SREBP-1 AND CELL SURFACE GRP78 IN DIABETIC NEPHROPATHY

SREBP-1 AND CELL SURFACE GRP78 ARE IMPORTANT MODULATORS OF TGF-β1 IN THE PROGRESSION OF DIABETIC NEPHROPATHY

By

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TITLE: SREBP-1 and Cell Surface GRP78 are important modulators of TGF-β1 in the progression of Diabetic Nephropathy

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Lay Abstract

Diabetic kidney disease is the leading cause of end stage renal disease and represents an important risk factor for mortality. The goal of this thesis is to understand and describe the pathways and mechanisms that contribute to the development of diabetic kidney disease in order to identify novel therapeutic targets. This thesis has identified the protein sterol regulatory element binding protein (SREBP)-1 and the cell surface presentation of another protein, the 78 kDa glucose regulated protein (GRP78), as contributors to diabetic kidney disease. Furthermore, this thesis has demonstrated that anti-SREBP therapy with the drug fatostatin did not prevent diabetic kidney disease. These studies show that while inhibiting SREBP-1 and cell surface GRP78 may be effective in the treatment of diabetic kidney disease, the drug fatostatin should not be used for treatment.

Abstract

Diabetic nephropathy represents the leading cause of end stage renal disease worldwide and requires a kidney transplant or dialysis to survive. The number of patients suffering from diabetes is expected to increase, thus the number of patients with diabetic nephropathy is expected to concomitantly increase. Current treatment for diabetic nephropathy is not sufficient to prevent disease progression in most patients thus there is a need to develop novel therapies to treat diabetic nephropathy.

The earliest changes that occur during the pathogenesis of diabetes occur in the glomerulus. The mesangial cells are a subpopulation of cells in the glomerulus that are responsible for coordinating responses with other nearby cell types. Transforming growth factor (TGF)-β1 is a cytokine that mesangial cells secrete, and has been identified as a profibrotic factor during the pathogenesis of diabetic nephropathy. Concerns have been raised in the use of direct anti-TGF-β1 therapy due to adverse events (such as dyspepsia and diarrhea) and lack of efficacy of anti-TGF-β1 monoclonal antibody LY2382770 in patients with diabetic nephropathy. Thus, therapy aimed at modulating TGF-β1 expression or activity may be efficacious in the treatment of diabetic nephropathy while avoiding potential adverse effects.

The hypothesis of this thesis is that SREBP-1 and cell surface GRP78 are novel regulators of TGF-β1 signaling in mesangial cells. Our first study aims to define a novel pathway by which SREBP-1 regulates TGF-β1 signaling in kidney

mesangial cells. Our results indicate that SREBP-1 regulates the expression of the type I TGF-β1 receptor through its secretion in exosomes. Our second study expands on these findings and aims to determine if inhibition of SREBP *in vivo* with the inhibitor fatostatin may prevent diabetic nephropathy. Our results indicate that treatment with fatostatin does not prevent diabetic nephropathy, but accentuates kidney injury in non-diabetic mice. Preliminary results from our lab have indicated that under diabetic conditions, GRP78 is upregulated at the cell surface and may contribute to the activation of SREBP-1 in an ER-stress dependent mechanism. Our third study thus aims to characterize the expression of cell surface GRP78 in diabetic conditions, and to determine its pathological relevance in the development of diabetic nephropathy. Our results have established novel pathways by which TGF-β1 signaling is regulated in mesangial cells. This will assist in identification of novel therapeutic targets that may be of use in the treatment of diabetic nephropathy.

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Preface

This thesis is a 'sandwich' style thesis. Chapter 1 is a general introduction that provides an overview of concepts relevant to this thesis. Chapters 2-4 have been published, or will be submitted for publication in a peer-reviewed journal. A preface may be found at the beginning of these chapters, describing the work performed and contributions of all authors involved. A discussion of limitations and future directions is included in Chapter 5 to provide an overall analysis of the data presented.

Figure and Table Summary

List of Abbreviations

AGE	Advanced glycation end products
8-OHdG	8-Hydroxyguanosine
Ang II	Angiotensin II
C20	GRP78 C20 Antibody
ca	Constitutively active
Cav	Caveolin
CD	Cyclodextrin
CD3	Cluster of differentation 3
cs	Cell surface
CTGF	Connective tissue growth factor
DBP	Diastolic blood pressure
DM	Diabetes Mellitus
DN	Diabetic Nephropathy
dn	Dominant negative
ECM	Extracellular matrix
eGFR	Estimated GFR
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
ESRD	End stage renal disease
FAK	Focal adhesion kinase
Fato	Fatostatin
FN	Fibronectin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
Glu	Glucose
GM	GM6001
GRP78	78 kDa glucose regulated protein
HDL	High density lipoprotein
HG	High glucose
HMGCR	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
IF	Immunoflourescence
IHC	Immunohistochemistry

Insig	Insulin-induced gene
IP	Intraperitoneal
LAP	Latency associated peptide
LDL	Low density lipoprotein
Leu	Leupeptin
LTBP	Latent TGF-β binding protein
MC	Mesangial cell
MCP-1	Monocyte chemoattractive protein 1
MG	MG132
mRNA	Messenger RNA
mSREBP-1	Mature SREBP-1
mSREBP-1	Mature SREBP-1
MTJ-1	DnaJ-like protein 1
Mut	Subtilase cytotoxin A272 mutant
N20	GRP78 N20 Antibody
NC	NH4Cl
NOX	NADPH oxidase
PAI-1	Plasminogen activator inhibitor 1
PAS	Periodic acid schiff's reagent
PDGFR	Platelet-derived growth factor receptor
PF	PF573228
PSR	Picosirius Red
pSREBP-1	Parent SREBP-1
RAAS	Renin-angiotensin-adolsterone system
RAGE	Receptor for AGE
RANTES	Regulated on activation, normal T cell expressed and secreted
ROS	Reactive oxygen species
R-Smads	Receptor Smads
S1P	Site 1 protease
S2P	Site 2 protease
SBE	Smad binding element
SBP	Systolic blood pressure
SCAP	SREBP-cleavage activation protein
SD	Sprague-Dawley
siRNA	Small intefering RNA
SLC	Small latent complex
Smad	Small mothers against decapentaplegic

SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
STZ	Streptozotocin
SubA	Subtilase cytotoxin A
TACE	TNFα converting enzyme
TGF-β or T	Transforming growth factor β
TNFα	Tumor necrosis factor α
TβRI	Type I TGF-β receptor
TβRII	Type II TGF-β receptor
UPR	Unfolded protein response
UUO	Unilateral ureteral obstruction
VEGF	Vascular endothelial growth factor
α2M	α2 macroglobulin
α2M*	Methylamine activated α2 macroglobulin
αSMA	α smooth muscle actin

Chapter 1 – Introduction

Diabetic Nephropathy

Diabetes is a chronic disease of hyperglycemia and results from the body's inability to produce or utilize insulin properly to store glucose [1]. Type I diabetes is caused by an autoimmune reaction where the immune system targets the insulin-producing β-cells in the pancreas [1]. Type II diabetes occurs due to insulin resistance where the body is unable to properly respond to insulin and eventually unable to produce insulin [1]. Type II diabetes is the most common form of diabetes worldwide and is associated with the aging population, economic development, urbanisation, unhealthy diets, and reduced physical activity [1]. If left untreated, people with diabetes are at a high risk of developing micro and macrovascular diseases affecting the heart, eyes, kidneys and nerves[1].

Clinically, diabetic nephropathy (DN) is defined as a rise in urinary albumin excretion with a reduction in renal function in patients with diabetes [2]. Reduction of renal function is characterized by elevated plasma creatinine, reduced creatinine clearance and glomerular filtration rate (GFR) [2]. DN represents one of the major microvascular complications that arise in 20-40% of diabetic patients, and represents the leading cause of end-stage renal disease (ESRD) in the Western world [3]. Treatment and management of ESRD requires dialysis or transplantation, however these methods are costly and place a heavy burden on healthcare resources [4]. Furthermore, patients with DN are at a higher risk of all cause and cardiovascular disease mortality than patients with diabetes alone [5]. Although current treatment for DN are capable of slowing progression

of the disease, they are not sufficient in preventing the progression of DN in most patients [6]. Thus there is a need to better characterize the pathophysiology of DN in order to identify new potential targets for therapy.

Epidemiology of DN

As of 2015, it has been estimated that 415 million people worldwide have diabetes [1]. This estimate is expected to increase to 642 million by 2040 [1]. In Canada, up to 2.4 million Canadians were diagnosed with diabetes representing 6.8% of the population [7]. People with diabetes were 5.9 times more likely to be hospitalized with renal disease, and 12 times more likely to be hospitalized due to ESRD [7]. As of 2009, diabetes was reported in 34% of incident cases related to ESRD, representing the leading causing of ESRD in Canada [7]. Management of diabetes and diabetic complications was conservatively estimated to be around $2.5 billion in Canada in 2000 [7]. This number does not include treatment associated with management of long-term complications of diabetes such as DN which is expected to cost up to 3.6 times more [7]. Thus, DN is a pressing issue in Canada and is associated with a large burden on healthcare spending.

Physiology of the Kidney and Pathophysiology of DN

The purpose of the kidney is to filter out harmful toxins from circulation, and maintain proper homeostasis of the body [8]. The functional unit of the kidney is the nephron, which comprises of the renal corpuscle and the tubular systems that ultimately drain into the collecting duct [8].

The renal corpuscle is the subunit of the kidney responsible for filtration, and consists of the glomerulus and the Bowman's capsule [8]. The glomerulus consists of a tuft of capillaries that are held together by the mesangium and surrounded by the glomerular basement membrane (GBM) [8]. The mesangium is formed from mesangial cells (MCs) that secrete a matrix of extracellular matrix (ECM) proteins [8]. The GBM is one of the supports of the glomerular tuft and is mainly comprised of ECM proteins [8]. The GBM is part of the filtration barrier in the kidney, which is additionally comprised of fenestrated endothelium and foot processes derived from podocytes [8]. Together, the filtration barrier acts to filter macromolecules from circulation depending on their charge, size, and shape as well as ensuring that plasma proteins remain in circulation [8].

Development of DN in humans typically occurs over the span of many years and manifests as morphological changes to glomeruli during early stages of the disease [9]. The earliest changes in diabetic glomeruli include hyperfiltration, thickening of the GBM, expansion of the mesangial matrix, decrease in podocytes and effacement of foot processes, and glomerular hypertrophy **(Figure 1-1)** [10].

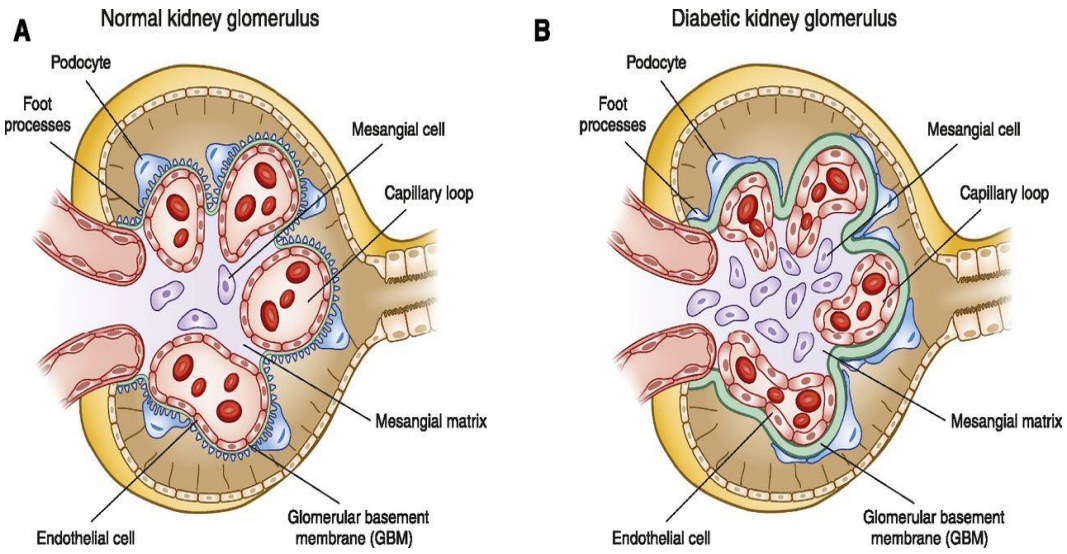

Figure 1-1 – Morphological changes to the glomerulus during DN.
The earliest morphological changes to the kidney in patients with DN occur within the glomerulus. These changes manifest as glomerular hypertrophy, expansion of the mesangium, thickening of the GBM, and loss of podocytes and foot processes. Taken from Alicic et al. (2017) [10]

Later in the disease progression, damage to the kidney spreads to the tubulointerstium. This is characterized by interstitial fibrosis and renal tubular atrophy [9, 11, 12]. These changes in kidney morphology manifest as a decline in GFR in the late stages of the disease, proteinuria, and hypertension [10]. A summary of the changes that occur to the kidney during the natural history of DN is presented in **Figure 1-2**.

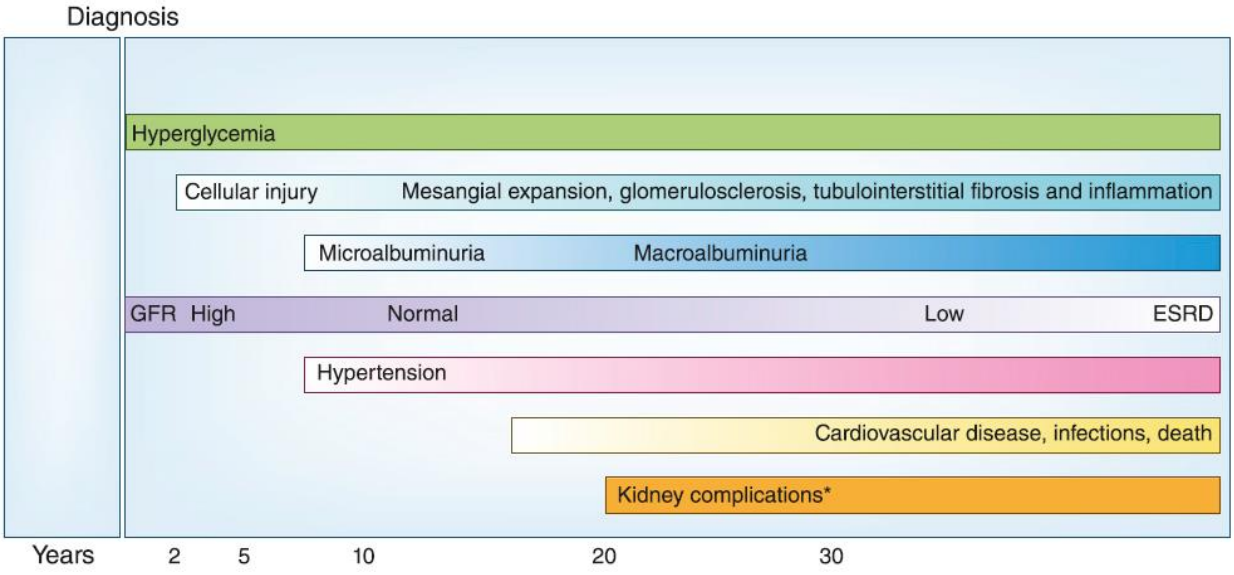

Figure 1-2 - Natural History of DN.

Length of diabetes since diagnosis is expressed on the horizontal axis in years. Damage occurs as hyperglycemia induces morphological changes in the glomerulus, manifesting as hyperfiltration, hypertension and microalbuminuria. Over a prolonged period the damage spreads to the tubulointerstium and results in advanced kidney injury. This manifests as reduced GFR, macroalbuminuria, and metabolic and other complications of advanced kidney disease. Taken from Alicic et al. (2017) [10]

Factors and signaling cascades involved in DN

The pathophysiology of DN extends from a complex interplay of hemodynamic and metabolic factors that occur due to the diabetic milieu [10]. Many of these pathways converge around the activation of a plethora of growth factors such as transforming growth factor (TGF)-β that ultimately drive the loss

of kidney function [13]. A short summary of key DN pathways are listed below (summarized in **Figure 1-3**). **Hemodynamic factors:** Early hemodynamic changes that occur due to DN include hyperfiltration and hyperfusion [13]. These hemodynamic changes lead to elevated intraglomerular capillary pressure and manifest as damage to the glomerulus (GBM thickening, expansion of the mesangium) [13]. **Oxidative Stress:** Hyperglycemia has been shown to induce the formation of reactive oxygen (ROS) and nitrogen species (RNS) in the kidney [13, 14]. ROS are reactive chemical species that contain oxygen, whereas RNS contain nitrogen. Sources of ROS production in the kidney include the mitochondria, expression of NAPDH oxidase enzymes, and depletion of antioxidants (such as glutathione), whereas RNS may originate from the expression of endothelial nitric oxide synthase [13, 14]. In turn, ROS and RNS may drive vascular dysfunction (such as activation of the renin-angiotensin-aldosterone system (RAAS)) leading to kidney injury [13, 14]. **Advanced glycation end-products (AGEs):** AGEs are compounds that form when glucose reacts with amino groups in proteins, nucleic acids or lipids [13]. The reactions form Schiff bases which are further modified into Amadori products and finally into AGEs [13]. AGEs bind to the receptor for AGE (RAGE), which in turn trigger the expression of pro-inflammatory and pro-fibrotic signaling in the kidney [13]. **Inflammation:** Human biopsy samples and animal models have shown accumulation of inflammatory cells in the glomerulus and tubulointerstium with diabetes [13]. Expression of pro-inflammatory cytokines, such as monocyte

chemoattractant protein-1 (MCP-1) and RANTES, are known to be upregulated during the pathogenesis of DN [13]. In turn, the recruited inflammatory cells release proteases and profibrotic cytokines that ultimately drive DN [13]. **RAAS:** The RAAS system is best characterized in its regulation of hemodynamic factors such as blood pressure [13]. However, angiotensin II (Ang II) has been shown to have direct pro-fibrotic and pro-inflammatory effects that promote DN [13].

Cytokines and Growth Factors: Many of the metabolic and hemodynamic pathways described converge on the expression of key growth factors that drive pro-fibrotic or pro-inflammatory signaling [13]. Some of these growth factors include insulin-like growth factors, TGF-β, connective tissue growth factor (CTGF), and vascular endothelial growth factor (VEGF). [15]

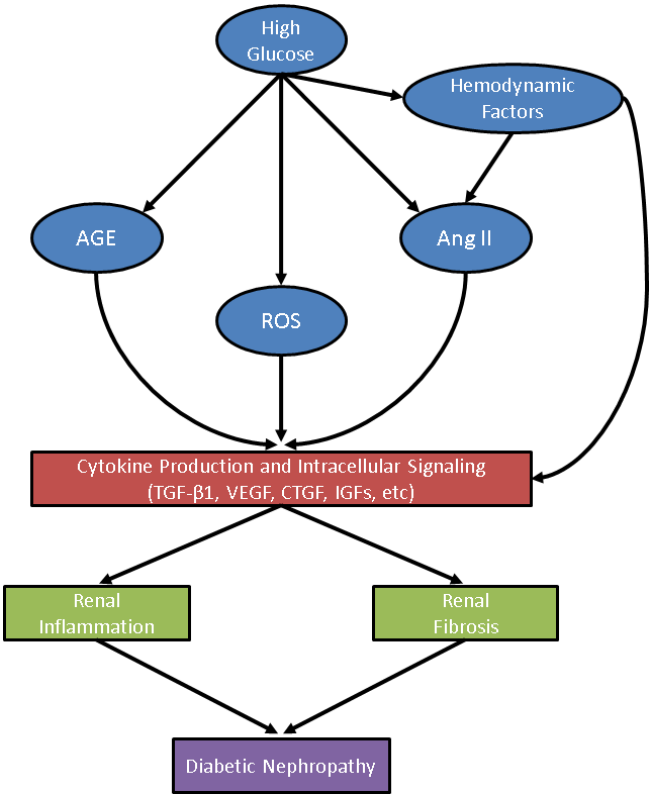

Figure 1-3 - Summary of mechanisms that promote the development of DN. The diabetic milieu stimulates hemodynamic changes, the production of metabolic (AGE, ROS) and hemodynamic (Ang II) changes that result in the production of pro-fibrotic and pro-inflammatory cytokines. These pathways ultimately result in induction of renal fibrosis and inflammation, leading to the development of DN.

MCs are mediators of glomerular damage in DN

MCs are found in the glomerulus and are important in the formation of the mesangium [16]. MCs contain contractile proteins that anchor them to the GBM and are in direct contact with endothelial cells [16]. In response to stretch or other factors that affect the mesangium, the MCs secrete a wide range of growth factors including TGF-β1, CTGF, and VEGF [16]. These factors allow MCs to cross-talk to endothelial cells and podocytes generating a coordinated response to stress in the glomerulus [16].

During the early pathogenesis of DN, glomerular hypertension occurs resulting in stretching of the MCs [16]. This leads to the release of growth factors and cytokines cumulating in the production of ECM [16]. This response manifests as MC hypertrophy, proliferation, and expansion of the mesangium [16]. Furthermore, the release of growth factors and cytokines may trigger a pro-inflammatory response by recruitment of leukocytes, and further exacerbation of

the profibrotic response in the glomeruli [16]. These pathways highlight the importance of MCs as mediators of DN due to their role in responding to early glomerular changes [16].

Current Treatments and Future Prospects of DN

Current treatment of DN focuses on glycemic control and inhibition of RAAS, which are aimed at slowing the progression of the disease [6]. Intensive glycemic control in patients with DN has been shown to reduce the risk of developing micro and macro-albuminuria, and development of ESRD [17-20]. However, intensive glycemic control has also been associated with increased cardiovascular mortality rates in high risk populations [21]. Similarly, RAAS inhibition reduces risk of ESRD and other clinical parameters associated with DN [22, 23]. However combination therapy using multiple RAAS inhibitors was associated with increased mortality, and risk of kidney injury [24, 25]. These trials have demonstrated that although current therapy is effective in reducing risk of ESRD, there still remains much room for the development of novel therapies to better manage DN with fewer severe adverse effects.

Transforming growth factor β (TGF-β)

TGF-β belongs to the TGF-β subfamily of the TGF-β superfamily [26]. The TGF-β subfamily regulates a wide variety of biological processes including cell growth, differentiation, adhesion, proliferation, tissue repair, morphogenesis, and apoptosis [27, 28]. In this subfamily, five TGF-β isoforms have been

identified in vertebrates, however TGF-β 1-3 have been shown to be expressed in mammals [29]. TGF-β1 is the dominant isoform in the kidney and evokes responses through signaling cascades involving the small mothers against decapentaplegic (SMAD)s, mitogen activated protein kinases, extracellular regulated kinase, p38 and Jun kinase [30]. In the context of kidney disease, activity of TGF-β1 has been strongly associated with renal fibrosis through three processes: (1) Increased production of ECM proteins such as type I and IV collagen, fibronectin, and laminin; (2) Decreased matrix degradation by upregulation of matrix metalloprotease inhibitors; and (3) Increased epithelial-mesenchymal transition (EMT) in the kidney leading to a pro-fibrotic phenotype associated with elevated ECM production [31, 32].

TGF-β1 Signaling

TGF-β1 is synthesized as a propeptide precursor that contains a prodomain (latency associated peptide (LAP)) and a mature domain [33, 34]. The LAP is cleaved but remains associated with the mature domain to form the small latent complex (SLC) [33, 34]. The SLC interacts with other latent TGF-β binding proteins (LTBP) to form the large latent TGF-β complex found in the ECM [33, 34]. Activation of TGF-β occurs due to proteases or environmental cues which release TGF-β from the LTBP, and allows for activation of TGF-β1 signaling [33, 34].

Canonical TGF-β1 signaling occurs through activation of the serine/threonine kinase type II (TβRII) and type I (TβRI) TGF-β receptors [34,

35]. Under basal conditions, TβRII and TβRI are found as homodimers on the cell surface [34, 35]. TGF-β1 binds and activates TβRII and promotes its association with TβRI [34, 35]. Activated TβRII phosphorylates TβRI on a GS sequence, leading to activation of TβRI [34, 35]. The receptor-ligand complex is then internalized through endosomes, and brought into close proximity with the receptor smads (R-Smads) Smad2 and Smad3 through interaction with the smad anchor for receptor activation (SARA) [34, 35]. TβRI interacts with the R-Smads leading to phosphorylation of the R-Smads on a conserved C-terminal SSXS motif [34, 35]. The activated R-Smads disassociate from TβRI and interact with the Co-Smad Smad4 [34, 35]. The R-Smad/Co-Smad complex is localized into the nucleus and drives the expression of TGF-β responsive genes through interaction with Smad binding elements (SBEs) [34, 35]. The R-Smads co-operate with many other coactivators such as CBP and p300 to generate a complicated mesh of regulation of target genes [34, 35].

Regulation of TGF-β1 signaling has been suggested to occur at many levels, offering a stringent method to control downstream events (summarized in **Figure 1-4**). **De-phosphorylation:** PPM1A, MTMR, PP2A, and PP1 have been identified as phosphatases for Smad2, Smad3 and TβRI [36, 37]. These phosphatases act to terminate TGF-β signaling by de-phosphorylating the R-Smads and TβRI [36, 37]. **Competitive Inhibition of TβRI:** Smad7 is an inhibitory Smad protein known to regulate TGF-β signaling in the kidney [38]. Smad7 actively competes with R-Smads (Smad2 and Smad3) for association with

TβRI, thus preventing activation of the R-Smads [38]. **Degradation:** Proteasomal and lysosomal degradation have been shown to regulate TGF-β signaling by controlling the expression of the R-Smads and TβRI [35]. Proteasomal degradation of TβRI and R-Smads is mediated by ubiquitination followed by recruitment of the proteasome [35]. In addition, TβRI has been shown to undergo lysosomal degradation which is dependent on the localization of TβRI into specialized vesicles capable of degradation referred to as lysosomes [39]. **Ectodomain Shedding:** In a process referred to as ectodomain shedding, metalloproteases such as TNFα converting enzyme (TACE) have been shown to cleave cell surface receptors, resulting in the release of the receptor's extracellular and intracellular domains [40]. TACE has been identified as a protease capable of selectively cleaving TβRI, which has been proposed to terminate TGF-β signaling [40]. **Endocytic regulation of TβRI:** The TβRII-TβRI receptors complexes are endocytosed and recycled to the plasma membrane by many endocytic pathways [40]. Clathrin-mediated endocytosis of the TβRII-TβRI receptors complexes is associated with activation of canocial TGF-β signaling [41]. Alternatively, the TβRII-TβRI receptors complexes may be internalized by lipid raft and/or caveolae endocytosis [41]. Endocytosis by lipid rafts has been associated with non-canonical TGF-β signaling and the degradation of the complex [42]. **Exosomal regulation of TβRI:** A novel mechanism of protein regulation has been suggested to occur by secretion of proteins in vesicles referred to as exosomes. Exosomes are microvesicles 30 to 100nm in size, consisting of a lipid bilayer containing

proteins and RNA [43, 44]. Secretion of proteins in exosomes has been shown to be a form of regulation in reticulocyte maturation and p53 signaling [45, 46]. Our data has suggested that TβRI may be localized and secreted in exosomes, as a novel form of protein regulation [47].

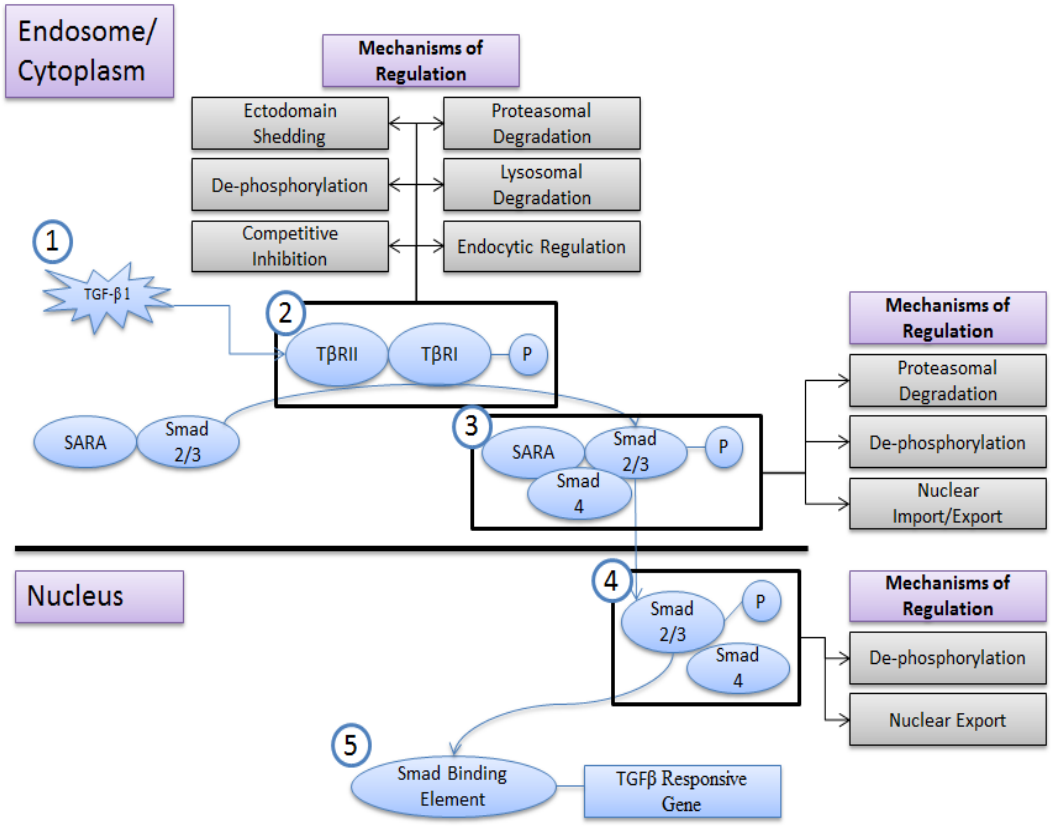

Figure 1-4 - Canonical TGF-β1 signaling and its regulation.

TGF-β1 binds to TβRII, causing association and cross-phosphorylation of TβRI. R-Smads (Smad2/3) are recruited to TβRI by interaction with SARA. This facilitates the phosphorylation of Smad2/3 and allows for their association with the Co-Smad (Smad4) leading to nuclear translocation of the protein complex. Within the nucleus, the phosphorylated R-Smads bind to Smad Binding Elements

in target genes, and drive expression of TGF-β responsive genes. Regulation of TGF-β1 signaling occurs at many levels that act to target the activation and levels of TβRII, TβRI, and the R-Smads.

TGF-β1 in DN

TGF-β1 expression and activation has been well described as an important pathway associated with the development of renal fibrosis in DN [48] .Furthermore many stimuli associated with the diabetic milieu, such as high glucose and AGE, are known to upregulate TGF-β1 expression and signaling in the kidney [49, 50]. The importance of TGF-β1 in the development of DN was recently studied by Hathaway et al [51]. This group generated mice with a graded mRNA expression of TGF-β1 (10-300% expression). Mice with low expression of TGF-β1 were generated through addition of the 3'-UTR of Fos onto the TGF-β1 allele, resulting in unstable TGF-β1 mRNA and reduced TGF-β1 expression [52]. This allele was converted to a high-expressing variant through exposure to Cre, leading to expression of the TGF-β1 allele with the 3'-UTR from bovine growth hormone [52]. This results in more stable TGF-β1 mRNA and elevated TGF-β1 expression [52]. These mice were crossed with Akita mice that contain a mutation in their insulin gene, resulting in type I diabetes, to generate diabetic mice with graded expression of TGF-β1 [51]. Hathaway et al. demonstrated that high expression of TGF-β1 exacerbated the DN phenotype (reduced GFR and

albuminuria), whereas reduced expression ameliorated the disease phenotype [51].

However, several studies have indicated that the pleiotropic effects of TGF-β1 make directly targeting it a difficult prospect. As noted previously, Smad2 and Smad3 are key mediators of TGF-β1 signaling, however their roles in mediating renal fibrosis differ. Type I diabetic mice knocked out for Smad3 demonstrated an improvement in markers for DN (such as renal hypertrophy, mesangial matrix expansion, GBM thickening), indicating a pathological role for Smad3 [53]. However, knockout of Smad2 in tubular cells exacerbated renal fibrosis in the unilateral ureteral obstruction (UUO) model and was shown to induce the expression of ECM proteins *in vitro*, indicating a protective role for Smad2 [54]. Furthermore, concerns have also been raised due to the anti-inflammatory actions of TGF-β1. Lan et al. has suggested that ectopic TGF-β1 expression prevented renal inflammation in DN and UUO models [32]. These studies suggest that targeting specific components of TGF-β1 signaling may be more beneficial than attempting to globally target TGF-β1.

Clinical trials into the effects of anti-TGF-β therapy on the development of DN have been slow in development [55]. A trial was conducted into the efficacy of Pirfenidone which acts as an anti-fibrotic (partially due to TGF-β1 inhibition) and anti-inflammatory agent, in a small population of type I and II diabetic patients with DN [56]. The study demonstrated that treatment with Pirfenidone improved estimated GFR (eGFR), but did not improve albuminuria [56].

Treatment with Prifenidone was associated with several adverse effects such as nausea, dyspepsia, and diarrhea [56]. By the end of the study, 8 (of the 51 patients) in the Pirfenidone treated groups withdrew from the study, while 1 (of 26 patients) in the placebo group withdrew [56]. More recently, a trial was conducted by Eli Lilly and Company on the efficacy of LY2382770 (monoclonal antibody against TGF-β1) in patients with DN [57]. However, the study was terminated due to lack of efficacy of treatment [57]. Further research into the efficacy of anti-TGF-β therapy in the treatment of DN is required before a final decision may be made, but the currently available data suggest that directly targeting TGF-β may not be efficacious in the treatment of DN [57].

Sterol regulatory element binding protein (SREBP)

The sterol regulatory element binding protein (SREBP)s belong to a family of basic helix-loop-helix-leucine zipper transcription factors that are important in the regulation of cholesterol and lipid metabolism [58]. Three SREBP isoforms are expressed in mammals: SREBP-1a, SREBP-1c, and SREBP-2 [59, 60]. SREBP-1a and SREBP-1c are under the regulation of separate promoters that produce an mRNA sequence with a different amino terminal [61]. As a result, SREBP-1a has a longer N-terminus domain which has been suggested to mediate interaction with co-factors such as CBP and thus act as a potent transcription factor. When SREBP-1a, 1c, and 2 are expressed at similar levels, SREBP-1c stimulates promoter activity 10-fold less than SREBP-1a and 2

[58]. This suggests that SREBP-2 and SREBP-1a activation domains are similar in strength, whereas SREBP-1c is a less potent transcription factor [58].

SREBP-1a is a regulator of fat metabolism and is the predominant isoform in cell culture, whereas SREBP-1c preferentially activates fatty acid synthesis genes and is the predominant isoform in most tissue [58]. SREBP-2 is important for cholesterol metabolism and is ubiquitous in expression [58].

SREBP Signaling

The SREBPs are synthesized and retained in the endoplasmic reticulum (ER) as large transcriptionally inactive precursor proteins [62]. Under basal conditions, SREBPs are retained in the ER through interaction with SREBP-cleavage activation protein (SCAP) and insulin-induced gene (Insig), two anchor proteins which retain SREBP in the ER [62]. There are two isoforms of Insig present in mammals, Insig-1 and Insig-2 [62]. Insig-1 is an unstable protein that is degraded by the proteasome pathway, whereas Insig-2 is stable and expressed at a lower level than Insig-1 [62]. Insig-1 has been most studied in the regulation of SREBP signaling and is an ER membrane protein that directly interacts with SCAP [62]. Interaction of Insig-1 with SCAP retains the SCAP-SREBP complex in the ER [62]. SCAP directly interacts with the SREBPs and contains a sterol-sensing domain that can directly bind cholesterol [62].

In response to stressors such as cholesterol deprivation and ER stress, SREBP is known to be activated. In the setting of cholesterol deprivation, SCAP undergoes a conformational change, leading to SCAP and Insig-1 release of

SREBP [62]. Insig-1 is marked for degradation by the ubiquitin-proteasome pathway, and an interaction motif in SCAP containing the MELADL sequence is revealed [62]. SCAP may then bind to Sec24, a subunit of COPII, which facilitates the translocation of the SCAP-SREBP complex from the ER to the Golgi [62]. At the Golgi, the membrane-bound proteases site-1 protease (S1P) and site 2 protease (S2P) cleave the precursor SREBP protein [62]. S1P cleaves within the luminal loop of the protein while S2P cleaves SREBP on the membrane spanning domain [62]. The proteolytic cleavage of SREBP results in the release of the N-terminal portion of SREBP [62].

ER stress occurs when unfolded or nascent proteins accumulate in the ER or when an impairment occurs to the proper protein folding machinery [63]. SREBP-1 and -2 are known to be activated in response to ER stress through three potential mechanisms: caspase-induced SREBP cleavage (via caspase-3 and caspase-7), ER stress-mediated degradation of Insig, and 78 kDa glucose regulated protein (GRP78) separation from SCAP-SREBP [64].

Activation of SREBP allows the N-terminal fragment of SREBP to act as a transcription factor and is targeted to the nucleus [62]. Active SREBPs drive expression of genes with sterol-response elements (SRE) or E-box motifs through interaction with cofactors such as NF-Y, CBP, and Sp1 [62, 65]. Interestingly, SRE elements are found in the promoters of SREBP and Insig-1 [66]. Thus, SREBP is capable of inducing its own expression and that of Insig-1, participating in a complicated feedback loop [66]. Furthermore, SREBPs are well known

regulators of genes involved in cholesterol and fatty acid biosynthesis such as the low density lipid receptor (LDLR), HMG-CoA reductase (HMGCR), and proprotein convertase subtilisin/kexin type 9 (PCSK9) [64].

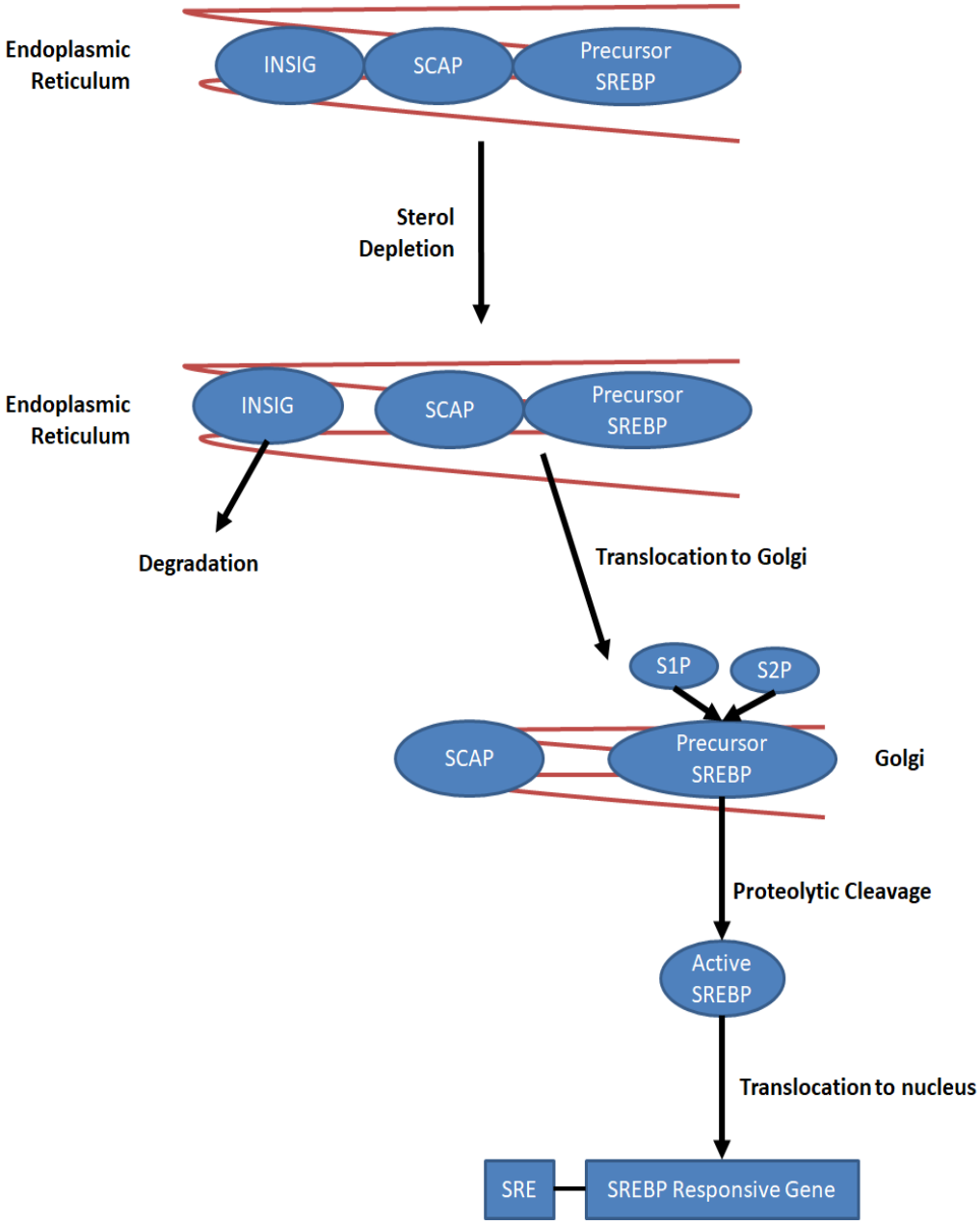

Figure 1-5 – Regulation of SREBP signaling.

Under basal conditions, SREBP is retained in the ER through interaction with SCAP and INSIG. When cells are deprived of cholesterol or undergo ER stress, the SCAP-SREBP complex disassociates from INSIG leading to SCAP-SREBP

translocation to the Golgi in COP II vesicles. In the Golgi, SREBP is cleaved by S1P and S2P, leading to the formation of active SREBP. Active SREBP is translocated into the nucleus, and acts as a transcription factor by binding to SRE elements in SREBP responsive genes.

SREBP in DN

Dyslipidemia is a state of high levels of low-density lipoprotein (LDL) cholesterol, or low levels of high-density lipoprotein (HDL) cholesterol, and has been linked to kidney damage and the development of DN [67]. However, the specific mechanisms by which dyslipidemia contributes to DN have not been well described. In the kidney, dyslipidemia may manifest as ectopic deposition of lipids in the kidney which has been demonstrated in diabetic humans and animals [68-70]. Lipid deposition in the kidney has been associated with glomerulosclerosis *in vivo*, and with the induction of profibrotic and proinflammatory signaling *in vitro* [71-74].

In diabetic rodents, the expression of SREBP-1 and SREBP-2 are concomitantly increased with the presence of lipid droplets suggesting that the SREBPs may be responsible for dyslipidemia in the kidney. The upregulation of SREBPs occurs primarily in renal tubules, but is also present in glomeruli [75, 76]. A correlation between SREBP-1 expression and DN was suggested by Sun et al [77]. The authors demonstrated that in a 2-week rat model of type I diabetes, lipid deposits occur in the kidney, even though serum cholesterol and triglyceride

levels were not elevated [77]. The increased kidney lipid deposits were associated with increased expression of SREBP-1 [77]. Furthermore, Sun et al. further showed that overexpression of SREBP-1a in the kidney led to albuminuria and glomerulosclerosis with a concomitant increase in profibrotic markers (TGF-β, type IV collagen, fibronectin) [77]. The role of SREBP-1 in the development of DN has also been recently supported by Ishigaki et al [78]. In this study, Ishigaki et al. overexpressed SREBP-1c in the liver and kidneys of mice [78]. Following induction of type I diabetes, overexpression of SREBP-1c led to a more profound DN phenotype that the controls and was associated with the increased expression of profibrotic markers (TGF-β, fibronectin, type IV collagen) [78]. Furthermore, they showed that type I diabetic mice knocked out for SREBP-1 did not develop a DN phenotype [78]. These results suggest that SREBPs may play a role in regulating profibrotic signaling in the kidney during the pathogenesis. Previous studies from our lab have indicated that in response to diabetic stimuli, activation of SREBP-1 is important for the upregulation of TGF-β1 in MCs [79]. Furthermore, we found that SREBP-1a interacted and facilitated the transcriptional activity of Smad3 [80].

These results indicate that increased expression of SREBP-1 in the kidney may facilitate the development of a profibrotic environment in the kidney that contribute to progressive renal failure in DN. Targeting SREBP-1 and SREBP-2 *in vivo* may provide novel therapeutic targets that are well suited for the treatment of DN due to their role in dyslipidemia and profibrotic signaling.

Glucose regulated protein 78 (GRP78)

78 kDa glucose regulated protein (GRP78) belongs to the heat shock protein family that is induced during conditions of glucose starvation [81]. GRP78 is best described as a 78 kDa ER chaperone protein that is responsible for maintaining ER homeostasis by assisting in proper protein folding and by causing the degradation of misfolded proteins [63]. GRP78 is evolutionarily conserved from yeast to humans. GRP78 maintains ER homeostasis by regulating the cellular response to ER stress, referred to as the unfolded protein response (UPR) [63].

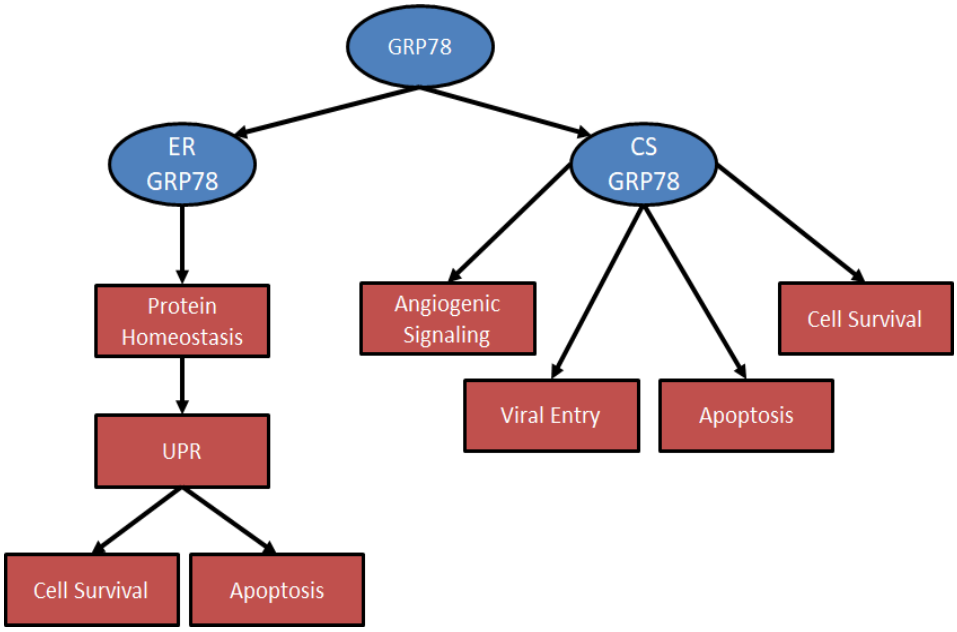

Figure 1-6 – Role of endoplasmic reticulum and cell surface GRP78.
The actions of GRP78 differ based on its intracellular location. GRP78 in the ER acts to maintain protein homeostasis. Under conditions of ER stress, GRP78 leads to the activation of the UPR in attempt to re-establish homeostasis. GRP78 at the

cell surface acts as a receptor that may facilitate viral entry into cells, or activation of signaling cascades involved in cell survival, apoptosis and angiogenesis.

Subcellular localizations of GRP78

ER stress has been shown to play a pathological role in the development of many diseases including DN [82]. Due to the pathological role of ER stress, cells retain a set of signaling pathways referred to as the UPR that attempt to maintain ER homeostasis by reducing the overall accumulation of proteins (by halting translation and degrading proteins) and increasing protein folding capacity in the ER [82]. If ER stress cannot be alleviated and persists the UPR may induce apoptotic pathways as well [82]. GRP78 is the master regulation of the mammalian UPR through interaction with three transmembrane ER stress sensors: inositol-requiring kinase 1 (IRE1), activating transcription factor 6 (ATF6), and PKR-like eukaryotic initiation factor 2α kinase (PERK). [81] When cells undergo ER stress, GRP78 dissociates from these three proteins, leading to the activation of the UPR [81].

GRP78 has also been shown to be localized to the cytoplasm, mitochondria, nucleus, and may be secreted from the cell [83]. Cytosolic GRP78 is formed from alternative splicing of GRP78 mRNA, resulting in a truncated GRP78 [83]. Cytosolic GRP78 has been suggested to regulate ER stress signaling, apoptosis, and viral entry [83]. Localization of GRP78 into the mitochondria has been suggested to occur in conditions of stress such as ER stress [83]. Within the

mitochondria, GRP78 may interact with GRP75 and regulate mitochondrial function (such as energy usage and mitochondrial homeostasis) [83]. GRP78 localization into the nucleus has been shown to occur when GRP78 is overexpressed or during ER stress [83]. Nuclear GRP78 has been shown to cross-link with DNA and has been suggested to play a role in DNA damage-mediated apoptosis [83]. Secretion of GRP78 was shown to occur in tumors, and has been identified in the sera of patients with gastric cancer [83]. It may act as a ligand for endothelial cell surface receptors and play a role in pro-survival signaling [83].

The expression of cell surface GRP78 (csGRP78) was initially described in an immortalized B lymphocyte line. In this experiment, Berger et al. were able to find a 78 kDa protein at the cell surface that was identified as GRP78 [84]. Since then studies have described the expression GRP78 at the cell surface from various cancer cell lines [83]. The actions of csGRP78 are variable and dependent on the cell on which it is expressed [83]. In tumor and endothelial cells, csGRP78 can trigger pro-apoptotic or pro-survival pathways and was shown to be important in activation of coagulation cascades [83]. In the context of viral infections, csGRP78 acts as receptor for viral entry [83].

Translocation of GRP78 to the cell surface

The precise mechanism by which GRP78 translocates to the cell surface remains to be elucidated; however, current studies highlight the importance of the KDEL sequence in GRP78 trafficking to the cell surface [83]. The KDEL

sequence is found at the C-terminal of GRP78 and allows GRP78 to interact with the KDEL receptors in the ER [83]. Interaction between GRP78 and the KDEL receptors retains GRP78 in the ER lumen, and thus prevents its trafficking to other locations [83]. Deletion of KDEL from GRP78 has been shown to induce the cell surface localization of GRP78, and to play a role in the secretion of GRP78 [83]. From these observations, two potential mechanisms have been suggested: (1) Production of GRP78 during cellular stress overwhelms the ER retention capacity of the KDEL sequence thereby GRP78 is shunted to the cell surface; and (2) Modification of the KDEL motif or other nearby sequences masks the KDEL sequence and allows transport to the cell surface [83].

GRP78 is also known to interact with other protein partners such as the DnaJ-like transmembrane protein MTJ-1 and the proteinase-activated receptor PAR4 [83]. Interaction with these proteins have been shown to be important for the cell surface localization of GRP78, suggesting that co-chaperone proteins are required for csGRP78 expression [83].

A recent study by Tsai et al. revealed that GRP78 is retained at the cell surface through interaction with GPI-anchored proteins [85]. GRP78 contains an ATPase binding domain, required for its ATPase activity, and a substrate binding domain required for its interaction with other protein [85]. Mutation of the substrate binding domain of GRP78 was found to attenuate cell surface localization of GRP78 [85]. However, mutation of the ATP binding domain did not affect the cell surface expression of GRP78 [85]. This provides further

evidence that cell surface localization of GRP78 requires interaction with other proteins [85]. At the cell surface, the N-terminal, the C-terminal, and a middle segment of GRP78 are exposed [83]. Tsai et al. have suggested that at the cell surface, two pools of GRP78 may exist [85]. One pool may be anchored to the cell surface through interaction with a GPI-anchored protein, thus the entirety of GRP78 would be exposed [85].

Signaling events regulated by cell surface GRP78

The cellular signaling events triggered by csGRP78 have been best described in tumor cells. In tumor cells, α2 macroglobulin (α2M) has been well-described as a ligand for csGRP78 [86-89]. Canonically, α2M has been described as an antiproteinase that binds and inhibits a wide range of proteinases. After α2M reacts with a proteinase, the receptor-binding domain of α2M is exposed, leading to enhanced biological activity as a ligand [90]. α2M activation of csGR78 promotes cellular proliferation and survival by stimulating ERK1/2, p38 MAPK, PI3K, Akt, and NF-kB signaling [83].

α2M has been reported to bind to the N-terminal domain of GRP78. The use of autoantibodies against the N-terminal domain of GRP78 showed similar pro-survival effects on *in vitro* tumor cell lines [89]. Conversely, the C-terminal domain of csGRP78, which may be activated by C-terminal targeting GRP78 autoantibodies, has been associated with pro-apoptotic signaling through upregulation of p53 and suppression of NF-kB, PI3K/Akt and Ras/MAPK

signaling [91-93]. These results suggest that activation of csGRP78 signaling is further dependent on which domain of GRP78 is activated.

csGRP78 may also signal through interaction with other receptors on the cell surface such as Cripto and integrin β1 [94]. Cripto is a GPI-anchored protein found on the cell surface that regulates signaling by members of the TGF-β family [94]. Cripto and GRP78 have been suggested to interact at the cell surface, leading to inhibition of Smad2/3 signaling in response to members of the TGF-β family [83, 95]. Integrin β1 is a cell surface receptor that is important in regulating signaling at focal adhesion points through activation of focal adhesion kinase (FAK). In MCs, the integrin heterodimer α5β1 has been shown to regulate the deposition of the ECM protein fibronectin; these results suggest that integrin β1 may play a role in ECM accumulation [96]. csGRP78 has been suggested to interact with integrin β1 in colorectal tumor cells, which in turn activates FAK signaling and cell migration and invasion [95]. Thus csGRP78 interaction with integrin β1 may be of relevance in ECM turnover in MCs.

Preliminary results from our laboratory have suggested that csGRP78 may play a role in the activation of SREBP-1 in kidney MCs. Treatment of MCs with inhibitors of csGRP78 prevented high glucose-mediated SREBP-1 activation. Furthermore, our results suggest that the cell surface expression of GRP78 may require ER stress. Thus, an attractive hypothesis is that under diabetic conditions, GRP78 is translocated to the cell surface due to high glucose-induced ER stress.

At the cell surface, GRP78 may activate SREBP-1 through activation of Akt signaling.

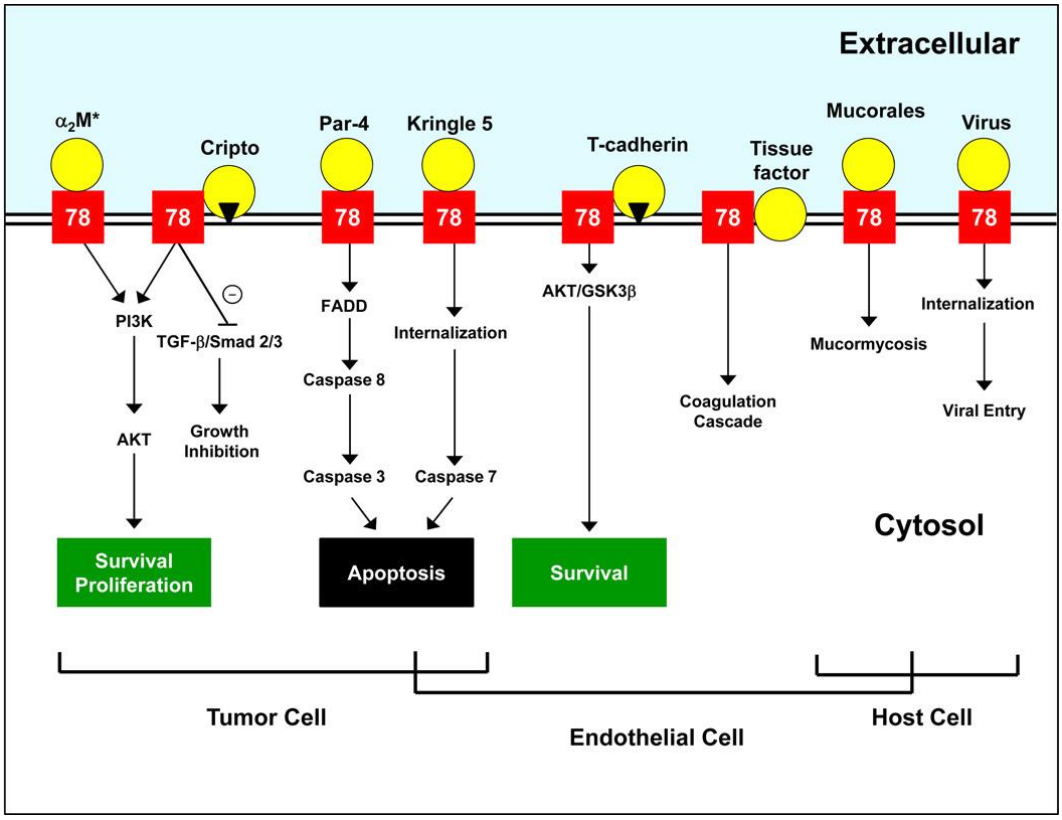

Figure 1-7 - Summary of signaling pathways regulated by csGRP78. csGRP78 may act as a receptor for extracellular ligands (α2M, Par-4, Kringle 5) and a co-receptor for cell surface receptors (Cripto, T-cadherin). Activation of these pathways lead to pro-survival or pro-apoptotic signaling depending on the cell type involved. Taken from Ni et al. (2011) [83]

Cell surface GRP78 as a therapeutic target

GRP78 was identified as an antigen that was highly expressed in patients with advanced prostate cancer in comparison to healthy patients [83]. This suggests that targeting GRP78 may be useful for treatment, and thus csGRP78 may offer a new method for ligand-directed therapy in cancer patients. GRP78 targeting peptides (WDLAWMFRLPVG and WIFPWIQL) have been previously used to identify csGRP78 *in vitro* and *in vivo* through IHC and IF [97]. Furthermore, conjugation of the GRP78 targeting peptides with a proapoptotic signal was capable of reducing tumor volume in mice with prostate or breast cancer [97]. More recently, GRP78 targeting peptides (WIFPWIQL and SNTRVAP) have been used to identify and treat aggressive variant prostate cancers and inflammatory breast carcinoma respectively [98, 99]. The authors expressed the GRP78 targeting peptides on adeno-associated virus/phage particles (AAVP) carrying the suicide gene HSVtk [98, 99]. Animals treated with the GRP78-targetting peptides with AAVP demonstrated reduction in tumor size [98, 99]. Future studies in the use of AAVP are still ongoing and are aimed at further optimization [100]. These data suggest that targeting csGRP78 *in vivo* is feasible. Furthermore, the use of csGRP78 targeted genetic therapy may be advantageous in the treatment of kidney disease by targeting pathological tissue and cells and inhibiting the gene expression of well-known pro-fibrotic or pro-inflammatory factors.

Main Objective

DN is the leading cause of ESRD worldwide, however current therapies are not capable of preventing disease progression in most patients. [6] TGF-β1 has been shown to play a key role in the progression and development of DN, and attempts have been made at targeting TGF-β1 for the treatment of DN in human clinical trials. [27, 30, 101] However, anti-TGF-β1 treatment has been associated with poor efficacy, or a wide range of side effects. [56, 57] Thus novel therapy aimed at modulating TGF-β1 expression or activity (rather than directly targeting the cytokine) may prove efficacious in the treatment of DN.

Research from our laboratory has demonstrated that SREBP-1 can regulate TGF-β1 signaling and expression in MCs. In these studies SREBP-1 may bind to the promoter of TGF-β1 (leading to increased TGF-β1 expression) and directly interact with Smad3, resulting in increased TGF-β1 signaling [79, 80]. Our first study is aimed at understanding if SREBP-1 can regulate TGF-β1 outside of transcriptional regulation, in the hopes of identifying new potential therapeutic targets. Furthermore, research from our lab has demonstrated that inhibiting SREBP activity with the small chemical inhibitor Fatostatin is capable of preventing renal fibrosis in a hypertensive and UUO model of kidney disease [102, 103]. It has also been shown that diabetic mice lacking SREBP-1 were protected from the development of early DN [78]. Our second study is aimed at determining the efficacy of the SREBP inhibitor Fatostatin in the treatment of DN.

Preliminary results from our laboratory have suggested that in response to high glucose, csGRP78 may activate SREBP-1 in kidney MCs. Furthermore, our results suggest that the cell surface expression of GRP78 may require ER stress. Since SREBP-1 has been shown to play a role in profibrotic signaling in the kidney, the aim of our study was to determine the role of csGRP78 in regulating profibrotic signaling in diabetic kidneys. However, csGRP78 has not been previously studied in the context of diabetes or kidney disease. It is known to play an important role in the pathogenesis of cancer and viral entry into cells [83]. Some studies have indicated that csGRP78 may interact with members of the TGF-β1 signaling family such as Cripto and the LAP of TGF-β1 [94, 104]. These studies suggest that csGRP78 may regulate TGF-β1 signaling in other tissues. The aim of our third study is to determine if csGRP78 is expressed as a result of the diabetic milieu, and if modulating csGRP78 expression will affect kidney function and health.

The purpose of my thesis is to: 1) Determine new cell signaling pathways (SREBP and csGRP78) that may regulate TGF-β1 signaling; and 2) Evaluate the significance of targeting these pathways as novel therapies for DN.

Research Aims

1) Determine how SREBP-1 regulates the TβRI and downstream TGF-β1 signaling.
2) Evaluate the efficacy of the SREBP inhibitor Fatostatin in the treatment of DN *in vivo*

3) Study the role of csGRP78 in DN and evaluate its importance in regulation of TGF-β1 signaling

Hypothesis

I hypothesized that SREBP-1 and csGRP78 positively regulate TGF-β1 signaling in MCs, leading to the progression and development of DN. Furthermore, targeting SREBP in vivo should attenuate DN. I also hypothesize that csGRP78 is a novel marker of DN.

Study Findings

1) SREBP-1 regulates the expression of TβRI through exosomal secretion
2) Inhibition of SREBP *in vivo* with Fatostatin does not affect the development of diabetic nephropathy
3) csGRP78 is a novel marker of DN

Chapter 2 – SREBP-1 1 is a novel regulator of TβRI through exosomal secretion

Title: Sterol Regulatory Element Binding Protein (SREBP)-1 is a novel regulator of the Transforming Growth Factor (TGF)-β receptor I (TβRI) through exosomal secretion

Authors: Richard Van Krieken, Guang Chen, Bo Gao, Jolene Read, Hassan A. Al Saleh, Khalid Al-Nedawi, Joan C. Krepinsky

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Preface

Significance to thesis

TGF-β1 is a key mediator of renal fibrosis during the pathogenesis of DN. Previous research from our lab has identified SREBP-1 as a regulator of TGF-β1 expression and signaling through its actions as a transcription factor. MCs have been recognized as an important intermediary in communication for cells in the glomerulus. The purpose of this study was to determine if SREBP-1 was capable of regulating TGF-β1 independent of its actions as a transcription factor in MCs. These results would demonstrate novel pathways by which SREBP-1 is capable of regulating TGF-β1 signaling, and reveal new potential therapeutic targets that may be developed to target TGF-β1. As noted earlier, anti-TGF-β1 has demonstrated mixed to lacking efficacy and has been suggested to have a wide range of adverse effects. Therapy aimed at modulating pathways involved in TGF-β1 expression may thus prove beneficial in the treatment of DN. Our results demonstrate that SREBP-1 regulates TGF-β1 signaling through TβRI by exosomal secretion. These results suggest that targeting SREBP-1 may prove beneficial in the treatment of DN by attenuating TGF-β1 signaling.

Author's contribution

Richard Van Krieken conducted most of the experiments, analyzed the results, and prepared the first draft of the manuscript.

Guang Chen conducted the experiments using the $CAGA_{12}$ and SBE_4 luciferase in figure 6C and 6D.

Bo Gao assisted in cell culture experiments.

Jolene Read, Hassan A. Al Saleh, and Khalid Al-Nedawi assisted in the extraction and purification of exosomes from MCs.

Joan Krepinsky conceived of the project and edited the manuscript.

Abstract

Accumulation of matrix in the glomerulus is a classic hallmark of diabetic nephropathy. The profibrotic cytokine transforming growth factor beta 1 (TGF-β1) plays a central role in the development of glomerular sclerosis. Recent studies have demonstrated that the transcription factor sterol regulatory element binding protein (SREBP)-1 is an important regulator of glomerular sclerosis through both induction of TGF-β1 as well as facilitation of its signaling. Here we have identified that SREBP-1 is also a novel regulator of TGF-β receptor I (TβRI) expression in kidney mesangial cells. Inhibition of SREBP activation with fatostatin or downregulation of SREBP-1 using siRNA inhibited the expression of the receptor. SREBP-1 did not regulate TβRI transcription, nor did it induce its proteasomal or lysosomal degradation or proteolytic cleavage. Disruption of lipid rafts with cyclodextrin, however, prevented TβRI downregulation. This was not dependent on caveolae since SREBP-1 inhibition could induce TβRI downregulation in caveolin-1 knockout mesangial cells. SREBP-1 associated with TβRI, and SREBP-1 inhibition led to the secretion of TβRI in exosomes. Thus, we have identified a novel role for SREBP-1 as a cell surface retention factor for TβRI in mesangial cells, preventing its secretion in exosomes. Inhibition of SREBP-1 *in vivo* may thus provide a novel therapeutic strategy for diabetic nephropathy which targets multiple aspects of TGFβ signaling and matrix upregulation.

Abbreviations: TGF-β1, transforming growth factor beta 1; SREBP-1, sterol regulatory element binding protein-1; TβRI, TGF-β receptor I

1. Introduction

Diabetic nephropathy is an important microvascular complication of diabetes characterized by glomerulosclerosis [101]. Glomerular mesangial cells (MC) are known to play a key role in the development of glomerulosclerosis through synthesis and regulation of extracellular matrix (ECM) proteins [16] . TGF-β1 has been identified as a key mediator of ECM accumulation in MCs [101]. A role for sterol regulatory element binding protein (SREBP)-1 has also been suggested, with its overexpression leading to glomerulosclerosis, and increased SREBP expression seen in diabetic nephropathy [77, 78]. We have recently demonstrated the importance of SREBP-1 in coordinating TGF-β1 signaling through its interaction with Smad3 [80]. However, whether SREBP-1 regulates TGF-β1 signaling upstream of Smad3 transcriptional activity is as yet unknown.

TGF-β1 signaling occurs through binding and activation of the type II receptor (TβRII), which in turn phosphorylates and activates the type I receptor (TβRI). TβRI then recruits and phosphorylates Smad2/3 on a conserved C-terminal SSXS motif. Activated Smad2/3 dissociate from the receptors, associate with Smad4, and migrate into the nucleus to activate TGF-β1 responsive genes [105]. Activation of Smad2/3 is controlled at multiple levels, including the

turnover of TβRI and TβRII. Expression of these receptors is known to be regulated dynamically through multiple mechanisms including transcription, translation, degradation, and proteolytic cleavage [37, 106, 107]. A role for SREBP-1 in the regulation of TβRI expression has not as yet been described.

SREBPs are a family of transcription factors that maintain cholesterol and lipid homeostasis. We have recently shown a role for SREBP-1 in matrix regulation through both a direct transcriptional effect on the TGF-β1 promoter as well as through its ability to enhance Smad3 transcriptional activity [79, 80]. Three SREBP isoforms are expressed in mammals: SREBP-1a and SREBP-1c are produced from alternative transcription start sites of the SREBP-1 gene, and SREBP-2 [59, 60]. SREBPs are synthesized and retained in the endoplasmic reticulum as large transcriptionally inactive precursor proteins. Canonically, SREBPs are activated in response to sterol deficiency, leading to their transport into the Golgi through interaction with SREBP-cleavage activation protein (SCAP). Within the Golgi, SREBPs are processed by membrane-associated site 1 (S1P) and site 2 (S2P) proteases which release the mature transcription factor. This is then translocated into the nucleus to drive expression of genes with sterol-response elements (SRE) in cooperation with transcriptional cofactors [65]. Although SREBP-1 regulation of TGF-β1 signaling has been attributed to transcriptional regulation, whether it can regulate TGF-β1 signaling independently of its transcriptional activity has not been previously explored.

In this study, we have identified a novel role for SREBP-1 as a cell surface retention factor for TβRI in MCs. Disruption of SREBP-1 leads to the downregulation of cell surface TβRI through its secretion in exosomes. This opens new avenues for the treatment of diabetic nephropathy by targeting SREBP-1.

2. Materials and Methods

2.1 Cell Culture

Primary MCs were isolated from male Sprague-Dawley (SD) rats and caveolin-1 wild-type and knockout mice as published [79, 108-111]. MCs were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100 μg/ml). Cells between passages 8 and 17 were used. R1BL17 cells (Mv1Lu cells lacking TβRI) [112] were a gift from Dr. Henis at Tel Aviv University. 293T and R1BL17 cells were cultured in a high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100 μg/ml). All cells were kept at 37˚C in 95% air, 5% CO_2. Cells were serum deprived at 80-90% confluence overnight, then treated with the following: Fatostatin (Chem Bridge), MG132 (Cayman), NH_4Cl (Sigma), Leupeptin (Sigma), GM6001 (Sigma), Cyclodextrin (Sigma), and TGF-β1 (Medicorp).

2.2 Protein Extraction

Whole cell expression of protein was determined as previously described [113]. Briefly, cells were lysed in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol and protease/phosphatase inhibitors. Cellular debris was cleared from cell lysate by centrifugation at 13,000 rpm for 10 minutes at at 4ºC.

Isolation of nuclear protein has been described previously [114]. Briefly, cells were lysed in a hypotonic lysis buffer and centrifuged at 500 rpm for 10 minutes. The pellet containing the nucleus was resuspended in whole cell lysis buffer and sonicated.

Proteins were separated by SDS-PAGE and Western blotting performed with antibodies against PDGFR (1:10000, Santa Cruz), TβRI (1:1000, Santa Cruz), TβRII (1:1000, Santa Cruz), Flotillin-1 (1:1000, Santa Cruz), SREBP-1 (1:1000, Santa Cruz), PAI-1 (1:1000, Santa Cruz), CTGF (1:1000, Santa Cruz), pSmad3 S423/S425 (1:1000, Millipore), Smad3 (1:1000, Abcam), caveolin-1 (1:500, BD Biosciences) and Tubulin (1:10000, Santa Cruz).

2.3 mRNA and qRT-PCR

mRNA was isolated using Trizol (Invitrogen), and 1 μg of RNA was reverse transcribed using qScript (Quanta Biosciences). Real-time PCR was carried out using primers for rat fatty acid synthase (FAS) (Fwd: 5’ CCAAGCAGGCACACACAATG 3’; Rev: 5’ GAGTGAGGCCGGGTTGATAC 3’), low density lipoprotein receptor (LDLR) (Fwd: 5’ AGTGCCCGGATGGCTCCGAT 3’; Rev: 5’ GCCACCGTTGGGGAGAACCG

3'), TβRI (Fwd: 5' GGGGCGAACGCATTACAGTGTTTCTGCCAC 3'; Rev: 5' TGGAATGCAGAGGAAGCAGACTGGACCAGC) and 18S (Fwd: 5' TGCGGAAGGATCATTAACGGA 3'; Rev: 5' AGTAGGAGAGGAGCGAGCG ACC 3'). Reactions were performed in an Applied Biosystem 7600 system. mRNA was determined relative to 18S of the same sample using the $\Delta\Delta C_T$ method.

2.4 Transfection

MCs were plated to 70% confluence and transfected with 5 μg of the active N-terminal fragment (constitutively active (ca)) of SREBP-1a or SREBP-1c, generously provided by Dr. H. Shimano, or a transcriptionally inactive (dominant negative (dn)) SREBP-1a (Y335A) and SREBP-1c (Y320A), generously provided by Dr. A. Schulze [115], using XFect (Clontech). After 48 hours, cells were serum-deprived for 24 hours and harvested for protein expression. 293T cells were plated to 40% confluence and transfected with His-tagged TβRI (Addgene plasmid 19161) and SREBP-1 eGFP C2 (5 μg) (kindly provided by Dr. W. Chow [116]) overnight by calcium-phosphate transfection.

2.5 RNA Interference

Rat SREBP-1 On-Target Plus Smart Pool siRNA and non-specific control siRNA were obtained from Dharmacon (Lafayette, CO). MCs were plated to 70% confluence and then transfected with 100 nM of the respective siRNA utilizing GeneEraser siRNA reagent (Stratagene). After 48 hours, cells were serum-deprived for 24 hours and harvested for mRNA and protein.

2.6 Luciferase

MCs and R1BL17 cells were plated to 50% confluence and transfected with 0.5 μg of a Smad3-responsive luciferase (SBE4 (Addgene plasmid 16495) or $CAGA_{12}$ (kindly provided by Dr. M. Bilandzic)) and 0.05 μg pCMV β-galactosidase (Clontech) using Effectene (Qiagen). Cells were serum-deprived overnight following transfection, then treated with TGF-β1 (2 ng/ml) for 16 hours. Lysis was attained with a Reporter Lysis Buffer (Promega) with one freeze-thaw cycle. Luciferase and β-gal activity were determined using respective kits (Promega) with a Berthold luminometer and plate reader (420 nm). β-Gal activity was used to normalize for transfection efficiency.

2.7 Biotinylation

Cells were washed with ice-cold PBS and incubated with EZ-link Sulfo-NHSLC-Biotin (0.5 mg/ml in PBS, Fisher) for 20 minutes. Biotinylation was stopped with 0.1 M glycine in PBS. Cells were lysed in IP lysis buffer (PBS pH 7.4, 5mM EDTA, 5mM EGTA, 10mM sodium pyrophosphate, 50mM NaF, 1mM $NaVO_3$, 1% Triton, protease inhibitors). Biotinylated proteins were precipitated with 50% neutravidin slurry (Fisher) overnight, after which the beads were washed, boiled in PSB and proteins assessed by immunoblotting.

2.8 Immunoprecipitation

Cells were washed with ice-cold PBS and lysed in IP lysis buffer as mentioned above. Cellular debris was clear through centrifugation at 13,000 rpm for 10 minutes and equal amounts of lysate were incubated overnight with 2μg of

an SREBP-1 (SC13551) antibody overnight. The next day, proteins associated with SREBP-1 were precipitated through incubation with protein G agarose beads (Invitrogen, KIT0204) for 1.5 hours. The beads were then washed, boiled in PSB and protein was assessed by immunoblotting.

2.9 Exosome Isolation

Exosomes were isolated as published [117]. Briefly, conditioned medium was collected from control or treated MCs, then subjected to two consecutive centrifugations. The first was at 300g for 5 minutes followed by 12,000g for 20 minutes to eliminate cellular debris. Exosomes were then isolated after centrifugation for 2 hours at 100,000g and washed twice with large volumes of PBS. Exosome protein concentration was measured using the Bradford assay.

2.10 Statistical Analysis

Statistical analysis was performed using the two-tailed t-test for experiments with only two experimental groups. Experiments with more than two groups were analyzed by one-way ANOVA with Tukey's HSD. A $P<0.05$ was considered significant. Data are presented as the mean ± SEM.

3. Results

3.1 SREBP-1 regulates the expression of TβRI

We have previously shown that SREBP-1 is a novel mediator of TGF-β1 signaling through its interaction with Smad3 [80]. However, it has yet to be determined whether SREBP-1 may also regulate TGF-β1 signaling further upstream of Smad3 activation. To this end, we have assessed the importance of

SREBP-1 in the regulation of TβRI, a key effector of TGF-β1 signaling. Treatment with the SCAP inhibitor fatostatin, which prevents SREBP transport to the Golgi for activation [118], attenuated the whole cell expression of TβRI. To determine if this effect was selective for TβRI or may be affecting the overall expression of other cell surface protein the expression of TβRII, a co-receptor for TGF-β1 with TβRI [106], and an unaffiliated cell surface protein PDGFR were assessed. However, treatment with fatostatin did not regulate the expression of other cell surface receptors such as TβRII or PDGFR (Figure 1A). The efficacy of fatostatin as an SREBP-1 inhibitor was demonstrated through its antagonism of the expression of SREBP-1 regulated genes (FAS and LDLR) (Figure 1B) and inhibition of the cleaved activated form of SREBP-1 (mSREBP-1, Figure 1C). Selective knockdown of SREBP-1 using siRNA also led to the downregulation of TβRI, but not TβRII or PDGFR (Figure 1D). These data indicate that SREBP-1 selectively regulates the expression of TβRI.

3.2 SREBP-1 does not regulate TβRI expression through canonical mechanisms

Regulation of TβRI expression may occur at multiple levels, including transcription, translation, degradation, and proteolytic cleavage. Although SREBP-1 has been well described as a transcription factor, its inhibition using fatostatin or siRNA did not alter the expression of TβRI mRNA (Figures 2A and 2B). We further assessed the importance of the transcriptional activity of SREBP-1 in the regulation of TβRI expression through overexpression of the transcriptionally active N-terminal of SREBP-1 (constitutively active (ca)

SREBP-1a or caSREBP-1c) or the transcriptionally inactive N-terminal of SREBP-1 (dominant negative (dn) SREBP-1a (Y335A) or dnSREBP-1c (Y321A)) [80]. Overexpression of ca/dnSREBP1a or 1c did not affect basal TβRI protein expression (Figure 2C).

Turnover of TβRI at the protein level has been attributed to degradation and proteolytic cleavage [37, 107]. Degradation of TβRI has been attributed to proteasomal and lysosomal dependent pathways [119-123]. However, antagonism of proteasomal degradation using MG132, or lysosomal degradation using NH_4Cl or leupeptin, failed to reverse the downregulation of TβRI by SREBP-1 inhibition (Figures 3A-C). Aside from degradation, proteolytic cleavage of TβRI has been shown to be mediated by the metalloprotease ADAM17. Cleavage of TβRI at the cell surface by ADAM17 has been shown to prevent TGF-β1 signaling through attenuation of Smad3 activation [107]. However, general inhibition of metalloproteases using GM6001 did not attenuate the downregulation of TβRI by fatostatin, excluding a role for SREBP-1 in the proteolytic cleavage of TβRI (Figure 3D). In aggregate, these data show that SREBP-1 does not regulate the transcription, degradation or cleavage of TβRI.

3.3 SREBP-1 regulates the cell surface expression of TβRI in a lipid raft-dependent manner

TGF-β1 signaling is regulated by TβRI/TβRII endocytic pathways. While clathrin-mediated endocytosis enables signaling, lipid raft and caveolar endocytosis has been shown to terminate canonical signaling. Although lipid raft

and caveolar endocytosis require the formation of sterol and sphingolipid-enriched domains, lipid rafts differ from caveolae in that expression of caveolin-1 is not required. Lipid raft and caveolar endocytosis have been associated with the termination of TGF-β1 signaling through the downregulation of TβRI. [124] Disruption of lipid rafts and caveolae with the cholesterol depleting agent cyclodextrin attenuated the downregulation of TβRI mediated by SREBP inhibition (Figure 4A). Basal levels of TβRI were also increased. However, the absence of caveolae in caveolin-1 knockout mesangial cells had no effect on TβRI downregulation (Figure 4B), suggesting a role for rafts, but not caveolae, in SREBP-1 effects.

Since our data suggest that lipid raft endocytosis mediates SREBP-1 regulation of TβRI, we further analyzed the effects of SREBP-1 on cell surface expression of TβRI. Routing and regulation of the cell surface expression of TβRI has been highlighted as an important regulator of the complexity of TGF-β1 responses [125]. Cell surface TβRI was detected by immunoblotting after immunoprecipitation of biotinylated cell-surface proteins. We first overexpressed TβRI-His in 293T cells and confirmed that SREBP inhibition with fatostatin decreases its expression at the cell surface (Figure 4C). Endogenous expression of cell surface TβRI in 293T cells was also observed (data not shown). Since inhibition of SREBP-1 lead to the downregulation of TβRI, we next determined the effects of overexpressing full length SREBP-1 on the cell surface expression of TβRI. In MC, depletion of lipid rafts using cyclodextrin also reversed the

fatostatin-induced downregulation of cell surface TβRI (Figure 4E). Interestingly, we also found overexpressed full length SREBP-1 at the cell surface (Figure 4D). However, we were unable to detect endogenous SREBP-1 at the cell surface of MCs (Figure 4F). It is thus likely that increased association of overexpressed SREBP-1 with TβRI enabled its detection after immunoprecipitation of biotinylated cell-surface proteins. Finally, we tested whether TβRI interacts with SREBP-1. Figure 4G shows that this is indeed the case, and that this interaction is, as expected, diminished by fatostatin.

3.4 SREBP-1 regulates TβRI expression through secretion via exosomes

Our data show that SREBP-1 mediates TβRI stability at the cell surface and this retention requires lipid rafts. The secretion of exosomes has recently been identified as a mechanism to allow cells to shed proteins as an alternative to degradation. This was demonstrated to play an important role in regulation of transferrin, p53, and Wnt/β-Catenin signaling. Furthermore, the formation of exosomes has been linked with the maturation of endosomes from lipid rafts [126-128]. We thus sought to determine whether SREBP-1 might regulate the downregulation of TβRI through its secretion by exosomes. Figure 5A demonstrates the appearance of TβRI in exosomes of cells treated with fatostatin, but not in control cells. Since exosomes have been highlighted as an important mediator of cell-to-cell communication through transfer of their cargo to neighboring cells [44], we tested if exosomes enriched with TβRI, due to SREBP inhibition, may facilitate TGF-β1 signaling. Exosomes isolated from fatostatin-

treated MC were used to treat the TβRI-deficient cell line R1BL17, which have been previously demonstrated to lack responsiveness to TGF-β1 [112]. Activity of the downstream mediator of TβRI signaling, Smad3, was used to assess TβRI activity. Figure 5B shows that exosomes isolated from fatostatin-treated MC, but not those from control cells, re-established the ability of R1BL17 cells to increase Smad3 activation, determined by the Smad3-responsive reporter $CAGA_{12}$-luc (Figure 5B). Last, we confirmed that R1BL17 cells do not respond to TGF-β1 at baseline, but that this response can be restored with TβRI re-expression. This is shown in Figure 5C. Taken together, these data show that SREBP-1 regulates TβRI expression through a novel mechanism involving the secretion of TβRI in exosomes.

3.5 Downregulation of TβRI expression by SREBP-1 inhibition attenuates TGF-β1 signaling

We assessed the importance of TβRI downregulation by SREBP-1 inhibition through analysis of downstream targets of TGF-β1 signaling. Inhibition of SREBP-1 attenuated TGF-β1 mediated activation of Smad3 as assessed through its C-terminal activating phosphorylation (Figure 6A), nuclear accumulation of total Smad3 (Figure 6B), and activation of the Smad3-responsive reporters $CAGA_{12}$-luc (Figure 6C) and SBE4-luc (Figure 6D). Furthermore, downregulation of SREBP-1 using siRNA inhibited expression of the TGF-β1 responsive genes PAI-1 and CTGF (Figure 6E). These data demonstrate that inhibition of SREBP-1 attenuates TGF-β1 signaling.

4. Discussion

TGF-β1 signaling is known to play a key role in the progression and development of diabetic nephropathy [101]. TβRI has been identified as an important target for inhibition of TGF-β1 signaling and prevention of renal fibrosis [106]. Our study has now identified SREBP-1 as a novel regulator of TβRI through its actions as a TβRI cell surface retention factor. SREBP-1 inhibition and downregulation led to its loss from the cell surface through secretion in exosomes, a process which likely utilizes lipid rafts. This results in decreased cell surface receptor expression of TβRI available for signaling. Our study demonstrates an additional novel level of the regulation of TGF-β1 signaling by SREBP-1 and highlights the potential importance of targeting SREBP-1 to inhibit renal fibrosis.

SREBP-1 is best known for its role in regulating fatty acid and lipid metabolism [129]. However recent studies have implicated SREBP-1 in the development of renal fibrosis. SREBP-1a or -1c overexpression in the kidney induced glomerular sclerosis with upregulation of TGF-β and matrix proteins including fibronectin and collagen (3, 4). Conversely, SREBP inhibition with fatostatin attenuated angiotensin II-induced glomerular fibrosis [103], and SREBP-1c deletion protected against the development of early diabetic nephropathy (4). We have previously shown that SREBP-1 directly mediates TGF-β1 transcript expression through binding to SRE sites in the promoter [79, 103]. Interestingly, SREBP-1 also facilitates TGF-β1-induced Smad3

transcriptional activity in cooperation with CBP [80]. We now show that SREBP-1 additionally regulates TGF-β1 signaling at the level of the TβRI, highlighting the existence of a positive feedback cycle in which SREBP-1 mediates both expression of TGF-β1 and facilitates its signaling.

SREBP-1 is best known as a transcription factor that directly binds to SRE sites and coordinates the expression of target genes through interaction with other transcription factors such as Sp1 and CBP [62]. Indeed, fatostatin, which prevents the processing of SREBP to the mature transcription factor, effectively decreased TβRI expression. Surprisingly, overexpression of transcriptionally active or inactive SREBP-1 did not alter the expression of TβRI, nor did inhibition of SREBP-1 alter TβRI transcript levels. This suggests that SREBP-1 regulates TβRI independently of its transcriptional activity. Importantly, SREBP-1 is known to induce its own transcription through SRE sites in its promoter [130]. Thus, while fatostatin is an inhibitor of SREBP activation, it also effectively downregulates SREBP-1 expression, consistent with the potential involvement of the precursor form of SREBP-1 in TβRI regulation.

The turnover of TβRI protein has been attributed to the endocytic pathways by which it is internalized. Receptor degradation was shown to occur following endocytosis through rafts/caveolae which promotes the colocalization of TβRI with ubiquitination machinery [119-123]. Our data show that although the absence of caveolin-1/caveolae does not affect TβRI downregulation by SREBP inhibition, disruption of lipid rafts restores TβRI levels. This would

suggest that SREBP regulates the degradation of TβRI by altering its localization into lipid rafts. Contrary to expectations, however, antagonism of the proteasome or lysosome was unable to restore TβRI expression after inhibition of SREBP-1. Overall, these data suggest that SREBP-1 regulates TβRI internalization but does not affect its expression through degradation. Ectodomain shedding of TβRI by the metalloprotease ADAM17 has recently been described in cancer cells as an alternate mechanism for downregulation of TβRI cell surface expression [107]. Our data, however, also exclude this as a mechanism for the observed downregulation of TβRI after SREBP inhibition.

We thus sought alternate pathways by which SREBP might regulate cell surface TβRI. Secretion of proteins in exosomes is becoming increasingly recognized as an alternative mechanism for protein regulation independent of degradation and proteolytic cleavage. Exosomes are vesicles ranging from 30 to 100nm in diameter which consist of a lipid bilayer, transmembrane proteins, and a hydrophilic core enriched in proteins and RNA [43, 44]. Formation of exosomes has been associated with maturation of multivesicular endosomes derived from lipid-raft endocytosis [126-128]. Secretion of receptor proteins as a form of protein regulation has been documented and suggested to play an important role for reticulocyte maturation and p53 signaling [45, 46]. Chairoungdua et al. had shown that localization of β-catenin into exosomes play a role in the negative regulation of Wnt signaling due to stimulation of CD9 and CD82 [43]. Similar to

Chairoundua et al., our study supports the novel concept that SREBP-1 regulates the localization and secretion of TβRI in exosomes to regulate TGF-β1 signaling.

Exosomes have been recognized as messengers which mediate communication between cells by promoting the transfer of intracellular components such as RNA and proteins [44]. We have shown that exosomes enriched in TβRI restore the ability of a TβRI deficient cell line to respond to TGF-β1, indicating that the exosomes which are secreted upon SREBP inhibition contain functional TβRI which may be exchanged with other cells. Little is known about how exosomes fuse with the cell membrane of recipient cells, but it has been hypothesized that exosomes are targeted to cells based on the expression of specific adhesion proteins such as integrins (34). Although our data show that SREBP inhibition prevents TGF-β1 signaling, they do not exclude the possibility of fusion of secreted exosomes to neighboring MC given that SREBP-1 is also important in regulating Smad3 activity downstream of TGF-β1 [80]. Future studies will determine whether re-fusion of secreted exosomes occurs in MC, and the efficiency with which this occurs. In this regard, it is interesting to note that although exosomes restored some TGF-β1 signaling capability to TβRI cells; this was still significantly less than signaling obtained by overexpression of TβRI in these cells (compare Figure 5B to C). Thus, even if re-fusion does occur to some extent in MC, it is unlikely to contribute significantly to overall TGF-β1 signaling and profibrotic effects.

5. Conclusions

Our study has demonstrated a novel role for SREBP-1 in the regulation of TβRI through attenuation of its membrane expression by secretion in exosomes. Together with our previous studies [79, 80], we have shown that SREBP-1 plays an important role in TGF-β1 signaling. Targeting SREBP-1 *in vivo* may thus present a potential novel therapeutic strategy for diabetic nephropathy and other fibrotic kidney diseases.

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions: RV conducted most of the experiments, analyzed the results, and wrote the first draft of the manuscript. GC conducted $CAGA_{12}$ and SBE_4 luciferase experiments. KA, JR and HAA assisted in the purification of exosomes. BG assisted in cell culture experiments. JK conceived the idea and

edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

6. Tables and Figures

Figure 1

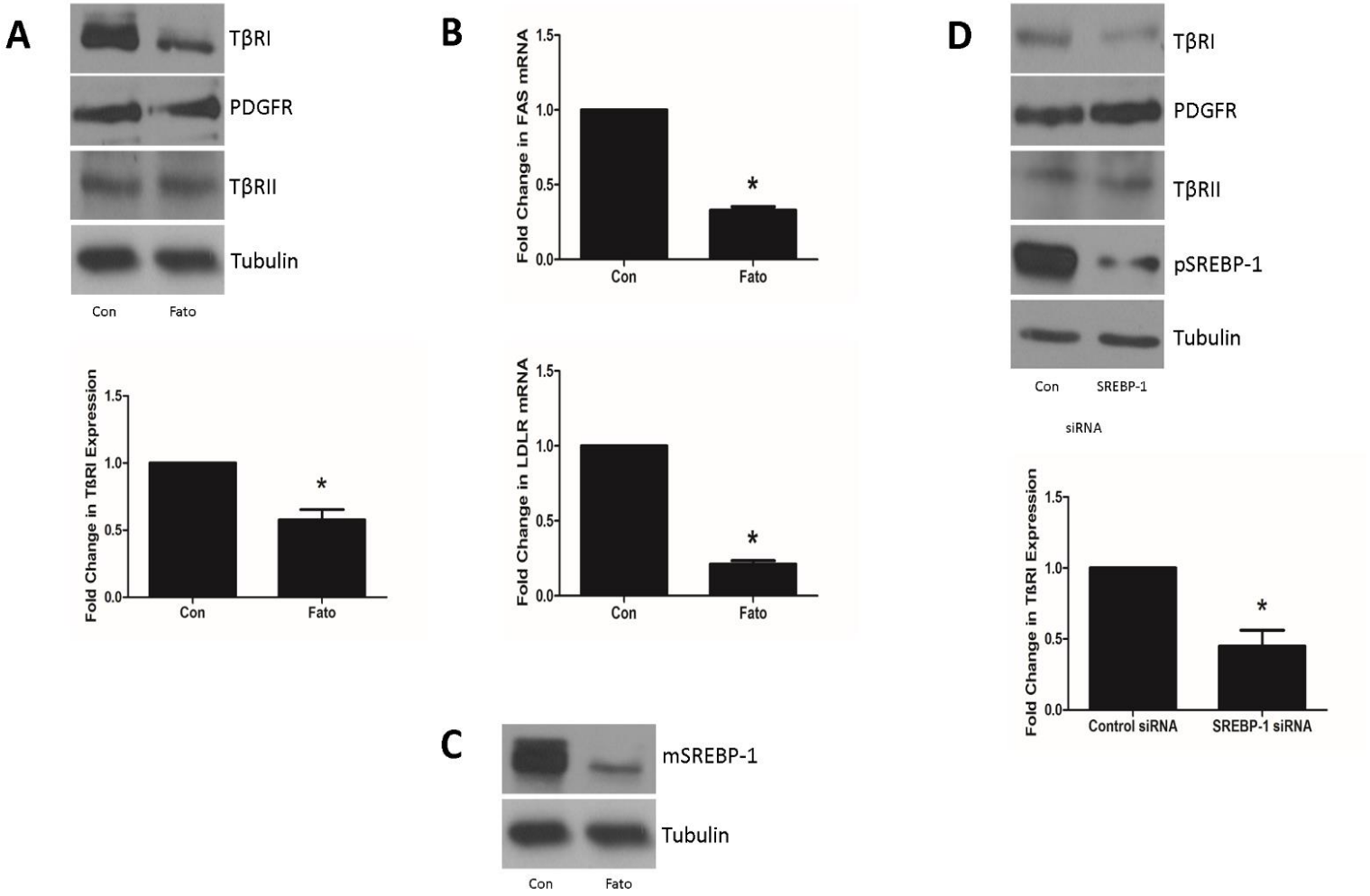

Figure 2-1 - SREBP-1 Regulates the expression of TβR1.
(A and B) MCs were treated with the SCAP inhibitor fatostatin (Fato, 20 μM) for 5 hrs. (A) Whole cell expression of TβRI, but not PDGFR or TβRII, were decreased by fatostatin (n=3). (B) mRNA expression of SREBP-1-responsive genes FAS and LDLR and expression of mature SREBP-1 was decreased by Fatostatin, confirming its efficacy (n=3). (C) MCs were transfected with non-specific siRNA (Con) or SREBP-1 siRNA. TβR1, but not PDGFR or TβRII, was decreased by SREBP-1 downregulation (n=3). * $P < 0.05$ Treatment versus Control.

Figure 2

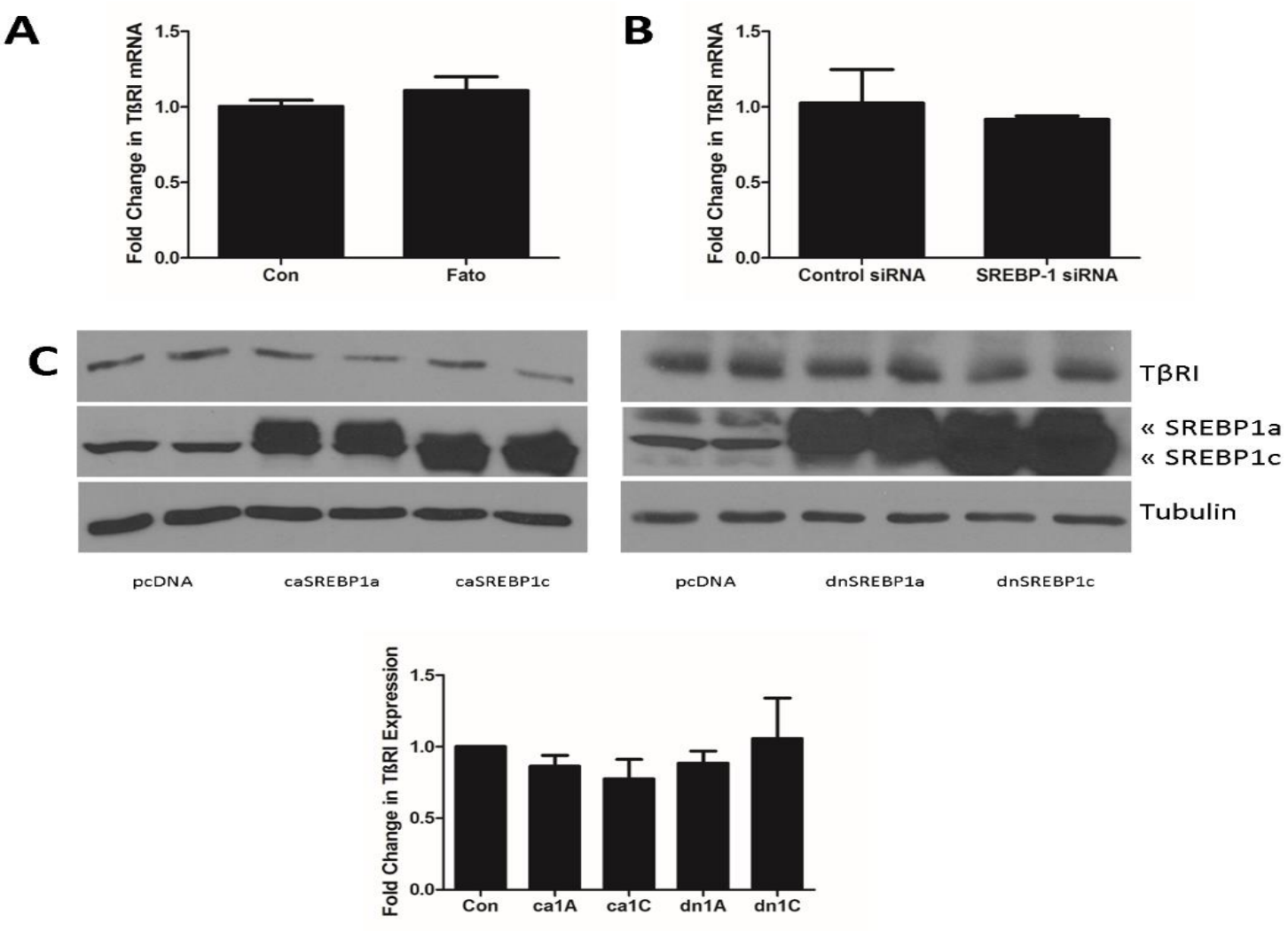
A
Fold Change in TßRI mRNA
1.5
1.0
0.5
0.0
Con
Fato
B
Fold Change in TßRI mRNA
1.5
1.0
0.5
0.0
Control siRNA
SREBP-1 siRNA
C
TβRI
« SREBP1a
« SREBP1c
Tubulin
pcDNA
caSREBP1a
caSREBP1c
pcDNA
dnSREBP1a
dnSREBP1c
Fold Change in TßRI Expression
1.5
1.0
0.5
0.0
Con
ca1A
ca1C
dn1A
dn1C

Figure 2-2 - SREBP-1 does not regulate TβR1 expression through transcription or translation.
(A) TβR1 mRNA was not affected by fatostatin (20 μM, 5 hrs; n=3) nor by (B) SREBP-1 downregulation (n=3). (C) MCs were transfected with pcDNA (control), transcriptionally active (constitutively active (ca)) or inactive (dominant negative (dn)) SREBP-1a or SREBP-1c. None of these altered expression of TβR1 (n=5).

Figure 3

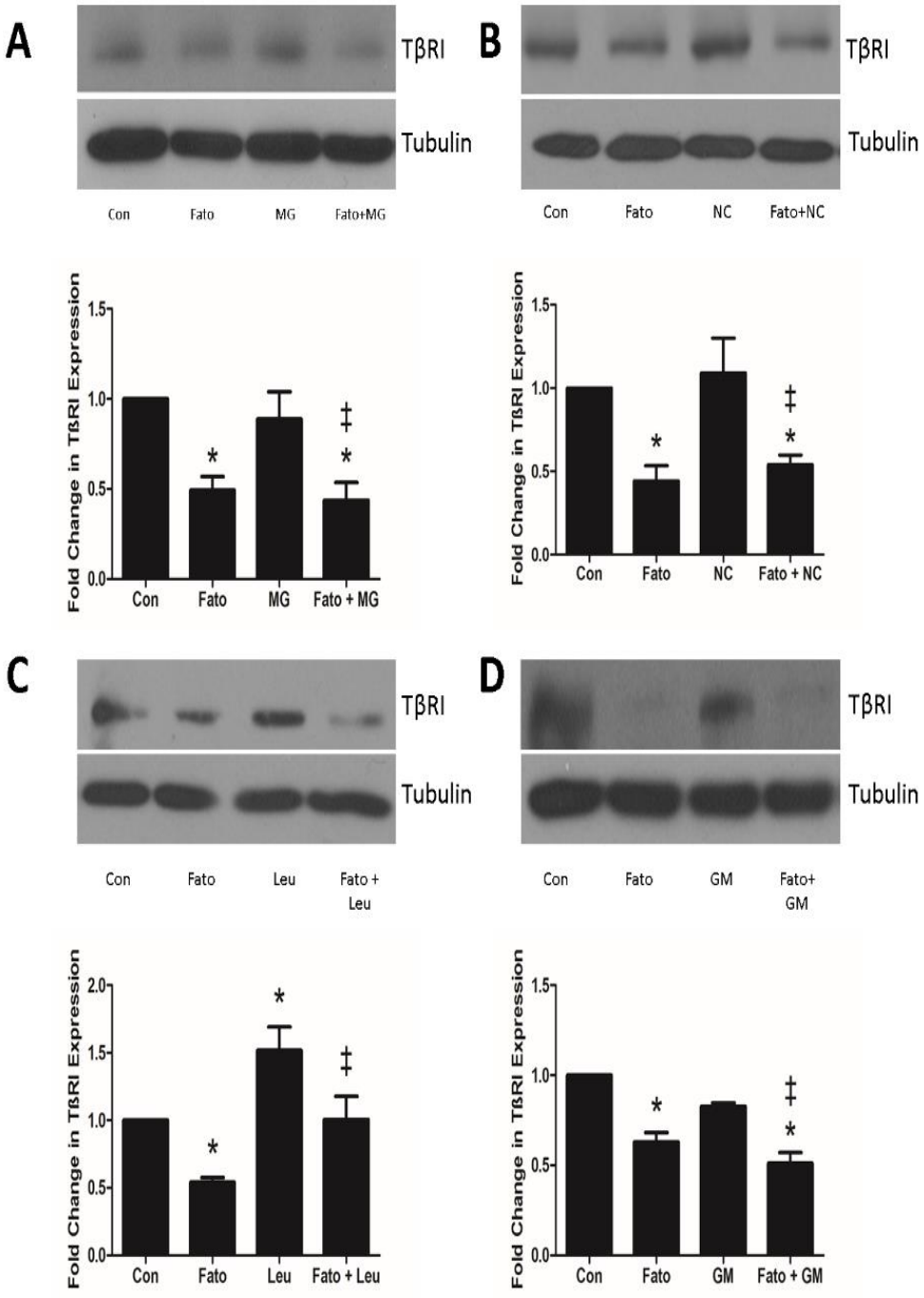
A
TβRI
Tubulin
Con
Fato
MG
Fato+MG
Fold Change in TßRI Expression
1.5
1.0
0.5
0.0
*
‡
*
Con
Fato
MG
Fato + MG
B
TβRI
Tubulin
Con
Fato
NC
Fato+NC
Fold Change in TßRI Expression
1.5
1.0
0.5
0.0
*
‡
*
Con
Fato
NC
Fato + NC
C
TβRI
Tubulin
Con
Fato
Leu
Fato + Leu
Fold Change in TßRI Expression
2.0
1.5
1.0
0.5
0.0
*
*
‡
Con
Fato
Leu
Fato + Leu
D
TβRI
Tubulin
Con
Fato
GM
Fato+ GM
Fold Change in TßRI Expression
1.5
1.0
0.5
0.0
*
‡
*
Con
Fato
GM
Fato + GM

Figure 2-3 - SREBP-1 does not regulate TβR1 expression through degradation, or cleavage
MCs treated with Fatostatin (20 μM) for 5 hrs were pretreated with: (A) proteasomal inhibitor MG132 (MG, 10 μM, 1 hour) (n=6); (B-C) lysosomal inhibitor NH_4Cl (NC, 20 μM, 2 hours)(n=9) or leupeptin (Leu, 100 μM, 18 hours)(n=5); or (D) metalloprotease inhibitor GM6001 (GM, 20 μM, 1 hour)(n=3). None could rescue the downregulation of TβRI by SREBP inhibition. * $P < 0.05$ Treatment versus Control; ‡ $P < 0.05$ Fato+Treatment versus Treatment.

Figure 4

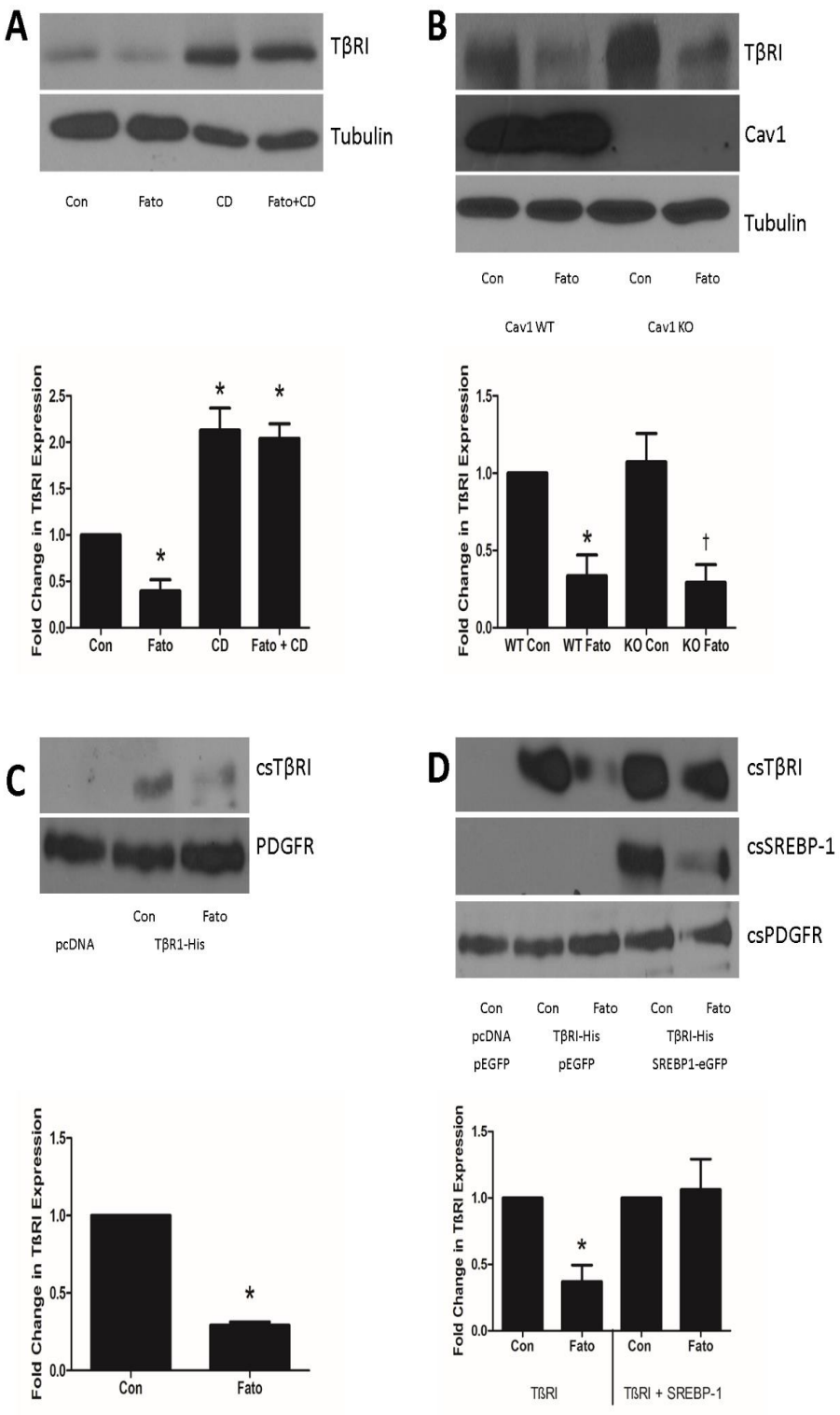
A
TβRI
Tubulin
Con
Fato
CD
Fato+CD
B
TβRI
Cav1
Tubulin
Con
Fato
Con
Fato
Cav1 WT
Cav1 KO
Fold Change in TßRI Expression
Con
Fato
CD
Fato + CD
Fold Change in TßRI Expression
WT Con
WT Fato
KO Con
KO Fato
C
csTβRI
PDGFR
Con
Fato
pcDNA
TβR1-His
D
csTβRI
csSREBP-1
csPDGFR
Con
Con
Fato
Con
Fato
pcDNA
TβRI-His
TβRI-His
pEGFP
pEGFP
SREBP1-eGFP
Fold Change in TßRI Expression
Con
Fato
Fold Change in TßRI Expression
Con
Fato
Con
Fato
TßRI
TßRI + SREBP-1

Figure 4

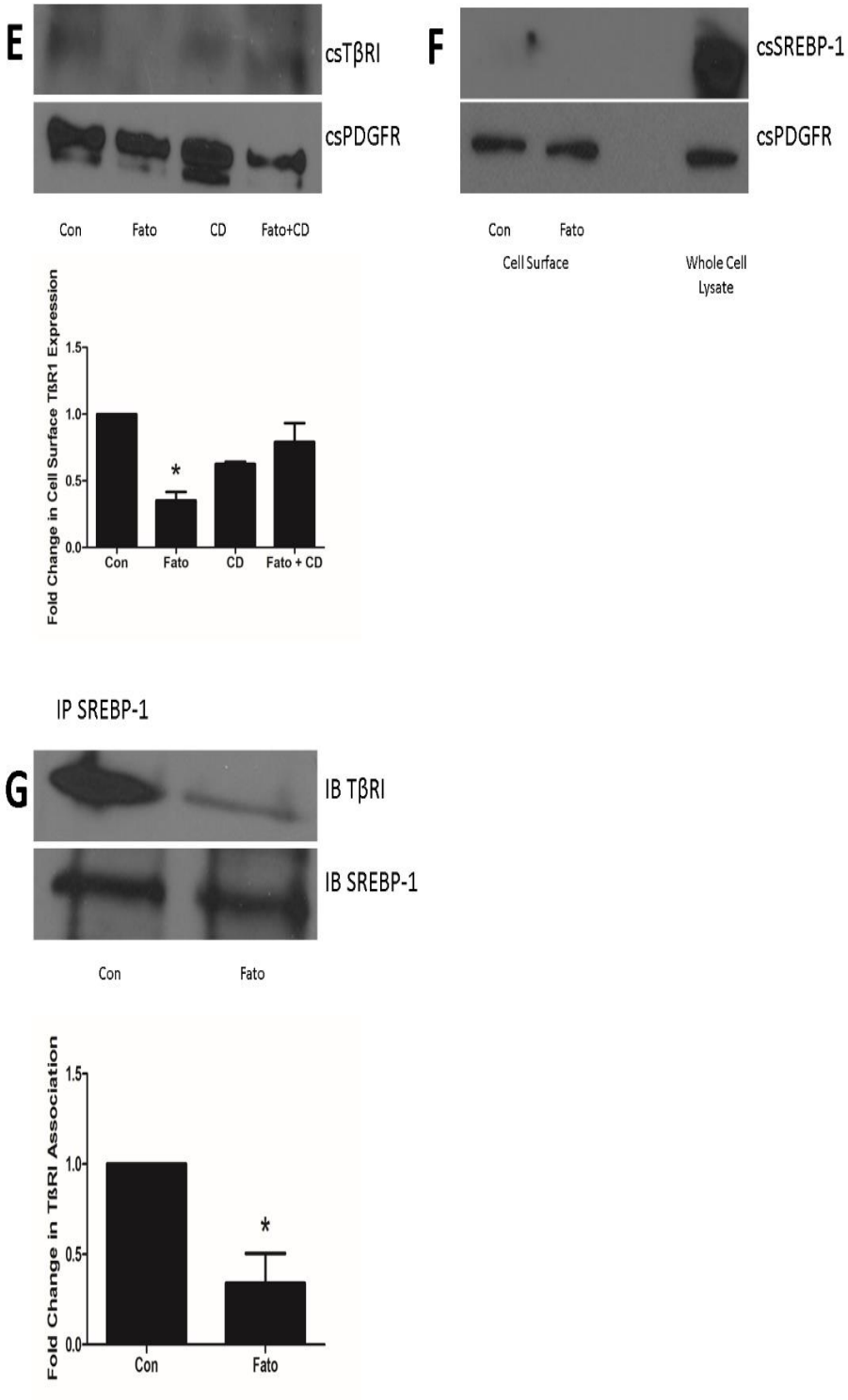
E
csTβRI
csPDGFR
Con
Fato
CD
Fato+CD
Fold Change in Cell Surface TßR1 Expression
1.5
1.0
0.5
0.0
*
Con
Fato
CD
Fato + CD
F
csSREBP-1
csPDGFR
Con
Fato
Cell Surface
Whole Cell Lysate
IP SREBP-1
G
IB TβRI
IB SREBP-1
Con
Fato
Fold Change in TßRI Association
1.5
1.0
0.5
0.0
*
Con
Fato

Figure 2-4 - SREBP-1 regulates the cell surface expression of TβR1 in a lipid-raft dependent mechanism.
(A) and (E) MCs were treated with the cholesterol-depleting drug cyclodextrin (CD, 10 mM, 1 hour) prior to Fatostatin (20 μM) for 5 hrs and were for TβR1 expression in total lysate (A) or on the cell surface (E). (A) and (E)Cyclodextrin rescued the expression of TβRI (n=3). (B) Caveolin-1 wild-type (WT) and knockout (KO) MCs were treated with Fatostatin (20 μM) for 5 hrs. Absence of caveolin-1/caveolae did not rescue the expression of TβRI (n=3). (C and D) 293T cells overexpressing empty vectors pcDNA/pEGFP, TβRI-His, or TβRI-His and full length SREBP1-eGFP were treated with fatostatin (20 μM) for 5 hrs (n=3). Fatostatin decreased cell surface (cs) expression of TβR1-His, which was rescued by the overexpression of SREBP-1. (F) TβR1-deficient R1BL17 cells were treated with fatostatin (20 μM) for 5 hrs. SREBP-1 was not detected at the cell surface. * $P < 0.05$ Treatment versus Control. ‡ $P < 0.05$ Treatment+Fato versus Treatment.

Figure 5

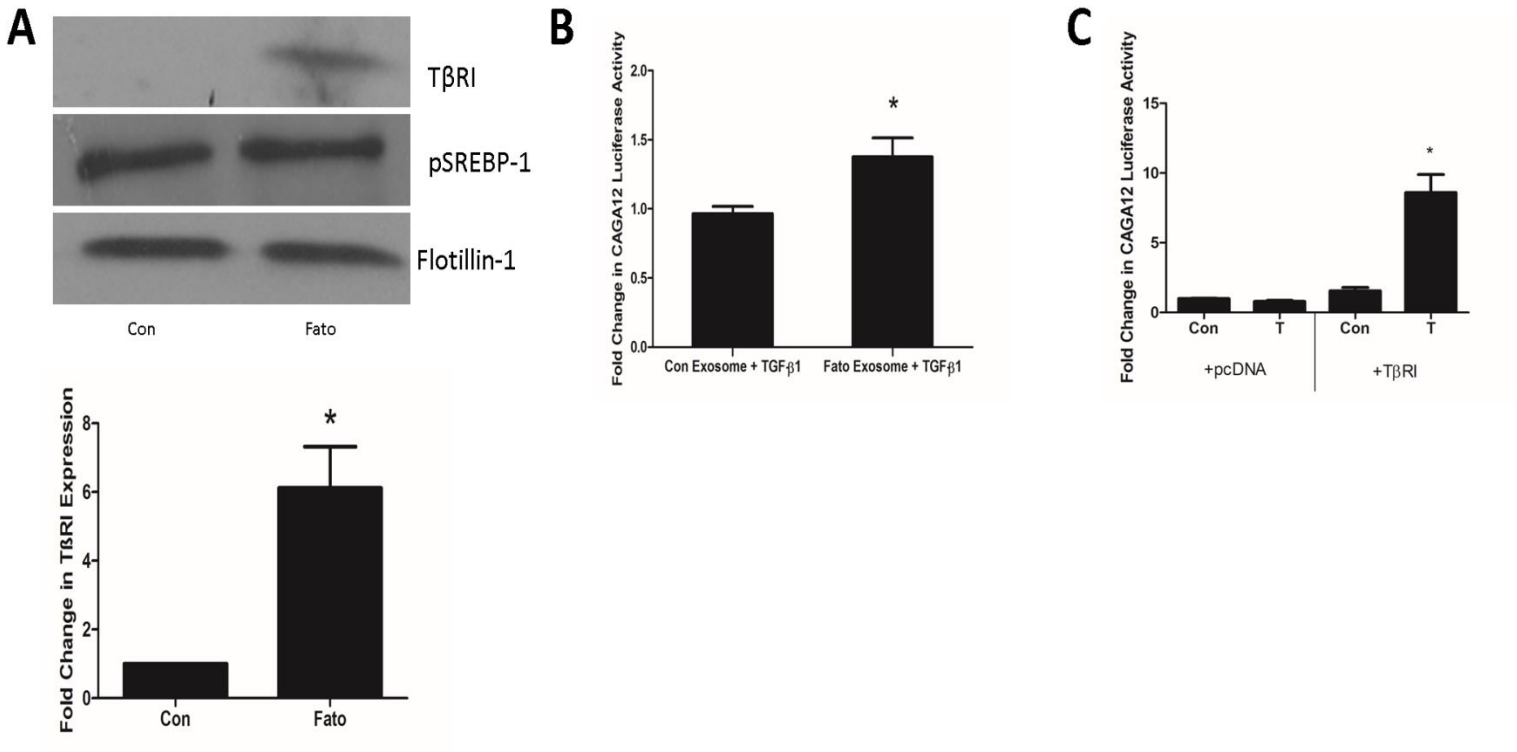

Figure 2-5 - SREBP-1 regulates the localization of TβR1 into exosomes.
(A) After treatment with fatostatin (Fato, 20 μM) for 5 hrs, SREBP-1 was immunoprecipitated and association with TβR1 assessed by immunoblotting. SREBP inhibition decreased its association with TβR1 (n=3). (B) Conditioned media was collected and purified for exosomes. Fatostatin increased TβR1 found in exosomes (n=3). (C,D) TβR1-deficient R1BL17 cells were transfected with the TGFβ1-responsive $CAGA_{12}$-luciferase. (C) Treatment with exosomes

(25 μg, 24 hrs) purified from media of MCs treated with Fatostatin increased luciferase activity (n=4). (D) R1BL17 cells were cotransfected with pcDNA or TβR1 followed by treatment with TGFβ1 (5 ng/ml) for 18 hours (n=4). Re-expression of TβR1 restored TGFβ1 responsiveness. * $P < 0.05$ Treatment versus Control

Figure 6

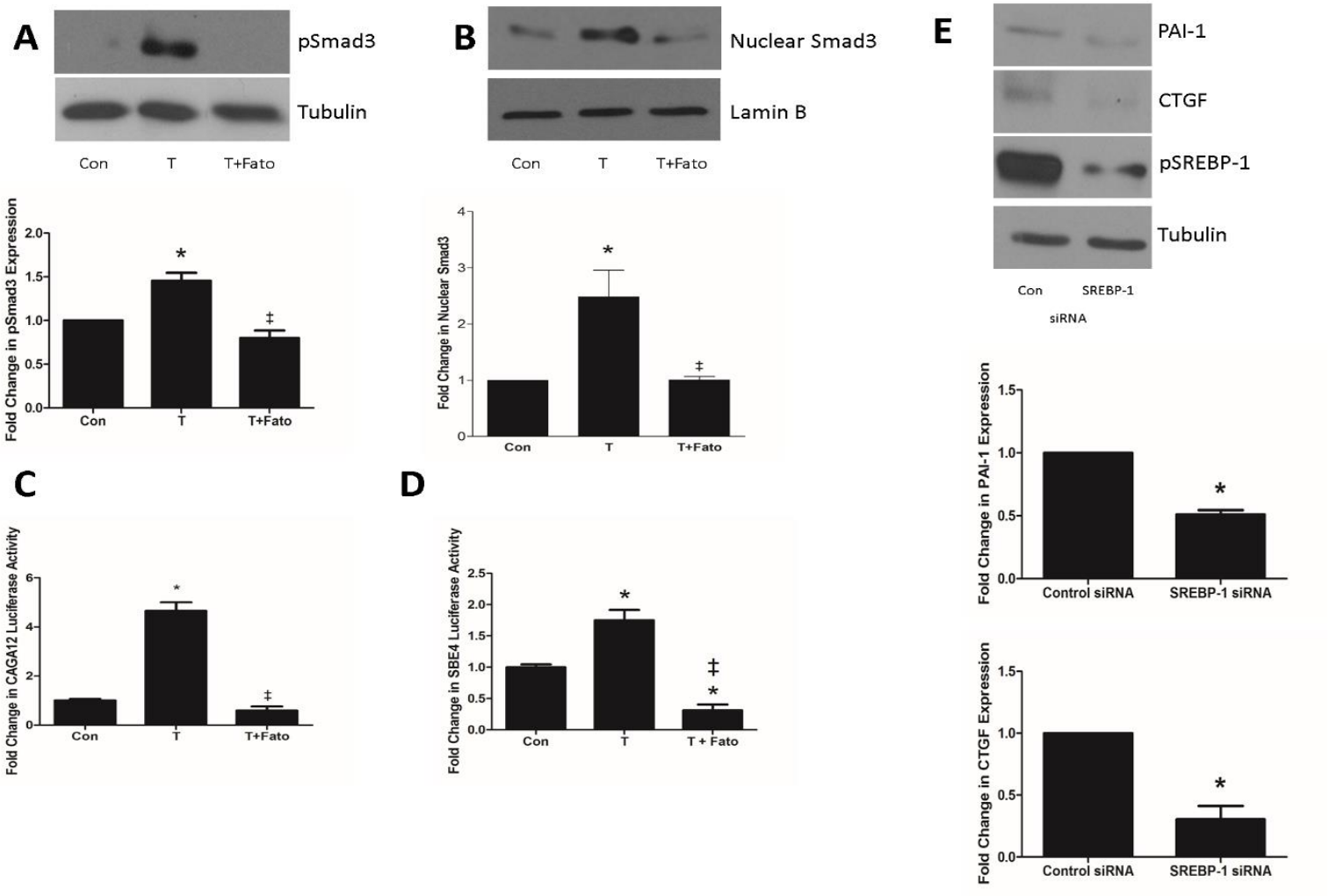
A
pSmad3
Tubulin
Con
T
T+Fato
B
Nuclear Smad3
Lamin B
Con
T
T+Fato
E
PAI-1
CTGF
pSREBP-1
Tubulin
Con
SREBP-1 siRNA
Fold Change in pSmad3 Expression
Fold Change in Nuclear Smad3
Fold Change in PAI-1 Expression
Control siRNA
SREBP-1 siRNA
C
D
Fold Change in CAGA12 Luciferase Activity
Fold Change in SBE4 Luciferase Activity
T + Fato
Fold Change in CTGF Expression

Figure 2-6 - Downregulation of TβRI expression by SREBP-1 inhibition attenuates paracrine and autocrine TGF-β1 signaling.
(A, B) MCs were treated with fatostatin (Fato, 20 μM) for 4 hrs, followed by TGFβ1 (T, 2 ng/ml) for 1 hour. SREBP inhibition decreased TGFβ1 activation of its downstream mediator Smad3, as assessed by its phosporylation (n=5) (A) and nuclear accumulation (n=3) (B). (C, D) MCs were transfected with the Smad3-responsive reporters SBE4-lucif (C) (n=7) or $CAGA_{12}$-lucif (D) (n=4), then treated with Fatostatin (Fato, 20 μM) for 4 hrs followed by TGFβ1 (2 ng/ml) for 18 hours. SREBP inhibition prevented the activation of both luciferases. (E) MCs were transfected with non-specific siRNA (Con) or SREBP-1 siRNA. Downregulation of SREBP-1 decreased the expression of TGF-β1-responsive proteins PAI-1 and CTGF (n=3). * $P < 0.05$ Treatment versus Control. ‡ $P < 0.05$ Treatment+Fatostatin versus Treatment.

Chapter 3 – Inhibition of SREBP does not attenuate diabetic nephropathy

Title: Inhibition of sterol regulatory element binding protein (SREBP) does not attenuate diabetic nephropathy

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Citation: In preparation for submission.

Preface

Significance to thesis

SREBP has been shown to be an important mediator of renal fibrosis, and is known to be upregulated during the pathogenesis of DN. Our previous studies have demonstrated that treatment with the SREBP inhibitor fatostatin was capable of preventing kidney injury in a hypertensive and UUO model of renal dysfunction. Furthermore, our results from study 1 indicate that SREBP-1 regulates the actions of the pro-fibrotic cytokine TGF-β1, suggesting that inhibition of SREBP-1 will be beneficial in the treatment of DN. The purpose of this study is to determine if inhibition of SREBP with fatostatin is an effective treatment for DN. These results would demonstrate the effects of SREBP inhibition on diabetic kidney function and structure, and highlight any potential adverse effects that may be expected. Our results have indicated that SREBP inhibition with fatostatin does not prevent DN, and furthermore leads to kidney dysfunction in non-diabetic mice. These results demonstrated that fatostatin is not an effective treatment for DN, thus other methods to inhibit SREBP should be investigated to determine their efficacy and safety profiles.

Author's contribution

Richard Van Krieken conducted most of the experiments, analyzed the results, and prepared the first draft of the manuscript.

Bo Gao assisted in extraction of mRNA and protein from animal tissue, and in maintenance of the animals.

Joan Krepinsky conceived of the project and edited the manuscript.

Abstract

Sterol regulatory element binding protein (SREBP)s has been recognized as an important mediator of renal fibrosis, and are known to be upregulated during the pathogenesis of diabetic nephropathy (DN). Previous studies have identified SREBP-1 as an important mediator of diabetic nephropathy, however the effectiveness of SREBP inhibition as treatment for diabetic nephropathy has not been evaluated. We have thus studied the effect of the SREBP inhibitor fatostatin in the development of diabetic nephropathy in type 1 diabetic CD1 mice. Treatment with fatostatin did not improve the glomerular filtration rate and albuminuria in diabetic mice. Furthermore, treatment of diabetic mice with fatostatin did not improve renal fibrosis. However, we found that treatment of non-diabetic mice with fatostatin led to kidney dysfunction characterized by hyperfiltration and increased renal inflammation. We found that the renal inflammation caused by fatostatin was found to be related to the increased expression of the pro-inflammatory cytokine monocyte chemoattractive protein -1 in renal tubular cells. Thus, we have demonstrated that treatment with fatostatin is not an effective therapy for diabetic nephropathy, and leads to kidney dysfunction in non-diabetic mice. Further research into the efficacy of other SREBP inhibitors in the treatment of DN may prove beneficial, and remain to be evaluated.

Abbreviations: SREBP, sterol regulatory element binding protein-1; DN, diabetic nephropathy

Introduction

Diabetic nephropathy is the leading cause of end stage renal disease, characterized by accumulation of extracellular matrix proteins (ECM) in the glomerulus and in later stages in the tubulointerstial space [48]. Current therapy, comprising glycemic control, blood pressure regulation, and use of inhibitors of angiotensin II signaling, is only capable of slowing the progression of diabetic nephropathy (DN) [131]. Thus, better understanding the mechanisms leading to the development and progression of DN is important to the identification of new treatment strategies.

SREBPs are members of the basic helix-loop-helix leucine zipper family of transcription factors that primarily regulate fatty acid and cholesterol levels. [59, 60]. SREBPs are retained in the endoplasmic reticulum (ER) as large transcriptionally inactive precursors through interaction with SREBP cleavage-activated protein (SCAP) and Insig. In response to sterol-deficient conditions, the interaction between Insig and SCAP is disrupted, leading to translocation of the SREBP-SCAP complex into the Golgi. Within the Golgi, SREBP is cleaved by membrane associated site 1 (S1P) and site 2 (S2P) proteases to form the transcriptionally active fragment which then translocates to the nucleus to affect signaling [77]. We have previously demonstrated a role for SREBP-1 in the

regulation of transforming growth factor β1 (TGF-β1) signaling in kidney mesangial cells (MCs) [47, 79, 80]. This illustrates that outside of fatty acid signaling, SREBP-1 is capable of directly regulating profibrotic signaling in the kidney.

Expression of SREBP-1 is increased in the kidney in models of both type 1 and type 2 diabetes [77, 78, 132-134]. The SREBP-1 gene can be expressed through the use of an alternative start site that leads to the expression of SREBP-1a and SREBP-1c. SREBP-1a is a potent regulator of fat metabolism and is the predominant isoform in cell culture. SREBP-1c preferentially activates fatty acid synthesis genes, and is the predominant isoform in most tissue. [58] Renal overexpression of the isoform SREBP-1a led to albuminuria and glomerulosclerosis, while very early DN (assessed by albuminuria) was inhibited in type 1 diabetic SREBP-1c knockout mice [77, 78]. These studies suggest an important role for SREBP-1 in the progression of DN.

We have previously demonstrated that the SREBP inhibitor fatostatin attenuates renal fibrosis and improves renal function in a hypertensive model of kidney disease induced by angiotensin II infusion [103]. Fatostatin also attenuated interstitial fibrosis in the unilateral ureteral obstruction model [102]. Whether fatostatin could prevent or delay the development of DN has not as yet been examined, and is the focus of the current study. Surprisingly, fatostatin did not ameliorate renal pathology or improve renal function in type 1 DN, but rather

increased renal inflammation in both diabetic and control mice. SREBP inhibition with fatostatin is thus not a feasible option for the treatment of type 1 DN.

Methods

Animal Studies

Animal studies were carried out in accordance with McMaster University and the Canadian Council on Animal Care guidelines. Male CD1 mice (Charles River), 9 weeks of age, underwent a left nephrectomy. At 10 weeks of age, diabetes was induced by a single intraperitoneal (IP) injection of streptozotocin (STZ) at 200 mg/kg. Control mice were injected with an equal volume of citrate buffer. Blood glucose was tested the following week and mice with blood glucose values of > 17 mM were enrolled in the study. Diabetic mice that developed ketonuria (assessed by dipstick [Bayer Multistix, Toronto, ON, Canada]) were implanted with an insulin pellet (LinShin Canada, Toronto, ON, Canada) to maintain body weight but not correct hyperglycemia.

Mice were administered fatostatin (30 mg/kg) or vehicle (7.5% DMSO in saline) IP daily after confirmation of hyperglycemia. They were sacrificed after 12 weeks of diabetes. At the end of the study urine was collected, blood pressure was assessed by tail cuff plethysmography (Kent Scientific, CODA 2 system) and glomerular filtration rate (GFR) assessed as described below. Urine 8-OHdG (Cell Bio Labs, STA320), creatinine (Crystal Chem, 80350) and albumin (Exocell, 1011) were measured using kits.

Glomerular Filtration Rate

GFR was assessed by clearance of fluorescein isothiocyanate (FITC)-labeled sinistrin (Fresenius Kabi Linz, Austria). A 5% FITC-Sinistrin solution in saline was injected retro-orbitally. Blood was collected into heparinized test tubes from the saphenous vein at 3, 7, 10, 15, 35, 55, and 75 minutes post-injection. Plasma fluorescence was assessed using a fluorometer (Gemini EM, Molecular Devices) at $\lambda = 485$ nm excitation, and $\lambda = 538$ nm emission.

Imaging

Formalin-fixed sections (4 μm) were stained with periodic acid schiff's reagent (PAS), trichrome (Sigma H15), or picrosirius red (Polyscience 24901). Glomerular hypertrophy was assessed by measuring the glomerular cross-sectional area (G(A)) of 40 glomeruli per animal. Glomerular volume was estimated from the formula $V(G) = 1.38/1.10 * (G(A))^{3/2}$ [135]. Glomerular scarring was scored by Dr. A. J. Ingram by randomly assessing 10 glomeruli per animal (0 = none; 1+ ≤ 25%; +2 25-50%; +3 ≥ 50% of glomerular area affected). Picrosirius red stained tissue was visualized under polarized light. Picrosirius red and trichrome staining was assessed by measuring the percentage of positive area using ImageJ. For immunohistochemistry, sections were deparaffinised and exposed to heat-induced epitope retrieval. SREBP-1 (Abcam, Ab44153) and CD3 (Dako, A0452) were used for staining. Images were taken at x20 magnification and staining quantified as above. Electron microscopy (EM) was performed on a small piece of kidney cortex fixed in 0.2 M glutaraldehyde pH 7.4/0.1 M sodium cacodylate, and processed by the McMaster University EM facility. Basement

membrane thickness was assessed on glomerular peripheral loops at x12500 magnification through calculation of the harmonic mean of 100 randomly selected points.

Nanostring Analysis

mRNA was isolated from kidney tissue using Trizol (Invitrogen) and sent for analysis using nCounter (nanoString Technologies, Seattle, WA, USA) at the Farncombe Metagenomics Facility at McMaster university. Data were analyzed using nSolver 2.5 provided by the manufacturers. Samples were normalized using background subtraction with the negative control and the geometric means of the positive control and the housekeeping gene (ACTB).

Protein Analysis

Protein from renal cortex tissue was performed as published [136]. Briefly, tissue was homogenized in lysis buffer through sonication. Lysate was centrifuged for 10 minutes at 13,000 rpm to clear debris. The supernatant was extracted and ran on a SDS-PAGE gel, and used for western blotting. Antibodies against FN (BD Transduction, 1:5000), pSmad3 (Millipore, 1:3000), α smooth muscle actin (SMA) (Fischer, 1:10000), and CD3 (Dako, 1:1000) were used.

Cell Culture

Primary MCs were isolated from male Sprague-Dawley (SD) rats as published [79]. MCs were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100 μg/ml). Cells between passages 8 and 17 were used. HK2 cells

were cultured in a high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100 μg/ml). All cells were kept at 37˚C in 95% air, 5% CO_2. Cells were serum deprived at 80-90% confluence overnight, then treated with fatostatin (20 μM) for 24 hours.

mRNA analysis

mRNA was isolated using Trizol (Invitrogen), and 1 μg of RNA was reverse transcribed using qScript (Quanta Biosciences). Real-time PCR was carried out using primers for rat MCP-1 (Fwd: 5' GATGCAGTTAATGCCCCACT3'; Rev: 5' TTCCTTATTGGGGTCAGCAC 3'), rat 18S (Fwd: 5' TGCGGAAGGATCATTAACGGA 3'; Rev: 5' AGTAGGAGAGGAGCGAGCG ACC3'), human MCP-1 (Fwd: 5' ACTGAAGCTCGCACTCTC 3'; Rev: 5' CTTGGGTTGTGG AGTGAG 3'), and human ACTB (Fwd: 5' ACCGAGCGCGGCTACAG 3'; 5' CTTAATGTCA CGCACGAT TTCC 3'). Reactions were performed in an Applied Biosystem 7600 system. mRNA was determined relative to 18S (for rat samples) or ACTB (for human samples) of the same sample using the $\Delta\Delta C_T$ method.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism (version 5.0) using one-way ANOVA with Tukey's HSD for post hoc analysis. Survival curve analysis was conducted by a log-rank (Mantel-Cox) test. Analysis of data with only 2

groups was performed using a student's t-test. A $P<0.05$ was considered significant. Data are presented as mean ± SEM.

Results

Fatostatin inhibits SREBP-1 and SREBP-2 in vivo

Following 12 weeks of type 1 diabetes, mice were assessed for development of diabetic nephropathy. Clinical characteristics of the mice are presented in **Table 1**. Blood glucose of diabetic mice was beyond the measurement range of the glucometer, and was thus recorded as greater than 27.0 mM. Fatostatin significantly reduced blood glucose in non-diabetic mice, which is consistent with reports that identify SREBP-1c as an important determinant in hepatic glucose regulation [137]. Systolic and diastolic blood pressure in both diabetic groups were significantly increased compared to both non-diabetic groups. Fatostatin did not significantly affect blood pressure in diabetic mice. Diabetic mice weighed significantly less than untreated non-diabetic mice. Fatostatin did not affect weight in diabetic mice, but attenuated weight gain in non-diabetic mice. A similar decrease in weight was seen in obese db/db mice treated with fatostatin [118]. Renal hypertrophy, as assessed by kidney to body weight ratio, was not significantly affected by fatostatin in diabetic mice.

Fatostatin is a potent inhibitor of SREBP-1 and SREBP-2 activation through its inhibition of SREBP cleavage-activating protein (SCAP) [118]. Since both also induce their own transcriptional expression through sterol regulatory element (SRE) sites, fatostatin also attenuates the expression of the precursor

proteins [118]. To assess the efficacy of fatostatin in our model system, we first assessed the expression of SREBP. Expression of both SREBP-1 and -2 was increased in diabetic kidneys (Figure 1A, B) as seen by others in various models of type 1 diabetic nephropathy [77, 78, 132-134], with attenuation of expression by fatostatin which also decreased renal mRNA expression of SREBP-1 (**Figure 1C**) and SREBP-2 (**Figure 1D**), although we did not observe upregulation in these transcripts by diabetes.

Fatostatin does not improve renal function or albuminuria in diabetic mice

As shown in **Figure 2A**, there was a significant increase in mortality after induction of diabetes which was unaffected by treatment with fatostatin. Similar mortality rates of CD1 diabetic mice have been demonstrated by others [138, 139].

Albuminuria, a major hallmark of diabetic nephropathy, was significantly elevated in diabetic mice, but was not affected by treatment with fatostatin (**Figure 2B**). Increased thickening of the glomerular basement membrane, another characteristic feature of diabetic kidney disease, was assessed by EM. Interestingly, fatostatin prevented basement membrane thickening in diabetic mice (**Figure 2C**).

Early diabetic nephropathy is characterized by hyperfiltration, manifest as elevated GFR. As shown in **Figure 2D**, diabetic mice developed significant hyperfiltration, but this was not affected by fatostatin. Interestingly, fatostatin significantly increased GFR in non-diabetic mice. Glomerular hypertrophy, also

seen in early diabetic nephropathy, was assessed by estimating the volume of glomeruli as described in Methods. Figure 2E shows that diabetic mice developed glomerular hypertrophy, and this was not affected by fatostatin (**Figure 2E**). Similar to its effects on GFR, fatostatin induced glomerular hypertrophy in non-diabetic mice.

Fatostatin does not attenuate renal fibrosis or profibrotic signaling in diabetic kidneys

Glomerulosclerosis and tubulointerstitial fibrosis are key hallmarks in the pathogenesis of diabetic nephropathy [101]. Diabetic mice developed both glomerulosclerosis and tubulointerstitial fibrosis, as assessed by PAS and trichrome staining respectively. Neither was affected by fatostatin (**Figure 3A, B)**. Deposition of collagen was further assessed by staining with Picrosirius red. This was increased in diabetic mice, but unaffected by fatostatin (**Figure 3C**). Corresponding to the increased collage deposition, mRNA expression of collagens I, III, and IV showed a trend towards an increase in diabetic kidneys without attenuation by fatostatin (**Figure 3D**). Fibronectin (FN) is a ubiquitous ECM protein that directs and facilitates the deposition of additional ECM proteins [140]. FN expression was significantly increased in diabetic mice, and was not affected by fatostatin (**Figure 3E**). Of interest, fatostatin led to a significant increase in FN expression in non-diabetic mice. A similar pattern was seen with FN mRNA (**Figure 3D**). Elevated expression of α smooth muscle actin (αSMA) is a marker of activated renal myofibroblast [141], and can act as an indicator of the

degree of renal fibrosis. Expression of αSMA was significantly increased in non-diabetic and diabetic mice treated with fatostatin as assessed by western and IHC (**Figure 3G** and **3H** respectively).

TGF-β1 has been identified as a key mediator of renal fibrosis in diabetic nephropathy through activation of its downstream mediator Smad3 [142]. Renal mRNA expression of TGF-β1 was elevated in diabetic mice, with expression unaffected by fatostatin (**Figure 4A**). Phosphorylation of Smad3 (pSmad3), indicative of Smad3 activation, was elevated in diabetic mice, and was not also unaffected by fatostatin (**Figure 4B**). There was a non-significant increase in TGF-β1 and pSmad3 in non-diabetic mice given fatostatin. Our previous study had demonstrated that treatment with fatostatin was capable of reducing expression of the type I TGF-β receptor (TβRI) [47]. Expression of TβRI was significantly reduced in diabetic mice treated with fatostatin (**Figure 4C**), however this decrease was not enough to attenuate TGF-β signaling in the kidney.

Fatostatin promotes renal inflammation

Renal inflammation is a major contributor to the pathogenesis of diabetic nephropathy, and is characterized by recruitment of macrophages and T-cells [143]. SREBPs have been suggested to play a critical role in the inflammatory response through regulation of inflammatory cytokines and facilitating clonal expansion [144-146].

Elevated mRNA expression of the chemokines MCP-1, RANTES, TNF-α as well as the T-cell marker CD3 and macrophage marker F4/80 were increased in

diabetic kidneys. Fatostatin did not significantly affect their expression in diabetic mice, but tended to increase expression in non-diabetic kidneys (**Figure 6A**). Accumulation of CD3 cells in diabetic kidneys as well as fatostatin-treated non-diabetic mice was also demonstrated using IHC (**Figure 6B**) and immunoblotting (**Figure 6C**). Furthermore, treatment with fatostation led to accumulation of macrophages in diabetic kidneys as assessed by IHC (**Figure 6D**).

MCP-1 is a chemokine that regulates the trafficking and recruitment of macrophages to the kidney, and is secreted by both MCs and tubular cells [147]. Mice treated with fatostatin demonstrated an increase in the expression of MCP-1 to a similar level of diabetic mice as assessed by western blot and IHC (**Figure 7A** and **7B** respectively). *In vitro*, treatment of primary MCs with fatostatin led to decreased mRNA expression of MCP-1 (**Figure 7C**). However, treatment of HK2 cells, an immortalized renal tubular cell line, increased MCP-1 mRNA (**Figure 7D**) and protein expression (**Figure 7E**). This suggests that an effect of fatostatin on renal tubular cells are may be responsible for driving inflammation in our model.

Fatostatin does not inhibit renal oxidative stress

Renal oxidative stress has been implicated in the development of diabetic microvascular complications including diabetic nephropathy [148]. NADPH oxidase has been identified as a major contributing source of reactive oxidative species in diabetic kidneys, with NOX-2 an important inducible component of this enzyme complex [149]. Expression of the NOX-2 transcript was elevated in

diabetic mice, but was not altered by fatostatin (**Figure 8A**). There was a trend towards increased expression of NOX-2 in non-diabetic mice treated with fatostatin. There was no significant difference in expression of the NOX-4 transcript in any of the groups (data not shown). Associated with the increased Nox2 expression, there was a trend towards an increase in oxidative stress in diabetic kidneys as measured by urinary 8-OHdG which was unaffected by fatostatin (**Figure 8B**). Fatostatin increased urinary 8-OHdG in nondiabetic mice, although this was not statistically significant.

Discussion

Despite recent advances in the understanding of the pathophysiology of DN, development of novel therapies has been elusive. SREBP-1 and SREBP-2 have been suggested to play an important role in the pathogenesis of DN [76-78, 134], but the therapeutic potential of SREBP inhibition in the treatment of DN has not been previously examined. Our study now shows that fatostatin, an inhibitor of both SREBP-1 and -2, does not abrogate the development of DN. Conversely, fatostatin promoted renal inflammation in both non-diabetic and diabetic mice, and induced glomerular hypertrophy and hyperfiltration in non-diabetic mice. These findings do not support a therapeutic role for SREBP inhibition with fatostatin in the treatment or prevention of DN, and suggest an important deleterious effect of longer-term treatment on kidney function.

The importance of SREBP in the progression of DN has been attributed to its importance to renal fibrosis. Overexpression of SREBP-1a and SREBP-1c was shown to lead to phenotypic changes reminiscent of DN including albuminuria, glomerulosclerosis and upregulation of profibrotic factors [77, 78]. Furthermore, knockout of SREBP-1 attenuated the early development of albuminuria and renal oxidative stress in diabetic mice [78]. At the molecular level, we have previously shown that in mesangial cells SREBP-1 directly mediates fibrosis through upregulation and increased signaling of the profibrotic factor TGF-β1 [79, 80]. Thus SREBP-1 mediated upregulation of profibrotic factors may cumulate in glomerulosclerosis and fibrosis ultimately leading to decline in kidney function. The connection between SREBP-2 and DN has not been well studied. A correlation between elevated expression of SREBP-2 and DN has been shown [76, 134]. However, the molecular mechanism by which SREBP-2 may contribute to DN has not been established.

Surprisingly, our study has demonstrated that fatostatin, which inhibits both SREBP-1 and -2, did not improve key features of DN (albuminuria, glomerular hypertrophy, and hyperfiltration). These conflicting results between our study and Ishigaki et al. [78] may be due to differences in the methods used to inhibit SREBP activity *in vivo*. In our study fatostatin acts as a SCAP inhibitor to decrease the expression of SREBP-1 and SREBP-2 [118]; however SREBP-1 KO mice are deficient in SREBP-1 expression but also have a concomitant up regulation in SREBP-2 expression [150]. The expression of SREBP-2 in the

milieu of the SREBP-1 KO mice may provide a protective effect in DN by providing a potential source of necessary fatty acid and cholesterol biosynthesis required for kidney function.

Surprisingly, our study showed that fatostatin increased GFR and glomerular volume in non-diabetic mice but improved basement membrane thickness in diabetic mice. In unpublished data, we tested the effects of acute treatment (2 days) of fatostatin on GFR in healthy CD1 mice. Treatment with fatostatin reduced GFR in these mice to levels similar to uninephrectomized CD1 mice two days post-surgery. This may represent a direct hemodynamic effect that fatostatin is exerting on the kidney. With chronic treatment of fatostatin, the hemodynamic changes may lead to a maladaptive response in the glomerulus resulting in elevated GFR and glomerular hypertrophy. The reason why fatostatin improves basement membrane thickness in diabetic mice is unknown.

Renal inflammation has been strongly implicated in the pathogenesis of diabetic nephropathy. Under diabetic stress, macrophages and CD4+ T-cells have been shown to be recruited in the kidney and have been suggested to exacerbate profibrotic and proinflammatory responses through secretion of cytokines such as TNF-α and TGF-β. [151, 152] Treatment with fatostatin led to increased expression of proinflammatory chemokines and infiltration of macrophages and CD4+ T-cells in diabetic and non-diabetic mice. Inflammation was detected primarily around the renal capillaries and in the tubulointerstitium, and was notably absent from the glomerulus. MCP-1 is a cytokine that promotes the

recruitment of monocytes, neutrophils, and lymphocytes, and regulates the expression of adhesion molecules and proinflammatory cytokines. MCP-1 has been described to be expressed by podocytes, mesangial and tubular cells in the kidney. [152, 153] We demonstrated that in mesangial cells, fatostation suppressed expression of MCP-1; conversely, tubular cells treated with fatostatin showed an increase in MCP-1 protein and mRNA expression.

SREBP-1 and SREBP-2 have been implicated in mediating inflammation through expression of key cytokines required for the inflammasome. Mice deficient for SREBP-1a had an impaired innate immune response characterized by a decrease in LPS-driven Nlrp1a inflammasome induction accompanied with decreased caspase-1 activity and IL-1β production in their macrophages [145]. Mice that overexpressed SREBP-2 in endothelial cells were shown to have accelerated development of atherosclerotic plaques by promoting the expression of NLRP3 and induction of the inflammasome [144]. Furthermore, antagonism of SREBP-1 and SREBP-2 through treatment with 25-hydroxycholesterol lead to reduced inflammation by inhibition of IL-1β expression [154]. In contrast, our results have indicated that inhibition of SREBP-1 and SREBP-2 with fatostatin lead to elevated inflammation in the kidney related to induction of MCP-1 expression in tubular cells. The elevated inflammation may represent an adverse event due to chronic administration of fatostatin. Our previous study assessed the efficacy of fatostatin in treating renal injury following unilateral ureteral obstruction and demonstrated that fatostatin treatment was able to reduce

macrophage and CD4+ T-cells recruitment in response to UUO [102]. However, this study was shorter (14 days of injection) in comparison to our current study (3 months of injection). Thus, chronic administration of fatostatin may be mediating a proinflammatory response in the kidney.

ROS is a known contributor to inflammation and the stimulation of profibrotic cytokines and ECM proteins [155]. NAPDH oxidase subunits Nox2 and Nox4 are upregulated during the pathogenesis of DN [149]. Overexpression of Nox2 in type I diabetic Akita mice led to elevated ROS production in the kidney and excaberation of DN within 8 weeks [156]. However, knockout of Nox2 was not capable of reversing the DN phenotype, but this may be attributed to the elevated expression of Nox4 in these mice leading to compensation [157]. Whole-body and podocyte-specific knockout for Nox4 improved renal function in diabetic mice by improving albuminuria and markers of glomerulosclerosis [158, 159]. Our results showed that fatostatin increased Nox2 mRNA expression in non-diabetic mice without a change in Nox4 levels, and this correlated with an increase in renal oxidative stress. Nox2 has been suggested to play an important role in macrophages by regulating inflammation and assisting in cross-talk with neutrophils [160]. Our results suggest that treatment with fatostatin promotes renal oxidative stress through recruitment of macrophages, which may play an important role in promoting fibrosis and inflammation.

Conclusions

In summary, our data shows that, contrary to what would be expected from our previous studies in fibrotic renal models and *in vitro* studies [47, 79, 80, 102, 103], fatostatin did not improve type 1 diabetic nephropathy and induced a maladaptive responses in non-diabetic mice. Fatostatin increased the recruitment of inflammatory cells, and this was associated with an increase in oxidative stress. Although the reasons underlying this are as yet to be fully elucidated, our data suggest that differential effects of fatostatin on different cell types are important. Specifically, fatostatin promoted chemokine expression in renal tubular cells. As the effects of mouse genetic background might also be important modifiers of the response to fatostatin, future studies should determine whether similar effects are seen in other type 1 diabetes models. Finally, since fatostatin inhibits both SREBP-1 and -2, it will be important to define the functional contribution of each of these isoforms to diabetic kidney disease. This may enable the development of a more specific therapeutic agent which can target the profibrotic actions of SREBP without induction of the adverse effects seen in this study.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

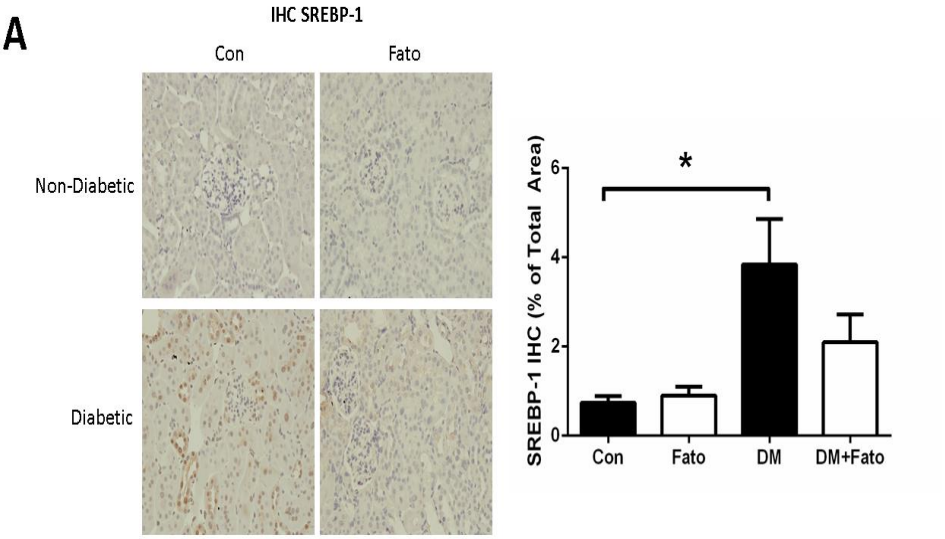
Author Contributions: RV conducted most of the experiments, analyzed the results, and wrote the first draft of the manuscript. BG assisted in animal care. JK conceived the idea and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

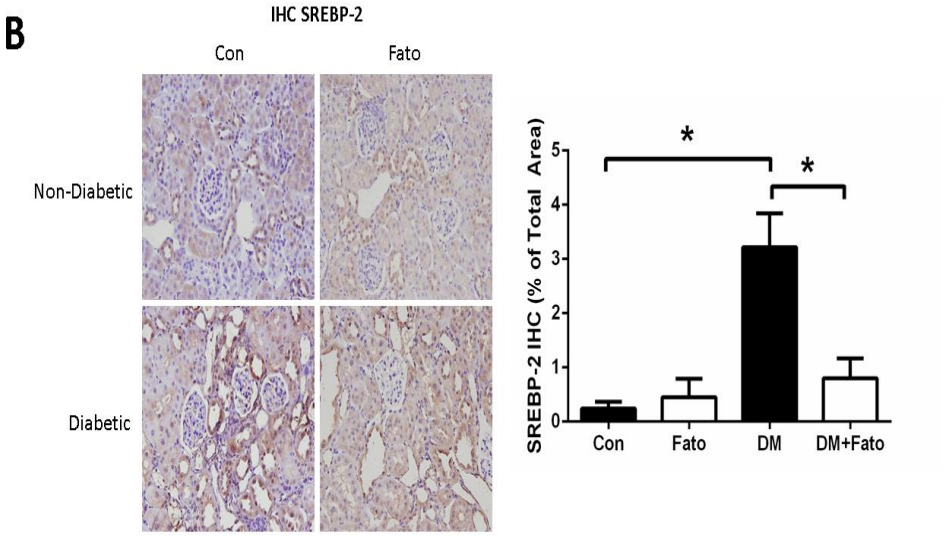
Tables and Figures

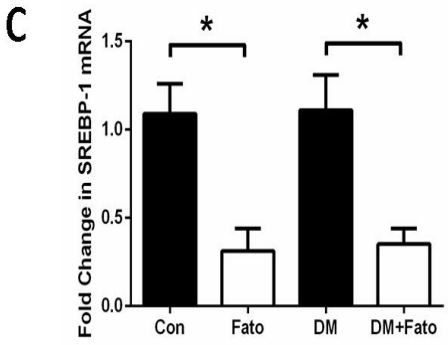
	Non-Diabetic		Diabetic	
	Control (n=6)	**Fatostatin (n=6)**	**Control (n=7)**	**Fatostatin (n=7)**
Glu (mM)	9.5±0.3	6.7±0.3*	≥ 27.0*	≥ 27.0*
SBP (mm Hg)	128±6	110±10	148±5	149±5
DBP (mm Hg)	98 ±9	74 ±13	113 ±7	112 ±4
Weight (g)	39.3±1.2	35.7±1.5	34.0±0.9*	32.4±1.3‡
Kidney: Body Weight (g/100g)			1.49±0.13	1.65±0.11

Table 3-1 - Clinical characteristics of mice.

Data are presented as mean ± SEM. * $P < 0.05$ Non-diabetic control versus others; ‡ $P < 0.05$ Diabetic Control versus others.


A
IHC SREBP-1
Con
Fato
Non-Diabetic
Diabetic
SREBP-1 IHC (% of Total Area)
0
2
4
6
*
Con
Fato
DM
DM+Fato


B
IHC SREBP-2
Con
Fato
Non-Diabetic
Diabetic
SREBP-2 IHC (% of Total Area)
0
1
2
3
4
5
*
*
Con
Fato
DM
DM+Fato


C
Fold Change in SREBP-1 mRNA
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0.5
1.0
1.5
*
*
Con
Fato
DM
DM+Fato

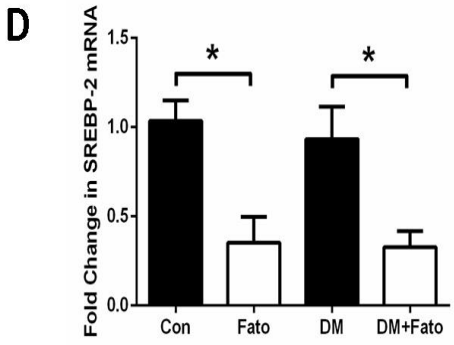
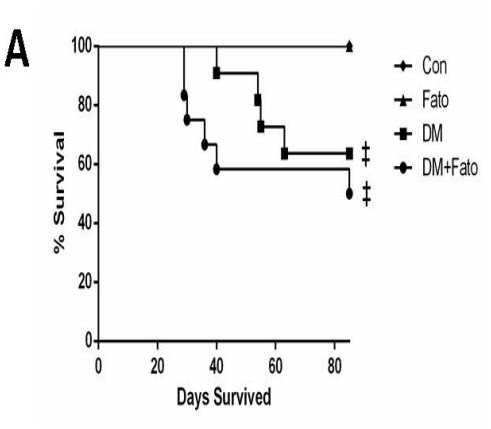

D
Fold Change in SREBP-2 mRNA
0.0
0.5
1.0
1.5
*
*
Con
Fato
DM
DM+Fato

Figure 3-1 - Fatostatin inhibits the expression of SREBP-1 and SREBP-2 in mice.

(A) SREBP-1 and (B) SREBP-2 was assessed by IHC, with images taken at 400x. This is quantified in the accompanying graph. Diabetes induced protein expression of SREBP-1 and -2, and this was attenuated by fatostatin. (C) and (D) Fatostatin decreased renal mRNA expression of both SREBP-1 and SREBP-2. * $P < 0.05$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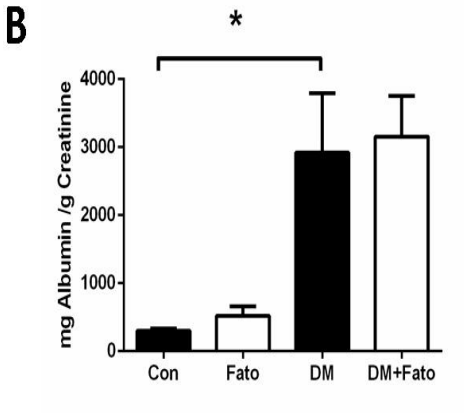
% Survival
Days Survived
Con
Fato
DM
DM+Fato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mg Albumin /g Creatinine
Con
Fato
DM
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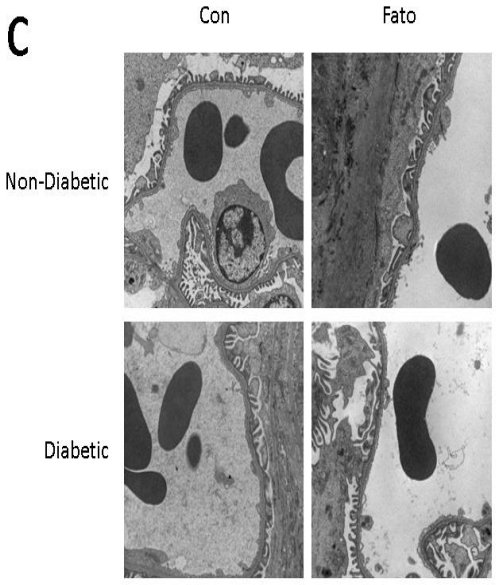
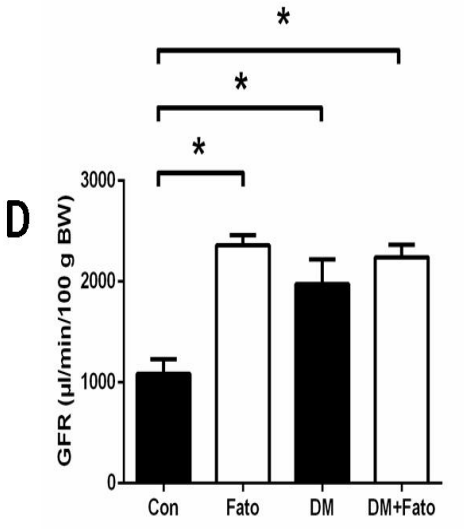
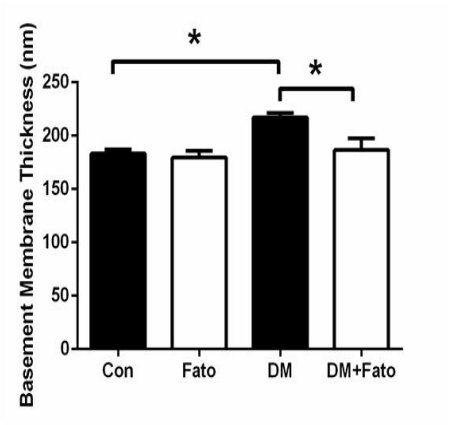

C
Con
Fato
Non-Diabetic
Diabetic


D
GFR (µl/min/100 g BW)
Con
Fato
DM
DM+Fato


Basement Membrane Thickness (nm)
Con
Fato
DM
DM+Fato

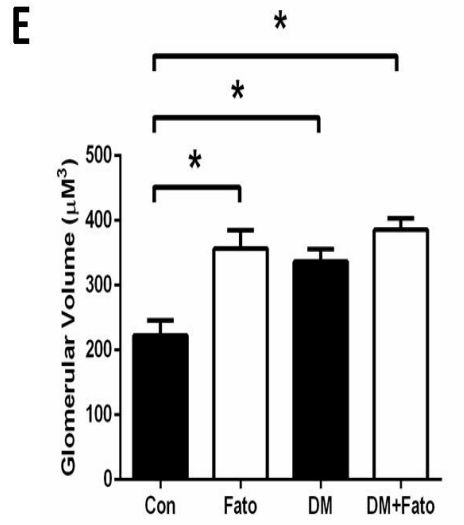
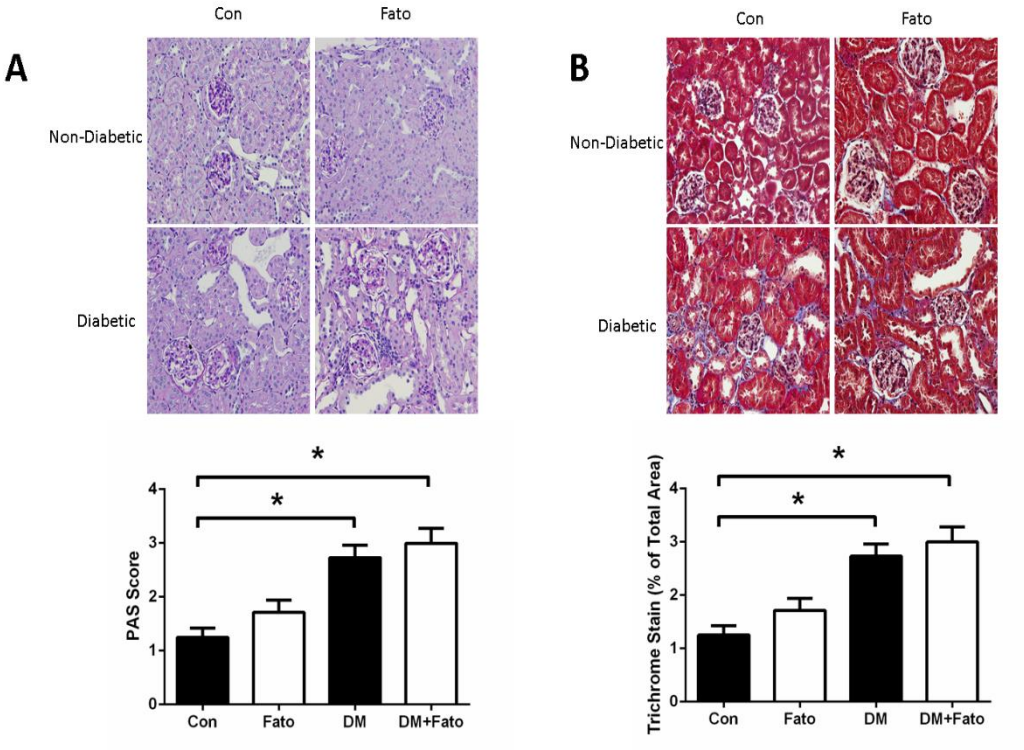
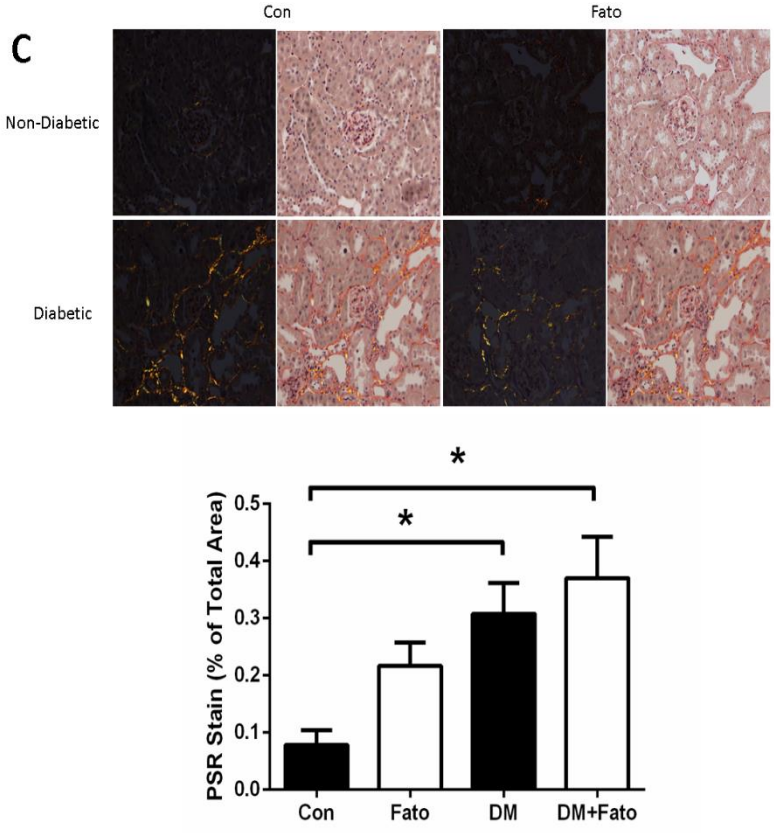

E
Glomerular Volume (µM³)
Con
Fato
DM
DM+Fato

Figure 3-2 - Fatostatin does not improve renal function or albuminuria in diabetic mice.

(A) Diabetic mice had lower survival rates than non-diabetic mice, and this was not affected by fatostatin. (B) The urine albumin-creatinine ratio was elevated in diabetic mice, but was not affected by fatostatin treatment. (C) Fatostatin reduced basement membrane thickening in diabetic mice. (D) Fatostatin elevated GFR in non-diabetic mice, but did not improve the GFR of diabetic mice. (E) Fatostatin promoted glomerular hypertrophy in non-diabetic mice, but did not improve the glomerular hypertrophy of diabetic mice. ‡ $P < 0.05$ Non-Diabetic Control versus others; * $P < 0.05$


A
Con
Fato
Non-Diabetic
Diabetic
B
Con
Fato
Non-Diabetic
Diabetic
PAS Score
Con
Fato
DM
DM+Fato
Trichrome Stain (% of Total Area)
Con
Fato
DM
DM+Fato


C
Con
Fato
Non-Diabetic
Diabetic
PSR Stain (% of Total Area)
Con
Fato
DM
DM+Fato

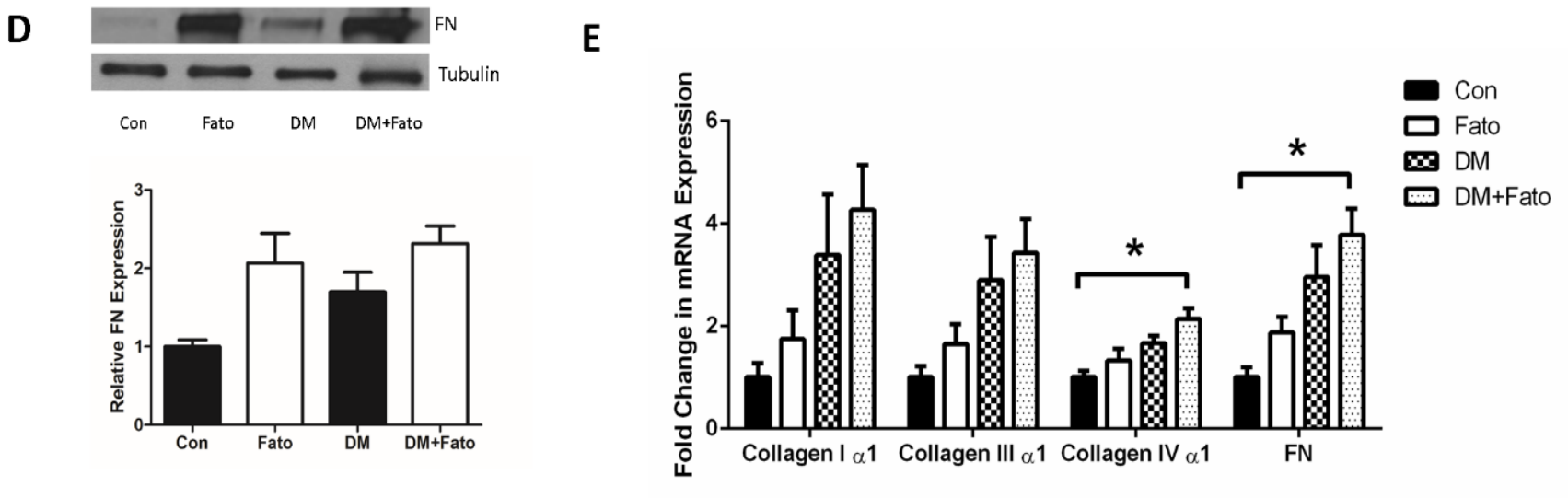
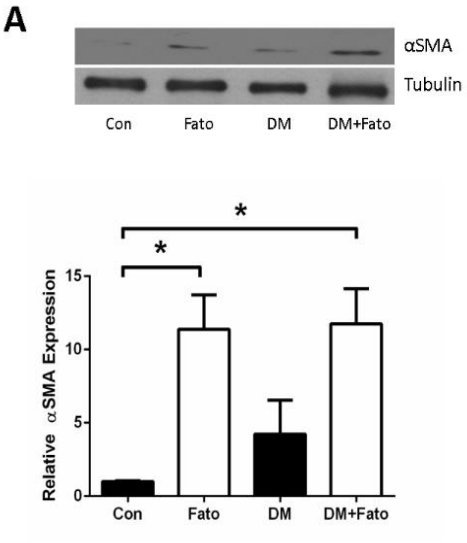
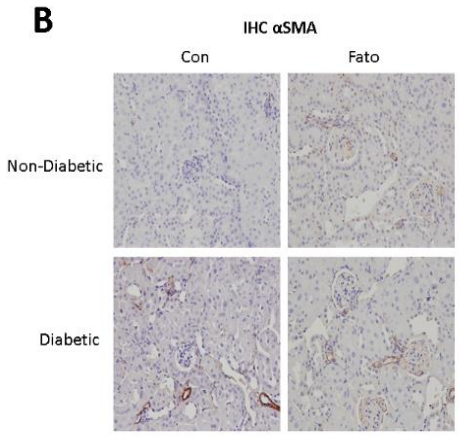
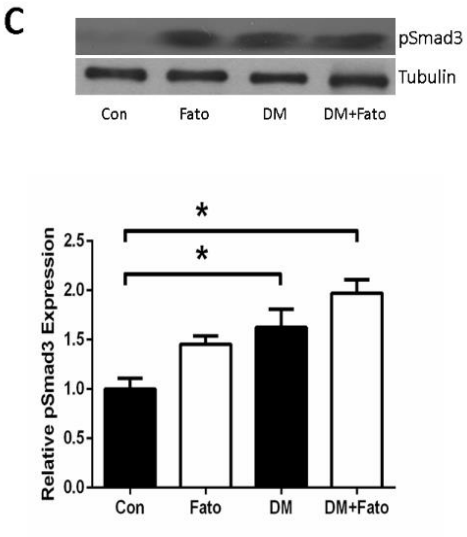


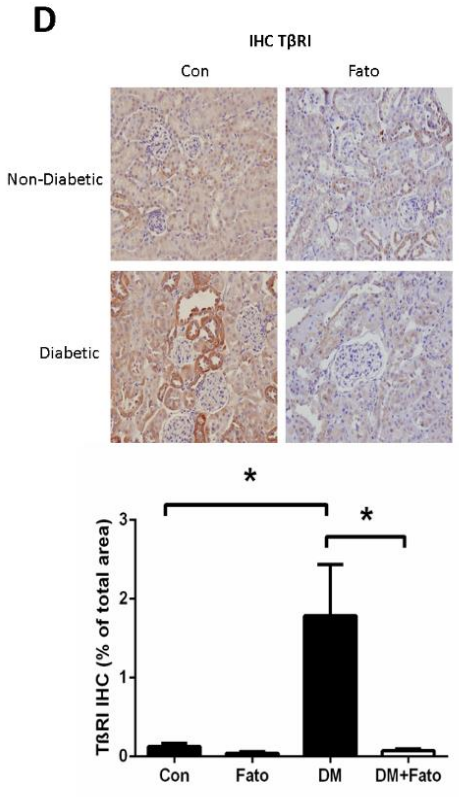
Figure 3-3 - Fatostatin does not attenuate renal fibrosis.

(A) Matrix accumulation, as assessed by PAS staining was increased in diabetic mice, and was not affected by fatostatin. (B) Tubulointerstitial fibrosis was assessed by trichrome staining, with images taken at 400x. The increase in diabetic kidneys was unaffected by fatostatin. (C) Collagen accumulation was assessed by picosirius red staining. Fatostatin had no effect on the increased collagen deposition seen in diabetic kidneys. There was a nonsignificant trend towards more collagen accumulation in kidneys of mice treated with fatostatin alone.(D) Immunoblotting for fibronectin shows a significant upregulation in diabetic kidneys. Fatostatin increases fibronectin production in both control and diabetic (E) Transcript levels for collagens Iα1, IIIα1 and IVα1 and fibronectin were increased in diabetic kidneys. Fatostatin did not reduce this. * $P < 0.05$


A
αSMA
Tubulin
Con
Fato
DM
DM+Fato
*
*
Relative αSMA Expression
15
10
5
0
Con
Fato
DM
DM+Fato


B
IHC αSMA
Con
Fato
Non-Diabetic
Diabetic


C
pSmad3
Tubulin
Con
Fato
DM
DM+Fato
*
*
Relative pSmad3 Expression
2.5
2.0
1.5
1.0
0.5
0.0
Con
Fato
DM
DM+Fato


D
IHC TβRI
Con
Fato
Non-Diabetic
Diabetic
*
*
TßRI IHC (% of total area)
3
2
1
0
Con
Fato
DM
DM+Fato

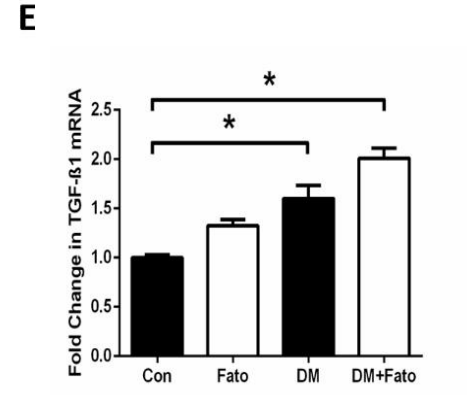
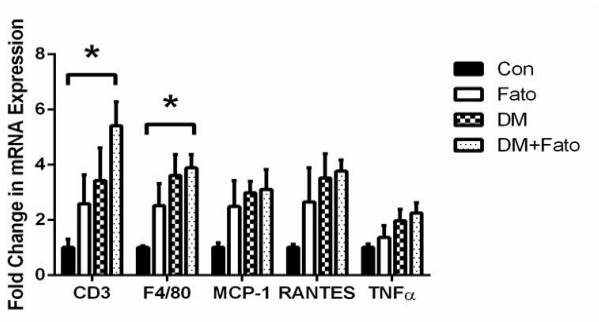
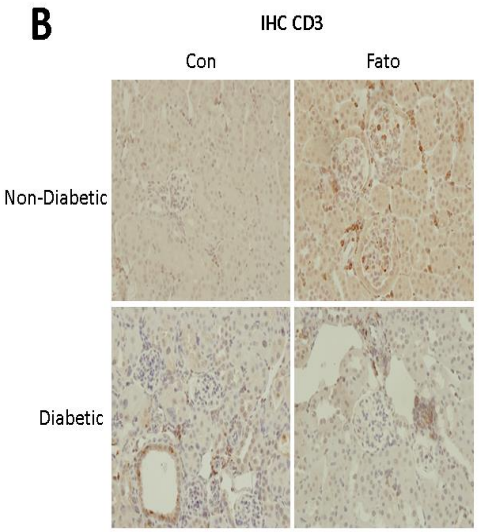
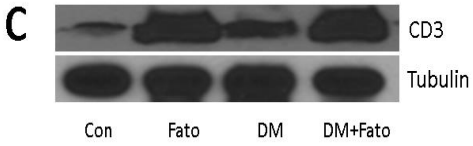

E
*
*
Fold Change in TGF-ß1 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
Con
Fato
DM
DM+Fato

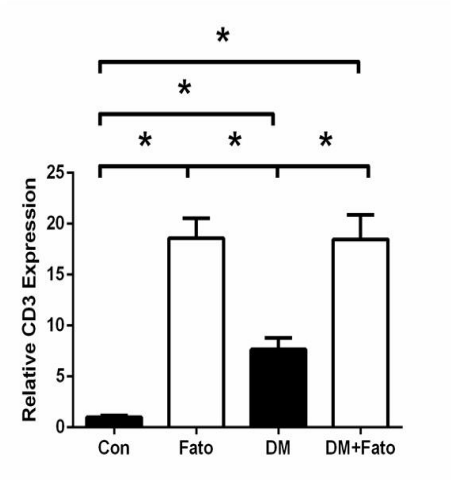
Figure 3-4 - Fatostatin does not affect the actions of renal TGF-β1.

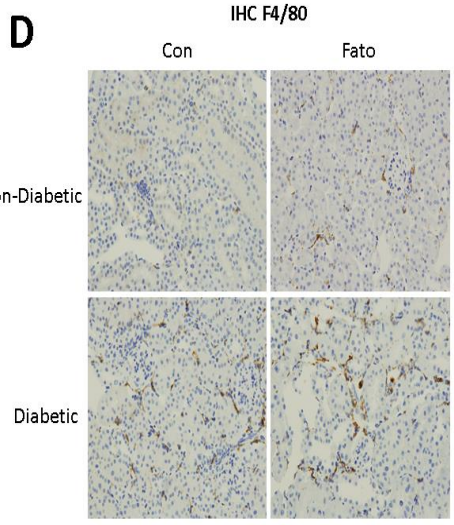
(A) and (B) Expression of α smooth muscle actin (αSMA) was assessed by western blot and IHC respectively. αSMA expression was elevated in mice treated with Fatostatin, indicative of elevated renal fibrosis. (C) Smad3 activation, assessed by its C-terminus phosphorylation, was increased in diabetic kidneys. (D) Expression of TβRI was significantly decreased by treatment with Fatostatin in diabetic kidneys, which corresponds with our previous study. (E) Upregulation of TGF-β1 transcript levels by diabetes was not inhibited by fatostatin. * $P < 0.05$


A
Fold Change in mRNA Expression
Con
Fato
DM
DM+Fato
CD3
F4/80
MCP-1
RANTES
TNFα


B
IHC CD3
Con
Fato
Non-Diabetic
Diabetic


C
CD3
Tubulin
Con
Fato
DM
DM+Fato


Relative CD3 Expression
Con
Fato
DM
DM+Fato


D
IHC F4/80
Con
Fato
Non-Diabetic
Diabetic

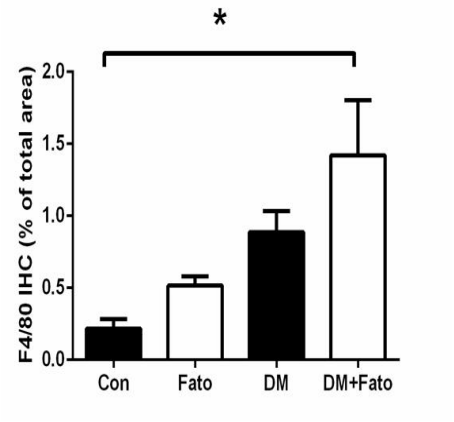

F4/80 IHC (% of total area)
Con
Fato
DM
DM+Fato

Figure 3-5 - Fatostatin promotes renal inflammation.

(A) Transcript expression of inflammatory cell markers and proinflammatory cytokines was assessed by Nanostring. (B) and (C) T-cell infiltration was assessed by IHC and western blot for CD3 respctively. CD3 staining was increased in diabetics, as well as with fatostatin treatment alone. (D) Infiltration of macrophage was assessed by staining for F4/80. F4/80 staining was increased by treatment with fatostatin. * $P < 0.05$

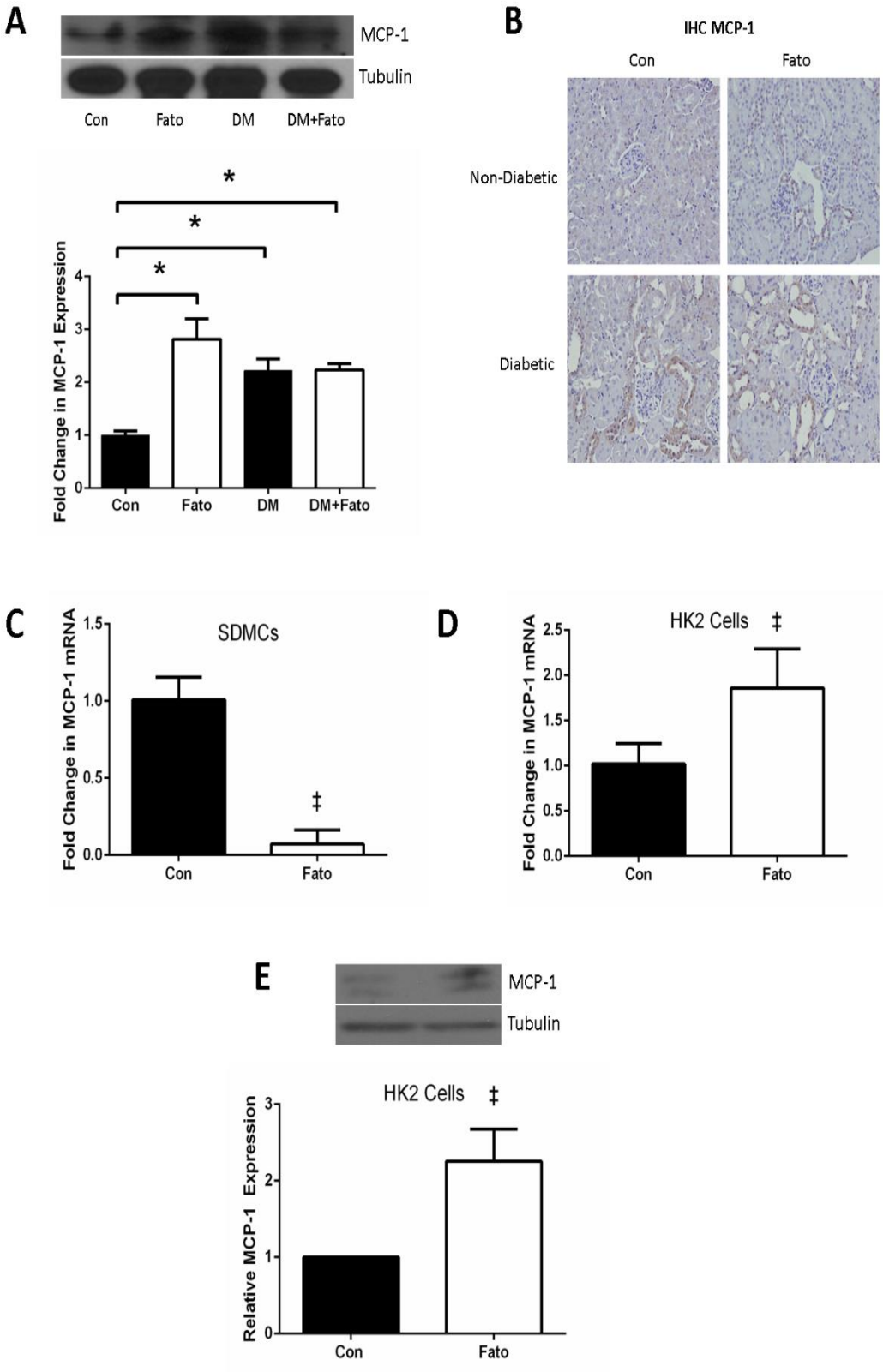

A
MCP-1
Tubulin
Con
Fato
DM
DM+Fato
Fold Change in MCP-1 Expression
B
IHC MCP-1
Con
Fato
Non-Diabetic
Diabetic
C
SDMCs
Fold Change in MCP-1 mRNA
D
HK2 Cells
Fold Change in MCP-1 mRNA
E
MCP-1
Tubulin
HK2 Cells
Relative MCP-1 Expression

Figure 3-6 - Fatostatin promotes the expression of MCP-1.

(A) and (B) Expression of MCP-1 was assessed by western blot and IHC respectively. Treatment with Fatostatin increased expression of MCP-1. (C) and (D) mRNA expression of MCP-1 was assessed by qRT-PCR in mesangial and tubular cells respectively. Treatment with Fatostatin inhibited expression of MCP-1 in mesangial cells, but induced its expression of renal tubular cells. (E) Expression of MCP-1 in tubular cells was assessed by western blot. Protein expression of MCP-1 was elevated in tubular cells following treatment with Fatostatin. * $P < 0.05$; ‡ $P < 0.05$ Treatment versus control

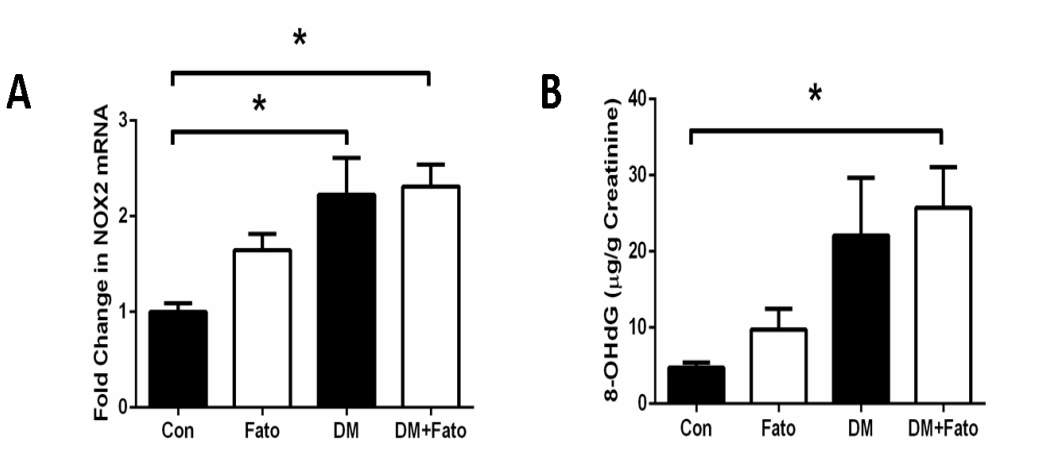


Figure 3-7 - Fatostatin promotes renal oxidative stress.

(A) Nox2 mRNA expression was increased in diabetic kidneys. This was not inhibited by Fatostatin. (B) Renal oxidative stress was assessed by determining the levels of oxidized DNA in the urine (8-OHdG). The increase in diabetic kidneys was not inhibited by fatostatin. * $P < 0.05$

Chapter 4 – Cell surface GRP78 is a novel marker of DN

Title: Cell surface (cs) glucose regulated protein 78 (csGRP78) is a novel marker of diabetic nephropathy

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Citation: In preparation for submission.

Preface

Significance to thesis

csGRP78 has been suggested to play a pathogenic role in the development of cancer, however it has not been previously examined in the context of diabetic nephropathy. Furthermore csGRP78 has been suggested to modulate TGF-β1 signaling in cancer. Since TGF-β1 is an important profibrotic cytokine strongly involved in the pathogenesis of DN, we hypothesized that by extension csGRP78 may also regulate DN. There are two main purposes of this study: (1) To discern if csGRP78 is upregulated in MCs in response to diabetic stimuli; and (2) To determine if csGRP78 is found in the kidneys of diabetic mice. These results would demonstrate a novel role for csGRP78 in intracellular signaling within MCs, and may establish a connection between csGRP78 and the profibrotic actions of TGF-β1. Furthermore, by examining the expression of csGRP78 in the kidneys of diabetic mice we would be able to determine if csGRP78 may act as a novel marker of DN. Our results have indicated that csGRP78 is upregulated in MCs in response to high glucose, and acts to increase the expression of TGF-β1. Furthermore, csGRP78 was found in the glomeruli of diabetic mice. These results demonstrate csGRP78 as a novel marker of DN, and suggest that therapy targeting csGRP78 may prove beneficial in the treatment of DN.

Author's contribution

Richard Van Krieken conducted most of the experiments, analyzed the results, and prepared the first draft of the manuscript.

Tong Wang conducted the experiments using the TGF-β1 luciferase and ELISA in figure 4A-4D.

Bo Gao assisted in cell culture experiments and maintenance of the animals.

Ehab Ayaub and Kjetl Ask assisted in the analysis of samples by flow cytometry.

Joan Krepinsky conceived of the project and edited the manuscript.

Abstract

The cell surface expression of glucose regulated protein 78 (csGRP78) has been shown to play a pathological role in many forms of cancer. However, a role for csGRP78 in kidney function and diabetes has not been previously examined. Our study is the first to show that in kidney mesangial cells (MCs), treatment with high glucose induces the expression of csGRP78. High glucose activation of csGRP78 signaling was found to occur through FAK and Akt, and requires the ligand α2-macroglobulin. Furthermore, inhibition of csGRP78 was found to prevent the expression and secretion of the profibrotic cytokine transforming growth factor β-1. We have further demonstrated that in the Akita and streptozotocin models of type I diabetes, expression of csGRP78 is increased in the glomeruli of diabetic mice. Collectively our results demonstrate that csGRP78 is upregulated during the pathogenesis of diabetic nephropathy, and is expressed in MCs in response to high glucose. Targeting of csGRP78 may provide a novel therapeutic target to treat diabetic nephropathy.

Abbreviations: csGRP78, cell surface glucose regulated protein 78; mesangial cell, MC

Introduction

Diabetic nephropathy (DN) is a microvascular complication of diabetes characterized by glomerulosclerosis [101]. Glomerular mesangial cells (MC) are known to play a key role in the development of glomerulosclerosis through synthesis and degradation of extracellular matrix (ECM) proteins [16]. Recent research has indicated that glucose regulated protein 78 (GRP78) may be translocated to the cell surface and play a pathological role in the development of diseases such as cancer [161]. Although a protective role for GRP78 has been suggested in the pathogenesis of DN [162], the relevance of cell surface GRP78 (csGRP78) in DN has not been previously examined.

GRP78 was originally described as a 78 kDa chaperone protein that assists in the proper folding of proteins in the endoplasmic reticulum (ER). It is ubiquitously expressed in mammalian cells and acts to control ER stress through regulation of the unfolded protein response. [161] In response to stessors such as ER stress [85], GRP78 has been also been reported to translocate to the cell surface through association with co-chaperone proteins like MTJ-1 [163] and Par-4 [164]. At the cell surface, the C-term and N-term of GRP78 are exposed allowing csGRP78 to act as a receptor for agonists such as α2-macroglobulin (α2M) and auto-antibodies against GRP78. Activation of csGRP78 promotes its association with other cell surface proteins, such as Integrin β1 [95], leading to a diverse collection of intracellular signaling events depending on the cell type and interacting partners [83]. Expression of csGRP78 has been described to occur in

pathological tissue such as tumors, and *in vitro* in carcinoma, endothelial, liver, and immune cells [83], indicating its prevalence among the pathogenesis of a growing number of disorders.

Induction of ER stress and elevated expression of GRP78 are well recognized features of rodent models [165] and humans with DN [162]. This is thought to be an adaptive response to maintain homeostasis [162]. The expression of csGRP78, however, has not been examined in the context of kidney function or diabetes. Our results suggest that under diabetic stress, GRP78 is translocated to the cell surface in renal mesangial cells (MCs). We demonstrate that csGRP78 acts as a receptor to α2M, and regulates the expression of the profibrotic cytokine TGF-β. Furthermore, diabetic mice demonstrate the expression of csGRP78 in the glomerulus and tubule structures of the kidney. Our results suggest that csGRP78 acts as important marker of DN through regulation of profibrotic signaling. Our data suggest that targeting csGRP78 may prove useful in the treatment of DN.

Materials and Methods

Cell Culture

Primary MCs were isolated from Sprague-Dawley (SD) rats as noted in previous work completed by our laboratory [79, 108-111]. MCs were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100 μg/ml) at 37˚C in 95% air, 5% CO_2. Cells between passages 8 to 17 were used. PC3 and 1-LN cells were

cultured in a high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100 μg/ml). Cells were serum deprived at 80-90% confluence overnight. Cells were treated with 24.4 mM glucose (to a final concentration of 30 mM glucose) for HG treatment. Prior to treatment with HG, cells were treated with the inhibitors: GRP78 C20 (Santa Cruz, SC1051), GRP78 N20 (Santa Cruz, SC1050), Subtilase Cytotoxin A (SubA), $SubA_{272}$, PF573228 (Tocris), and $\alpha2M^*$. SubA and $SubA_{272}$ was generously provided by Dr. James Paton. $\alpha2M^*$ was generously provided by Dr. Salvatore Pizzo.

Protein Extraction

Whole cell expression of protein was determined as previously described [113]. Briefly, cells were lysed in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol and protease/phosphatase inhibitors. Cellular debris was cleared from cell lysate by centrifugation at 13,000 rpm for 10 minutes at 4ºC. Proteins were separated by SDS-PAGE and probed for: PDGFR (1:10000, Santa Cruz), GRP78 (1:1000, BD Transduction), pAkt Ser473 (Cell Signaling, 1:1000), Akt (Cell Signaling, 1:1000), pFak Y397 (Upstate, 1:1000), FAK (Santa Cruz, 1:1000), MTJ-1 (Santa Cruz, 1:1000), KDEL (Santa Cruz, 1:500), and GAPDH (Cell Signaling, 1:1000).

Biotinylation

Cells were washed with ice-cold PBS and incubated with EZ-link Sulfo-NHSLC-Biotin (Pierce, 21331) (0.5 mg/ml in PBS) for 20 minutes. Biotinylation was

stopped with 0.1 M glycine in PBS. Cells were lysed in Cell Surface IP lysis, and biotinylated proteins were precipitated with 50% slurry neutravidin (Fisher, PI29200) overnight. The following day, the neutravidin beads were washed in wash buffer and assessed for protein expression.

ELISA

Conditioned media from MCs was collected after treatment. The amount of TGF-β1 in the media was determined through the TGF-beta 1 Quantikine ELISA Kit (R&D Systems, MB100B) as per manufacturer's instructions.

Flow Cytometry

MCs, 1-LN, and PC3 cells were allowed to grow until 90% confluence. Following their respective treatments, cells were washed three times with cold PBS and harvested with accutase (Innovative cell technologies, AT104). Cells were washed three times with FACS buffer (1% FBS in PBS) and counted manually using a hemocytometer. Cells were incubated with 5 μg/10^6 cells of the C20 GRP78 antibody (Santa Cruz, SC1050) for 1 hour at 37°C. Cells were washed with FACS buffer, and then incubated with 1 μg/10^6 cells of AF488 anti-goat secondary antibody (Molecular Probes, A11055) for 1 hour at 37°C in the dark. Cells were washed with FACS buffer, and resuspended in 1% paraformaldehyde in PBS. Staining was assessed using the LSRII software from BD Biosciences (San Jose, CA, USA) and analyzed using FlowJo software from Treestar (Ashlan, OR, USA).

Non-denaturing gel

1 μg of recombinant α2M (Cedarlane, 1938-PI) or α2M* (provided by Dr. Pizzo) were diluted in 2x loading buffer (62.5 mM Tris HCl pH 6.8, 25% glycerol, 1% bromophenol blue). Samples were ran on a 6% non-denaturing acrylamide gel for 6 hours at 100 V. Protein was fixed on gel using a fixing solution (10% methanol, 7% acetic acid), stained with Sypro Ruby (Sigma) overnight at 4°C, and then imaged under ultraviolet light.

mRNA and qRT-PCR

mRNA was isolated using Trizol (Invitrogen), and 1 μg of RNA was reverse transcribed using qScript (Quanta Biosciences). Real-time PCR was carried out using primers for α2M (Fwd: 5' ACCAGGACACGAAGAAGGAG 3'; Rev: 5' CCACTTCACGATGAGCATGG 3') and 18S (Fwd: 5' TGCGGAAGGATCATTAACGGA 3'; Rev: 5' AGTAGGAGAGGAGCGAGCGACC 3'). Reactions were performed in an Applied Biosystem 7600 system. mRNA was determined relative to 18S of the same sample using the $\Delta\Delta C_T$ method.

RNA Interference

Rat MTJ-1 and α2M on-target plus Smart Pool siRNA and non-specific control siRNA were obtained from Dharmacon (Lafayette, CO). MCs were plated to 70% confluence and then transfected with 100 nM of the respective siRNA (MTJ-1 and α2M) utilizing GeneEraser siRNA reagent (Stratagene). After 48 hours, cells were serum-deprived for 24 hours and harvested for mRNA and protein.

Luciferase

MCs were plated to 50% confluence and transfected with 0.5 μg of the TGFβ-luciferase (kindly provided by Dr. N. Kato) and 0.05 μg pCMV β-galactosidase (Clontech) using Effectene (Qiagen). Cells were serum-deprived overnight following transfection and then treated. Lysis was attained with a Reporter Lysis Buffer (Promega) with one freeze-thaw cycle. Luciferase and β-gal activity were determined using respective kits (Promega) with a Berthold luminometer and plate reader (420 nm). β-Gal activity was used to normalize for transfection efficiency.

Animals

Animal studies were carried out in accordance with McMaster University and the Canadian Council on Animal Care guidelines. Male CD1 (Charles River), 9 weeks of age, underwent a left nephrorectomy. At 10 weeks of age, diabetes was induced by a single intraperitoneal (IP) injection of streptozotocin (STZ) at 200 mg/kg. Control mice were injected with an equal volume of citrate buffer. Blood glucose was tested the following week and mice with blood glucose values of > 17 mM were enrolled in the study. Diabetic mice that developed ketonuria (assessed by dipstick [Bayer Multistix, Toronto, ON, Canada]) were implanted with an insulin pellet (LinShin Canada, Toronto, ON, Canada) to maintain body weight and hyperglycemia.

Imaging

Immunohistochemistry and immunofluorescence were performed on 4 μm paraffin sections that were deparaffinised. Tissue was stained with GRP78 C20

(Santa Cruz) overnight. For immunofluorescence, tissues were stained with AF488 anti-goat antibody (Molecular Probes, A11055). The plasma membrane of the tissue was visualized by staining with wheat germ agluttin-CF594 (WGA) (Biotium, 29073). Images were taken at 20x and 40x magnification. Colocalization between GRP78 and the plasma membrane stain was determined through the colocalization plugin available for ImageJ.

Cell Surface preparation from animal tissue

Frozen animal tissue was processed with the Minute^{TM} Plasma Membrane protein isolation kit (Invent Biotechnologies, SM005) per manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using the two-tailed t-test for experiments with only two experimental groups. Experiments with more than two groups were analyzed by one-way ANOVA with Tukey's HSD post hoc analysis. A $P<0.05$ was considered significant. Data is presented as mean ± SEM.

Results

High Glucose induces the translocation of GRP78 to the cell surface

The translocation of GRP78 to the cell surface has been noted under conditions of stress in carcinoma, endothelial, liver, and immune cells [83]. However, the effect of diabetic stimuli on the cell surface localization of GRP78 in kidney MCs have not been determined. Treatment of MCs with high glucose (HG) induced the expression of csGRP78 as assessed by cell surface biotinylation (Figure 1A). To provide further evidence that high glucose induced csGRP78

expression, GRP78 expression was assessed by flow cytometry (Figure 1B). In agreement with our cell biotinylation data, high glucose induced the expression of csGRP78. PC3 and 1-LN cells are prostate cancer cell lines that have been reported to express low and high levels, respectively, of csGRP78 [166]. As a proof of concept experiment to demonstrate that the detection of csGRP78 by flow is representative of cellular effects, expression of GRP78 in PC3 and 1-LN cells were determined. 1-LN cells demonstrated elevated csGRP78 expression compared to PC3 cells (Figure 1C).

High glucose-induced FAK and Akt activation occur through csGRP78

We have previously demonstrated that Akt is phosphorylated in MCs in response to diabetic stress and is important in expression of ECM protein [167]; furthermore, activation of Akt has been previously established as a downstream regulator of csGRP78 signaling [168, 169]. GRP78 neutralization antibodies have been used to manipulate the activity of csGRP78. Binding of ligands against the C-terminus of GRP78 have been reported to inhibit csGRP78 signaling and induce apoptosis [91], whereas N-terminal binding promoted csGRP78 signaling and survival [89]. Surprisingly, treatment of MCs with antibodies targeting the C-terminal (C20) or N-terminal (N-20) of GRP78 both attenuated the phosphorylation of Akt in response to HG (Figure 2A). This suggests that the C- and N-terminal domains of csGRP78 are playing an important role in signaling in MCs.

The enzyme subtilase cytotoxin A (SubA) is a proteinase that selectively cleaves GRP78 but is unable to enter the cell. Thus SubA, and the $SubA_{272}$ mutant that is unable to cleave GRP78, have been utilized as tools to selectively target C-terminal signaling by csGRP78 [166]. In line with our previous results, proteolytic cleavage of csGRP78 attenuated HG-induced Akt activation (Figure 2B). The chaperone protein DnaJ-like protein 1 (MTJ-1) has been shown to act as a co-chaperone for GRP78 to promote its localization to the cell surface. [163] Knockdown of MTJ-1 attenuated HG-induced Akt activation (Figure 2C).

Activation of focal adhesion kinase (FAK) has been suggested to link activation of csGRP78 to phosphorylation Akt [95]. Inhibition of csGRP78 with neutralization antibodies, proteolytic cleavage, and knockdown of co-chaperone protein attenuated phosphorylation of FAK in response to HG (Figure 2A-2C). Furthermore, treatment with a FAK inhibitor attenuated activation of Akt in response to HG (Figure 2D). Together our data suggests that csGRP78 acts as a regulator of FAK and Akt signaling in response to diabetic stimuli in MCs.

α2M mediates csGRP78 signaling in MCs

α2M has been reported as a ligand that stimulates csGRP78 signaling through interaction with the C-terminal domain of GRP78. α2M may be biologically activated by proteinases to generate $\alpha2M^*$ which possesses enhanced biological activity. [86] To determine if the recombinant $\alpha2M^*$ we used in our studies were biologically active, we ran recombinant $\alpha2M^*$ and α2M a non-denaturing gel. $\alpha2M^*$ has been reported to run faster than α2M [170], and in

corroboration with this we showed that our recombinant α2M* had a predominant 'fast' band when compared to α2M (Figure 3A).

We next decided to determine if MCs may respond to α2M*, and found that treatment of MCs with 50 pM of recombinant α2M* stimulated phosphorylation of Akt (Figure 3B). Furthermore, inhibition of cell surface GRP78 with neutralization antibodies for GRP78 (Figure 3B) or proteolytic cleavage of csGRP78 with SubA (Figure 3C) prevented α2M* activation of Akt. Surprisingly, treatment with both C- and N-terminal antibodies against GRP78 attenuated Akt activation despite α2M* known binding to the C-terminal domain. We hypothesize that the ability of the N-terminal GRP78 antibody to antagonize α2M* signaling may be due to steric hindrance of the N-20 antibody, thus preventing α2M* from binding to its nearby binding domain.

We then attempted to assess the role of α2M in regulating HG signaling in MCs, which has not been previously examined. We demonstrated that knockdown of α2M with siRNA inhibited activation of Akt in response to HG (Figure 3D). Together our data demonstrates that α2M regulates the activation of csGRP78 in response to HG in MCs.

csGRP78 regulates the expression of TGF-β1

TGF-β1 is recognized as a key mediator of ECM accumulation in MCs and a key mediator of DN [101]. It has been previously established that HG drives the expression of TGF-β1 in MCs [171]. We have shown that treatment with neutralization antibodies against GRP78 decreased the secretion and expression of

TGF-β1 (Figure 4A and 4C respectively). In agreement with this data, prevention of accumulation of csGRP78 through depletion of MTJ-1 also prevented the secretion and expression of TGF-β1 (Figure 4B and 4D respectively). Collectively, this data demonstrates that antagonism of csGRP78 prevents the expression of TGF-β1 in response to HG. This suggests that targeting csGRP78 may be useful in reversing the renal fibrosis events that occur with DN.

csGRP78 is upregulated in the kidneys of type I diabetic mice

Since we had demonstrated that GRP78 is expressed at the cell surface *in vitro*, we examined the expression of csGRP78 *in vivo*. Akita mice carry a mutation in the Ins2 gene leading to improper formation of insulin and pancreatic injury resulting in type I diabetes [172]. Plasma membrane isolated from 44 week old Akita demonstrated an increased expression of GRP78 when compared to wild-type control (Figure 5A). Proper separation of the plasma membrane from the total organelle fraction was demonstrated by expression of the cell surface protein PDGFR primarily on the plasma membrane and expression of KDEL primarily in the organelle fraction (Figure 5B).

We next tested the expression of GRP78 in CD1 mice that were made diabetic through injection with streptozotocin. IHC staining for GRP78 revealed that there was elevated GRP78 expression in glomeruli of diabetic mice (Figure 6A). Furthermore, the staining was found surrounding the mesangium (shown in black arrows) suggesting the presentation of GRP78 at the plasma membrane. These results were further examined through immunofluorescence for GRP78 (in

FITC), wheat germ agluttin (WGA; in Texas Red), and DAPI. WGA is a lectin that binds to gylcoproteins and glycolipids on plasma membranes that contain sialic acid and N-acetylglucosamine [173]. We used the colocalization plugin for ImageJ to assess for areas where GRP78 and WGA were both present. Areas where both stains were present are presented as a colocalization mask highlighted in white. Glomeruli from diabetic mice demonstrated increased presentation of GRP78 on the plasma membrane in comparison to non-diabetic mice. In summary, our data demonstrates GRP78 is upregulated at the plasma membrane of two different models of type 1 diabetes in mice. Our data suggest that csGRP78 is upregulated during the pathogenesis of DN.

Discussion

Studies have shown that csGRP78 may be found in patients with prostate cancer and atheromatous lesions. Furthermore, its expression on cells ranging from cancer, endothelial, and macrophage cells have been suggested to play a pathological role in cell signaling, viral entry, and antigen presentation. [174] For the first time, our study has demonstrated that under diabetic stress, GRP78 translocates to the cell surface *in vitro* and *in vivo* in the kidney. At the cell surface, α2M activates csGRP78 leading to elevated expression of TGF-β1. These findings extend our knowledge of csGRP78 to diabetes, and suggest that csGRP78 acts as a novel marker of DN through regulation of profibrotic signaling in MCs.

It has been shown previously that treatment with HG induces the expression of GRP78 in kidney cells [175], however the impact of HG on the redistribution of GRP78 to the cell surface has not been previously examined. We have demonstrated that treatment with HG causes the expression of GRP78 on the cell surface. However, a point of interest was that csGRP78 was found on untreated MCs. This suggests that in MCs there is a pool of GRP78 that is constitutively found at the cell surface, however its role and importance in regulating the basal activity of MCs has not been determined.

The mechanisms by which GRP78 translocates to the cell surface remains an area of research. In our model, treatment with HG induced the translocation of GRP78 through an MTJ-1 dependent mechanism. MTJ-1 is a transmembrane protein that can act as a co-chaperone for GRP78 in protein folding [176]. Furthermore, knockdown of MTJ-1 has been demonstrated to inhibit the accumulation csGRP78 thus attenuating csGRP78 signaling [163]. However, the mechanism by which stressors such as HG cause the interaction between GRP78 and MTJ-1 remains unknown. Under basal conditions, GRP78 is typically retained in the ER through interaction with the KDEL receptor [177]. It has been hypothesized that translocation of GRP78 to the cell surface may be attributed to modification of the KDEL sequence on GRP78, or by overwhelming the KDEL receptors in the ER through elevated GRP78 expression [177]. Thus an attractive hypothesis may be that treatment with HG leads to modification of GRP78 in the ER resulting in its interaction with MTJ-1 and presentation on the cell surface.

At the cell surface, GRP78 has been shown to act as a receptor for a wide range of ligands. Canonically, activation of csGRP78 leads to the phosphorylation of Akt [168]. Our study has similarly shown that inhibition of csGRP78 prevented HG-mediated Akt activation. Topological analysis of csGRP78 has shown that the C- and N-terminal of GRP78 are exposed to external stimuli [178]. Activation of the C-terminal domain of csGRP78 is associated with apoptosis [91], whereas activation of the N-terminal domain is associated with cellular proliferation [89] in cancer cells. Surprisingly, treatment with C-terminal and N-terminal antibodies against GRP78 both antagonized activation of Akt in response to HG. Furthermore, treatment with SubA that cleaves GRP78 on Leu^{416} and Leu^{417} also antagonized Akt activation [166]. Together this suggests that the C- and N-terminal of csGRP78 in MCs signal in a similar matter to trigger Akt activation.

FAK is a protein tyrosine kinase localized at focal adhesion points that contributes to growth factor and integrin signaling [179]. Integrin β1 and FAK have been previously identified as important mediators for csGRP78 signaling in cancer cells [95]. Our study has shown that HG-mediated FAK activation is dependent on csGRP78 signaling, and that inhibition of FAK prevents activation of Akt. Our results suggest that csGRP78 activates FAK that leads to further downstream activation of Akt.

At the cell surface, GRP78 has been shown to act as a receptor for a wide range of ligands including α2M, vaspin, and autoantibodies against GRP78

[87-89]. Canonically, α2M has been described as an antiproteinase that binds and inhibits a wide range of proteinases. After α2M reacts with a proteinase, the receptor-binding domain of α2M is exposed leading to its enhanced biological activity as a ligand. [90] Our results have demonstrated that treatment of MCs with $\alpha2M^*$ induces activation Akt. These results are in agreement with other published data which has described α2M as a ligand for csGRP78 [86, 87, 163, 169, 180]. Increased secretion of α2M into the serum has been described in several disease including diabetes [181, 182]. Furthermore, α2M expression has been shown to be upregulated in patients with DN [183]. Our results demonstrate that downregulation of α2M with siRNA attenuated HG-mediated activation of csGRP78. These results indicate that α2M may play a pathological role in development of DN through the activation of csGRP78.

The expression of csGRP78 has been thus far demonstrated in human cancer (multiple myeloma and prostate) and atheromatous lesions, however its expression in the context of diabetes has not been examined. [184-186] For the first time, our study has demonstrated that GRP78 is found at the plasma membrane in diabetic mice. Collectively our data demonstrates that in diabetic mice, GRP78 is found at the plasma membrane and may act as a novel marker of DN.

Conclusion

csGRP78 has been shown to play a pathological role in human diseases such as cancer and atherosclerosis [174]. Our study has extended these findings to

include csGRP78 as a novel marker of DN. We have demonstrated that treatment with HG induced the cell surface expression of GRP78, and that targeting csGRP78 *in vitro* prevents HG-induced expression of TGF-β1. Furthermore, recent studies have demonstrated that targeting csGRP78 *in vivo* may be of therapeutic use in the treatment of cancer [97-99]. Future studies will be aimed at assessing the role of csGRP78 in mediating DN *in vivo*, further characterizing potential ligands for csGRP78, and the assessment of the efficacy of potential anti-csGRP78 therapy.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

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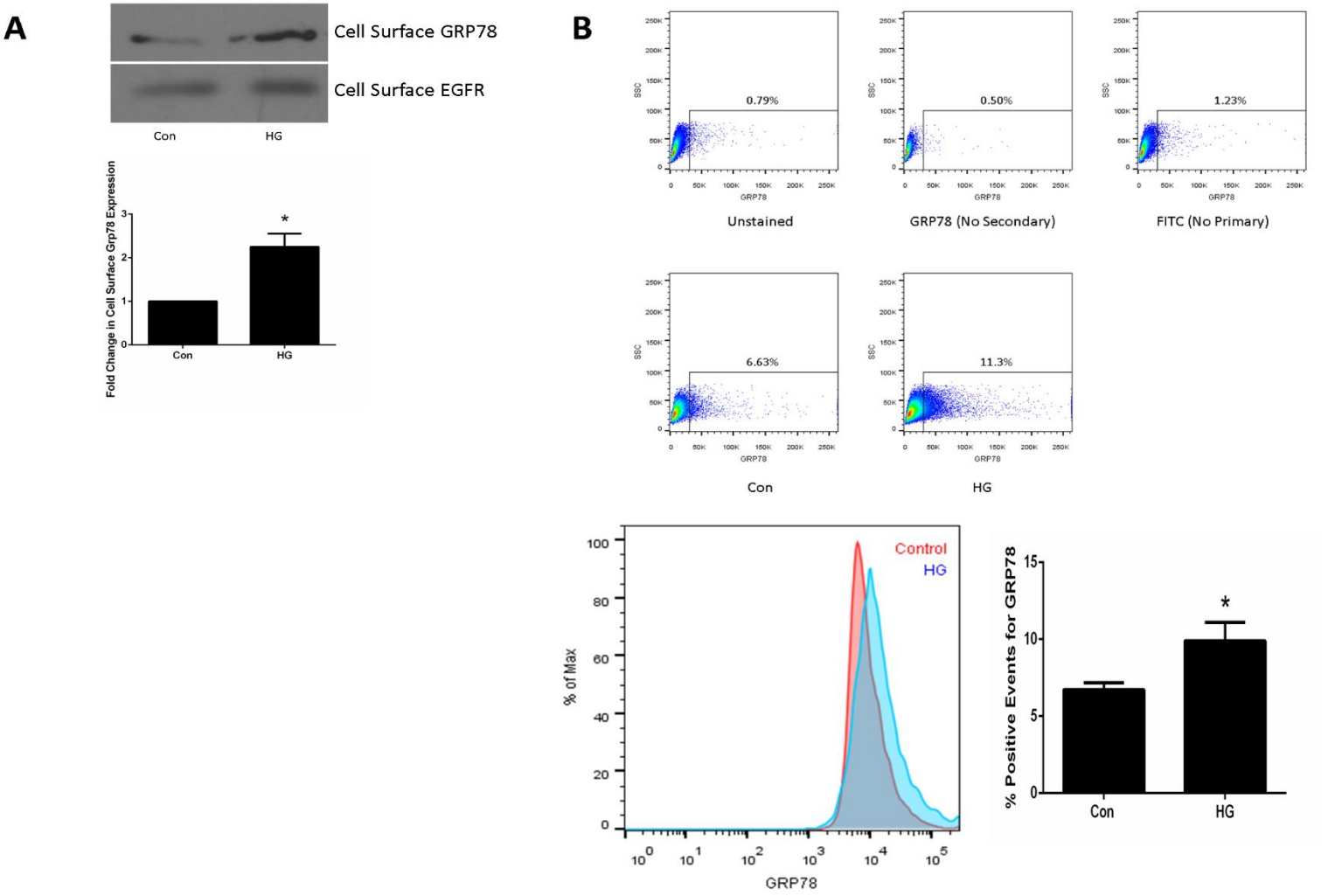
Author Contributions: RV conducted most of the experiments, analyzed the results, and wrote the first draft of the manuscript. TW conducted the ELISA and luciferase assays testing TGF-β1 expression. EA assisted in analyzing samples by flow cytometry. BG assisted in the cell experiments and animal care. JK conceived the idea and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Tables and Figures



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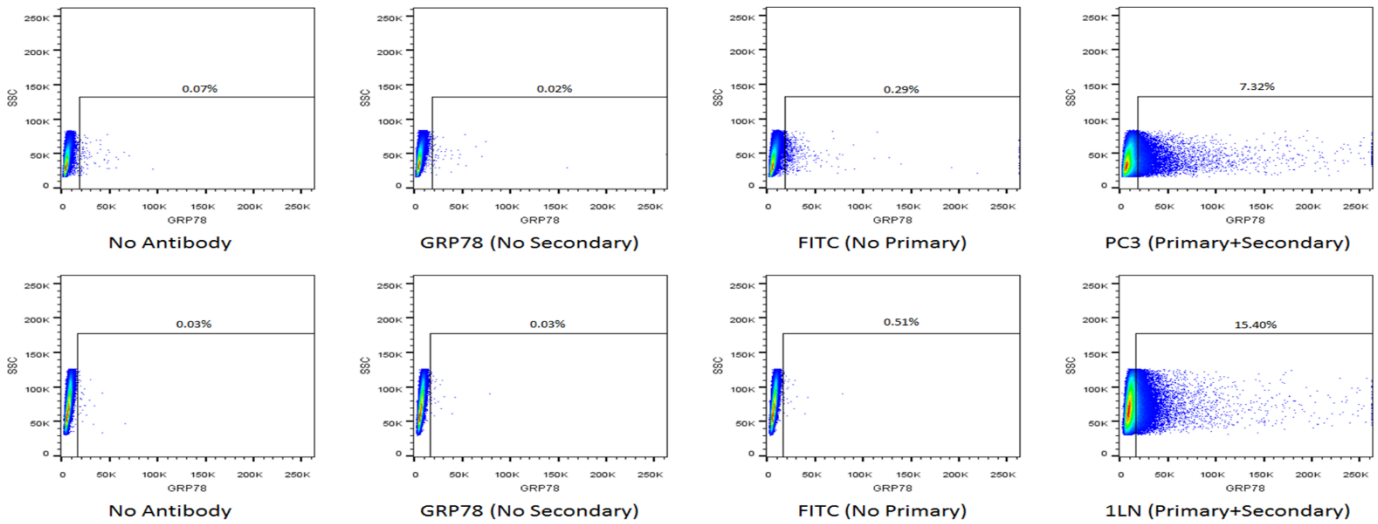

0.07%
No Antibody
0.02%
GRP78 (No Secondary)
0.29%
FITC (No Primary)
7.32%
PC3 (Primary+Secondary)
0.03%
No Antibody
0.03%
GRP78 (No Secondary)
0.51%
FITC (No Primary)
15.40%
1LN (Primary+Secondary)
SSC
GRP78

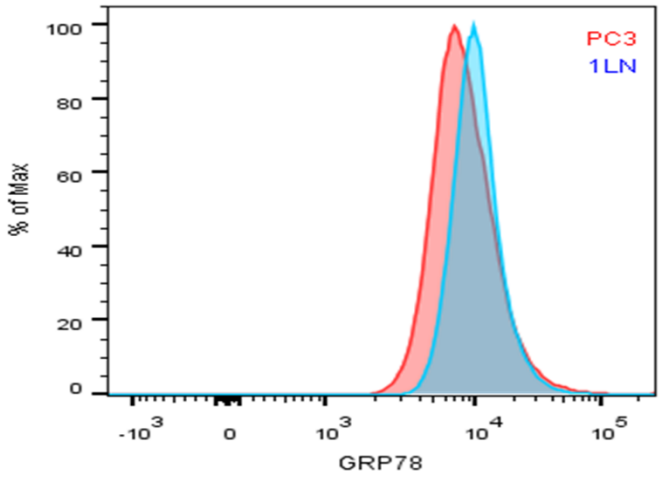

PC3
1LN
% of Max
GRP78

Figure 4-1 - High glucose promotes the cell surface expression of GRP78.

(A-B) MCs were treated with high glucose (HG, 30 mM) for 6 hours. (A) Cell surface proteins were labelled with biotin, pulled down, and ran on a Western. High glucose promoted the expression of cell surface GRP78 (n=4). (B) Expression of csGRP78 was assessed by flow cytometry in MCs. High glucose induced the expression of cell surface GRP78 as assessed by flow. (C) PC3 and 1-LN cells were labelled with (1) No antibody, (2) GRP78 primary antibody alone, (3) FITC Anti-goat secondary antibody alone, or (4) GRP78 antibody + FITC Anti-goat secondary antibody. 1-LN cells demonstrate higher csGRP78 expression in comparison to PC3 cells. * $P < 0.05$ Treatment versus Control.

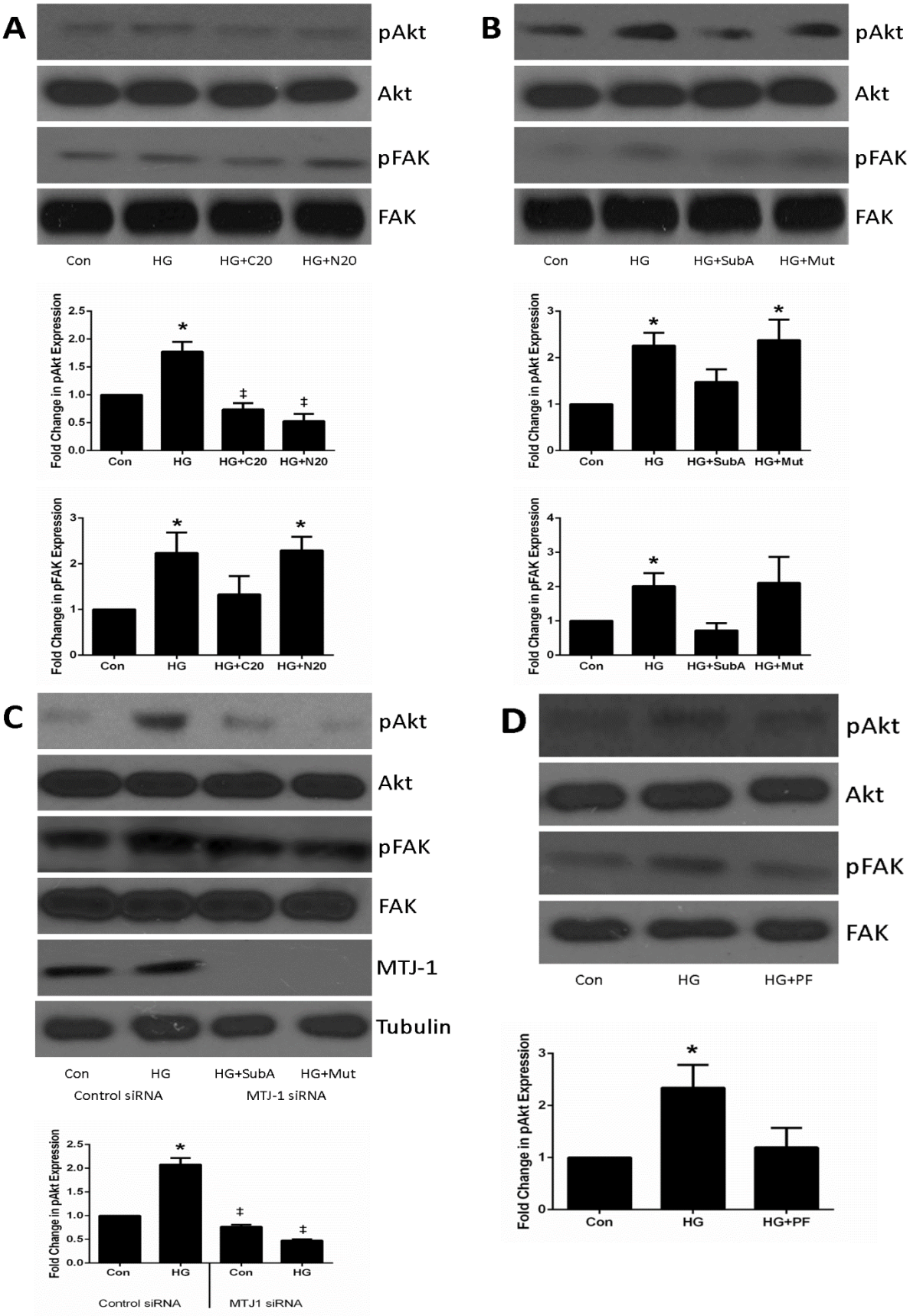
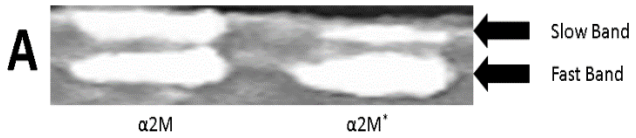
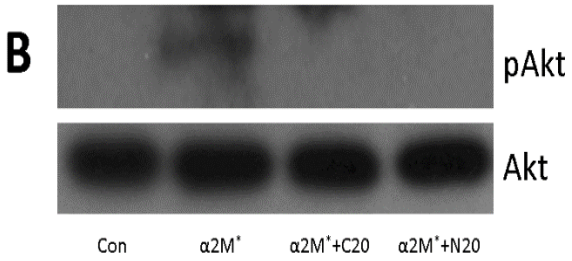
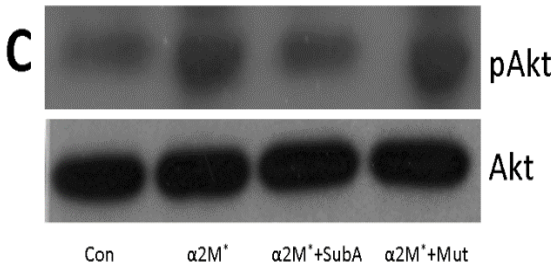

A
pAkt
Akt
pFAK
FAK
Con
HG
HG+C20
HG+N20
Fold Change in pAkt Expression
Fold Change in pFAK Expression
B
pAkt
Akt
pFAK
FAK
Con
HG
HG+SubA
HG+Mut
Fold Change in pAkt Expression
Fold Change in pFAK Expression
C
pAkt
Akt
pFAK
FAK
MTJ-1
Tubulin
Con
HG
HG+SubA
HG+Mut
Control siRNA
MTJ-1 siRNA
Fold Change in pAkt Expression
Con
HG
Con
HG
Control siRNA
MTJ1 siRNA
D
pAkt
Akt
pFAK
FAK
Con
HG
HG+PF
Fold Change in pAkt Expression
Con
HG
HG+PF

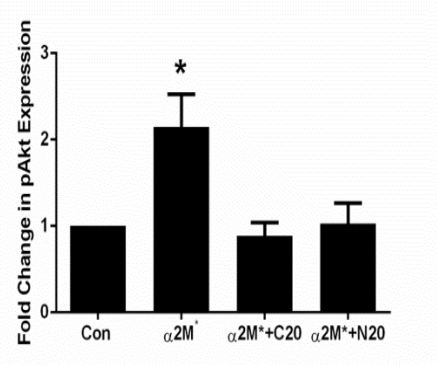
Figure 4-2 - High glucose signals through cell surface GRP78 to activate FAK and Akt.

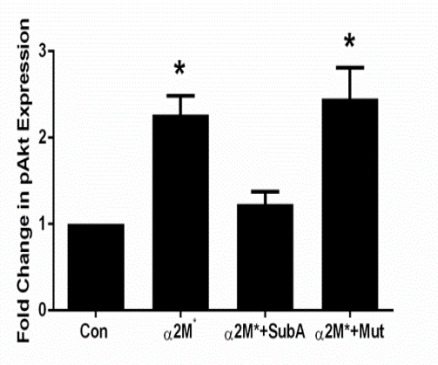
(A-D) MCs were treated with high glucose (HG, 30 mM) for 3 hours. (C) MCs were pretreated with 10 μg/ml of a GRP78 neutralizing antibody targeting the C-terminal (C20) or N-terminal (N20) domains. Treatment with the C20 and N20 antibodies prevented high glucose (n=5) mediated Akt activation, and attenuated activation of FAK in response to high glucose. (B) MCs were pretreated with 25 ng/ml of subtilase cytotoxin A (SubA) or an inactive mutant (SubA A272B; Mut) for 1 hour. Treatment with SubA prevented high glucose mediated Akt activation, and attenuated activation of FAK in response to high glucose (n=11). Treatment with the mutant SubA did not affect Akt or FAK activation. (C) MCs were transfected overnight with scramble or MTJ-1 siRNA (50 nM), and then serum starved and treated the next day (n=3). Knockdown of MTJ-1 prevented Akt and FAK activation in response to high glucose. (D) MCs were pretreated with the FAK inhibitor PF573228 (PF, 1 μM) for 1 hour (n=6). Treatment with PF573228 attenuated high glucose mediated Akt and FAK activation. * $P < 0.05$ Treatment versus Control. ‡ $P < 0.05$ HG versus Other

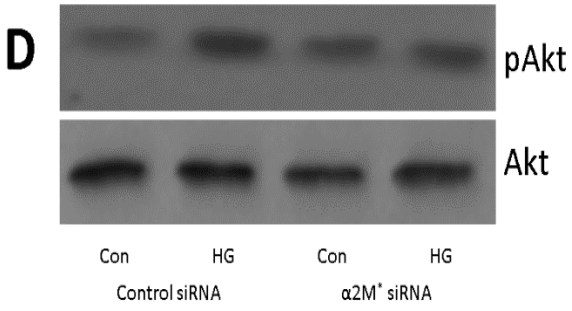

A
Slow Band
Fast Band
α2M
α2M*


B
pAkt
Akt
Con
α2M*
α2M*+C20
α2M*+N20


C
pAkt
Akt
Con
α2M*
α2M*+SubA
α2M*+Mut


Fold Change in pAkt Expression
0
1
2
3
*
Con
α2M*
α2M*+C20
α2M*+N20


Fold Change in pAkt Expression
0
1
2
3
*
*
Con
α2M*
α2M*+SubA
α2M*+Mut


D
pAkt
Akt
Con
HG
Con
HG
Control siRNA
α2M* siRNA

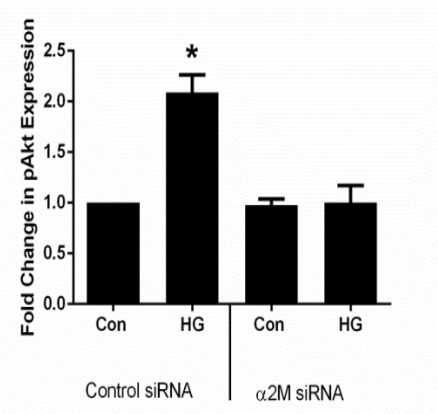

Fold Change in pAkt Expression
0.0
0.5
1.0
1.5
2.0
2.5
*
Con
HG
Con
HG
Control siRNA
α2M siRNA

Figure 4-3 - α2M* signals through cell surface GRP78 to activate Akt.

(A) Recombinant α2M and methyl-amine activated α2M* were run on a non-denaturing gel to test their bioactivity. The increased prevalence of the 'fast band' in the α2M* lane, in comparison to the α2M lane, indicates the high bioactivity of the methyl-amine active α2M*. (B-C) MCs were treated α2M* (100 pM) for 30 minutes. (B) MCs were pretreated with 20 μg/ml of a GRP78 neutralizing antibody targeting the C-terminal (C20) or N-terminal (N20) domains. Treatment with the C20 and N20 antibodies prevented α2M* (n=3) mediated Akt activation. (C) MCs were pretreated with 25 ng/ml of subtilase cytotoxin A (SubA) or an inactive mutant (SubA A272B; Mut) for 1 hour. Treatment with SubA prevented α2M* (n=3) mediated Akt activation. Treatment with the mutant SubA did not affect Akt signaling. (D) MCs were transfected overnight with scramble or α2M siRNA (100 nM), and then serum starved and treated with high glucose (30 mM, 3 hr treatment) the next day (n=3). Knockdown of α2M prevented Akt activation in response to high glucose. * $P < 0.05$ Treatment versus Control. ‡ $P < 0.05$ HG versus Other

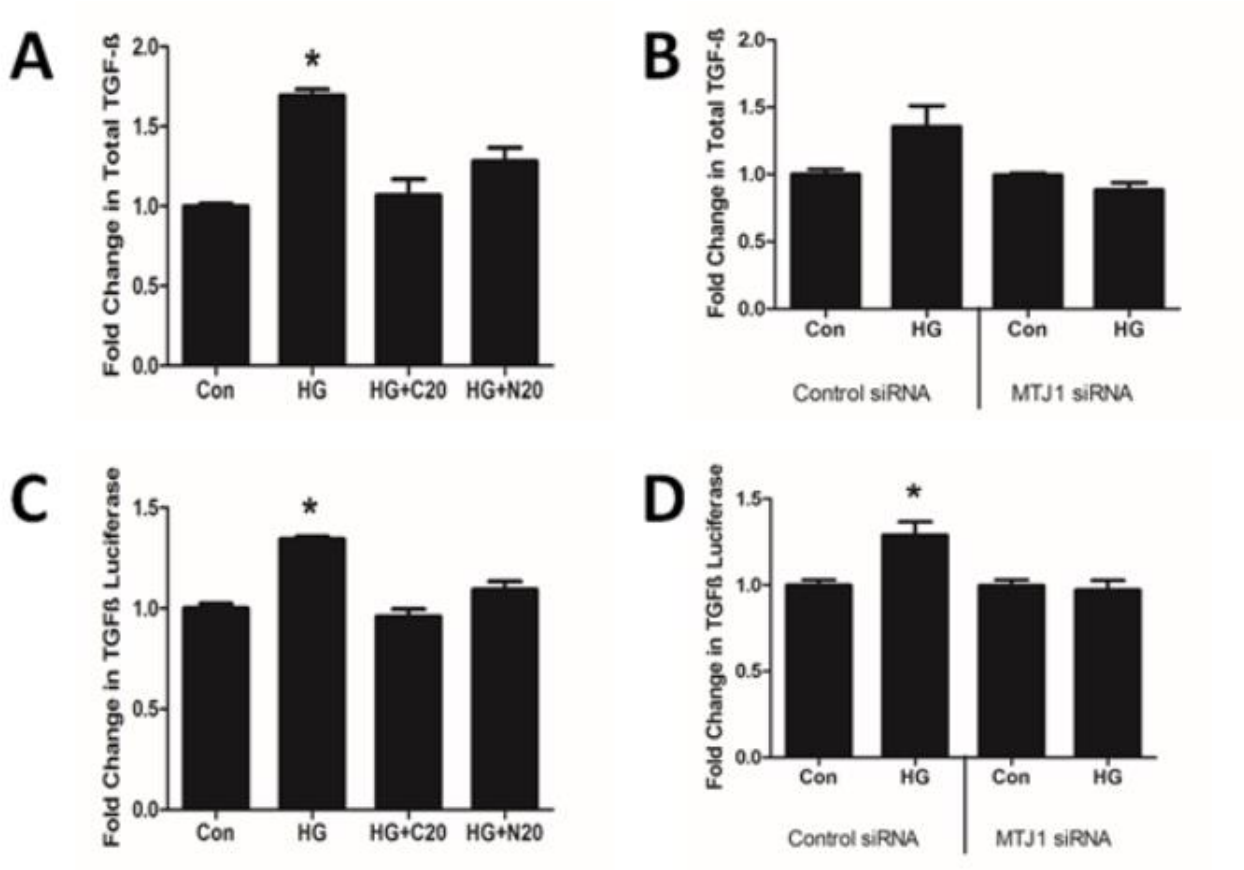


Figure 4-4 - Cell surface GRP78 facilitates the expression of TGF-β1.

(A-B) MCs were treated with high glucose (30 mM) for 24h, and conditioned media was harvested for analysis of active TGF-β by ELISA. (C-D) MCs were transfected with a TGF-β1 luciferase and treated with high glucose (30 mM) for 24 hours. Luciferase readings were normalized against β-Gal. (A,C) MCs were pretreated for 24 hours with 100 μg/ml ?dose of the C20 or N20 GRP78 neutralizing antibody (n=3). The C20 antibody decreased the luciferase activity and production of TGF-β1. (B,D) MCs were transfected with non-specific siRNA (Con) or MTJ-1 siRNA (n=3). Inhibition of MTJ1 decreased expression of TGF-β. * $P < 0.05$ Treatment versus Control.

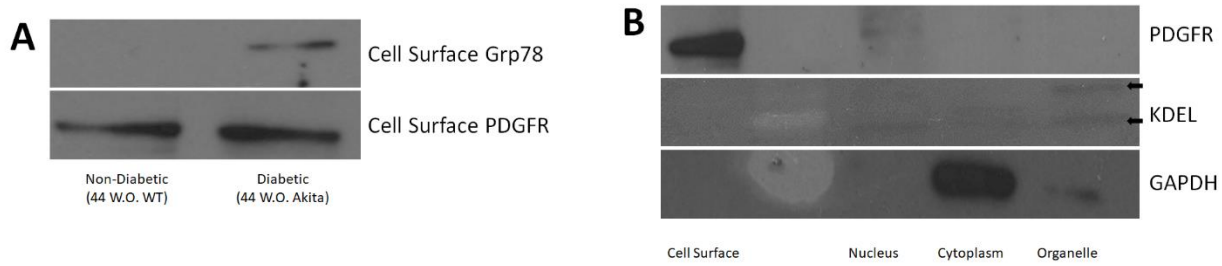
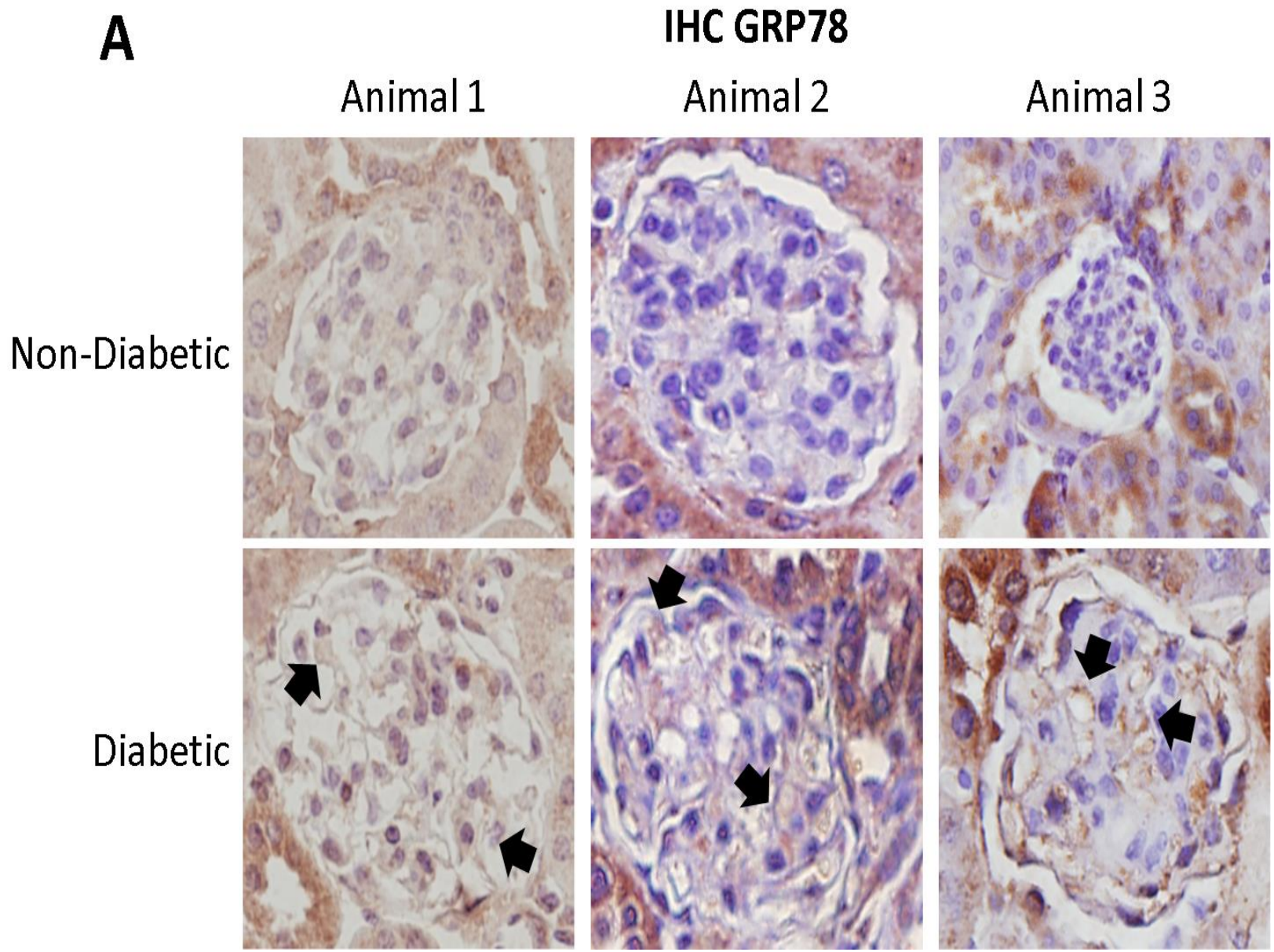


Figure 4-5 - GRP78 is upregulated at the cell surface in the kidney of diabetic mice.

Frozen kidney tissue from 44 week old male non-diabetic (C57BL/6) and type 1 diabetic (C57BL/6-Ins^{Akita}/J) mice were kindly provided by Dr. Thomas Hawke. The cell surface, organelle, cytoplasmic, and nuclear fractions were isolated from frozen samples using the Minute^{TM} Plasma membrane isolation kit (as mentioned in Methods). (A) Cell surface proteins were isolated. PDGFR was assessed as a loading control. (B) Proper fractionation of the tissue was assessed by probing for markers of the cell surface (PDGFR), organelles (KDEL), and cytoplasm (GAPDH).


A
IHC GRP78
Animal 1
Animal 2
Animal 3
Non-Diabetic
Diabetic

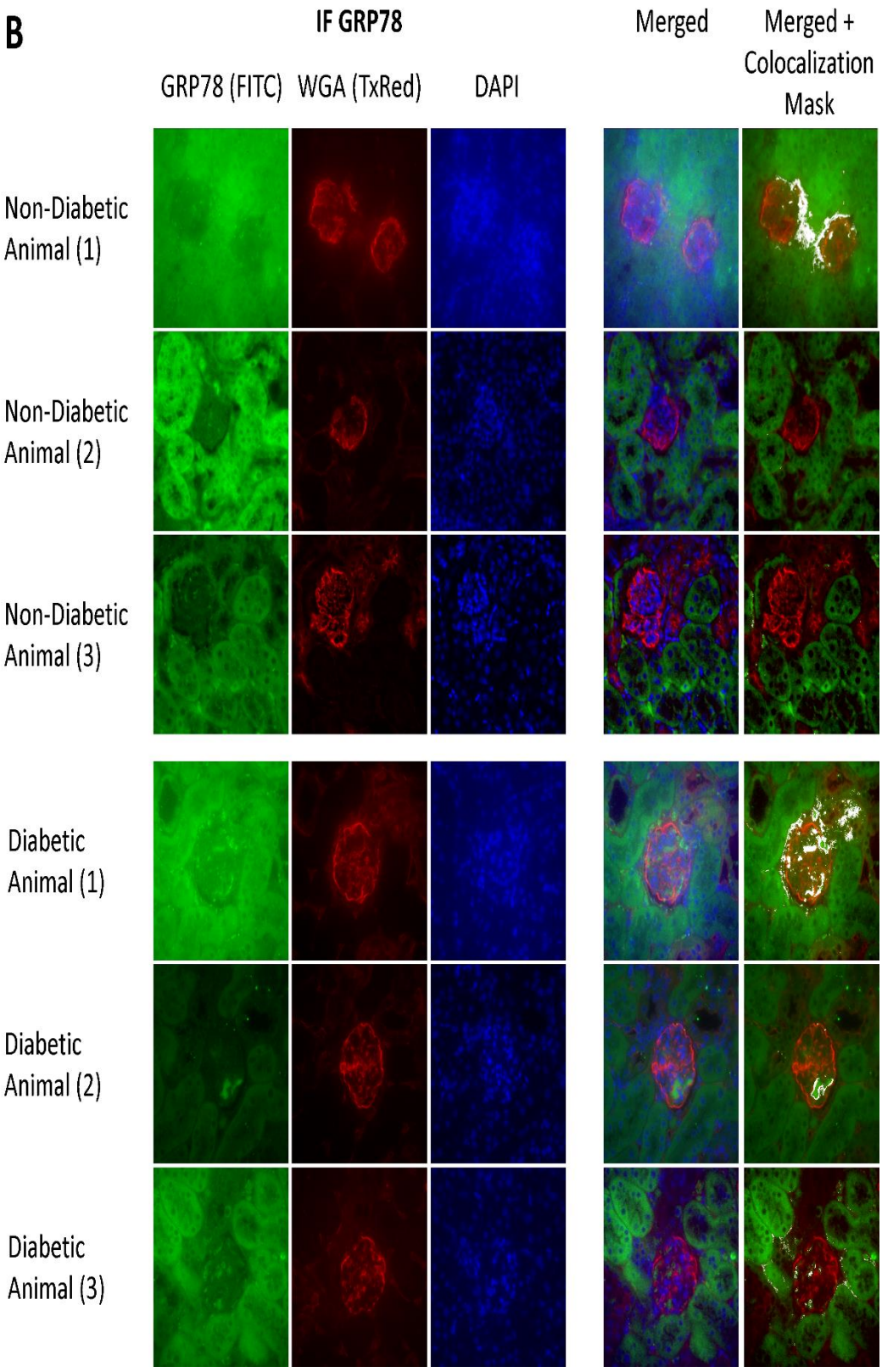

B
IF GRP78
GRP78 (FITC)
WGA (TxRed)
DAPI
Merged
Merged + Colocalization Mask
Non-Diabetic Animal (1)
Non-Diabetic Animal (2)
Non-Diabetic Animal (3)
Diabetic Animal (1)
Diabetic Animal (2)
Diabetic Animal (3)

Figure 4-6 - GRP78 is upregulated at the cell surface in the kidney of diabetic mice.

(A) Mice were stained for GRP78, and pictures were taken at 400x magnification. Black arrows indicate areas of the cell surface localization of GRP78. (B) Mice were stained for GRP78 (FITC), a marker for the cell surface WGA (Texas Red), and the nucleus. Pictures were taken at 400x. Co-expression of GRP78 and the WGA stain was assessed by the colocalization plugin available for ImageJ. Areas that coexpressed GRP78 and the WGA stain are shown in white. Three mice each from the non-diabetic and diabetic groups were stained for GRP78. Diabetic mice demonstrated elevated expression of cell surface GRP78 through IF and IHC.

Chapter 5 - General Discussion and Conclusions

DN is a major complication that arises in 20-40% of diabetic patients and is the leading cause of ESRD [3]. Furthermore, patients with DN are at a higher risk of all cause and cardiovascular-disease mortality than diabetic patients without nephropathy [5]. Current treatment for DN focuses on glycemic control and inhibition of RAAS [6]. However, these treatments are aimed at slowing disease progression but are not enough to prevent disease progression in most patients [6]. Thus there is a strong demand to better characterize the pathophysiology of DN in order to identify novel therapeutic targets.

TGF-β1 is a member of the TGF-β superfamily and is the dominant isoform present in the kidney [30]. TGF-β1 has been well characterized as a profibrotic factor that drives renal fibrosis through: (1) Increased production of ECM proteins; (2) Decreased production of matrix degradation proteins; and (3) Induction of EMT. TGF-β1 has been shown to play an important role in DN [27, 30, 101], however clinical trials examining the effects of anti-TGF-β1 therapy have been slow in development [55]. Early trials utilizing anti-TGF-β1 therapy in patients with DN have demonstrated mixed results. Furthermore, concern have been raised that targeting TGF-β1 may be associated with severe adverse effects due to the pleiotropic actions of TGF-β1 [32]. Thus an attractive area of study is to examine the mechanisms by which TGF-β1 signaling is activated and regulated during the pathogenesis of DN. This is done with the hopes to reveal new potential therapeutic targets that may modulate TGF-β1 expression or activity, rather than directly targeting TGF-β1, thus avoiding potential adverse effects.

The SREBPs are transcription factors that regulate cholesterol and lipid metabolism in the body [58]. The SREBPs have been shown to be upregulated during the pathogenesis of DN, and have been suggested to contribute to DN progression by promoting dyslipidemia and renal fibrosis in the kidney [75, 76]. This was further supported in a study conducted on mice lacking SREBP-1 in which knockout of SREBP-1 attenuated early manifestations of DN [78]. We have also demonstrated that inhibiting SREBPs *in vivo* improved renal fibrosis and kidney function in a hypertensive and UUO model of kidney disease [102, 103]. Furthermore, studies from our laboratory have revealed that SREBP-1 is important in co-ordinating profibrotic TGF-β1 signaling in MCs [79, 80]. Better characterizing the mechanisms by which SREBP-1 and TGF-β1 crosstalk may provide new potential therapeutic targets to modulate TGF-β1 signaling. Furthermore, due to earlier studies that have demonstrated SREBP-1 to be important in DN, evaluating the efficacy of anti-SREBP therapy *in vivo* is of high importance.

GRP78 has been well characterized as an ER chaperone protein, however it has been shown GRP78 may be translocated to the cell surface in diseases such as cancer [83]. csGRP78 has been associated with the pathogenesis of these diseases, however the role of csGRP78 in diabetes and kidney disease has not been previously described. Studies have suggested that there is an interaction between csGRP78 and TGF-β1 signaling in cancer and immune cells [94, 104]. Furthermore, unpublished data from our laboratory have suggested that csGRP78

may play a role in the activation of SREBP-1 in kidney MCs. Since SREBP-1 has been shown to play an important role in the regulation of profibrotic signaling, regulation of SREBP-1 signaling by csGRP78 may play an important role in renal fibrosis. This suggests that better understanding of the mechanisms of csGRP78 signaling may reveal novel therapeutic targets that may modulate TGF-β1 signaling. Furthermore, examining the expression of csGRP78 in DN may lead to the development of novel biomarkers for DN.

Our current studies are aimed at: (1) Determining if SREBP-1 influences TGF-β1 signaling outside of transcriptional regulation; (2) Evaluating the efficacy of SREBP inhibition *in vivo* in the treatment of DN; and (3) Studying the expression of csGRP78 in DN and its role in regulating of TGF-β1. Our *in vitro* studies were carried out in MCs due to their importance in the pathogenesis of DN by mediating glomerulosclerosis [16]. Our major findings are: (1) SREBP-1 regulates TGF-β1 signaling through the expression of TβRI via exosomal secretion; (2) Inhibition of SREBP *in vivo* with the SCAP inhibitor fatostatin does not reverse DN; and (3) csGRP78 is a novel marker of DN and regulates TGF-β1 expression *in vitro*. Our studies have revealed novel mechanism by which TGF-β1 is regulated by SREBP-1 and csGRP78. However, we have determined that treatment with fatostatin does not confer protective effects against DN in mice, but rather leads to kidney dysfunction in non-diabetic mice. This suggests that inhibition of SREBP with fatostatin is not a viable treatment for DN, and that other anti-SREBP therapies should be evaluated.

Fatostatin does not attenuate DN despite the role of SREBP-1 in TGF-β1 signaling

SREBP-1 is a novel regulator of TβRI through exosome secretion

Our lab has previously shown that SREBP-1, outside of its role in dyslipidemia, has a direct role on TGF-β1 signaling. Uttarwar et al. [79] showed that SREBP-1a and SREBP-1c expression and activity was induced in response to the diabetic stimuli HG. Antagonism of SREBP-1 attenuated HG-mediated TGF-β1 expression by preventing SREBP-1 from binding to the TGF-β1 promoter. Chen et al. demonstrated that treatment with TGF-β1 drives the expression and activation of SREBP-1a and SREBP-1c, and in turn SREBP-1 was found to drive activation of TGF-β1 signaling [80]. Furthermore, SREBP-1a was found to act as a co-factor for Smad3 by facilitating its transcriptional activity. These results collectively demonstrate that the transcriptional activity of SREBP-1 is required for its regulation of TGF-β1 expression and signaling. Our current study extends these results and demonstrates that independent of its transcriptional activity, SREBP-1 is required for TGF-β1 signaling in MCs through regulation of TβRI.

TβRI is a Ser/Thr kinase that belongs to the family of type I TGF-β receptors that activates the R-Smads (Smad2/3) in response to TGF-β1 [34]. Our results have indicated that inhibition of SREBP-1 led to downregulation of TβRI. Surprisingly, the regulation of TβRI by SREBP-1 does not occur through canonical mechanisms involving transcription, degradation (proteasomal and lysosomal), or proteolytic cleavage. By pursuing alternative pathways, we

discovered that inhibition of SREBP-1 led to the accumulation of TβRI in exosomes secreted from MCs. Exosomes are vesicles ranging from 30 to 100nm in diameter consisting of a lipid bilayer, transmembrane proteins, and a hydrophilic core with proteins and RNA [43, 44]. Formation of exosomes has been associated with maturation of endosomes cycled from lipid-raft endocytosis [126-128]. In agreement with this observation, we demonstrated that disruption of lipid rafts, but not caveolae, prevents the downregulation of TβRI by SREBP-1 inhibition.

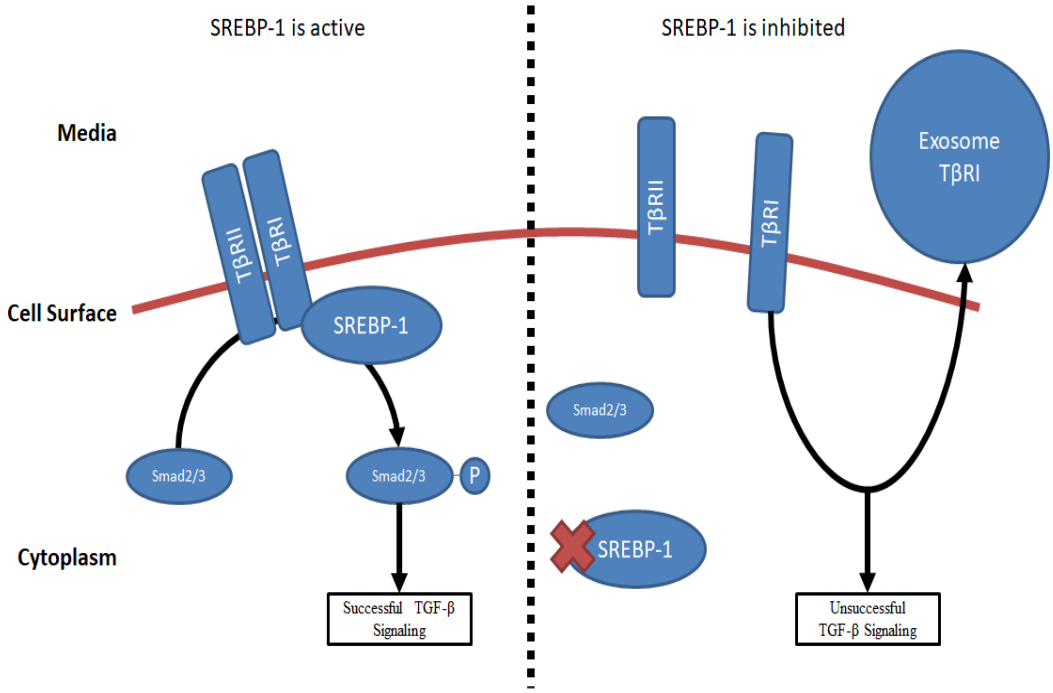


Figure 5-1 – Model of SREBP-1 regulation of TβRI.

In basal conditions, when SREBP-1 is active, it binds to TβRI and retains the receptor at the cell surface. This promotes activation of TGF-β1 signaling and activation of Smad3. When SREBP-1 is disrupted, TβRI is endocytosed by lipid

rafts and is secreted from the cell in exosomes. Secretion of TβRI prevents the activation of TGF-β1 signaling and leads to inhibition of Smad3 phosphorylation. Taken from Van Krieken et al. (2017) [47].

Our first study demonstrates a novel mechanism of action of SREBP-1 and a novel mechanism of regulation of TβRI. Our data demonstrates for the first time that SREBP-1 acts as a retention factor for TβRI that is independent of its actions as a transcription factor. Furthermore, we have demonstrated that secretion of TβRI in exosomes as a new mechanism to regulate TGF-β1 signaling. This form of regulation has been shown to be relevant in the reticulocyte maturation and p53 signaling, but overall have not been well characterized [45, 46]. Although beyond the scope of our current study, delineating the mechanism by which SREBP-1 regulates TβRI localization into exosomes may provide additional insight as to how cargo is sorted into exosomes. Furthermore, future studies will aim to study how SREBP-1 is localized outside of the nucleus and how it interacts with TβRI. This may prove useful when studying new potential therapeutic targets by attempting to manipulate the contents of exosomes to regulate intracellular signaling. Furthermore, our results suggest that SREBP-1 is deeply-rooted with TGF-β1 expression and signaling, and may be a useful target when treating DN developed renal fibrosis as part of their pathophysiology.

Inhibition of SREBP-1 does not attenuate DN

Due to the importance of TGF-β1 signaling in DN, we expected inhibition of SREBP *in vivo* to be protective role due to its close relationship to TGF-β1 signaling. To this end, we used the SCAP inhibitor fatostatin. Fatostatin is a small chemical inhibitor that binds to SCAP at a location distinct from SCAP's sterol-binding domain. This prevents the SCAP-SREBP complex from translocating into the Golgi, thus stopping its activation by S1P and S2P [118]. We have previously established the efficacy of fatostatin therapy in fibrotic kidney disease using two models: (1) Ang II [103]; and (2) UUO [102]. Ang II is a well-known pathogenic factor in ESRD and DN that mediates the expression of profibrotic cytokines (such as TGF-β1) and ECM accumulation. Treatment of Ang II-infused mice with fatostatin prevented renal fibrosis with notable attenuation of ECM accumulation (collagen I, fibronectin) and inhibition of profibrotic signaling (reduced pSmad3 and TGF-β1 expression) [103]. The UUO model is a well-establish model of CKD with prominent renal tubular injury and fibrosis. Treatment with fatostatin in UUO mice attenuated ECM accumulation (collagen I, fibronectin), and inhibited of profibrotic signaling (reduced pSmad3 and α smooth muscle actin expression) [102].

These data would suggest that fatostatin is efficacious in the treatment of fibrotic kidney disease by preventing profibrotic signaling. However, treatment with fatostatin in CD1 diabetic mice did not improve parameters of renal function (GFR and albuminuria) or fibrosis (trichrome, PSR, TGF-β1 expression, phosphorylation of Smad3, and expression of ECM genes). Furthermore,

treatment of non-diabetic mice with fatostatin induced hyperfiltration, glomerular hypertrophy, and demonstrated a non-significant trend towards increased albuminuria and renal fibrosis. Our current study demonstrates that treatment with fatostatin in diabetic mice is not protective in the development of DN, and induces kidney dysfunction in healthy mice. Future studies will assess the efficacy of the SREBP inhibitor betulin and use knockdown models of SERBP-1 and SCAP to assess the impact of their inhibition on DN progression.

Our second study is the first to investigate the effects of anti-SREBP treatment in the development of DN. Ishigaki et al had demonstrated that SREBP-1 knockout diabetic mice had reduced albuminuria, but did not fully characterize the DN phenotype in their mouse model [78]. Our current study has characterized the effects of SREBP inhibition on renal pathology, and revealed that treatment with fatostatin did not improve key clinical features of DN (such as albuminuria, GFR, renal fibrosis) and furthermore mediated a pro-inflammatory response in the kidney. We have characterized the effects of fatostatin on renal inflammation in the UUO model, and demonstrated that treatment reduced recruitment of macrophage and CD4+ T-cells [102]. However, this study is shorter (3 weeks of treatment) in duration versus our current study (12 weeks of treatment), and thus the effects of fatostatin on inflammation may represent an adverse event related to chronic administration which has not been previously described. Our study demonstrates that treatment with fatostatin is not an effective treatment to manage DN due to its pro-inflammatory effects. Other treatments to inhibit SREBP

activity *in vivo* may present an attractive alternative to further investigate the efficacy of SREBP inhibition for treatment of DN.

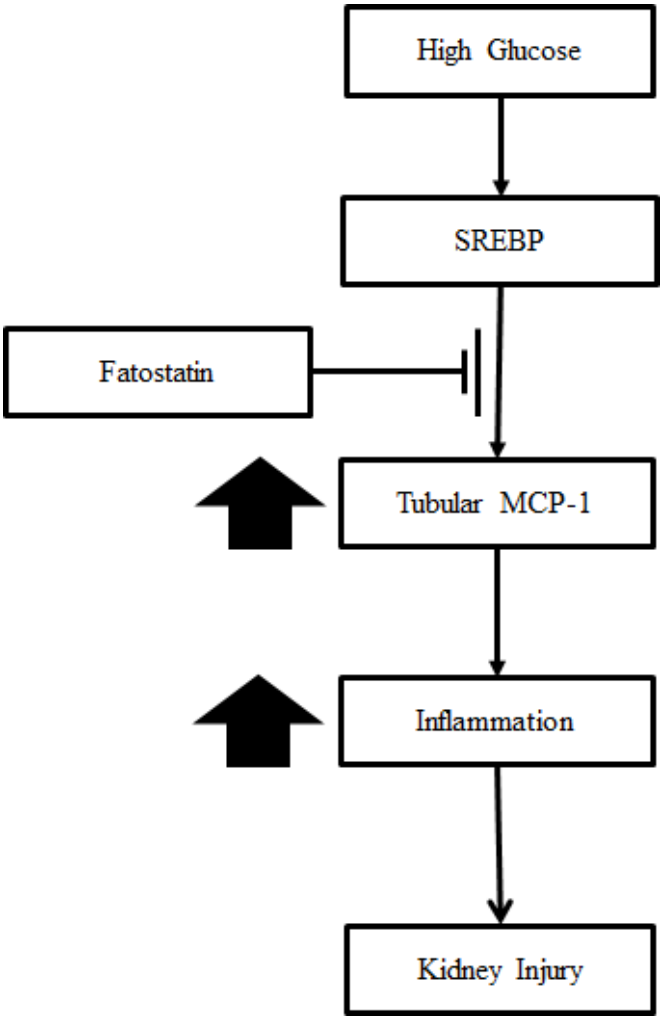


Figure 5-2 – Model of fatostatin effect on kidney function.
SREBP-1 and SREBP-2 are upregulated during the pathogenesis of DN, which is antagonized by treatment with fatostatin. Fatostatin causes an adverse event associated with elevated MCP-1 expression in renal tubular cells. This leads to

renal infiltration by CD3 cells and macrophages resulting in renal inflammation. Renal inflammation is expected to lead to kidney dysfunction by driving renal fibrosis.

SREBP inhibition does not prevent DN, despite the importance of SREBP-1 in TGF-β1 signaling

TGF-β1 is a well-established profibrotic factor that drives DN [27, 30, 101]. SREBP-1 has been shown as a regulator of TGF-β1 expression and signaling through transcriptional regulation [79, 80], and has been shown to play an important role in the development of DN [78]. Our first study has revealed that SREBP-1 regulates TGF-β1 signaling, independent of its transcriptional activity, through regulation of TβRI. However, despite the role of SREBP-1 in TGF-β1 signaling, our second study demonstrated that inhibition of SREBP *in vivo* with fatostatin does not attenuate DN and furthermore drives renal dysfunction in non-diabetic mice. Collectively these data demonstrate that SREBP and TGF-β1 crosstalk, however targeting this crosstalk with fatostatin is not an effective method to target DN. Other drugs that target SREBP may prove efficacious in the treatment of DN, furthermore attempting to target the interaction between SREBP-1 and TβRI may provide novel treatment as well.

csGRP78 promotes TGF-β1 expression *in vitro* and acts as a novel marker of DN

csGRP78 has been suggested to play a pathogenic role in the development of cancer, and has been shown to play an important role in cell signaling, viral entry, and antigen presentation [174]. The role of csGRP78 in the development of kidney disease and diabetes has not been studied; however, csGRP78 has been suggested to play a role in modulating TGF-β1 signaling which has been implicated in DN. Oida et al. showed that in P3U1 cells presentation of the LAP (of TGF-β1) on the cell surface required GRP78 [104]. Furthermore, isolation of cell surface proteins identified GRP78 at the cell surface in association with LAP. Shani et al. has demonstrated that the glycosylphosphatidylinositol-anchored signaling protein Cripto was associated with GRP78 at the cell surface [187]. Association between Cripto and csGRP78 attenuated TGF-β1 induced phosphorylation of Smad2 and downstream activation of TGF-β1 signaling. These data suggest that csGRP78 may interact with and regulate TGF-β1. Our third study is aimed at understanding the impact of diabetes on the expression of csGRP78 and understanding the potential role of csGRP78 in regulating TGF-β1.

Our results have indicated that treatment with high glucose promotes the translocation of GRP78 to the cell surface in MCs. csGRP78 propagates intracellular signaling through phosphorylation of FAK and Akt, which have been previously described as downstream mediators of csGRP78 signaling in cancer cells [95, 168]. α2M has been shown to act as a ligand for csGRP78 in cancer cells, and we have shown that in MCs α2M acts on csGRP78 to induce activation of Akt [90]. Furthermore, inhibiting csGRP78 activation prevented high glucose-

induced expression of TGF-β1. These data demonstrate that in response to diabetic stimuli MCs express csGRP78 that binds to α2M leading to increased expression of TGF-β1. We next sought to determine if csGRP78 was upregulated in the glomeruli of diabetic mice. We showed that in two models of type 1 diabetes, GRP78 was upregulated at the cell surface by WB, IHC, and IF. Collectively, our data from this study suggest that csGRP78 mediates TGF-β1 expression in MCs, and is expressed in kidneys of diabetic mice.

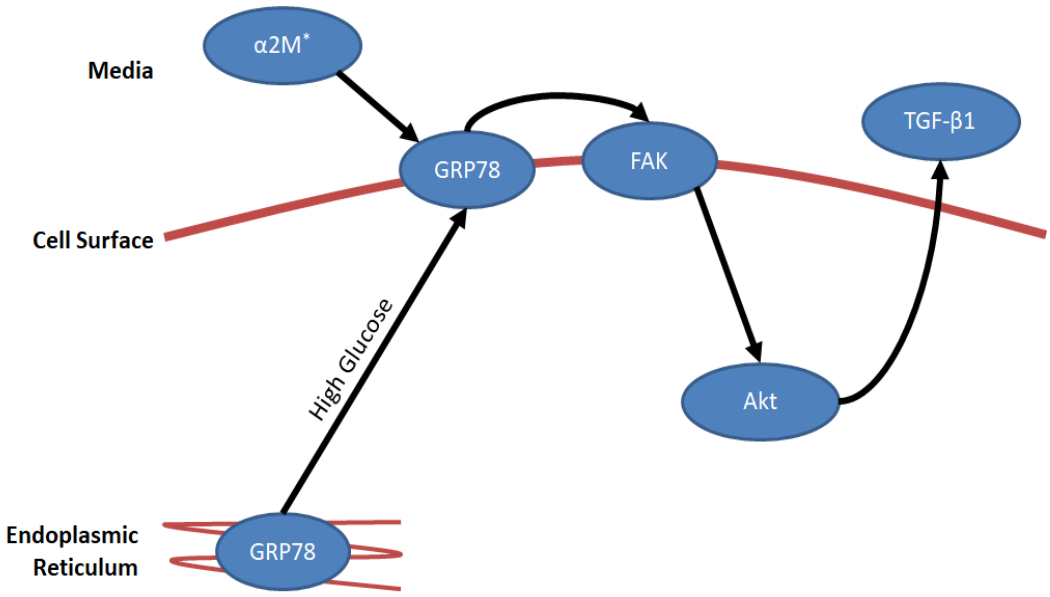


Figure 5-3 – Model of csGRP78 signaling in MCs.
We suggest that under high glucose conditions GRP78 is translocated from the ER to the cell surface, likely through an ER stress dependent mechanism. At the cell surface, we hypothesize that GRP78 may binds to circulating α2M resulting in the activation of FAK and Akt. Activation of Akt may in turn lead to

expression of the profibrotic cytokine TGF-β1 and downstream activation of SREBP-1.

Our third study is the first to investigate the role of csGRP78 in mediating diabetic signaling in MCs, and to examine its expression in the kidneys of diabetic mice. For the first time, we have demonstrated that GRP78 is expressed at the cell surface in MCs and in diabetic glomeruli. Furthermore, we have shown that csGRP78 regulates TGF-β1 expression, indicating that targeting csGRP78 may prove beneficial for the treatment of DN. The use of neutralization antibodies (C38, N88, C107 antibodies against GRP78) [188], GRP78 targeting protease [189], and GRP78 targeting peptides [97] have demonstrated efficacy in treating mouse models of different cancer types. Although these therapies have been shown to be efficacious in the treatment of tumors, their mechanisms of actions typically involve apoptosis of affected cells, which is not a favourable outcome in the treatment of DN. Recently, Ferrara et al. and Dobroff et al. had shown that by combining a GRP78 targeting peptide with AAVP particles form a platform to enable genetic therapy on cells expressing csGRP78 [98, 99]. Use of this technology in treatment of diseases requires further validation, although it may be an attractive method to evaluate the expression of csGRP78 in diabetic models.

Overarching Hypothesis and Future Directions

SREBP-1 and csGRP78 regulate TGF-β1 signaling in MCs

TGF-β1 is a pro-fibrotic cytokine that mediates renal fibrosis and injury during the pathogenesis of DN [30]. However, clinical trials testing the efficacy of TGF-β1 inhibition have been slow in development due to concerns of adverse events [32]. Furthermore, current anti-TGF-β1 treatment has demonstrated mixed efficacy in DN [56, 57]. Thus discovering novel pathways that regulate TGF-β1 signaling and expression is of importance to identify new potential therapeutic targets that may prove efficacious in the treatment of DN.

Our first and third study have suggested that SREBP-1 and csGRP78 are novel regulators of TGF-β1 in MCs. MCs are found in the glomerulus, and have been suggested to be important in the development of glomerulosclerosis in DN through expression of cytokines such as TGF-β1 [16]. Since MCs play a role in coordinating the response of cells in the glomerulus to damaging stimuli, we hypothesize that targeting pathways that regulate TGF-β1 in MCs will prevent the spread of damage in the glomeruli. Glomerular damage is typically an early pathological change that occurs during the development of DN, thus we expect that prevention of glomerular damage to prevent further damage to the kidney [9].

Our first study demonstrated that inhibition of SREBP-1 results in decreased TGF-β1 signaling and expression of TGF-β1 responsive genes through reduced expressed of TβRI in MCs. Our third study demonstrated that inhibition of csGRP78 results in reduced expression and secretion of TGF-β1 in MCs. Collectively, these data suggest that targeting of SREBP-1 and csGRP78 will prevent TGF-β1 signaling and expression in MCs. Future studies will aim to study

the interaction between csGRP78 and SREBP-1 signaling. Understanding the interaction between csGRP78, ER stress, and activation of SREBP-1 may provide novel methods to treat DN.

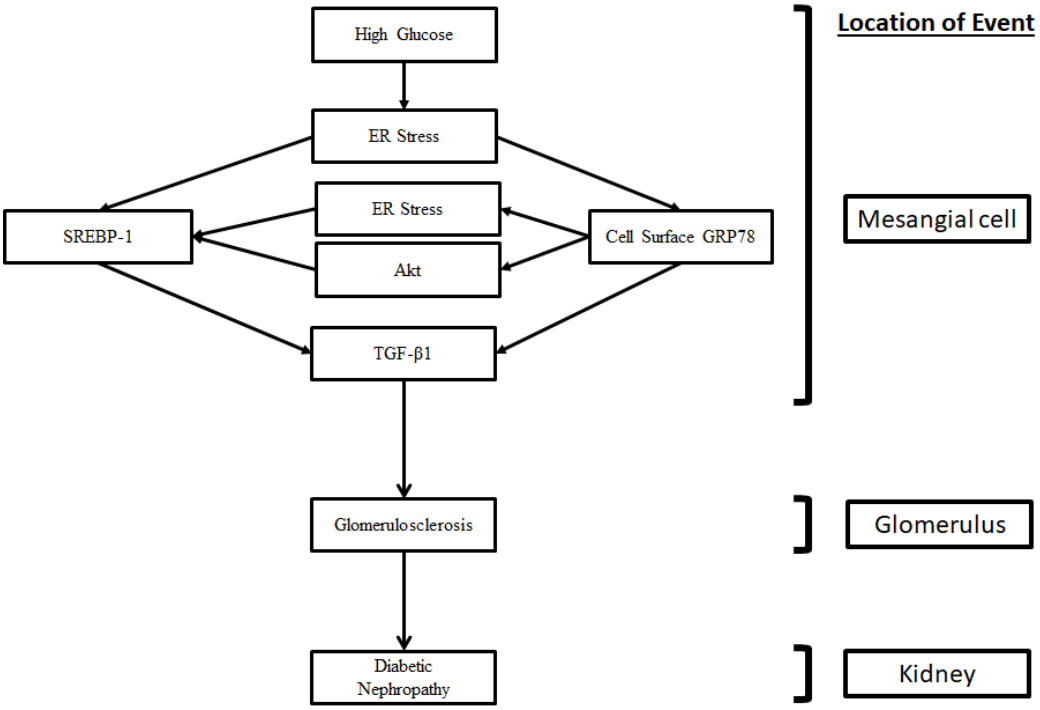


Figure 5-4 – Model of overarching hypothesis.
Under diabetic conditions, SREBP-1 and csGRP78 are upregulated in MCs. The activation of SREBP-1 and csGRP78 signaling promotes the activity and expression of the profibrotic cytokine TGF-β1 in MCs. Activation of MCs through TGF-β1 leads to a fibrotic phenotype resulting in further secretion of profibrotic cytokines. This cumulates in the dysregulation of the glomerulus and the development of glomerulosclerosis, ultimately resulting in DN.

Fatostatin does not prevent DN, however csGRP78 acts as a novel marker of DN

Contrary to our expectations from our first study, the results from our second study have indicated that treatment of mice with the SREBP inhibitor fatostatin does not prevent the development of DN. Furthermore, treatment of non-diabetic mice with fatostatin resulted in renal inflammation and injury. In corroboration with our first study, treatment of mice with fatostatin reduced the expression of TβRI; however, despite reduced TβRI expression the kidneys still displayed a fibrotic phenotype. This study highlights the adverse events associated with chronic treatment with fatostatin, and demonstrates that not all the cell types in the kidney respond in a similar manner to fatostatin therapy. Our study demonstrated that treatment with fatostatin inhibited the expression of the pro-inflammatory cytokine MCP-1 in MCs, but induced MCP-1 expression in renal tubular cells. This study demonstrates that inhibition of SREBP with fatostatin is not an efficacious therapy for DN due to adverse events. An important limitation to this study is that the efficacy of other SREBP inhibitors has not been studied. Thus the effects of fatostatin on renal inflammation may represent adverse events associated with the formulation of the drug rather than adverse events associated with SREBP inhibition.

In line with the *in vitro* data from our third study, *in vivo* analysis of diabetic mice from the third study suggests that csGRP78 is expressed glomeruli of diabetic mice. These results suggest that csGRP78 presents as a novel marker of DN, and may be of interest when developing therapy to treat DN. A key limitation to these results are that they are early in their design, and require further

validation through other experimental techniques and analysis in other models of diabetes. The expression of csGRP78 in diabetic mice should be further assessed through other methods such as flow cytometry and the use of peptides/antibodies that selectively recognize csGRP78 for staining purposes. Furthermore, although our *in vitro* studies show that inhibition of csGRP78 attenuates HG-induced TGF-β1 expression in MCs although previous studies have demonstrated opposite effects of csGRP78 on TGF-β1 signaling [187]. Further studies will be required to evaluate the efficacy of targeting csGRP78 *in vivo* and to further understand how csGRP78 regulates TGF-β1 expression.

Future Directions

Our current studies have indicated that although SREBP-1 is important for TGF-β1 signaling, however targeting SREBP activity through fatostatin is not an effective method to treat DN. The drug betulin has been described as an SREBP inhibitor by promoting the interaction between SCAP and Insig [190]. The effects of betulin treatment on kidney health has not been extensively studied, however a study by Zhao et al. demonstrated that treatment with betulin attenuated kidney injury in sepsis by inhibition of inflammation [191]. Research into using betulin, or other methods to inhibit SREBP activity (such as SCAP knockdown in the kidney), are of interest in the treatment of DN to better determine if SREBP inhibition is a viable treatment to manage DN.

Future studies into csGRP78 will be focused on improving our understanding the mechanisms of csGRP78 translocation to the cell surface, and

activation of its downstream pathways. Previous studies have suggested that targeting csGRP78 using genetic therapy is feasible, however this therapy is still in development for humans and not easily accessible [98, 99]. Our future studies will focus on identifying potential antagonists for csGRP78 that may prevent its activation. Examples include the adipokine vaspin which has been reported to prevent α2M binding to csGRP78 [88]. Further research will focus on the efficacy of targeting csGRP78 in *in vivo* in models of diabetes.

Conclusions

DN is a major microvascular complication that arises in diabetic patients and is the leading cause of ESRD in the Western world [3]. Furthermore, patients with DN are at a higher risk of all cause and cardiovascular-disease mortality than diabetic patients [5]. Current treatment of DN involve inhibition of RAAS and glycemic control, however these therapies are not enough to prevent DN progression in most patients [6].

Results from our first and third study have identified SREBP-1 (through regulation of TβRI secretion in exosomes) and csGRP78 (through activation of FAK/Akt) as regulators of TGF-β1 in *in vitro* studies using kidney MCs. TGF-β1 has been recognized as a pro-fibrotic cytokine that leads to kidney damage in DN [48]. Thus we hypothesized that SREBP and csGRP78 may exert pathogenic effects on DN progression due to their role in TGF-β1 regulation. Results from our second study revealed that inhibition of SREBP using the SCAP inhibitor fatostatin did not prevent DN and led to kidney dysfunction in non-diabetic mice. These results demonstrate that fatostatin is not an effective therapy for DN. However, it is important to note that our results do not conclusively demonstrate SREBP inhibitors are non-effective for treatment of DN, thus other methods of SREBP inhibitors will need to be investigated to determine their potential therapeutic effects. Our third study suggests that csGRP78 is found in the glomeruli in diabetic mice, and is upregulated in the whole kidney during the

development of DN. Future studies will evaluate the efficacy of csGRP78 targeted therapy in the treatment of DN.

Collectively, our results have revealed novel mechanisms involved in the progression and development of DN. We hope that these results will reveal new potential targets for therapy in order to improve the health and well-being of patients suffering from DN.

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