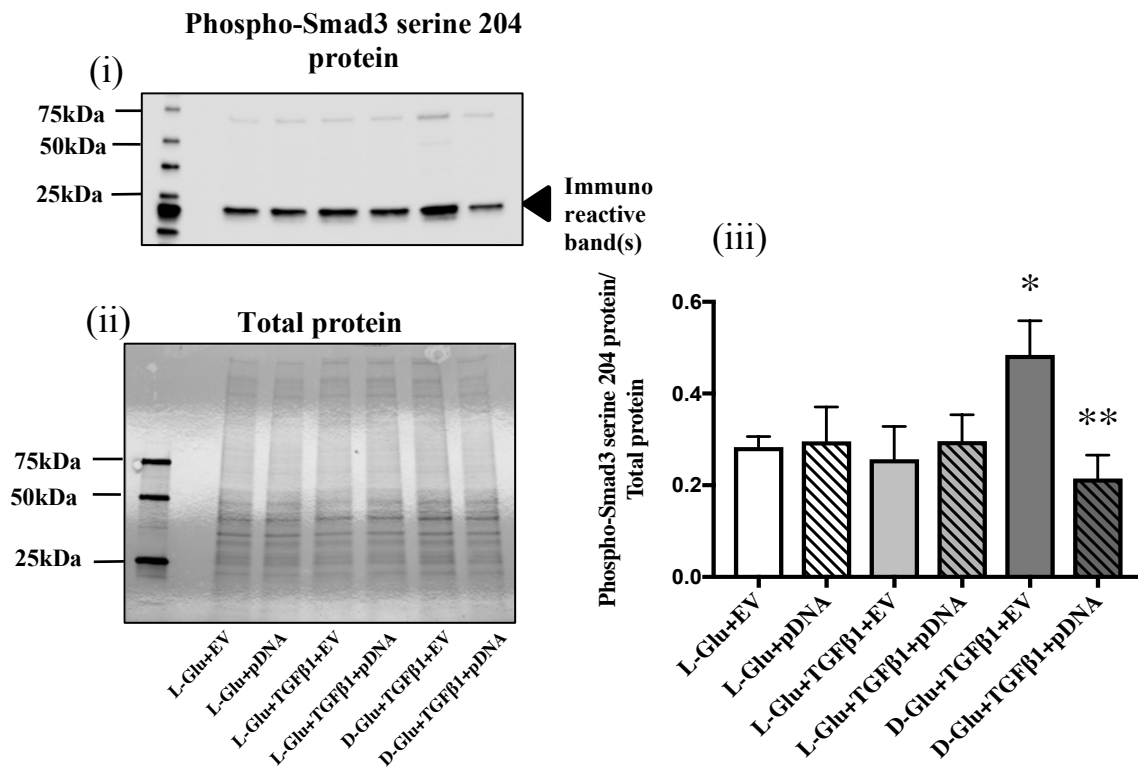


Figure 5.4F: Phospho-Smad3 serine 204 protein abundance in PTECs treated with glucose and TGF-β1 after mutant plasmid DNA transfection.

Key: **L-Glu+EV**= 7mM D-glucose+18mM L-glucose+2μg empty vector plasmid DNA, **L-Glu+pDNA**= 7mM D-glucose+18mM L-glucose+2μg mutant plasmid DNA, **L-Glu+TGFβ1+EV**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGFβ1+2μg empty vector plasmid DNA, **L-Glu+TGFβ1+pDNA**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGFβ1+2μg mutant plasmid DNA, **D-Glu+TGFβ1+EV**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGFβ1+2μg empty vector plasmid DNA, **D-Glu+TGFβ1+pDNA**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGFβ1+2μg mutant plasmid DNA. (i) Western blot showing immunostained for phospho-Smad3 serine 204 protein (~25kDa) in PTECs under various interventions and 30 min treatment, (ii) corresponding total protein stain free gel which is used as a loading control, (iii) plotted data points after quantification of phospho-Smad3 serine 423/425 protein (phospho-Smad3 serine 423/425 protein abundance/total protein abundance) presented in graph format. D-Glu+TGF-β1 significantly increased phospho-Smad3 serine 204 protein abundance; this was reversed after mutant plasmid DNA transfection. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. $P^* < 0.05$ vs L-Glu+EV, L-Glu+pDNA, L-Glu+TGFβ1+EV, L-Glu+TGFβ1+pDNA, $P^{**} < 0.01$ vs D-Glu+TGFβ1+EV, all $n=3$. Data are shown as mean $\pm$ SD.

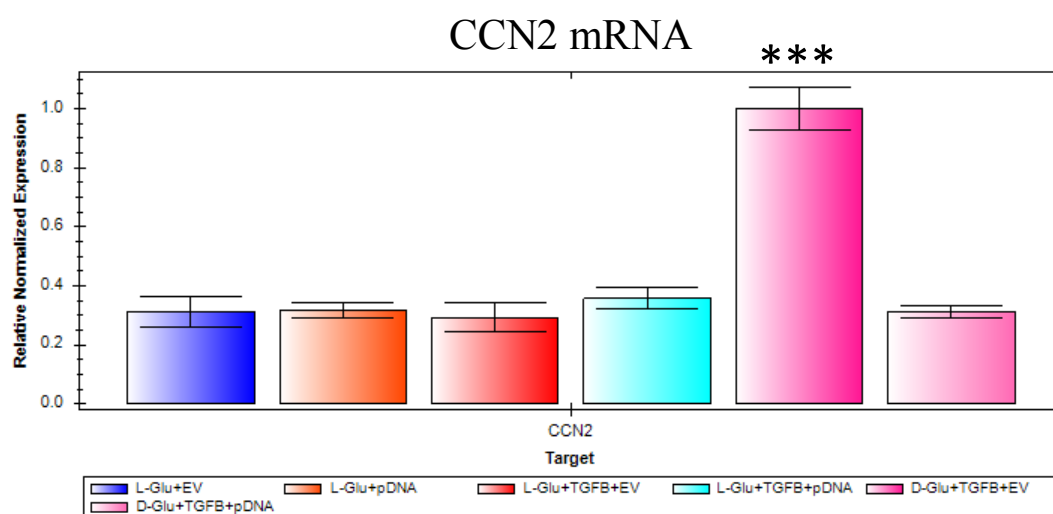


Figure 5.4G: CCN2 mRNA abundance in PTECs treated with glucose and TGF- β 1 after mutant plasmid DNA transfection.

Key: **L-Glu+EV**= 7mM D-glucose+18mM L-glucose+2 μ g empty vector plasmid DNA, **L-Glu+pDNA**= 7mM D-glucose+18mM L-glucose+2 μ g mutant plasmid DNA, **L-Glu+TGFB β 1+EV**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGF β 1+2 μ g empty vector plasmid DNA, **L-Glu+TGFB β 1+pDNA**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGF β 1+2 μ g mutant plasmid DNA, **D-Glu+TGFB β 1+EV**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGF β 1+2 μ g empty vector DNA, **D-Glu+TGFB β 1+pDNA**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGF β 1+2 μ g mutant plasmid DNA. The figure shows plotted data points after quantification of CCN2 mRNA (CCN2 mRNA abundance/GAPDH mRNA abundance) presented in graph form. Mutant plasmid DNA transfected PTECs significantly reduced serine 204 phosphorylation after 24h D-Glu+TGF- β 1 treatment. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. ***P<0.001 compared to all other treatments, all n=3. Data are shown as mean $\pm$ SD.

5.4. Discussion

This chapter was focused on better understanding the high D-glucose mediated induction of pro-fibrotic marker CCN2. I examined signalling pathways potentially involved in the induction of CCN2 protein and mRNA.

The first result demonstrated that exposure to high D-glucose alone increased phospho-ERK protein abundance, indicating a specific role for ERK in my DKD model. This data is consistent with findings in literature over the years that have also explored markers involved in hyperglycaemia-mediated complications. Fujita et al discovered high glucose induced cell hypertrophy observed in proximal tubule cells could be attenuated by administration of MAPK/ERK inhibitor PD98059 ((Fujita *et al.*, 2004). Ha et al found that MAPK/ERK inhibitors PD98059 and U0126 significantly decreased CCN2 abundance in vascular smooth muscle cells (VSMCs) exposed to elevated glucose (Ha *et al.*, 2013). Hayashida et al found that high glucose and TGF- β 1 co-incubation induced fibrotic activity was promptly subdued by inhibition of ERK (Hayashida, Decaestecker and Schnaper, 2003).

The upregulation of phospho-ERK was ameliorated by dapagliflozin, indicating SGLT2 dependent phosphorylation or inhibition of dephosphorylation by SGLT2. Similarly, another group found that SGLT inhibitor phlorizin treatment significantly reduced ERK phosphorylation in rat kidneys (Wang *et al.*, 2013). Conversely, two other groups found that dapagliflozin treatment significantly propagated phospho-ERK protein abundance in their colon cancer and HK-2 cells. However, it should be noted that these studies utilised a 0.25 μ M and 1 μ M concentration of dapagliflozin, with the latter experiment having exposed the cells to hypoxic medium (Saito *et al.*, 2015; Chang *et al.*, 2016). The IC₅₀ of SGLT2 inhibition by dapagliflozin is stated to be an estimated 1nM (Kanwal *et al.*, 2012), which I have used in my experiments.

My results have detailed a series of cellular signalling cascades activated by the combined stimulation of high D-glucose and 0.75ng/ml TGF- β 1 that is SGLT2 dependent. As SGLT2 facilitated glucose uptake across proximal tubule cells is complemented with sodium uptake at an equimolar rate, it should be noted that an equal influx of sodium will

parallel the glucose influx in PTECs. However, it is doubtful that alterations in intracellular sodium levels contributed towards any of the changes observed in my results. There are a variety of sodium channels found in the apical and basolateral membranes of PTECs including the sodium amino acid transporter, sodium hydrogen exchanger isoform 3, and sodium/potassium adenosine triphosphate pump (Wang *et al.*, 2009). Contrarily, glucose uptake is monopolised by SGLT in PTECs.

Threshold TGF- β 1 stimulation significantly induced the phosphorylation of serines 423 and 425 (SSXS) on Smad3 in PTECs. TGF- β 1 modulated activation of Smad3 has been long established both by ourselves and others (Hu, Wu and Phan, 2003; Phanish *et al.*, 2005), although these studies use a TGF- β 1 concentration of higher than 2ng/ml. The significance of the threshold 0.75ng/ml concentration that I have used in my experiments was discussed in the previous results chapter. Phospho-ERK protein was induced by high D-glucose alone at 30 min, whilst phospho-Smad3 serine 204 protein was significantly induced at 30 min after combined high D-glucose and 0.75ng/ml TGF- β 1 treatment. There are studies in literature that have consistently demonstrated ERK phosphorylation of the LR on R-Smads (Kretzschmar, Doody and Massagué, 1997; Matsuura *et al.*, 2005; Hough, Radu and Jules J.E. Doré, 2012). Interestingly, ERK mediated R-Smad LR phosphorylation also potentiates TGF- β 1 and Smad target gene transcription ((Hough, Radu and Jules J E Doré, 2012; Browne *et al.*, 2013).

My results show that phospho-Smad3 serine 204 protein abundance was significantly reduced upon dapagliflozin and U0126 administration. Data previously published by our group show that CCN2 protein abundance induced by TGF- β 1 is Smad3 dependent after transfection with Smad3 siRNA (Phanish *et al.*, 2006). This indicates that the SGLT2 dependent ERK-mediated phosphorylation of Smad3 serine 204 is potentially where high D-glucose and TGF- β 1 converge to induce CCN2 in my DKD model. There are several sites on the LR of Smad3 available for phosphorylation including serine 204, serine 208, serine 213 and threonine 179 (Ooshima, Park and Kim, 2019). I have focused my experiments on phospho-Smad3 serine 204 as the Hayashida/Schnader group discovered that ERK blockade in their renal mesangial cells caused both collagen I promoter activation and a significant increase in phospho-Smad3 serine 204 protein. They then transfected the mesangial cells with the same mutant plasmid DNA that I have used in my

experiments and found that collagen I transcriptional activity (Browne *et al.*, 2013) was significantly reduced. This supports the notion that Smad3 serine 204 may be an important mediator of renal fibrosis. The same group also found that high ambient glucose exposure increases ERK activity and potentiates TGF- β 1 response (Hayashida and H. William Schnaper, 2004). Although their experiments were conducted on different cells, I propose that phospho-Smad3 serine 204 is of similar importance in my DKD PTECs.

Mutant plasmid DNA transfection confirmed that there were no changes in phospho-Smad3 serine 423/425 and P-ERK protein abundance after the inhibition of Smad3 serine 204 phosphorylation. This indicates that the transfection of the mutant plasmid did not alter the signalling cascades observed in my DKD model. Inhibition of phospho-Smad3 serine 204 phosphorylation in high D-glucose+0.75ng/ml TGF- β 1 treated PTECs attenuates CCN2 mRNA abundance back down to levels comparable to the osmotic control. This demonstrates the direct involvement of serine 204 in the activation of CCN2.

Unexpectedly, my data shows that combined high D-glucose and TGF- β 1 treatment phosphorylates the truncated Smad3 isoform (~25kDa) instead of the full length Smad3 (~53kDa). This truncated isoform is likely dimerised to the full length Smad3, producing a protein product of approximately 75kDa as seen on my phospho-Smad3 serine 423/425 and serine 204 western blots. There is limited literature regarding Smad3 truncated isoforms as the majority of studies have reported the common full length Smad3 protein. Further research would be required to characterise the properties of the former. The truncated isoform is only present on the immunoblots when probed with antibodies whose immunogen sequences are between amino acids 200 to 432, implying the absence of the Smad3 MH1 N-terminal domain.

Archetypally, it is the Smad3 MH1 domain that controls DNA binding through the CAGAC Smad binding element (SBE) (Kusanagi *et al.*, 2000). Nevertheless, there have also been reports more recently substantiating that DNA sequences in G/C rich areas can also act as a Smad binding motif and that Smad3 can bind to these G/C rich motifs which do not have the traditional CAGAC element (Martin-Malpartida *et al.*, 2017). Smad3 also has the ability to bind with Smad4 and form either homo or heterotrimers (Chacko *et al.*, 2004; Macias, Martin-Malpartida and Massagué, 2015). I hypothesise that my DKD model

first binds together the full length phospho-Smad3 and truncated MH1 domain deficient Smad3 to form a heterotrimer with Smad4 before being translocated to the nucleus.

From my data, I propose that this novel TGF- β 1/Smad3 mediated signalling cascade comprises of the phosphorylation of the Smad3 full length/truncated heterotrimer initially at serines 423/425 and later at serine 204 by high D-glucose induced ERK (Figure 5.5).

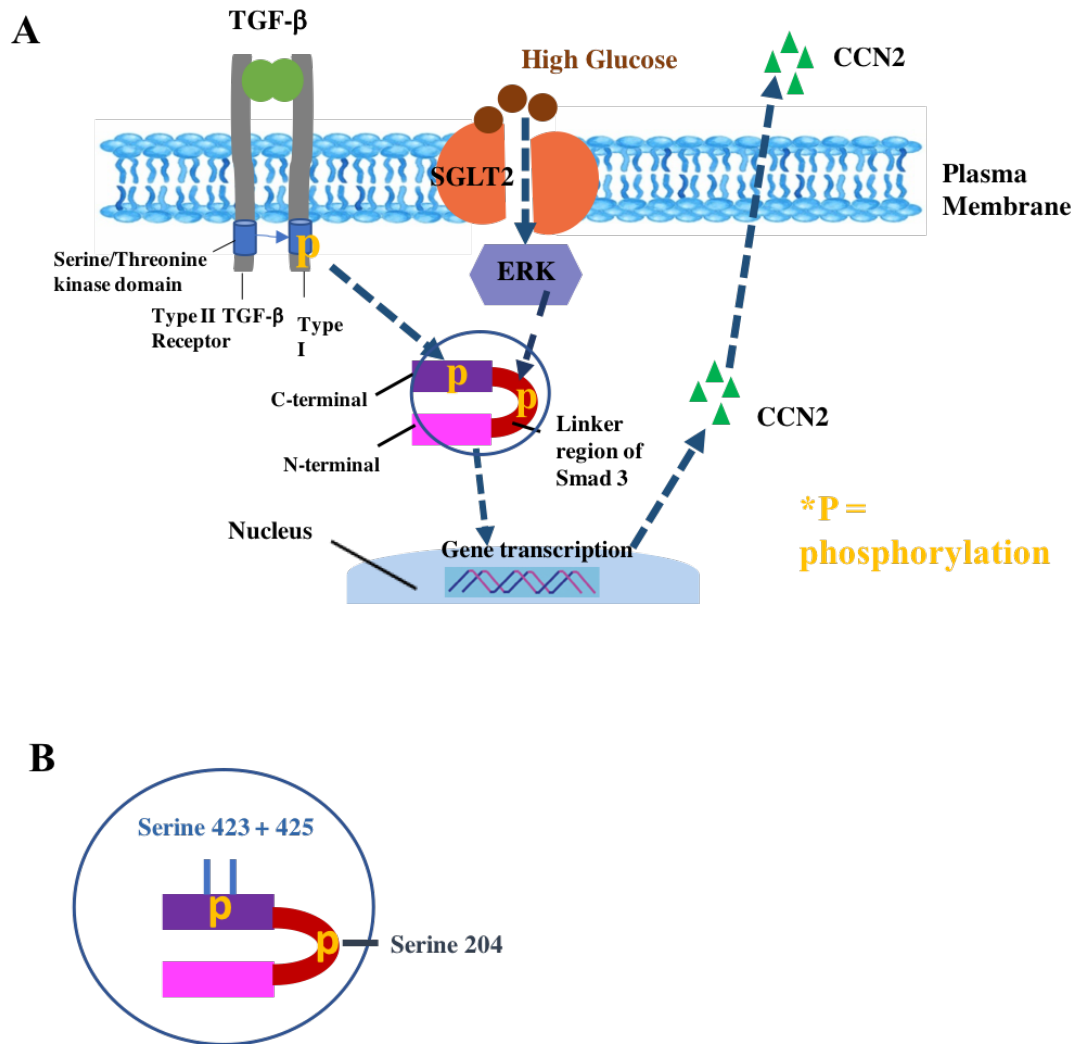


Figure 5.5: Proposed mechanism for high glucose and TGF- β 1 mediated induction of CCN2.

(A) Under normoglycaemic conditions the type 2 TGF- β 1 receptor typically phosphorylates the cytoplasmic domain of the type 1 receptor. This in turn phosphorylates R-Smads (as circled on the figure) on the C terminal/MH2 domain. This Smad homo/heterotrimer complex is activated and then translocated to the nucleus where it can control gene transcription. Under high D-glucose exposure in my DKD model, ERK is activated and induces LR phosphorylation at serine 204. This then potentiates TGF- β specific signalling of the Smad complex, thus increasing CCN2 transcriptional activity. (B) Serines 423/425 are phosphorylated first and then serine 204 is phosphorylated later on the LR that links the C and N terminals.

Chapter 6:

The Effects of Glucose, TGF- β 1 and Dapagliflozin on Fibronectin

6.1. Introduction

This thesis chapter will focus on fibronectin, an important glycoprotein ECM component that acts as a communicative link between the intracellular and extracellular environment through the binding of cell surface receptors. The ECM is a three-dimensional network made up of extracellular macromolecules like collagen, laminin, proteoglycans and various other fibrous proteins and serves as a scaffold for cellular structures (Yue, 2014). It is a vital and complex structural component necessary in all living organisms to carry out fundamental cellular process like differentiation, adhesion and proliferation (Parisi *et al.*, 2020). The importance of the network is demonstrated through the occurrence of various diseases that develop upon ECM dysfunction (Järveläinen *et al.*, 2009). Collagen fibrils are also a key part of the early ECM and their deposition and retention requires fibronectin. During renal fibrosis, the ECM is rich in fibrillar collagen, demonstrating the importance of fibronectin in the development of the DKD (Duffield, 2014; Holmes et al., 2018; Musiime et al., 2021).

Structurally, it is comprised of a multifunctional glycoprotein dimer made up of two ~220 to 250kDa subunits joined at the carboxyl terminus via anti-parallel disulphide bonds. Approximately 90% of all fibronectin molecules are divided into three homologous domains: type I, type II and type III repeats. Generally, there are ~twelve type I, two type II and ~15 to 17 type III repeats connected together in the form of β -strand sheets (To and Midwood, 2011) (Figure 6.1). Similar to CCN2, these domains are also capable of interacting/binding with various cell receptors and other matrix proteins (Aziz-Seible and Casey, 2011). Fibronectin also has alternatively spliced repeats extra domain A (EDA) and extra domain B (EDB) encoded by single exons. The ECM protein is split into cellular and plasma fibronectin, where the former is produced by fibroblasts and endothelial cells

whilst the latter by hepatocytes. Alternative splicing is common in cellular fibronectin, resulting in approximately 20 different fibronectin isoforms, including EDA and EDB. Plasma fibronectin, which does not contain EDA or EDB isoforms, is usually found circulating in the blood in an inactive form (Patten and Wang, 2021). There are very low levels of the EDA fibronectin isoform in healthy tissue but elevated levels in fibrotic organs (Van Der Straaten *et al.*, 2004; Muro *et al.*, 2008). Consequently, the experiments described in this final results chapter will demonstrate the effects of glucose and TGF- β 1 on fibronectin and investigate whether SGLT2 regulates this pro-fibrotic signalling pathway in a similar way to CCN2.

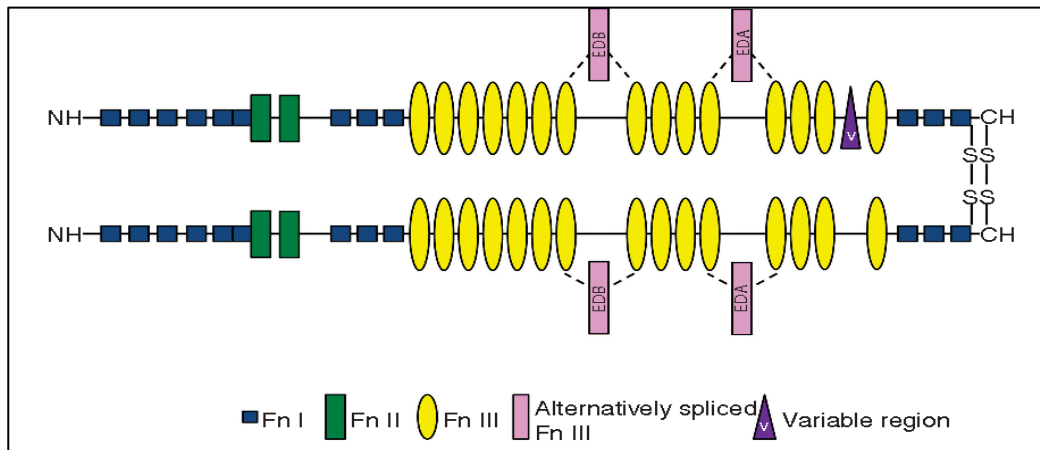


Figure 6.1. Structure of Cellular Fibronectin.

Fibronectin consists of two ~250kDa subunits linked by strong disulphide bonds at the CH terminus. It is made up of three repeating homologous domain structures: type I, type II and type III. Fibronectin can undergo gene splicing, which results in extracellular domain A&B fibronectin domains. Figure taken from (Kelsh, McKeown-Longo and Clark, 2015).

6.2. Aims and Objectives

1. To investigate the changes in fibronectin protein and mRNA abundance in PTECs following glucose and TGF- β 1 treatment.
2. To investigate the effect of SGLT2 inhibition on fibronectin protein and mRNA abundance in PTECs using dapagliflozin, a drug that selectively inhibits the transporter.
3. To determine the breakdown of dapagliflozin over time using high performance liquid chromatography (HPLC).
4. To investigate the changes in fibronectin mRNA abundance following exclusive TGF- β 1 treatment of PTEC.
5. To investigate the presence of urinary fibronectin protein in diabetic kidney disease patient samples.
6. To investigate whether inhibition of Smad3 serine 204 phosphorylation prevents fibronectin transcription in PTECs following combined high D-glucose and TGF- β 1 treatment.

6.3. Results

6.3.1. EDA fibronectin protein abundance in PTECs treated with glucose, TGF- β 1 and dapagliflozin.

For my western blot analysis, I probed for EDA fibronectin protein in my PTECs as it is the main splice variant that is upregulated in renal fibrosis (Phanish *et al.*, 2015). There is no commercially available EDA fibronectin gene expression assay for PCR analysis, so the fibronectin mRNA examined was unaltered by splicing.

There was a significant increase in fibronectin mRNA abundance after 24h in the high D-glucose (7mM D-glucose+18mM D-glucose)+ 0.75ng/ml TGF- β 1 treated PTECs (Figure 6.2A). Contrarily, there was no change in EDA-fibronectin protein abundance after 24h under any of the treatments (Figure 6.2B). There was a significant increase in EDA fibronectin protein after 72h in the high D-glucose+ 0.75ng/ml TGF- β 1 treated PTECs (Figure 6.2C).

In order to determine whether this significant increase in fibronectin mRNA and protein abundance after combined high D-glucose and 0.75ng/ml TGF- β 1 treatment was SGLT2 dependent, these PTECs were also administered with dapagliflozin (0.1,1,10nM). The two higher concentrations of dapagliflozin significantly decreased fibronectin mRNA abundance to osmotic control levels after 24h treatment (Figure 6.2D). Dapagliflozin did not change EDA fibronectin protein abundance after 72h treatment (Figure 6.2E).

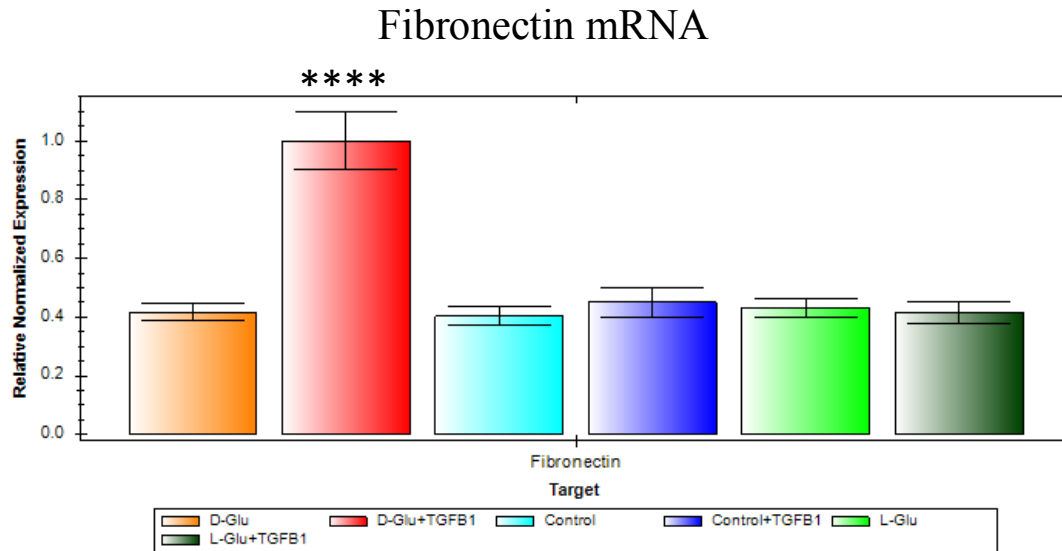


Figure 6.2A: Fibronectin mRNA abundance in PTECS treated with glucose and TGF- β 1 after 24h.

Key: **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGFB1**=7mM D-glucose+18mM D-glucose+0.75ng/ml TGF- β 1, **Control**= 7mM D-glucose, **Control+TGFB1**= 7mM D-glucose+0.75ng/ml TGF- β 1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGFB1**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGF- β 1. The figure shows plotted data points after quantification of fibronectin mRNA (fibronectin mRNA abundance/GAPDH mRNA abundance) presented in graph form. Fibronectin mRNA was significantly increased in D-Glu+TGF- β 1 treated PTECs. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. ****P<0.001 compared to all other treatments, all n=4. Data are shown as mean $\pm$ SD.

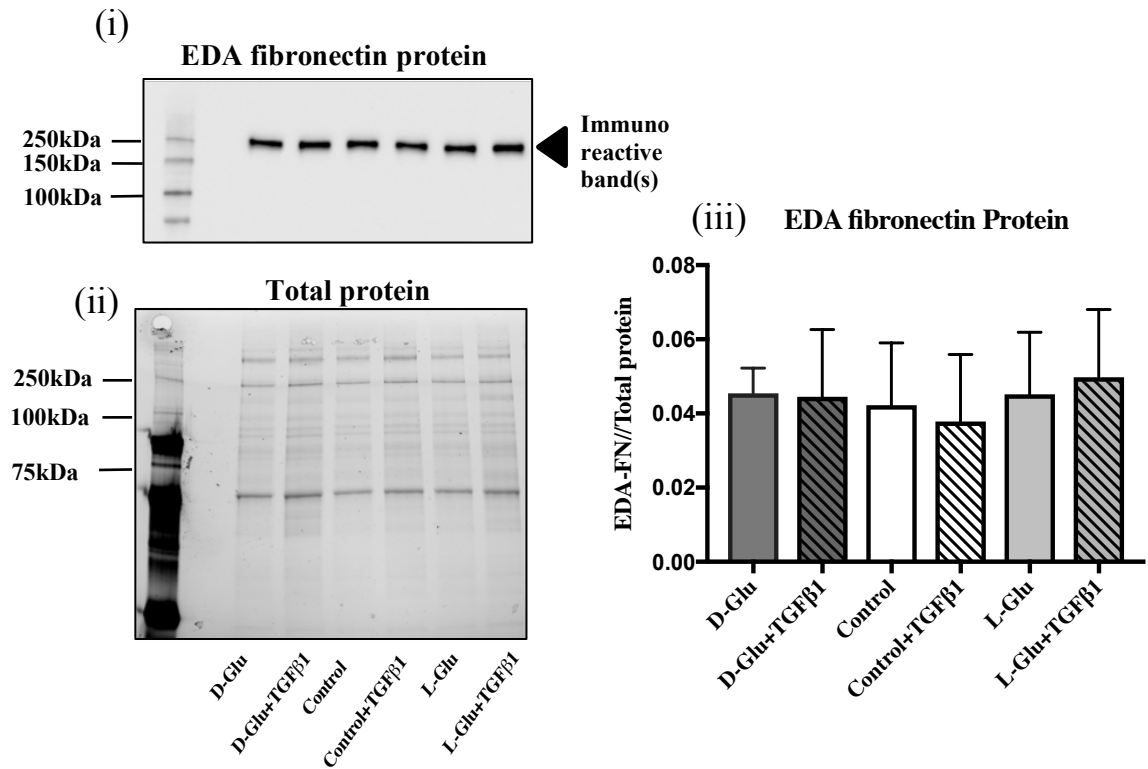


Figure 6.2B: EDA-fibronectin protein abundance in PTECs treated with glucose and TGF-β1 after 24h.

Key: **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGFβ1**=7mM D-glucose+18mM D-glucose+0.75ng/ml TGF-β1, **Control**= 7mM D-glucose, **Control+TGFβ1**= 7mM D-glucose+0.75ng/ml TGF-β1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGFβ1**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGF-β1. (i) Western blot showing immunostained EDA-fibronectin protein (~250kDa) in PTECs under various treatments, (ii) corresponding total protein stain free gel which is used as a loading control, (iii) plotted data points after quantification of EDA-fibronectin protein (EDA-fibronectin protein abundance/total protein abundance) presented in graph format. There were no detectable changes in EDA-fibronectin protein in any of the treatments. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. Data are shown as mean +/- SD.

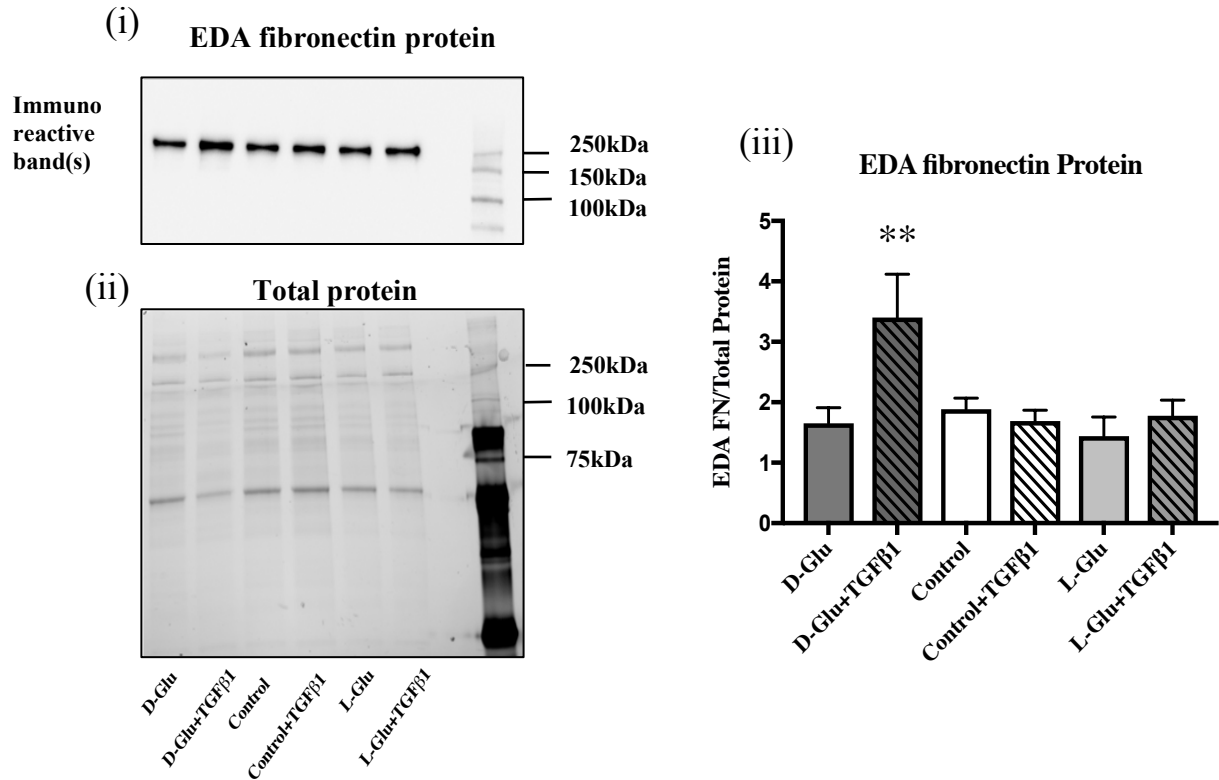


Figure 6.2C: EDA-fibronectin protein abundance in PTECS treated with glucose and TGF-β1 after 72h.

Key: **D-Glu**= 7mM D-glucose+18mM D-glucose (high D-glucose), **D-Glu+TGFβ1**=7mM D-glucose+18mM D-glucose+0.75ng/ml TGF-β1, **Control**= 7mM D-glucose, **Control+TGFβ1**= 7mM D-glucose+0.75ng/ml TGF-β1, **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **L-Glu+TGFβ1**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGF-β1. (i) Western blot showing immunostained EDA-fibronectin protein (~250kDa) in PTECs under various treatments, (ii) corresponding total protein stain free gel which is used as a loading control, (iii) plotted data points after quantification of EDA-fibronectin protein (EDA-fibronectin protein abundance/total protein abundance) presented in graph format. EDA-fibronectin protein was significantly increased in the D-Glu+TGF-β1 treated PTECs. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. **P<0.01 compared to all other treatments, all n=3. Data are shown as mean +/- SD.

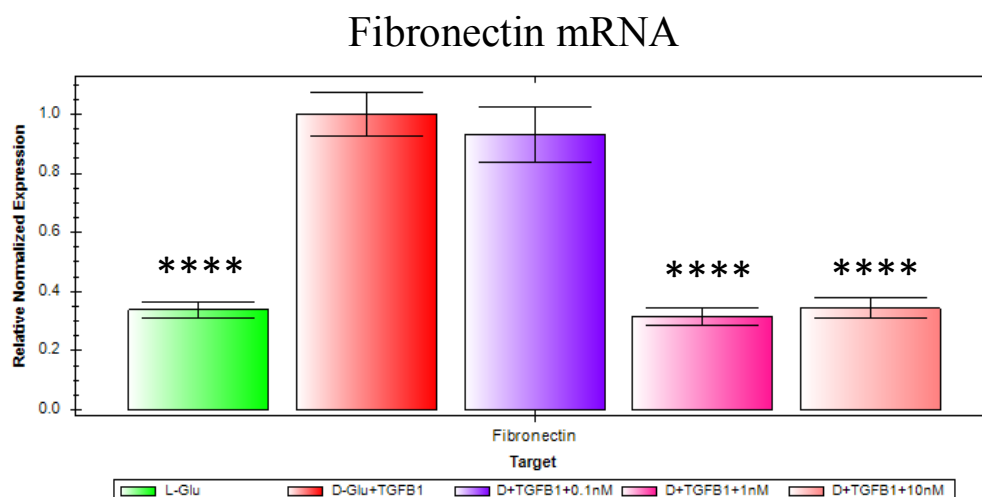


Figure 6.2D: Fibronectin mRNA abundance in PTECS treated with glucose, TGF- β 1 and dapagliflozin after 24h:

Key: L-Glu= 7mM D-glucose+18mM L-glucose (osmotic control), **D-Glu+TGF β 1**=7mM D-glucose+18mM D-glucose+0.75ng/ml TGF- β 1 (high D-glucose), **D+TGF β 1+0.1nM**=7mM D-glucose+18mM D-glucose+0.75ng/ml TGF- β 1+0.1nM dapagliflozin, **D+TGF β 1+1nM**=7mM D-glucose+18mM D-glucose+0.75ng/ml TGF- β 1+1nM dapagliflozin, **D+TGF β 1+10nM**=7mM D-glucose+18mM D-glucose+0.75ng/ml TGF- β 1+10nM dapagliflozin. The figure shows plotted data points after quantification of fibronectin mRNA (fibronectin mRNA abundance/GAPDH mRNA abundance) presented in graph form. Fibronectin mRNA was significantly increased in D-Glu+TGF- β 1 treated PTECs. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. ****P<0.001 compared to D-Glu+TGF- β 1, all n=4. Data are shown as mean +/- SD.

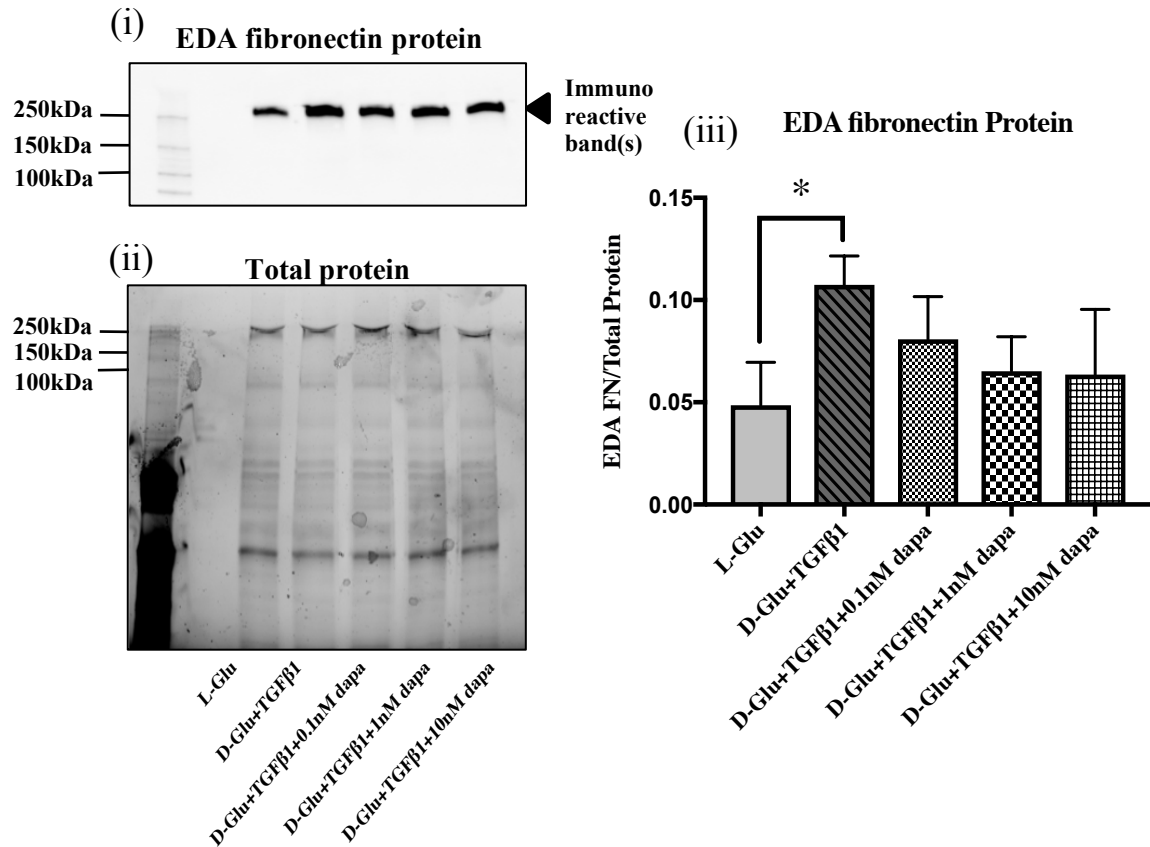


Figure 6.2E: EDA-fibronectin protein abundance in PTECs treated with glucose and TGF-β1 after 72h.

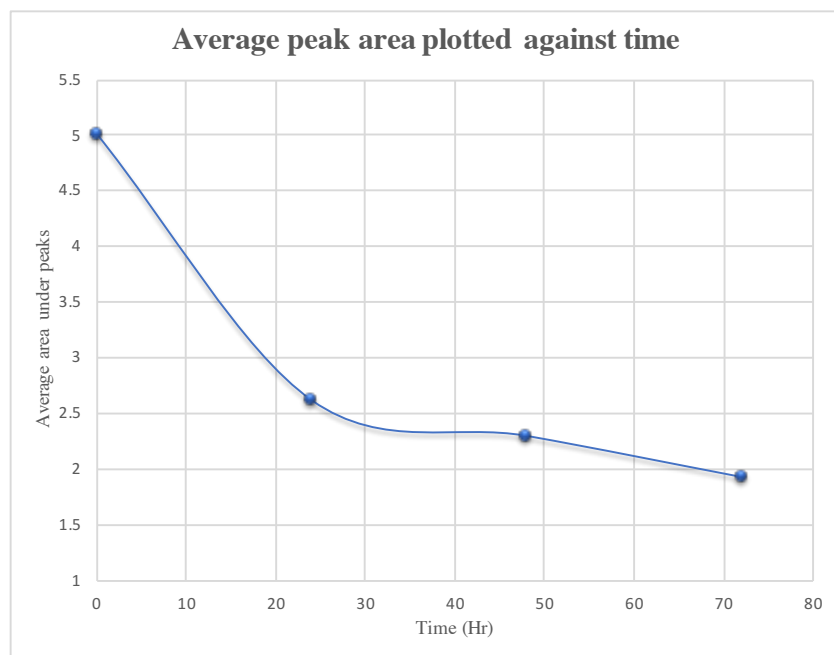
Key: **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **D-Glu+TGFβ1**=7mM D-glucose+18mM D-glucose+0.75ng/ml TGF-β1 (high D-glucose), **D-Glu+TGFβ1+0.1nM dapa**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGF-β1+0.1nM dapagliflozin, **D-Glu+TGFβ1+1nM dapa**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGF-β1+1nM dapagliflozin, **D-Glu+TGFβ1+10nM dapa**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGF-β1+10nM dapagliflozin. (i) Western blot showing immunostained EDA-fibronectin protein (~250kDa) in PTECs under various treatments, (ii) corresponding total protein stain free gel which is used as a loading control, (iii) plotted data points after quantification of EDA-fibronectin protein (EDA-fibronectin protein abundance/total protein abundance) presented in graph format. EDA-fibronectin protein was significantly increased in the D-Glu+TGF-β1 treated PTECs compared to the L-Glu treated PTECs. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. *P<0.05, all n=4. Data are shown as mean +/- SD.

6.3.2. The breakdown of dapagliflozin over 72h using high performance liquid chromatography

My results have demonstrated that fibronectin mRNA at 24h is significantly reduced back down to osmotic control levels upon 1nM dapagliflozin administration. However, this concentration of dapagliflozin did not reduce EDA-fibronectin protein abundance at 72h. I propose that this is because the dapagliflozin has broken down over time and is no longer effective at inhibiting SGLT2 after 24h. This theory was tested using HPLC, a technique commonly utilised in analytical chemistry to identify, separate and quantify components in a liquid mixture.

High D-glucose, 0.75ng/ml TGF- β 1 and 10nM dapagliflozin treated medium was collected at 0, 24, 48, and 72h and concentrated in preparation for HPLC. The average peak area and peak height of dapagliflozin was plotted against the 4 timepoints. There was a sharp decline in peak dapagliflozin area and height from 0 to 24h, which then levelled off from 24 to 72h (Figure 6.3). It is important to note that the two graphs are for illustration purposes only and no statistical analysis was performed.

(i)



(ii)

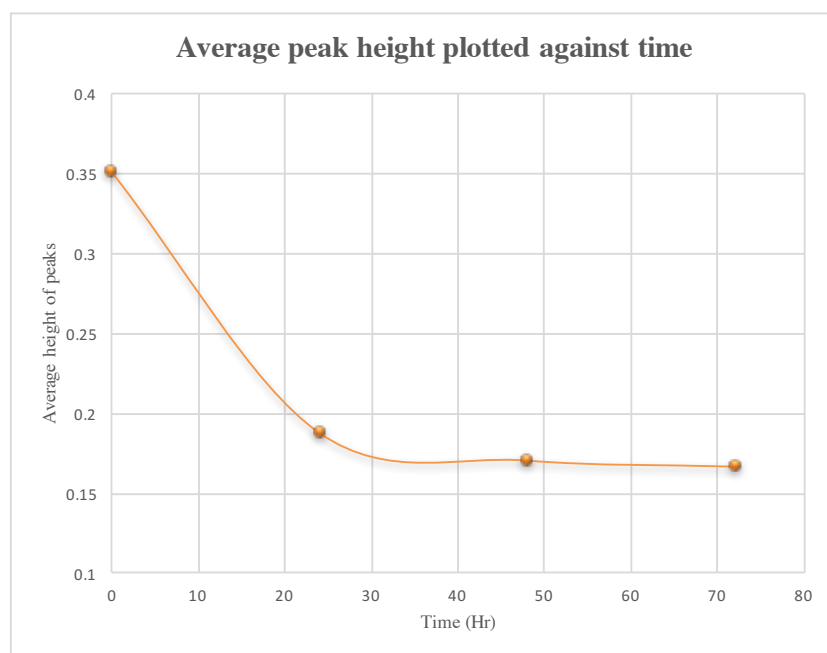


Figure 6.3: Plots of average dapagliflozin peak area and height versus time.

(i) Plotted graph of dapagliflozin peak area over 0, 24, 48, and 72h treatment, (ii) plotted graph of dapagliflozin peak height over 0, 24, 48, and 72h treatment. There is a steep decline in dapagliflozin concentration from 0h to 24h for both peak area and height.

6.3.3. Fibronectin mRNA abundance in PTECs treated with TGF- β 1 and dapagliflozin

To investigate whether fibronectin mRNA was upregulated with TGF- β 1 stimulation alone under osmotic control conditions, PTECs were treated with additional higher concentrations of TGF- β 1 (0.75, 2.5, and 5ng/ml) and cultured in osmotic control media. Fibronectin mRNA abundance was significantly increased with 2.5ng/ml and 5ng/ml TGF- β 1 (Figure 6.4A).

Dapagliflozin (1nM) was administered to PTECs treated with 2.5ng/ml and 5ng/ml TGF- β 1 under osmotic control conditions in order to investigate whether SGLT2 inhibition could affect TGF- β 1 signalling, independent of elevated D-glucose treatment. Dapagliflozin did not change the TGF- β 1-induced fibronectin mRNA abundance (Figure 6.4B).

Fibronectin mRNA

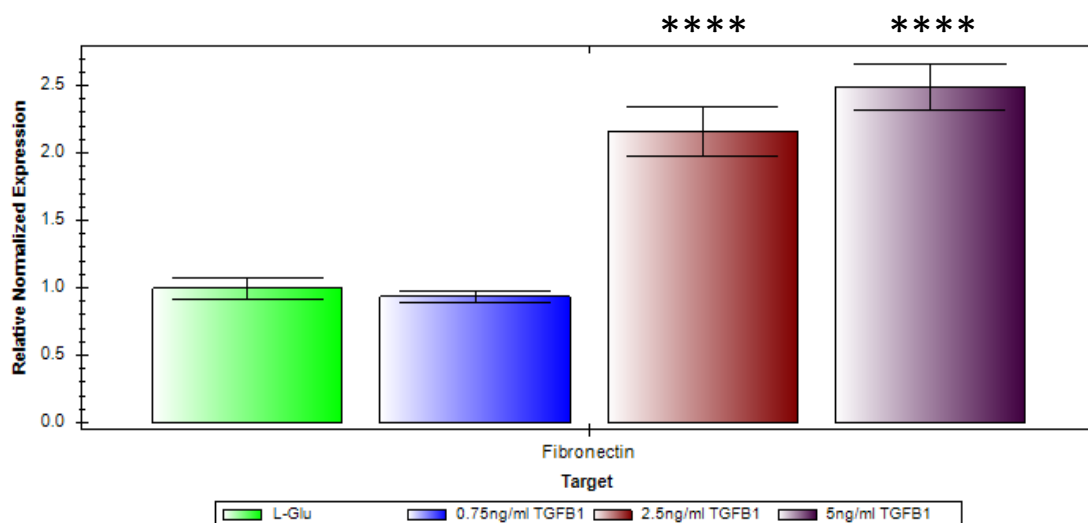


Figure 6.4A: Fibronectin mRNA abundance in PTECs treated with TGF-β1 after 24h.

Key: **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **0.75 ng/ml TGFβ1**=7mM D-glucose+18mM L-glucose+0.75ng/ml TGF-β1, **2.5ng/ml TGFβ1**=7mM D-glucose+18mM L-glucose+2.5ng/ml TGF-β1, **5ng/ml TGFβ1**=7mM D-glucose+18mM L-glucose+5ng/ml TGF-β1. The figure shows plotted data points after quantification of fibronectin mRNA (fibronectin mRNA abundance/GAPDH mRNA abundance) presented in graph form. Fibronectin mRNA was significantly increased in 2.5 and 5ng/ml TGF-β1 treated PTECs. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. ****P<0.001 compared to L-Glu and 0.75ng/ml TGF-β1, all n=4. Data are shown as mean +/- SD.

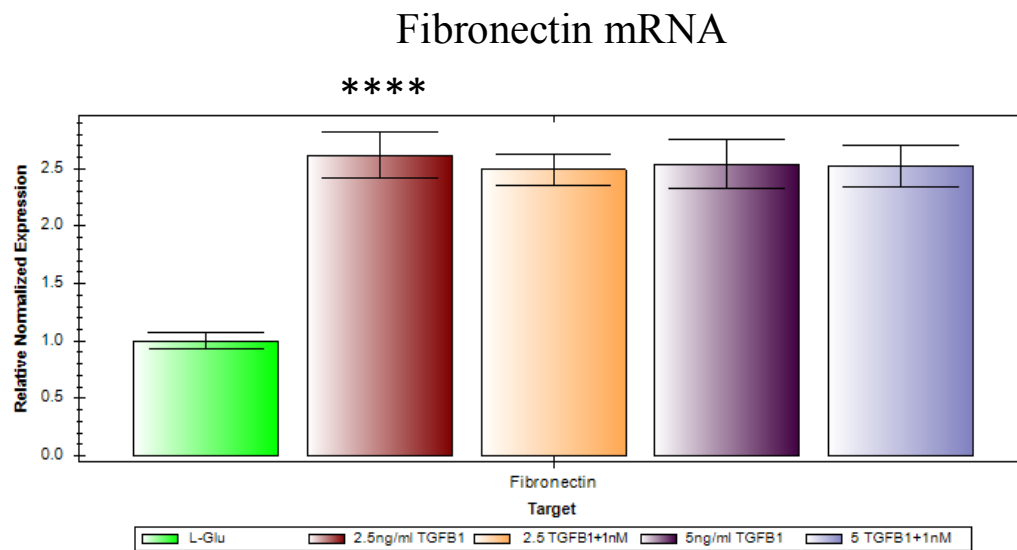


Figure 6.4B: Fibronectin mRNA abundance in PTECS treated with TGF- β 1 and dapagliflozin after 24h.

Key: **L-Glu**= 7mM D-glucose+18mM L-glucose (osmotic control), **2.5ng/ml TGF β 1**=7mM D-glucose+18mM L-glucose+2.5ng/ml TGF- β 1, **2.5 TGF β 1+1nM**=7mM D-glucose+18mM L-glucose+2.5ng/ml TGF- β 1+1nM dapagliflozin, **5ng/ml TGF β 1**=7mM D-glucose+18mM L-glucose+5ng/ml TGF- β 1, **5 TGF β 1+1nM**= 7mM D-glucose+18mM L-glucose+5ng/ml TGF- β 1+1nM dapagliflozin. The figure shows plotted data points after quantification of fibronectin mRNA (fibronectin mRNA abundance/GAPDH mRNA abundance) presented in graph form. Fibronectin mRNA was significantly increased in 2.5 and 5ng/ml TGF- β 1 treated PTECs. Dapagliflozin (1nM) did not reduce fibronectin. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. ****P<0.001 compared to all other treatments, all n=4. Data are shown as mean +/- SD.

6.3.4. Presence of urinary EDA-fibronectin protein in samples from patients with diabetic kidney disease

As EDA-fibronectin protein abundance was increased by high D-glucose and 0.75ng/ml TGF- β 1 treatment, I carried out a pilot clinical study to gain more insight into whether EDA-fibronectin would be an appropriate clinical biomarker of DKD. Urine samples were from the same pool of DKD patients that were studied in chapter 4, split equally into group A and B. Group A, in comparison to group B, had better DKD outcomes including lower HbA1C levels, lower albumin to creatinine ratios (ACR) and eGFR values of $>60\text{ml/min/1.73m}^2$ (refer to Table 4.1). Samples were previously segregated by DKD outcomes, but the two groups were blinded during this study. The results showed that none of the DKD patients in group A had any observable immuno-reactive EDA-fibronectin protein bands. All DKD patients in group B had immuno-reactive protein bands at approximately 50 and 25kDa, although only one of the samples contained full length EDA-fibronectin ($\sim 250\text{kDa}$) (Figure 6.5).

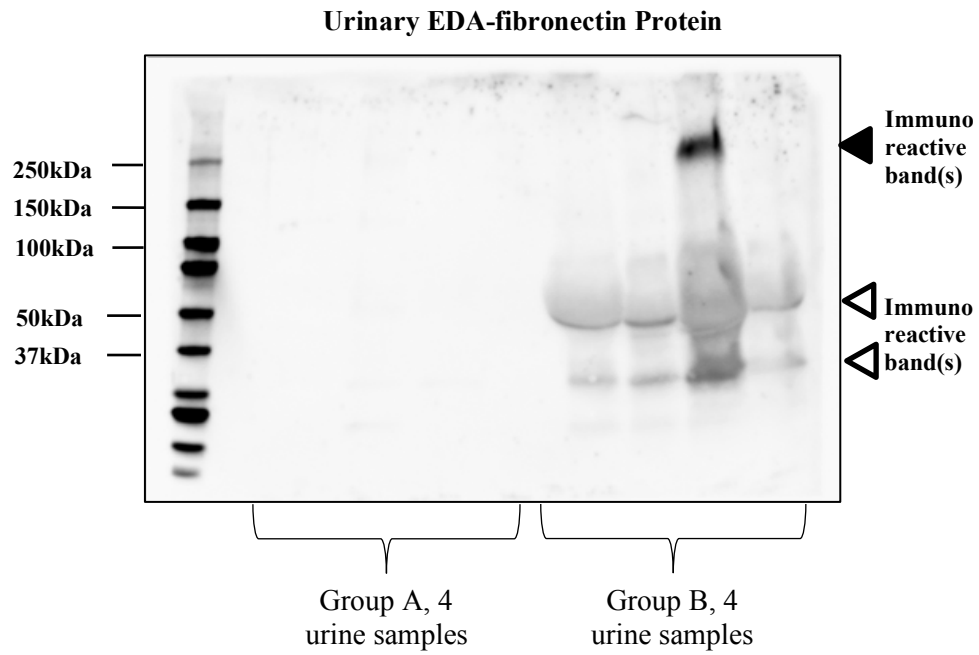


Figure 6.5: Western blot immuno-stained of for urinary EDA-fibronectin protein.

Western blot probed for EDA-fibronectin protein (~25kDa, ~50kDa, ~250kDa) in diabetic kidney disease urine samples. The black arrowhead indicates the full length band while the white arrowheads with black outlines indicate the cleaved fragments. There were no observable immuno-reactive protein bands in the samples from group A. There were immuno-reactive bands at ~50kDa and ~25kDa in all of the samples in group B, and one sample contained full length EDA-fibronectin (~250kDa).

6.3.5. Fibronectin mRNA abundance in PTECs after mutant plasmid DNA transfection

The previous chapter demonstrated that phospho-Smad3 serine 204 is where high D glucose and 0.75ng/ml TGF- β 1 converge to induce CCN2. The inhibition of serine 204 phosphorylation prevented CCN2 transcription. In order to examine whether inhibition of Smad3 serine 204 phosphorylation could also prevent fibronectin transcription, PTECs were again transfected with the dominant negative Smad3 serine 204A mutant plasmid. There were no changes in fibronectin mRNA under osmotic control and osmotic control+0.75ng/ml TGF- β 1 treatment, with or without mutant plasmid DNA transfection. Fibronectin mRNA abundance was also significantly upregulated in the high D-glucose+0.75ng/ml TGF- β 1 treated PTECs. This significant rise was not changed upon mutant plasmid DNA transfection (Figure 6.6).

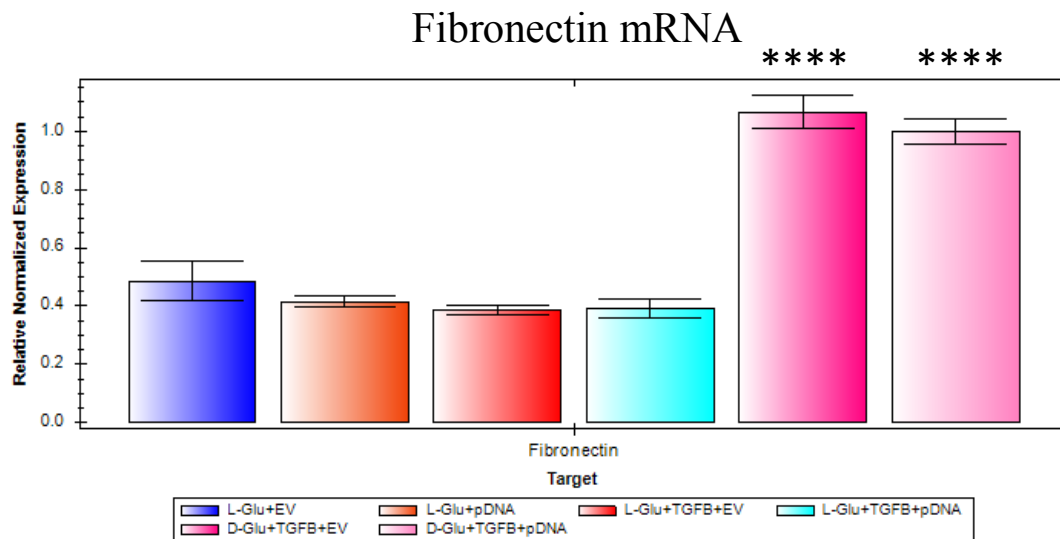


Figure 6.6: Fibronectin mRNA abundance in PTECs treated with glucose and TGF- β 1 after mutant plasmid DNA transfection.

Key: **L-Glu+EV**= 7mM D-glucose+18mM L-glucose+2 μ g empty vector plasmid DNA, **L-Glu+pDNA**= 7mM D-glucose+18mM L-glucose+2 μ g mutant plasmid DNA, **L-Glu+TGF β 1+EV**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGF β 1+2 μ g empty vector plasmid DNA, **L-Glu+TGF β 1+pDNA**= 7mM D-glucose+18mM L-glucose+0.75ng/ml TGF β 1+2 μ g mutant plasmid DNA, **D-Glu+TGF β 1+EV**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGF β 1+2 μ g empty vector plasmid DNA, **D-Glu+TGF β 1+pDNA**= 7mM D-glucose+18mM D-glucose+0.75ng/ml TGF β 1+2 μ g mutant plasmid DNA. The figure shows plotted data points after quantification of fibronectin mRNA (fibronectin mRNA abundance/GAPDH mRNA abundance) presented in graph form. Fibronectin mRNA was significantly increased in the 24hr D-Glu+TGF- β 1 transfected PTECs. This increase was not attenuated by mutant plasmid transfection. Data were analysed using the Bio-Rad CFX1000 statistical software package and the formula used to generate the P value is given in equation 2.2. ****P<0.001 compared to L-Glu+EV, L-Glu+pDNA, L-Glu+TGF β 1+EV, and L-Glu+TGF β 1+pDNA, all n=3. Data are shown as mean $\pm$ SD.

6.4. Discussion

This chapter examined the effects of combined glucose and TGF- β 1 treatment on fibronectin, an important ECM protein that accumulates during renal fibrosis (Genovese *et al.*, 2014).

My results indicate that fibronectin protein and mRNA abundance are unchanged in PTECs treated with 0.75ng/ml TGF- β 1. The induction of fibronectin by TGF- β 1, a key regulator of ECM remodelling, has been long established in literature by ourselves and others (Wrana, Overall and Sodek, 1991; Hocevar, Brown and Howe, 1999; Niculescu-Duvaz *et al.*, 2007; Phanish *et al.*, 2015). However, it should be noted that the concentrations of TGF- β 1 used in these experiments were all greater than 2ng/ml.

There was a significant induction of fibronectin observed in my data only after combined treatment of high D-glucose and 0.75ng/ml TGF- β 1. This is in line with what was discussed previously where Zorena *et al.* concluded that serum TGF- β 1 of over 0.44ng/ml concentration can point to the presence of diabetes related complications (Zorena *et al.*, 2013). This fibronectin result, similar to what was observed with CCN2 in the previous chapter, indicates that my DKD PTEC model induces another marker of fibrosis which is also contingent on a “two hit” model.

Interestingly, Lam *et al.* found that whilst both high glucose and TGF- β 1 were able to significantly increase fibronectin secretion from renal fibroblasts, the use of a neutralising anti TGF- β 1 antibody did not prevent the effects of the high glucose (Lam, Verhagen, *et al.*, 2003). This was an early indication that high glucose could regulate ECM production independent of TGF- β 1. Indeed, in the following years, there has been evidence in literature concluding that exclusive high glucose exposure induces fibronectin. Other groups have shown that subjecting cells to a D-glucose concentration of equal to or higher than 25mM stimulated fibronectin matrix assembly, protein expression and mRNA production (Park *et al.*, 2010; Zhang *et al.*, 2012; Miller *et al.*, 2014). However, these experiments were all carried out using mesangial cells. To the best of my knowledge, there are no reports in literature focusing on the effect of high glucose on fibronectin in primary PTECs.

The induction of EDA-fibronectin protein did not become significant until 72h treatment, unlike what was previously seen with CCN2, where the pro-fibrotic marker was induced significantly by 24h. This is likely due to the fact that CCN2 is an immediate early gene, whilst fibronectin, which forms part of the ECM, takes time to accumulate and deposit. This result seems to be in line with a study carried out by Montero *et al.*, where the group did not find a significant increase in fibronectin concentration until incubation with 30mM D-glucose for 48h in immortalised proximal tubule cells (Montero *et al.*, 2016). For future studies, it may be useful to analyse the fibronectin protein and mRNA abundance in my PTECs at 48h as well, to examine whether there is a time dependent effect.

The significant induction of fibronectin mRNA in high D-glucose+0.75ng/ml TGF- β 1 treated PTECs was attenuated by dapagliflozin at 24h. This means that induction of fibronectin protein seen at 72h cannot derive from the fibronectin RNA produced at 24h. I hypothesise that there may be a second activation of fibronectin transcription that produces the protein. Derynck *et al.* showed that matrix metalloproteinase 2 (MMP2) can mediate Smad3 induced activation of latent TGF- β (Wu and Derynck, 2009). As MMP2 can be activated independent of the Smad3/glucose pathway, it is plausible that my PTECs could have latent TGF- β which activates fibronectin protein at 72h.

There are a number of reports in literature which look into the effects of anti-diabetic drugs on DKD progression. Both dapagliflozin and empagliflozin were able to reduce the increased levels of fibronectin mRNA expression in HK-2 cells, as well as other ECM proteins and markers of inflammation like collagen type 1 and monocyte chemoattractant protein-1 (MCP-1) (Yao *et al.*, 2018; Ndibalema *et al.*, 2020). Dapagliflozin also significantly reduced diabetes-induced fibronectin protein and mRNA in the *db/db* mice kidneys (Tang *et al.*, 2017). Other therapies like angiotensin II inhibitor reduce fibronectin staining and proteinuria in nephritic rats (Gu *et al.*, 2014), suggesting that fibronectin may be an effective biomarker of diabetic kidney disease.

Dapagliflozin did not reduce EDA-fibronectin protein at 72h. This is likely due to the breakdown of dapagliflozin over time. The majority of dapagliflozin is converted to the metabolite 3-O-Glucuronide, which does not inhibit SGLT2 under clinically relevant conditions (Kasichayanula *et al.*, 2014). There was a steep decline in dapagliflozin

concentration over the first 24h seen in the HPLC data. Clinically, dapagliflozin is administered every 24h, so it was plausible in my experiments that by 72h, the EDA-fibronectin protein had accumulated again.

My pilot study demonstrated the presence of urinary EDA-fibronectin protein in all of the DKD patients with high HbA1c levels (median 12.2%), high ACR (median 336), and eGFR values of <60ml/min/1.73m². The full-length EDA-fibronectin band (~250kDa) was only observed in one sample whilst the other samples in this group had immuno-reactive bands at approximately 50 and 25kDa. I hypothesise that these two smaller bands may be the result of full-length EDA-fibronectin cleavage by serine protease thrombin. Quigley *et al.* established that thrombin cleaves fibronectin (Quigley *et al.*, 1987). The presence of thrombin in the urine is an indicator of glomerular inflammation (Kitamoto *et al.*, 2015), commonly observed during renal fibrosis in DKD (Lim and Tesch, 2012).

The biggest limitations for my pilot clinical study included the low sample size and lack of non-diabetic kidney controls. Increasing the sample size will increase the reliability of my results and incorporating non-diabetic kidney disease controls in the study will reveal whether urinary fibronectin or CCN2 (identified previously in section 4.4.3) is also present under normoglycaemic conditions. This should be considered for future work.

Increased urinary fibronectin levels in patients with type 1 and type 2 diabetes and macro-albuminuria was reported early on (Takahashi, 1995; Fagerudd *et al.*, 1997). Zheng *et al.* also found a positive correlation between urinary fibronectin protein abundance and worsening DKD (Zheng *et al.*, 2012). These findings implicate fibronectin as an indicator of early diabetic kidney disease. However, there needs to be future long-term clinical studies to provide better insight into whether fibronectin targeted therapy provides substantial clinical benefits.

Inhibition of Smad3 serine 204 phosphorylation did not prevent the upregulation of fibronectin transcription, unlike CCN2. This suggests that although both CCN2 and fibronectin are induced by high D-glucose and TGF- β 1 co-incubation, phospho-Smad3 serine 204 is not the convergence point for induction of fibronectin. Other potential convergence candidates include p38 MAPK, which has been well established as a

downstream mediator for glucose. High glucose is known to activate the p38 MAPK pathway in human mesothelial cells (Xu *et al.*, 2003). The same pathway also plays a critical role in high glucose induced EMT in renal tubule cells (Lv *et al.*, 2011). Examining p38 protein and mRNA abundance under the specific glucose and TGF- β 1 treatments I have used would be an interesting next step.

Chapter 7: Conclusion

Diabetic kidney disease is a major complication of type 1 and type 2 diabetes and the most common cause of ESRD in developed countries (Oba *et al.*, 2018). It is typically characterised by the thickening of the GBM, the presence of glomerular lesions, pathological quantities of albuminuria, and a progressive decline of eGFR (Lim, 2014; Pourghasem, Shafi and Babazadeh, 2015). Clinically, albuminuria is widely accepted as the gold standard marker for DKD advancement in patients diagnosed with diabetes (Lee and Choi, 2015). However, it is well recognised that a significant proportion of progressive DKD patients with declining renal function exhibit non-albuminuric phenotypes (Gross *et al.*, 2005; Laranjinha *et al.*, 2016). This establishes the importance of studying markers of DKD progression like tubulointerstitial fibrosis. Studies have indicated that tubulointerstitial fibrosis is a central hallmark of DKD and a final progression pathway to kidney failure (Tonolo and Cherchi, 2014; Menn-Josephy *et al.*, 2016). This has shifted the proximal tubule and its involvement in the development of DKD into prominent focus.

DKD is primarily driven by TGF- β 1 and hyperglycaemia. The TGF- β 1 signalling pathway is stimulated during DKD (Zhao, Zou and Liu, 2020) and inhibition of the growth factor has resulted in the amelioration of fibrosis in animal models (Fu *et al.*, 2008; Kim *et al.*, 2020). Elevated levels of glucose can also trigger inflammatory and fibrotic changes in PTECs (Tang *et al.*, 2008). There is increasing evidence implicating SGLT2 as the major regulator of the effects of chronically elevated glucose in PTECs (Vallon *et al.*, 2011; Novikov and Vallon, 2016). To this end, anti-diabetic drugs that target SGLT2, referred to as gliflozins, have produced promising clinical results (Bailey *et al.*, 2012). Though initially expected to only improve glycaemic control, these gliflozins have surpassed expectations and have provided other benefits like significant reductions in adverse cardiovascular events (Zinman *et al.*, 2015).

7.1. Key Findings

*Unless otherwise stated, concentration of TGF- β 1 is 0.75ng/ml (threshold) and concentration of high D-glucose is 7mM D-glucose+18mM D-glucose (“hyperglycaemia”). The “osmotic control” is defined as 7mM D-glucose +18mM metabolically inert L-glucose.

SGLT2 is expressed by PTECs grown on collagen IV under various treatments

Under osmotic control conditions, SGLT2 protein was detected in PTECs grown on collagen IV. Subsequent experiments were all conducted using the same PTECs cultured on collagen IV. Microscopic images of osmotic control and high D-glucose+TGF- β 1 treated PTECs indicated an archetypal cobblestone pattern. Similarly, distinct orange-red staining confirmed the presence of gamma-glutamyl transferase (GGT) enzyme in healthy and differentiated PTECs under both conditions.

SGLT inhibitors reduce glucose uptake in PTECs

The bioluminescent glucose uptake assay revealed significantly reduced glucose uptake by SGLT inhibitor phlorizin under osmotic control conditions while SGLT2 inhibitor dapagliflozin did not have any effect. Contrarily, dapagliflozin significantly reduced glucose uptake conditions under high D-glucose conditions while phlorizin did not. This was expected as SGLT1 is a high affinity/low capacity transporter and thus under “normoglycaemic” osmotic control conditions, glucose is predominantly taken up by SGLT1. SGLT2 is a low affinity/high capacity transporter (Baud *et al.*, 2016) and therefore would only become active and overtake SGLT1 function upon exposure of PTECs to higher D-glucose concentrations.

High D-glucose and TGF- β 1 induces p21 protein in PTECs while T β RII and K-Cadherin protein remain unchanged

The combined treatment of high D-glucose and TGF- β 1 significantly increased cell cycle inhibitor p21 protein in PTECs after 24h compared to all other treatments. This suggests that this combined treatment induces a reduction in PTEC proliferation activity. T β RII and K-Cadherin protein abundance was unchanged, indicating that the combined treatment did not increase receptor expression or cause loss of phenotype.

High D-glucose and TGF- β 1 induced CCN2 and fibronectin in PTECs is SGLT2 dependent

CCN2 mRNA and fibronectin mRNA were significantly upregulated in high D-glucose+TGF- β 1 treated PTECs after 24h compared to all other treatments. This CCN2 and fibronectin rise was significantly attenuated upon administration of 1nM/10nM dapagliflozin. Similarly, CCN2 and fibronectin protein were also significantly induced after 24 and 72h combined treatment, respectively. While the addition of dapagliflozin (0.1, 1, 10nM) significantly reduced CCN2 protein, there was no reduction observed in fibronectin protein. However, as fibronectin mRNA at 24h was significantly reduced, it is likely that the effect of dapagliflozin had worn off over time, allowing fibronectin protein to accumulate again by 72h. This was confirmed by HPLC. Dapagliflozin in high D-glucose+TGF- β 1 treated media was analysed at 0, 24, 48, and 72h. There was a sharp decline in dapagliflozin concentration in the first 24h before it began to level off between 24 to 72h, supporting the hypothesis that the potency of dapagliflozin wears off over time.

Smad3 serine 204 is where high D-glucose+TGF- β 1 treatment converge to induce CCN2

TGF- β 1 treatment increased cellular phosphorylation of ERK for 60 min, while high D-glucose treatment increased ERK phosphorylation at 30 min before abruptly reducing down to levels comparable to the osmotic control at 60 min. Smad3 serine 423/425

phosphorylation was increased by TGF- β 1 from 30 to 60 min, with or without high D-glucose stimulation. There was a significant increase in Smad3 serine 204 phosphorylation at 30 min in the combined high D-glucose+TGF- β 1 treatment while the exclusive high D-glucose, exclusive TGF- β 1, and exclusive osmotic control+TGF- β 1 treatments did not change phosphorylation. Smad3 serine 204 phosphorylation is also significantly reduced by both dapagliflozin (1nM) and U0126 (10 μ M), indicating the importance of SGLT2 and ERK in this pro-fibrotic pathway.

Inhibition of Smad3 serine 204 phosphorylation prevents CCN2 transcription but does not change fibronectin transcription

A Smad3 mutant construct that prevents serine 204 phosphorylation was transfected into PTECs. CCN2 mRNA was significantly reduced in the high D-glucose+TGF- β 1 treated, mutant plasmid DNA transfected PTECs when compared to the high D glucose+TGF- β 1 treated, empty vector plasmid DNA transfected PTECs. There were no changes detected with fibronectin mRNA. This data provides proves the crucial involvement of Smad3 serine 204 in the glucose/TGF- β 1 mediated pro-fibrotic induction of CCN2 but not fibronectin. This will be discussed further in the future directions section.

Urinary CCN2 and fibronectin protein are detected in DKD patient samples

A pilot clinical study where group A had comparatively better (HbA1c) percentages (median 6.7% vs median 12.2%), lower ACR (median 0.42 vs median 336), and higher eGFR rates (approx. >60ml/min vs <60ml/min) than group B, was conducted. There was urinary CCN2 protein present in all 4 patients of group B and none of group A. There was urinary fibronectin protein present in all 4 patients of group B and none of group A.

7.2. Final discussion

The DKD PTEC model that has been utilised throughout this project has been designed to mimic patients with established diabetes who are in the early stages CKD and likely have microalbuminuria, also known as tubular albuminuria. HbA1c levels are considered the most common predictor for the development of diabetic complications (Polhill *et al.*, 2004). However, in addition to the challenge of consistently controlling blood glucose levels, diabetic individuals also endure postprandial hyperglycaemia, characterised by short bouts of rapid hyperglycaemic spikes. There is evidence to indicate that these glucose spikes are a better predictor for microvascular diabetic complications independent of HbA1c measures (Node and Inoue, 2009; Giacco and Brownlee, 2010). The DKD model that I have developed was acutely exposed to elevated D-glucose (7mM D-glucose+18mM D-glucose, 24h) to simulate this effect.

Human serum albumin (HSA) produces a concentration dependent production of TGF- β 1, independent of its interaction with megalin. This effect becomes significant and begins to plateau at 1mg/ml HSA, comparable to approximately 0.75ng/ml TGF- β 1 protein (Diwakar, Alex L. Pearson, *et al.*, 2007), hence the use of this threshold concentration for my experiments. Interestingly, Shaker *et al.* also found that the serum TGF- β 1 levels at approximately 0.2ng/ml were significantly elevated in diabetic patients with microalbuminuria (Shaker *et al.*, 2014).

Furthermore, this threshold concentration of TGF- β 1 is particularly important for this model as TGF- β 1 concentrations higher than 1ng/ml have been shown to activate EMT in breast cancer and rat kidney cells (Tian and Schiemann, 2017; He *et al.*, 2018). To my current knowledge, there are no reports of EMT occurring in human DKD.

Derynck *et al.* found that exposure of 25mM glucose to rat kidney cells and fibroblasts significantly increased the expression of both T β RII and T β RI (Wu and Derynck, 2009). Therefore T β RII protein abundance was also analysed in my PTECs after high D-glucose + 0.75ng/ml TGF- β 1 treatment to ensure that the pro-fibrotic effects observed were not a direct result of an increase in receptor expression. T β RII protein was unchanged after

treatment. Additionally, the signalling data also showed that high D-glucose did not induce Smad3 signalling, and thus did not induce TGF- β 1.

Dapagliflozin inhibited both CCN2 and fibronectin abundance. However, whilst the Smad3 mutant construct proved that ERK/Smad3 serine 204 phosphorylation was critical for glucose/TGF- β 1 mediated CCN2 induction, this was not the case for fibronectin.

Therefore, it would be useful to investigate the convergence pathway that facilitates the glucose/TGF- β 1 mediated induction of fibronectin. ERK, p38 and JNK are all distinct signalling molecules found in the kidney that comprise the MAPK pathway responsible for mediating gene transcription and essential cellular functions (Gui *et al.*, 2012).

Whilst ERK is partly responsible for CCN2 induction, it is hypothesised that p38 and JNK may be responsible for fibronectin induction. There is evidence that TGF- β 1 can activate both p38 and JNK in a Smad-independent manner (Yamashita *et al.*, 2008). Zhang *et al.* reported that their STZ induced diabetic rats experienced aggravated aortic fibrosis after exposure to glucose spikes (5-25mM D-glucose every 12h) via the p38/MAPK pathway (Zhang *et al.*, 2020). Both fibronectin mRNA production and p38 MAPK activity were also significantly increased upon exposure to high glucose in human mesothelial cells (Xu *et al.*, 2003). Resveratrol, a polyphenol with renoprotective properties, significantly reduced fibronectin expression by inhibiting high glucose induced JNK activation in mesangial cells (Zhang *et al.*, 2012). Similarly, ginsenoside, an anti-diabetic agent, significantly reduced high glucose induced fibronectin expression by inhibiting the JNK signalling cascade (Park *et al.*, 2010). My hypothesis is that the combination of high D-glucose and TGF- β 1 may stimulate an additive effect on p38 and/or JNK, leading to increased fibronectin abundance (Figure 7.1).

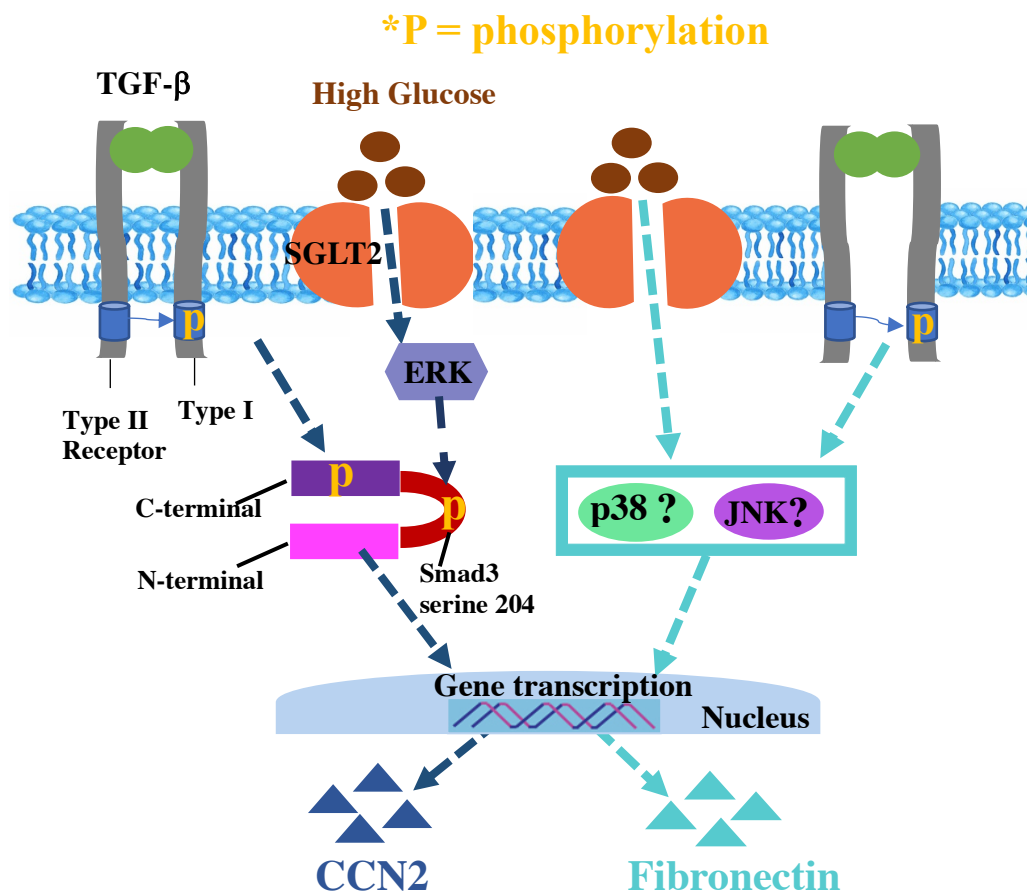


Figure 7.1: Proposed mechanism of glucose/TGF-β1 mediated CCN2 and fibronectin induction.

(A) The TGF-β1 receptor phosphorylates R-Smads on the C terminal. These R-Smads then bind to co-Smad4. This Smad homo/heterotrimer complex is activated and then translocated to the nucleus for gene transcription. High glucose enters via SGLT2 and in turn activates ERK. ERK then induces smad3 serine 204 LR phosphorylation. This then potentiates TGF-β specific signalling in the complex and increases CCN2 transcriptional activity. The induction of fibronectin is Smad independent. I propose that both high glucose and TGF-β1 can separately activate the p38 and/or JNK pathways. Thus, the combination of the two treatments will create an additive effect which then increases fibronectin transcriptional activity.

7.3. Future directions

It is hypothesised in my model that the truncated Smad3 isoform containing the phosphorylated serine 204 dimerises with the full length Smad3 which contains the phosphorylated serines 423 and 425. Immunofluorescence microscopy using phospho-Smad3 serine 204 and serine 423/425 antibodies on osmotic control and high D-glucose+TGF- β 1 treated PTECs would provide me with more information on this theory.

Clearly, high D-glucose spikes combined with microalbuminuria is damaging to PTECs and likely to be heavily involved with the long-term consequences of DKD. However, another question that remains is whether SGLT2 or Smad3 serine 204 is a better therapeutic target for DKD. From my results, SGLT2 inhibition prevents both CCN2 and fibronectin production while phospho-Smad3 serine 204 inhibition only prevents the former. It is not clear at the moment whether the reduction of CCN2 and fibronectin are both required for the prevention of DKD or whether one of the markers may be more important in the development of DKD.

My thesis has shown that it is possible to selectively target TGF- β mediated outcomes. I propose employing peptide aptamers to target phospho-Smad3 serine 204 in order to treat DKD in a pre-clinical model and observe whether this inhibition and subsequent CCN2 knockdown will be enough to prevent DKD. Peptide aptamers originate from an emerging class of stable molecules comprised of short polypeptides that include a specific sequence constrained with a scaffold protein which can then bind to and inactivate a target protein (Reverdatto, Burz and Shekhtman, 2015). If the CCN2 knockdown proves to be insufficient in reducing or preventing DKD, it may be the case that fibronectin is a more important molecule to target, perhaps through p38/JNK inhibition.

Inhibition of p38 as a treatment strategy for renal fibrosis was previously hypothesised by Prakash et al. Their group conjugated the SB202190 p38 inhibitor to a carrier lysosome which facilitated transport into the proximal tubules. The treatment resulted in a significant reduction in pro-collagenI α 1 in their HK-2 cells (Prakash *et al.*, 2006).

Further experiments to consider would include employing a glucose forster resonance energy transfer (FRET) sensor. Although the non-radioactive bioluminescent glucose uptake assay utilised in this project provided useful insights into the reduction of glucose uptake by glucose transport inhibitors, it was a relatively crude process that only allowed me to measure the 2DG6P accumulated at the end of the experiment. The glucose FRET sensor would allow the tracking of intracellular glucose signalling in real time. These sensors have been used to track live glucose measurements in both liver and ovarian cells (Takanaga, Chaudhuri and Frommer, 2008; Behjousiar, Kontoravdi and Polizzi, 2012).

Furthermore, it would be useful to conduct a larger clinical trial as a follow up to the pilot one carried out for this project. As there is urinary CCN2 and EDA-fibronectin protein present in DKD patients, the next step would be to take urine samples of DKD patients before and after they begin taking gliflozins. There could be a follow up period of 6 months to a year after the administration of gliflozins and the hypothesis would be that both urinary CCN2 and fibronectin protein would remain at low levels after drug intervention. This would strengthen the proposal that gliflozins are reno-protective.

The in vitro DKD model utilised in my experiments is a versatile tool that has been instrumental in the investigation of various scientific research questions asked throughout this thesis. Primary cell cultures continue to be invaluable for basic research as they allow the study of human and animal cells in a controlled environment, increasing the chances of high reproducibility and reliability. Their application is also advantageous for clinical research as genes and molecular pathways can be manipulated with greater ease within a cell culture system (Segeritz and Vallier, 2017). However, this type of 2D model also has various disadvantages as it cannot accurately mimic natural physiological conditions or the convoluted biological signalling mechanisms and cell to matrix interactions of in vivo tissues (Habanjar et al., 2021). Thus, utilising other technological advancements to evolve my DKD cell model should be considered as a following research step. These applications include but are not limited to transwell support systems, kidney organoids, and microfluidic systems.

Transwell support systems provide an updated method for culturing polarised renal epithelial cells. The transwell inserts fit into standard culture dishes by hanging off from the top edge, allowing the porous membrane to be enveloped in the medium but remain a

millimetre away from the bottom of the dish. This specialised structure separates lower and upper chambers and allows the cultivation of polarised cells with distinct apical and basolateral membranes. The membrane facilitates cellular metabolic activities in both fluid compartments, which better mimics what occurs in living epithelial tissues (Miller *et al.*, 2017; Vinaiphat, Charngkaew and Thongboonkerd, 2018).

Organoids are artificially grown, 3D mini structures generated from human induced pluripotent stem cells (iPSCs) which can continuously self-renew in culture and differentiate into diverse cell types (Yousef Yengej *et al.*, 2020). As these PSC-derived organoids mimic *in vivo* organs and have the capacity to construct tissue architecture through processes that only transpire during early development, they have important implications for the study of organ (patho)physiology (Wu *et al.*, 2021). As the generation of iPSCs is a laborious and invasive process, researchers have now devised a comprehensive and shortened procedure for generating these cells from isolated urinary epithelial cells. The yield of these urinary iPSCs are high and they demonstrate excellent differentiation capabilities (Zhou *et al.*, 2012).

Microfluidic systems, also referred to as organ-on-the-chip, describe a biomimetic cell culture built on clear polymer micro-devices engineered to recapitulate physiological and functional characteristics of the target organ. These micro-devices consist of hollow channels and continuously perfused chambers lined with living human tissue and blood vessel cells arranged in a 3D structure designed to replicate *in vivo* tissue geometry (Wu *et al.*, 2020; Kang, Park and Huh, 2021). These devices are also integrated with state-of-the-art features including mechanical/electrical stimuli, controlled flow profiles, and sensors to monitor cellular responses, thereby allowing it to mimic cell-cell interface (Paloschi *et al.*, 2021). Immortalised cells lines like MDCK (Madin-Darby canine kidney) tubular epithelial cells and HK-2 tubular epithelial cells are commonly used to develop kidney-on-a-chip. However, the proliferative ability of these cells can result in blockage of these hollow channels (Ashammakhi *et al.*, 2018). Primary cell lines like the PTECs used in my experiments offer a better alternative. As PTECs have limited doubling capacity, they are transfected with siRNA which can substantially increases doublings. Theses transfected cells are subsequently seeded onto the hollow channels where they quickly form a confluent layer (Sanechika *et al.*, 2011). More recently, microfluidic proximal tubule-on-a-chip models have been developed with success. Compared with 2D controls, these 3D

models demonstrated increased albumin uptake, stronger tight junction linkage, and greater microvilli density (Homan *et al.*, 2016).

These emerging technologies hold great potential for the study of kidney disease and will have broad applications in the future for precision medicine.

In my “two hit” glucose/TGF- β 1 DKD PTEC model which retains its phenotype, I have identified an SGLT2 mediated pro-fibrotic pathway anchored by a novel convergence mechanism. High glucose enters the PTEC via SGLT2 to stimulate the MAPK/ERK signalling cascade which in turn phosphorylates serine 204 on the Smad3 linker region. Simultaneously, TGF- β 1 phosphorylates its own receptor and then serines 423 and 425 on Smad3. This Smad heterotrimer complex then translocates to the nucleus where these combined events potentiate CCN2 gene transcription. This “two hit” DKD model also potentiates fibronectin transcription but the convergence mechanism for this gene has yet to be identified. My work especially highlights an important role for anti-diabetic SGLT2 inhibitors in reducing CCN2 and fibronectin abundance, as well as Smad3 serine 204 activity. I propose that SGLT2 is a promising candidate for anti-DKD therapies due to its crucial role in ameliorating renal fibrosis.

Chapter 8: References

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