CELL SURFACE GRP78 FACILITATES TGFβ1 SIGNALING BY HIGH GLUCOSE

CELL SURFACE GRP78 PARTICIPATES IN THE UPREGULATION OF TGFβ1 SIGNALING BY HIGH GLUCOSE

By MENGYU ZHENG, B.Sc.

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Descriptive Note

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AUTHOR: Mengyu Zheng, B.Sc. (McMaster University)

SUPERVISOR: Dr. J.C. Krepinsky

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Lay Abstract

Diabetic kidney disease affects around 40% of diabetic patients worldwide and is a major health concern. A major feature of the disease is glomerulosclerosis, which is the scarring of glomeruli. The glomeruli filter blood passing through blood vessels in the kidneys to remove waste, which will then be excreted into urine. In diabetic patients, high blood glucose causes the fibrosis of glomeruli and damages the filtration barrier. As a result, a large amount of proteins leak from the blood into the urine. It has been discovered that TGF-β1 is one of the key molecules mediating the generation of scar tissue in the glomerulus. It promotes the growth of mesangial cells, a major type of kidney glomerular cells, and stimulates their production of extracellular matrix proteins. Our results showed that GRP78, a protein that is primarily expressed in the endoplasmic reticulum and assists with protein folding, moves from the inside of cells to the surface in response to a high glucose environment. Here, we found that it facilitated TGF-β1 signaling. Based on our studies, we propose that when GRP78 is at the cell surface, it enables the release of latent TGF-β1, increasing TGF-β1 activity and thus promoting the development of disease.

Abstract

Diabetic nephropathy (DN) affects around 40% of diabetic patients worldwide and has become a major health concern due to its high morbidity and mortality. The progression of DN is characterized by the thickening of glomerular basement membrane, albuminuria and the development of glomerulosclerosis. Renal function is eventually compromised. Due to various hemodynamic and metabolic changes, especially the elevated blood glucose level in diabetic patients, glomerular mesangial cells have been shown to upregulate transforming growth factor-β1 (TGF-β1) level and signaling, resulting in the excessive production of extracellular matrix (ECM) proteins. The atypical expression of the 78-kDa glucose-regulated protein (GRP78) on the cell surface may be associated with this pro-fibrotic effect through its interaction with the TGF-β1 activation process. However, there is no current literature demonstrating the role of cell surface GRP78 (csGRP78) in the pathogenesis of diabetic renal diseases. The purpose of my MSc project was to determine the role of csGRP78 in TGF-β1 synthesis and activation and thereby in the progression of DN. We hypothesized that the increased expression of csGRP78 in response to high glucose exposure stimulates TGF-β1 upregulation through intracellular signaling, as well as its activation through interaction with the latent complex, which leads to the expansion of mesangial matrix.

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List of Abbreviation

DN – diabetic nephropathy

ACR – albumin/creatinine ratio

GFR – glomerular filtration rate

ECM – extracellular matrix

IL - interleukin

VEGF – vascular endothelial growth factor

TGF-β1 – transforming growth factor β1

MC – mesangial cell

LAP – latency associated peptide

SLC – small latent complex

LTBP – latent TGF-β binding protein

LLC – large latent complex

MMP – matrix metalloproteinase

TSP-1 – thrombospondin-1

TGFβRI/II – TGFβ receptor I/II

pSmad2/3 – phosphor-Smad2/3

R-Smad – receptor Smad

PAI-1 – plasminogen activator inhibitor-1

MAPK – mitogen-activated protein kinase

PI3K – phosphoinositide 3-kinase

GRP78 – 78 kDa glucose-regulated protein

ER – endoplasmic reticulum

UPR – unfolded protein response

csGRP78 – cell surface GRP78

GPI – glycosylphosphatidylinositol

α2M – alpha-2-macroglobulin

TF – tissue factor

Par-4 – protease-activated receptor 4

SubA – subtilase cytotoxin A

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

PBS – phosphate-buffered saline

BSA – bovine serum albumin

DMEM – Dulbecco's Modified Eagle's medium

PSB – protein solubilization buffer

IP - immunoprecipitation

ELISA – enzyme-linked immuno-sorbent assay

qRT-PCR – quantitative real-time reverse transcription polymerase chain reaction

SDS – sodium dodecyl sulfate

TBS-T – tris-buffered saline with 0.1% Triton X-100

HG – high glucose

STEC – Shiga toxin-producing Escherichia coli

Declaration of Academic Achievement

I, Mengyu Zheng, declare this thesis to be my own work. I am the sole author of this document. Data from Dr. Renzhong Li was used in the generation of Fig. 10. This work has not been published or submitted for publication or to another institution.

My supervisor, Dr. Joan Krepinsky, and the members of my supervisory committee, Dr. Richard Austin, Dr. Kjetil Ask and Dr. Suleiman A. Igdoura, have provided support and guidance throughout this project.

There are no conflicts of interest to my knowledge. Figure 1-5 were adapted from the indicated sources in figure legends with permission for republication obtained.

1 Introduction

1.1 Diabetic Nephropathy

1.1.1 Overview

Type 1, also termed insulin-dependent, diabetes mellitus is an autoimmune disease involving the destruction of insulin-producing pancreatic β cells by self-immunity (Atkinson, Eisenbarth, & Michels, 2014). The organ-specific chronic inflammation results in insulin deficiency and chronic hyperglycemia, meaning that patients will need a lifetime treatment of exogenous insulin replacement (Atkinson et al., 2014). In contrast, type II diabetes is characterized by deficient insulin production from pancreatic β cells as well as insulin resistance in target organs (Chatterjee, Khunti, & Davies, 2017). Due to various hemodynamic and metabolic changes occurring during disease progression, a series of microvascular and macrovascular complications were discovered in patients with long-term diabetes (Nathan, 2014). Diabetic nephropathy (DN) is a common microvascular complication of diabetes and is usually accompanied by other diabetic complications including retinopathy and neuropathy (Bennett & Aditya, 2015). As the leading cause of end-stage renal disease, DN currently affects 15-40% of type 1 diabetic patients with the peak incidence occurring at 15-20 years after the onset of diabetes, and 5-20% type 2 diabetic patients worldwide (Bennett & Aditya, 2015). It has become a major healthcare concern due to its high mortality and morbidity, as well as the increasing global prevalence of diabetes (Dugbartey, 2017). Therefore, great effort is needed to develop new

prevention and treatment strategies for DN and to resolve the colossal humanistic, societal and economic impact (Dasgupta, 2014).

1.1.2 Pathology

DN is characterized by persistent elevation in urinary albumin termed microalbuminuria (>30 mg/day or 20 μg/min; urinary albumin/creatinine ratio [ACR] >3.0 mg/mmol) (Bennett & Aditya, 2015). The disease progresses over time to macroalbuminuria, which represents leakage of the filtration barrier and an elevated urinary albumin excretion over 300 mg/day and urinary ACR >30 mg/mmol (Bennett & Aditya, 2015). The glomerular filtration rate (GFR) also declines over time, eventually leading to end-stage kidney disease (Reidy, Kang, Hostetter, & Susztak, 2014).

DN is morphologically characterized by the formation of glomerulosclerosis associated with mesangial expansion (Paulini, Higuti, Bastos, Gomes, & Rangel, 2016). The glomerular basement membrane also thickens, which reduces the filtration surface (Paulini et al., 2016). An illustration of structural changes during the development of glomerulosclerosis is shown in Fig. 1. Hypertrophy and hyperplasia are also seen in other cell types, such as glomerular podocytes and endothelial cells, interstitial fibroblasts and tubular epithelial cells (Kanwar et al., 2008). Consequently, renal function is compromised over time in association with albuminuria (Kanwar et al., 2008; Paulini et al., 2016). Glomerular mesangial cells are one of the first renal

cell types involved in the pathogenesis of DN and thus of relevance to this project (Abboud, 2012). Mesangial cell proliferation and hypertrophy, along with mesangial extracellular matrix (ECM) buildup, are directly associated with glomerulosclerosis, one of the earliest features of DN (Abboud, 2012).

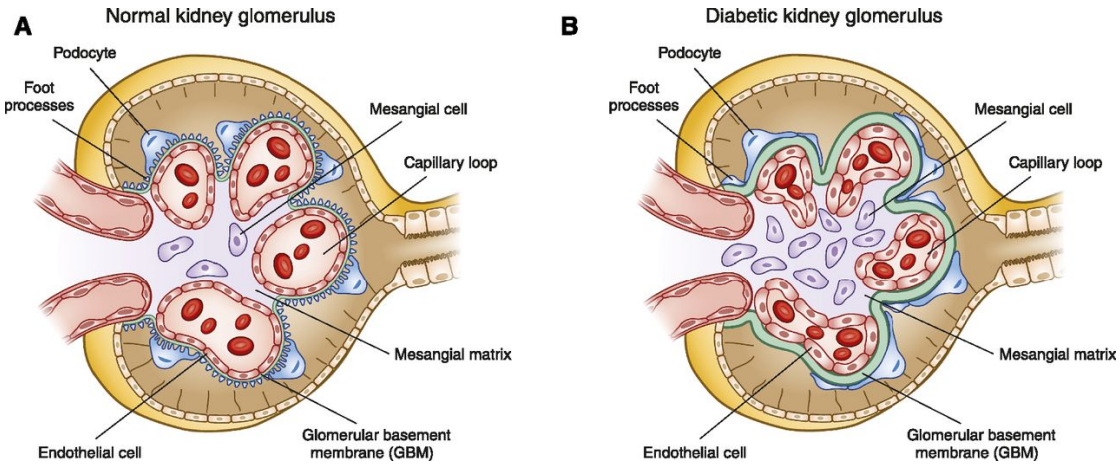

Figure 1. Morphological alterations in the diabetic kidney glomerulus.

Mesangial cell proliferation, mesangial matrix expansion, the thickening of basement membrane and the loss of podocytes lead to compromised renal function.

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Although both hyperglycemia and dyslipidemia are believed to participate in the pathogenesis and progression of DN, elevated blood glucose level is likely to be the key contributing factor through multiple molecular pathways (Dugbartey, 2017). For example, oxidative stress induced by high glucose exposure activates numerous pathogenic signaling pathways (Paulini et al., 2016). A variety of pro-fibrotic and inflammatory cytokines are also released, including vascular endothelial growth factor (VEGF), interleukin-1 (IL-1), IL-6, IL-18 and transforming growth factor-β1 (TGF-β1) (Paulini et al., 2016). The majority of these signaling mediators and pathways associated with hyperglycemia seem to converge to upregulate TGF-β1, the key mediator of fibrosis (Hills & Squires, 2011). However, in a phase II clinical trial, direct TGF-β1 neutralization using monoclonal antibodies failed to show any therapeutic benefits to DN progression with the chosen dosage (Voelker et al., 2017). The researchers suggested that more complete TGF-β1 inhibition may be required to interfere with disease progression, but this would also be associated with a higher risk of adverse events (Voelker et al., 2017). Therefore, a better understanding of TGF-β1 activation and signaling mechanism will be valuable for developing novel therapies to alleviate or reverse the damage caused by DN.

1.2 Mesangial Cells

Mesangial cells (MCs), accounting for about one-third of the glomerular cell population, play an important structural role in the glomerulus (Abboud, 2012). They

originate from the metanephric mesenchyme and form the mesangium and glomerular tuft to stabilize the afferent and efferent arterioles (Migliorini, Ebid, Rebecca Scherbaum, & Anders, 2013). As illustrated in Fig. 2, the mesangium fills the space both between glomerular capillaries and outside of the glomerulus at the vascular pole and the juxtaglomerular apparatus (Migliorini et al., 2013).

MCs are the primary target of various glomerular diseases related to inflammation and metabolic changes (Abboud, 2012). Major biological responses to injury include proliferation, apoptosis, migration, excessive ECM production, and release of cytokines and chemokines, etc. (Abboud, 2012). The secreted factors may act on MCs in an endocrine or autocrine fashion, and also on adjacent cells in a paracrine fashion, allowing for cross-communication between different cell types (Abboud, 2012). In the context of DN, MCs exhibit proliferation and matrix expansion as one of the early signs observed within 5-7 years after the diagnosis of type 1 diabetes (Alicic, Rooney, & Tuttle, 2017). Previous *in vitro* studies on cultured MCs also documented the effect of hyperglycemia on these cells on both transcriptional and post-transcriptional processes (Abboud, 2012). Therefore, it is essential to understand the pathogenic mechanisms of high glucose in MCs to develop selectively targeted therapeutic agents for glomerulosclerosis in DN.

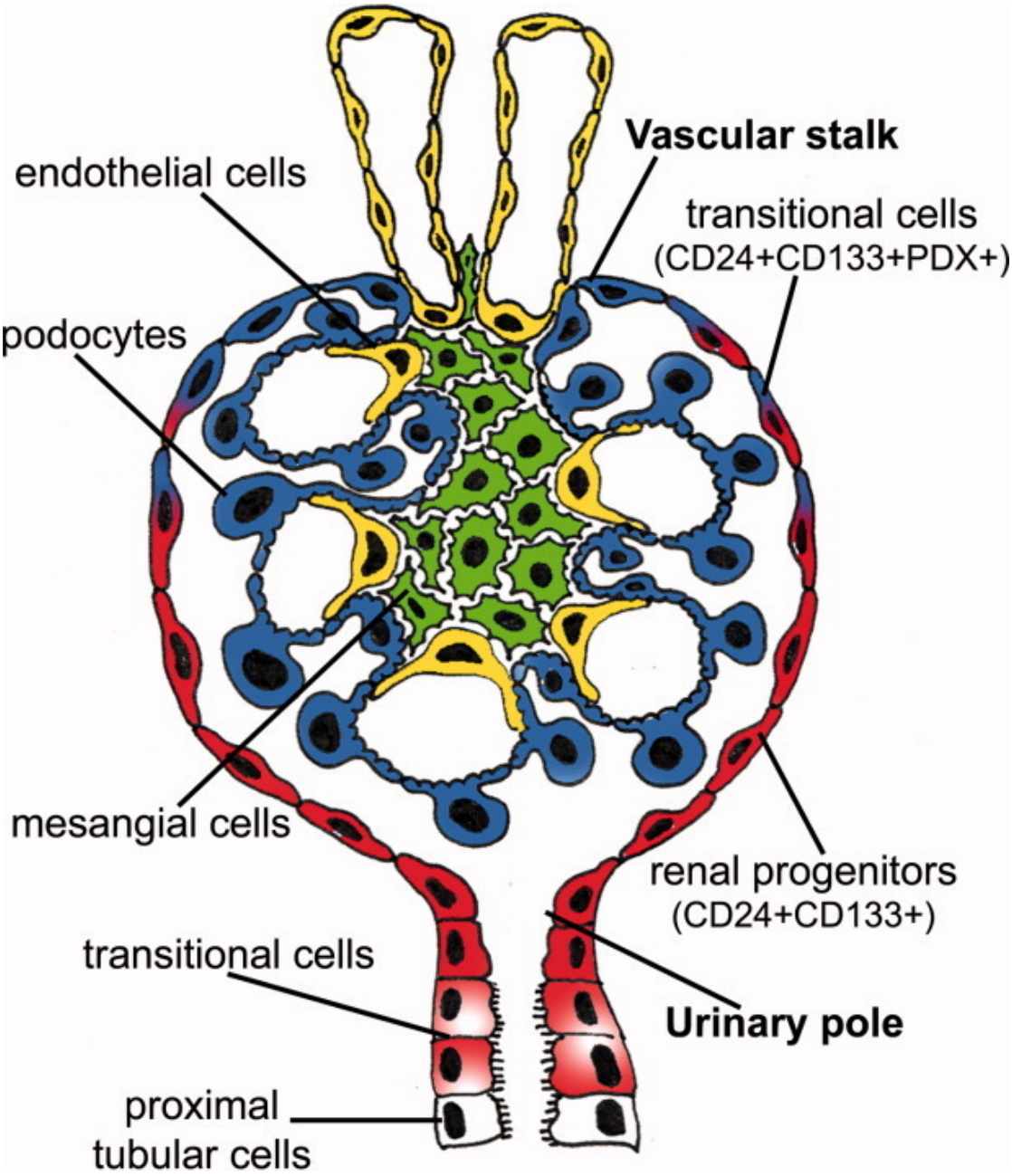

Figure 2. The structure of the glomerulus.

The overall structure of the glomerulus and the distribution of mesangial cells.

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1.3 TGF-β1

1.3.1 General Overview

TGF-β1 is a 25 kDa dimeric protein which functions as a pro-fibrotic cytokine, belonging to the TGF-β superfamily (Hills & Squires, 2011; Miyazono, Ichijo, & Heldin, 1993). Most of the TGF-β superfamily members play a role in the regulation of cell development, repair, proliferation and apoptosis (Hills & Squires, 2011). Three of the five distinct TGF-β isoforms are found to be ubiquitously expressed in mammalian cells, namely TGF-β1, β2, and β3, and are linked to various intracellular pathways (Hills & Squires, 2011). It has been well demonstrated that TGF-β1 upregulation and the activation of its Smad and non-Smad mediators are responsible for excessive ECM deposition in the progression of DN (Ziyadeh, 2004). In the normal physiological process, ECM proteins like collagen and fibronectin are necessary for wound healing and tissue scarring (Hills & Squires, 2011). The balance between the synthesis and breakdown of ECM has to be tightly regulated to maintain tissue function (Sutariya, Jhonsa, & Saraf, 2016). The overproduction of ECM protein that cannot be degraded properly is responsible for the mesangial matrix expansion and the generation of glomerulosclerosis in DN patients (Hills & Squires, 2011).

Previous *in vitro* and *in vivo* studies have demonstrated the protective effect of TGF-β1 neutralization or inhibition on the progression of glomerulosclerosis. Ziyadeh et al. showed that TGF-β1 neutralizing antibody blocked the high glucose-induced synthesis of α1 and α4 collagen in mouse MCs (Ziyadeh, Sharma, Ericksen, & Wolf,

1994). Antisense TGF-β1 oligodeoxynucleotides, which bind and prevent the translation of complementary mRNA, showed a similar anti-fibrotic effect of inhibiting proximal tubular cell hypertrophy *in vitro* under hyperglycemic conditions and attenuating extracellular matrix deposition *in vivo* in a diabetic mouse model (Cheol Han, Hoffman, Won Hong, Guo, & Ziyadeh, 2000). Smad3 gene knockout, as another approach to TGF-β1 signaling inhibition, can also limit the progression of diabetic glomerulosclerosis in mice (Wang et al., 2007). A number of DN-related defects was improved, including renal hypertrophy, mesangial matrix expansion, and glomerular basement membrane thickening (Wang et al., 2007). These studies further support the relevance of TGF-β1 signaling in DN pathology and suggest the therapeutic potential of TGF-β1 inhibition.

1.3.2 TGF-β1 Activation

Fig. 3 summarizes the process of TGF-β1 synthesis and activation. TGF-β1 is initially synthesized in the form of pro-TGF-β1 in conjunction with latency-associated protein (LAP) as a large N-terminal pro-domain (Sutariya et al., 2016). A signal peptide is also attached to pro-TGF-β1 to direct its translocation to the endoplasmic reticulum (ER), which will be removed afterward (Travis & Sheppard, 2014). Mature TGF-β1 peptide is cleaved by furin-type enzymes from pro-TGF-β1 but remains non-covalently bound with LAP, forming the small latent complex (SLC) (Travis & Sheppard, 2014). SLC is necessary for proper protein folding and is then secreted, in

association with latent TGF-β-binding protein (LTBP) through a covalent disulfide bond, as a large latent complex (LLC) (Sutariya et al., 2016). The latent TGF-β complex becomes sequestered between the cell surface and ECM and may be released through different mechanisms (Sutariya et al., 2016). Active TGF-β1 can be cleaved from LAP and LTBP by plasmin, matrix metalloproteinases (MMPs) or other proteases after the entire latent complex is released from ECM by proteases (Sutariya et al., 2016).

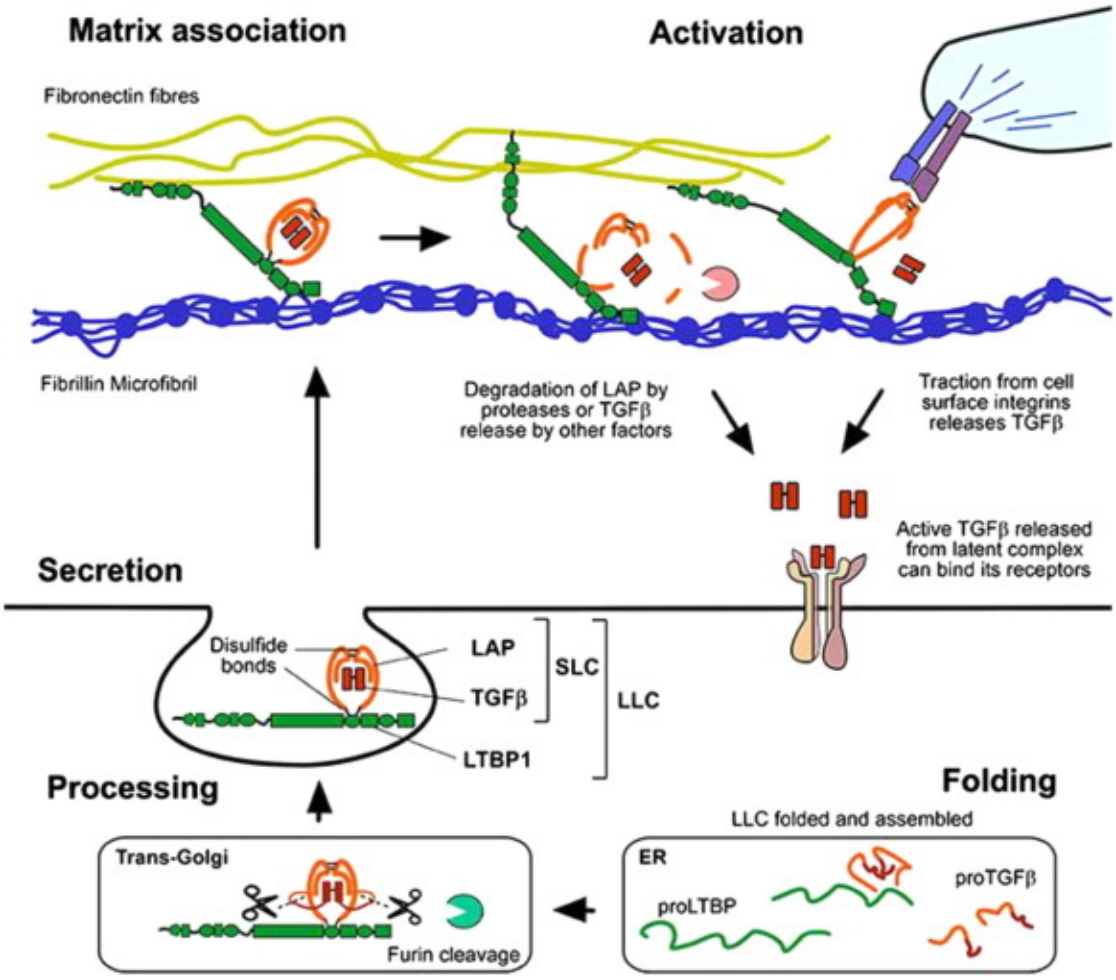

Figure 3. TGF-β1 synthesis, secretion and activation.

A summary of TGF-β1 synthesis, secretion, and activation processes.

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Thrombospondin-1 (TSP-1), a matri-cellular glycoprotein, has been shown to be a major activator of TGF-β1 in human renal MCs as well as in the glomerulus of experimental DN murine model (Daniel, Schaub, Amann, Lawler, & Hugo, 2007; Yevdokimova, Wahab, & Mason, 2001). It has a KREK sequence that can bind to the LSKL sequence of LAP in the extracellular matrix once it is correctly oriented by first binding to the mature TGF-β1 portion through its GGWSHW sequence (Ribeiro, Poczatek, Schultz-Cherry, Villain, & Murphy-Ullrich, 1999; Schultz-Cherry et al., 1995). The mature TGF-β1 molecule can then be released from its latent complex potentially through conformational changes without the participation of proteases (Schultz-Cherry & Murphy-Ullrich, 1993).

Other evidence suggests that the interaction between the LLC and integrins, which are cell surface adhesion receptors, may also help activate TGF-β1 in a mechanical force-dependent manner (Dong et al., 2017). Only after activation can mature TGF-β1 bind to its TGF-β receptor complex and initiate downstream signaling (Travis & Sheppard, 2014).

1.3.3 TGF-β1 Signaling Pathway

Extracellular TGF-β1 in its active form can bind to its transmembrane receptor kinases, TGF-β receptor I and II (TGF-βRI; TGF-βRII), to stimulate a series of intracellular pathways (Sutariya et al., 2016). Active TGF-β1 first binds to the TGF-βRII homodimer, which forms a tetrameric complex with a homodimer of TGF-βRI (Travis & Sheppard, 2014). Both receptor homodimers act as a serine-threonine receptor kinase (Travis & Sheppard, 2014). After TGF-βRII phosphorylates and activates TGF-βRI, intracellular receptor Smad (R-Smad) proteins, Smad2 and Smad3, are recruited and phosphorylated by the cytoplasmic domain of TGF-βRI (Travis & Sheppard, 2014). Phospho-Smad2/3 (pSmad2/3) form a complex with Smad4 and translocate to the nucleus, where they bind and regulate the transcription of proteins including ECM components (Sutariya et al., 2016). The Smad pathway is considered the canonical TGF-β1 signaling mediator, while non-canonical or non-Smad pathways triggered by TGF-βRI involve other signaling molecules such as mitogen-activated protein kinases (MAPK), growth and survival kinases phopatidylinositol-3-kinase (PI3K), and Rho GTPase (Travis & Sheppard, 2014). Both canonical and non-canonical pathways are summarized in Fig. 4.

After its translocation into the nucleus, the pSmad3/Smad4 complex acts as a transcription factor either directly interacting with its DNA binding site containing the CAGA box, or cooperating with other DNA binding proteins (Hu, Wu, & Phan, 2003). As a result, the expression of various pro-fibrotic molecules is induced, including

collagens, fibronectin, and plasminogen activator inhibitor-1 (PAI-1) (Hu et al., 2003; Meng, Nikolic-Paterson, & Lan, 2016).

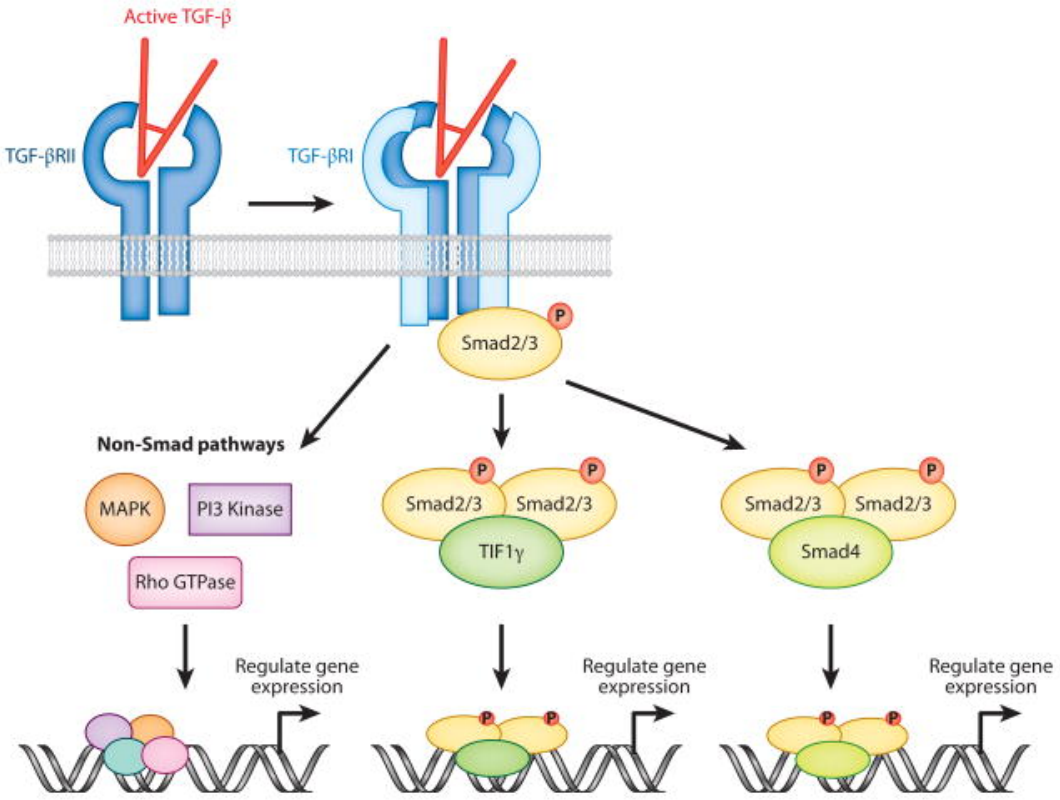

Figure 4. Smad and non-Smad signaling of TGF-β1.

Active TGF-β1 binds to TGF-βRII and TGF-βRI on the cell surface, triggering both Smad and non-Smad pathways, leading to gene expression regulation.

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1.4 Cell Surface GRP78

1.4.1 Overview

The 78 kDa glucose-regulated protein (GRP78) is an endoplasmic reticulum (ER) chaperone belonging to the heat shock protein 70 (HSP70) family (Ni, Zhang, & Lee, 2011). Its upregulation was discovered in chicken fibroblasts in response to glucose starvation (Shiu, Pouyssegur, & Pastan, 1977). GRP78 is originally documented to be responsible for proper protein folding in ER and acts as a marker for the unfolded protein response (UPR) and ER stress (Ni et al., 2011). The atypical expression of GRP78 on the cell surface was first noted in cancer cells and here it has been proposed to function as a co-receptor to mediate cell surface signaling (Ni et al., 2011; Oida & Weiner, 2010).

Cell surface GRP78 (csGRP78) has been identified on a variety of human cancer cell lines including neuroblastoma (SH-SY5Y), hepatoma (HepG2), and osteosarcoma (SJSA-1), as well as ovarian tumor cells obtained directly from patient ascites fluid (Ni et al., 2011). As GRP78 is also discovered on the plasma membrane of stressed epithelial cells, it shows a great therapeutic potential in the development of highly targeted interventions (Gopal, Gonzalez-Gronow, & Pizzo, 2016).

When expressed on the cell surface, GRP78 becomes a peripheral protein with its N- and C-terminus exposed to the extracellular space, interacting with transmembrane proteins such as glycosylphosphatidylinositol (GPI)-anchored proteins (Tsai et al., 2015). The different responses induced by csGRP78 may depend on which domain is

engaged in the interaction (Gonzalez-Gronow, Selim, Papalas, & Pizzo, 2009). Specifically, interaction with its N-terminal domain leads to stimulation of the PI3K/Akt pathway and promotion of cell proliferation and survival (Gonzalez-Gronow et al., 2009). On the contrary, csGRP78 can promote apoptotic activity through its COOH-terminal domain (Gonzalez-Gronow et al., 2009).

Fig. 5 outlines a number of proteins that have been proposed to interact with csGRP78. For example, the plasma protein α_2-macroglobulin (α_2M) is an identified ligand of the N-terminal domain of csGRP78 to promote cancer cell survival (Gonzalez-Gronow et al., 2009). Although the signaling mechanism is not fully understood, this proliferative effect is believed to involve the PI3K-Akt pathway (Gopal et al., 2016). The C-terminus of csGRP78 also interacts with tissue factor (TF) to inhibit its pro-coagulant activity, while the binding of auto-antibodies to its N-terminus has a promoting effect (Al-Hashimi et al., 2010; Gonzalez-Gronow et al., 2009). Finally, Cripto-1, or teratocarcinoma-derived growth factor 1, is a small GPI-anchored protein which exerts an inhibitory effect on TGF-β1 signaling upon binding to csGRP78.

A recent discovery in prostate, melanoma, and ovarian cancer patients suggests that csGRP78 acts as a major autoantigen, with the generation of anti-GRP78 autoantibodies correlated with disease progression (de Ridder, Gonzalez-Gronow, Ray, & Pizzo, 2011). However, the functions of anti-GRP78 autoantibodies isolated from

the sera of cancer patients still vary, potentially due to their different binding sites (Gonzalez-Gronow et al., 2009).

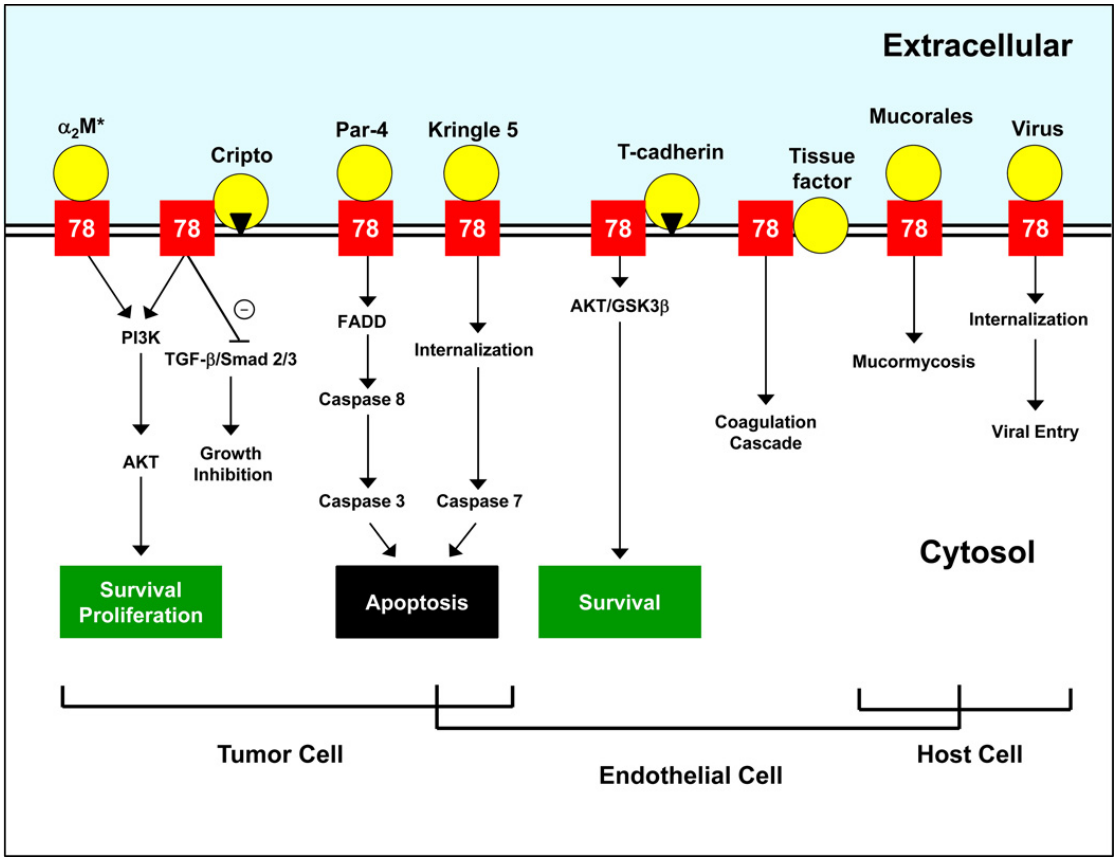

Figure 5. csGRP78 signaling and functions.

A brief overview of various binding partners and triggered signaling pathways associated with csGRP78. The signaling activity and biological effect of csGRP78 vary in accordance with the cell type and the nature of disease.

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1.4.2 csGRP78 Translocation Mechanisms

As the trafficking of GRP78 from ER to the plasma membrane is still not fully understood, different hypotheses have been proposed to explain its translocation. A tetra-peptide, KDEL, is expressed on the C-terminus of GRP78 and is responsible for its retention within ER by binding to the KDEL receptor in the Golgi apparatus and the intermediate ER-Golgi compartment (Capitani & Sallese, 2009; Ni et al., 2011). Since KDEL receptor is not upregulated in response to ER stress in HeLa cells, it is possible that the KDEL retrieval system is overwhelmed by the increased expression of intracellular GRP78, resulting in its escape to the cell surface (Llewellyn, Roderick, & Rose, 1997). It was recently discovered that ER stress resulted in the dispersion of KDEL receptors from *cis*-Golgi, interfering the retrieval system and thus allowing GRP78 to translocate to the cell surface (Tsai et al., 2018).

However, ER stress is not essential for GRP78 translocation, since GRP78 overexpression can also promote the translocation in the absence of ER stress (Zhang, Liu, Ni, Gill, & Lee, 2010). MTJ-1 is another protein demonstrated to be important in the translocation of GRP78 in macrophages (Misra, Gonzalez-Gronow, Gawdi, & Pizzo, 2005). On the other hand, prostate apoptosis inhibitor-4 (Par-4) was shown to be an essential member of the translocation mechanism in PC3 cells (Burikhanov et al., 2009). It has also been shown that it is unlikely for secreted GRP78 to bind to the plasma membrane, suggesting that csGRP78 is transported from the intracellular space (Zhang et al., 2010).

1.4.3 csGRP78 and TGF-β1 signaling

The association between csGRP78 and TGF-β1 signaling has been demonstrated in various cell lines. As mentioned above, the interaction between csGRP78 and Cripto promotes oncogenesis by inhibiting both canonical and non-canonical TGF-β1 signaling (Gray & Vale, 2012). csGRP78 is also a potential surface anchoring protein for the LAP/TGF-β1 complex in mouse myeloma cells, but how their interaction affects the release of mature TGF-β1 has not been demonstrated (Oida & Weiner, 2010). A recent discovery also proposes that GRP78 binds to CD109, another GPI-anchored protein belonging to the α_2M family, and induces the degradation of TGF-β receptors by routing to the caveolae. This disrupts the phosphorylation of Smad2 and its downstream signaling events, resulting in a pro-tumor environment (Tsai et al., 2018). Together, these studies suggest that in the tumor environment, csGRP78 inhibits TGF- β1 signaling.

Although there has not been any report on the role of csGRP78 in the context of DN, unpublished preliminary data from our lab have shown that high glucose can induce the upregulation of csGRP78 in mesangial cells. In contrast to cancer cells where TGF-β1 plays a tumor-suppressing role by inducing apoptosis, mesangial cells proliferate with elevated TGF-β1 signaling activity and produce an excessive amount of ECM proteins. By examining the role of csGRP78 in TGF-β1 signaling, we may be able to better understand the progression of DN and to develop novel therapeutic targets.

1.5 Objective of the Study

The purpose of my MSc project was to determine the role of csGRP78 in TGF-β1 synthesis and activation in response to high glucose, and thereby in the progression of DN. We hypothesized that the increased expression csGRP78 in response to high glucose exposure stimulates TGF-β1 upregulation, as well as its activation through direct interaction with the latent complex, which leads to expansion of mesangial matrix.

2 Materials and Methods

2.1 Proteins and Reagents

Subtilase cytotoxin A (SubA) was a kind gift from Dr. James Paton (University of New Mexico School of Medicine, Albuquerque, New Mexico, USA). Anti-GRP78 C38 and N88 antibodies were a kind gift from Dr. Rupa Ray (Duke University Medical Centre at Durham, North Carolina, USA). LEAF^{TM} purified anti-mouse/rat Integrin β1antibody (BioLegend) was used for co-immunoprecipitation. The following primary antibodies were used in western blotting: pSmad3 Ser423/425 (1:4000, Novus), total Smad3 (1:1000, Abcam), LAP (1:1000, R&D Systems), α-tubulin (1:40000, Sigma), GRP78 (1:1000, BD Biosciences), C20 GRP78 (1:1000, Santa Cruz), N20 GRP78 (1:1000, Santa Cruz), TSP-1 (1:1000, R&D Systems), Na+/K+ ATPase (1:1000, Novus), integrin β1 (1:10000, Abcam), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10000), lamin B (1:1000, Santa Cruz). The following secondary antibodies were used: goat anti-mouse secondary antibody (BioRad), goat anti-rabbit secondary antibody (BioRad), donkey anti-goat secondary antibody (BioRad).

2.2 Cell Culture

Primary MCs were obtained from Sprague-Dawley rats by our laboratory and were cultured in Dulbecco's modified Eagle's medium supplemented with 16% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 μg/ml penicillin. Cells were

maintained at 37 °C in 95% air and 5% CO_2 and passages between 10 and 15 were used. Cells were starved for 24 hours with 1% bovine serum albumin (BSA) for 24-hour treatment or 0.5% FBS medium for 48-hour treatment. 24.4 mM glucose was added to the cell culture at 80% confluence to reach the final concentration of 30 mM, referred to as high glucose treatment, with the selected GRP78 inhibitor (0.025 μg/ml SubA, 10 μg/ml anti-GRP78 C38 or N88 antibody).

HepG2 cells were cultured in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 μg/ml penicillin. Cells were plated at 40% confluence and starved with 0% FBS medium after reaching 80% confluence for 24 hours prior to treatment.

2.3 SubA treatment

SubA was tested in HepG2 cells to verify its ability to cleave the C-terminus of csGRP78 and impermeability through the plasma membrane. After 24-hour treatment with 0.025 μg/ml or 0.1 μg/ml SubA, both conditioned media and cell lysates were collected. Conditioned media was concentrated by centrifugation at maximum speed with Amicon™ ultra centrifugal filter units (Millipore) for 30 min. The expression of full-length GRP78, as well as its C-terminal and N-terminal domain after cleavage, was tested in conditioned media and cell lysates using Western blotting.

2.4 Transfection and Luciferase

MCs were plated to 50% confluence and transfected with 1 μg of the CAGA12-luciferase reporter construct (a kind gift from Dr. M. Bilandzic, Hudson Institute of Medical Research, Clayton, Victoria, Australia) and 0.05 μg pCMV β-galactosidase (β-Gal; Clontech) using Effectene (Qiagen). Cells were lysed after treatment with 1X Reporter Lysis Buffer (Promega) overnight at -80°C and luciferase and β-gal activity were measured with respective kits (Promega) and a Berthold luminometer on the next day. β-Gal activity was measured with a microplate spectrophotometer at 420 nm to test transfection efficacy. The same protocol was used for transfection of the TGF-β1-luciferase reporter construct (a kind gift from Dr. Hiroyoshi Taniguchi, University of Tokyo, Bunkyo-ku, Tokyo, Japan).

2.5 Protein Extraction

Cells were lysed with cell lysis buffer containing protease and phosphatase inhibitors, and whole-cell lysates were centrifuged at 14,000 rpm for 10 min. Protein concentration of each sample was calculated from the optical density measured at 595 nm using Bio-Rad Protein Assay. An equal amount of protein was obtained from each sample, and different protein expression was assessed by Western blotting as described in Section 2.10.

2.6 Biotinylation

MCs were treated with high glucose for 24 hours. After treatment, cells were washed three times with ice-cold phosphate-buffered saline (PBS) containing 1 g/L glucose, 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$ and incubated with EZ-linked Sulfo-Biotin (Pierce, 21331) (0.5 mg/ml in PBS) for 30 minutes. The excessive Sulfo-Biotin was removed by three consecutive washes with quenching buffer containing 0.1M glycine in PBS. Cells were then lysed in cell surface immunoprecipitation (IP) buffer. An equal amount of biotinylated protein from each sample was incubated in the 50% slurry Neutravidin (Fisher Scientific) overnight. On the next day, the Neutravidin beads were washed 5 times with co-IP washing buffer and cleaved from the cell surface proteins by 2X protein solubilization buffer (PSB). Protein expression was determined by Western blotting and normalized to Na^+/K^+ ATPase expression.

2.7 Active TGF-β1 Enzyme-Linked Immunosorbent Assay (ELISA)

MCs were treated with high glucose and C38 antibody for 24 hours. Conditioned media were collected from the cell culture after treatment and centrifuged at 3,000 rpm for 10 min. The amount of active TGF-β1 in the conditioned media was measured using the TGF-β1 Quantikine ELISA Kit (R&D Systems) as per the manufacturer's instruction. The step of TGF-β1 activation described in the protocol was omitted to measure the biologically active TGF-β1. All the samples were measured in duplicates with 1:2 dilution.

2.8 Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

MCs were treated with high glucose and C38 antibody for 48 hours. Cells were lysed with TRIzol reagent (Invitrogen) and chloroform was added to separate the aqueous phase from the organic phase. Total RNA was precipitated from the aqueous phase with isopropanol and washed with 75% ethanol before dissolving in RNase-free water (Applied Biosystems). 1 μg RNA from each sample was reverse transcribed into cDNA using qScript^{TM} cDNA SuperMix (Quanta Biosciences). For qRT-PCR analysis, cDNA was diluted 1:5 and TGF-β1 mRNA expression was detected with SYBR Green PCR master kit (Applied Biosystems). 18S ribosomal RNA expression was measured as an internal reference.

Table 1 Primers.

Primer	Sequence
TGF-β1 Forward	AAACGGAAGCGCATCGAA
TGF-β1 Reverse	GGGACTGGCGAGCCTTAGTT
18S Forward	GCCGCTAGAGGTGAAATTCTTG
18S Reverse	CATTCTTGGCAAATGCTTTCG

2.9 Surface Protein Co-Immunoprecipitation from Live Cells

MCs were treated with high glucose for 24 hours and washed with 1X PBS three times after treatment. Cells were then incubated in the tissue culture plate with 5 μg anti-GRP78 N88 antibody or anti-integrin β1 antibody diluted in 3 mL starvation medium (1% BSA) at 4 °C on a rocking plate for 2 hours. After incubation, cells were washed 3 times with 1X PBS and lysed with surface IP lysis buffer with protease inhibitors. Protein lysates passed through a 25-gauge needle 5 times to ensure complete lysis and centrifuged at 14,000 rpm for 10 min. Protein G beads were added to an equal amount of protein from each sample and incubated for 2 hours at 4 °C on a rocking plate. The beads were then washed 5 times with lysis buffer and eluted by boiling with 2X PSB for 5 min. Protein expression was measured by Western blotting.

2.10 Western Blotting

5-25 μg protein lysates were loaded into the wells of a sodium dodecyl sulfate (SDS) polyacrylamide gel and allowed to resolve at 100mV for approximately 1h at room temperature. Proteins loaded onto the gel carried negative charges and travelled toward the positive electrode while the voltage was applied (Mahmood & Yang, 2012). They were separated by their size, as the smaller proteins travelled more quickly than larger proteins (Mahmood & Yang, 2012). The gel percentage was determined according to the size of proteins of interest. The proteins were then transferred onto a

nitrocellulose membrane (GF Healthcare Life Science) at 260 mA on ice for 2h. The membrane was stained with 0.5% Ponceau S to confirm protein transfer and blocked with 5% skim milk in Tris-Buffered Saline with 0.1% Triton X-100 (TBS-T) for 1h at room temperature. After incubation, the membrane was washed three times with TBS-T and incubated in the selected primary antibody diluted in 5% BSA in TBS-T on a rocking plate at 4 °C overnight. On the next day, the membrane was washed with TBS-T three times for 5 min each and incubated with secondary antibody diluted in 5% milk in TBS-T for 90 min at room temperature. Chemiluminescent signal was detected with Clarity^{TM} western ECL substrate (Bio-Rad) using blue X-ray film (Carestream). The quantification of western blots was performed with Scion Image using *Gelplot2* macros and fold change in protein expression for each sample was calculated compared to control.

2.11 Statistical Analysis

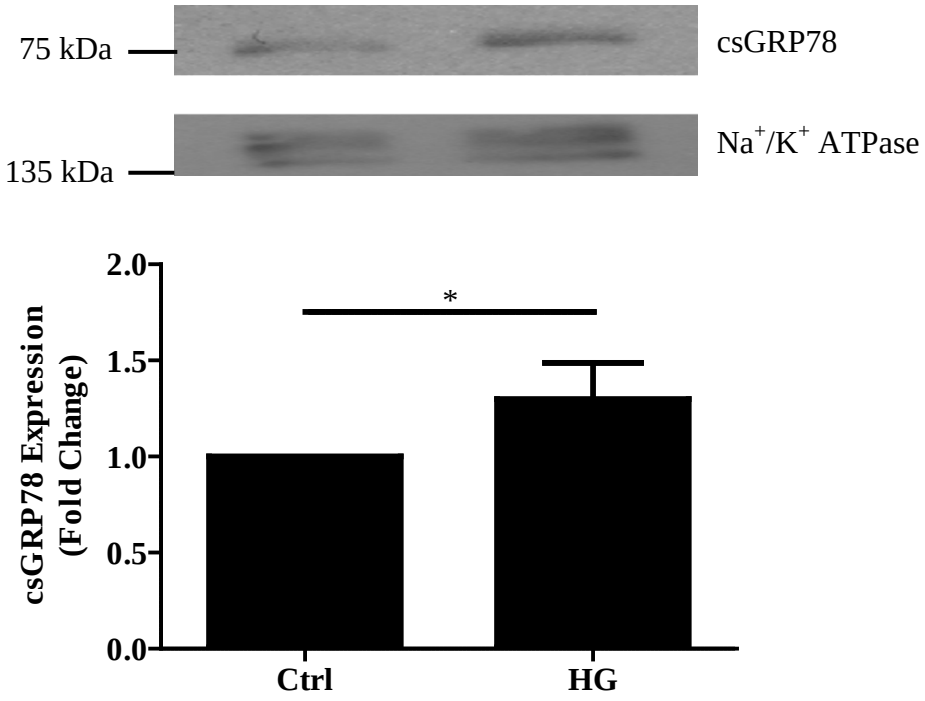
Statistical analysis was conducted using the two-tailed t-test and one-way ANOVA with Tukey's post hoc analysis for experiments with two or more than two groups respectively. $P<0.05$ was considered statistically significant. Outliers were determined using Grubb's test and removed from statistical analysis ($P<0.05$). Data were analyzed with GraphPad Prism 5 and are presented as mean ± SEM.

3 Results

3.1 GRP78 expression at the cell surface was increased in response to high glucose

To determine the role of csGRP78 in TGF-β1 signaling, it was essential to first confirm the increased expression of csGRP78 in MCs due to the high glucose exposure. As shown in Fig. 6A, membrane proteins were biotinylated and isolated from MCs after high glucose treatment for 24 hours. The expression of Na^+/K^+ ATPase was used as a loading control. An approximately 30% increase was observed in csGRP78 expression after high glucose treatment for 24 hours. Figure 6B demonstrated the purity of membrane protein purification by the absence of Lamin B, a nuclear protein, and the presence of the membrane protein Na^+/K^+ ATPase in the biotinylated protein lysate, as well as the absence of non-specific binding between non-biotinylated proteins and NeutrAvidin beads.

A)


75 kDa
csGRP78
135 kDa
Na+/K+ ATPase
csGRP78 Expression (Fold Change)
2.0
1.5
1.0
0.5
0.0
*
Ctrl
HG

B)

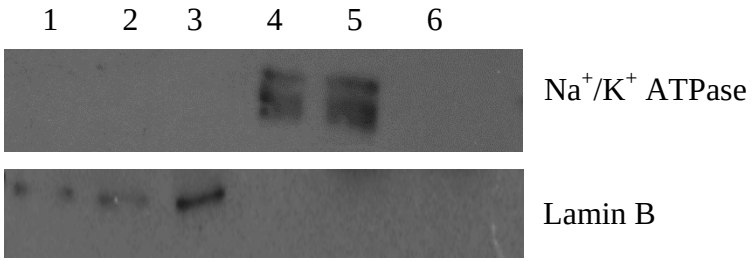

1
2
3
4
5
6
Na+/K+ ATPase
Lamin B

Figure 6. High glucose treatment resulted in the elevated expression of csGRP78.

MCs were treated with 30 mM glucose (HG) for 24 hours.

A) Plasma membrane proteins were labeled and isolated using biotinylation and the expression of csGRP78 was assessed with western blotting and normalized to the Na^+/K^+ ATPase level. The fold change of the HG-treated group compared to the control was calculated for each replicate and the average was illustrated (n=4) (*$p<0.05$).

B) Lysate was saved at various steps of the protein extraction procedure to test the purity of plasma membrane protein isolation. These were run on a gel for immunoblotting for the plasma membrane marker Na^+/K^+ ATPase or the nuclear marker lamin B.

Lane 1: Whole cell lysate;

Lane 2: Supernatant (un-biotinylated protein that were not pulled down by NeutrAvidin beads);

Lane 3: Supernatant from the biotin-negative lysate that were not pulled down by NeutrAvidin beads;

Lane 4 Ctrl membrane protein;

Lane 5 HG-treated membrane protein;

Lane 6 Biotin-negative protein exposed to NeutrAvidin beads.

3.2 csGRP78 inhibition attenuated secretion of active TGF-β1 in response to high glucose

We assessed the concentration of biologically active TGF-β1 in conditioned media using ELISA to evaluate the effect of csGRP78 inhibition on TGF-β1 activation by high glucose (Fig. 7). An approximately 2.5-fold elevation was observed in the active TGF-β1 concentration with high glucose treatment for 24 hours, which was attenuated by the C38 anti-GRP78 antibody. The C38 antibody binds to the COOH-terminal domain of csGRP78, but also acts as a non-competitive antagonist of α_2M, whose binding site is on the NH_2-terminal domain (de Ridder, Ray, & Pizzo, 2012). This result supported our hypothesis that csGRP78 upregulation may regulate active TGF-β1 secretion by high glucose, and the higher availability of active TGF-β1 in the extracellular space would promote increased downstream signaling activity.

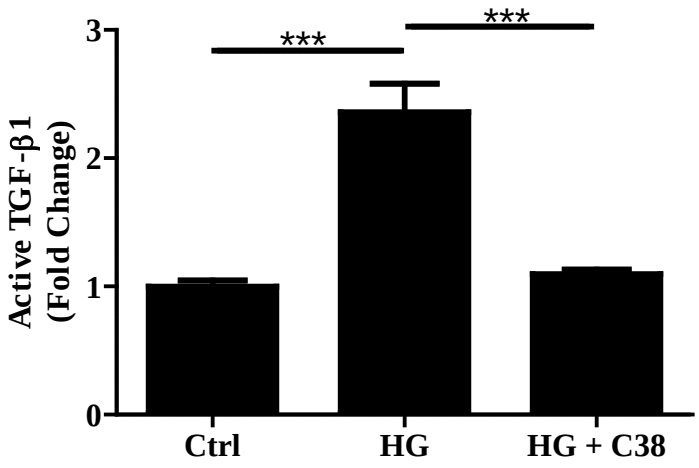


Figure 7. csGRP78 neutralization inhibited TGF-β1 activation induced by high glucose.

Conditioned media were collected from cell culture and the biologically active TGF-β1 concentration was assessed with ELISA. The average fold change of each treatment group compared to the control was calculated and illustrated in the figure. High glucose treatment resulted in the elevation of active TGF-β1 concentration in the conditioned media, and the effect was alleviated by the anti-GRP78 antibody C38. MCs were treated with 30 mM glucose (HG) and 10 μg/ml C38 antibody for 24 hours. (n=4) (***P<0.005).

3.3 csGRP78 interacted with the latent TGF-β1 complex in response to high glucose

A proposed mechanism of how csGRP78 affected mature TGF-β1 secretion was through its direct interaction with the TGF-β1 activation process. csGRP78 and its binding partners were isolated by surface co-IP, where the incubation of primary antibody was performed in live MCs. The upregulation of csGRP78 was demonstrated in response to high glucose treatment for 24 hours, as shown in Fig. 8. GRP78 located at the cell surface was shown to have a direct interaction with the LAP-TGF-β1 latent complex. It was also demonstrated that csGRP78 interacted with integrin β1 and TSP-1, both involved in TGF-β1 activation, in the context of high glucose (Massuto et al., 2010). Reverse IP of cell surface integrin β1 was performed to further confirm its interaction with csGRP78 and TSP-1 (Fig. 9). These data suggest that csGRP78 may exert its effect on TGF-β1 signaling by binding to integrin β1 and TSP-1 and thereby promoting traction-based TGF-β1 activation.

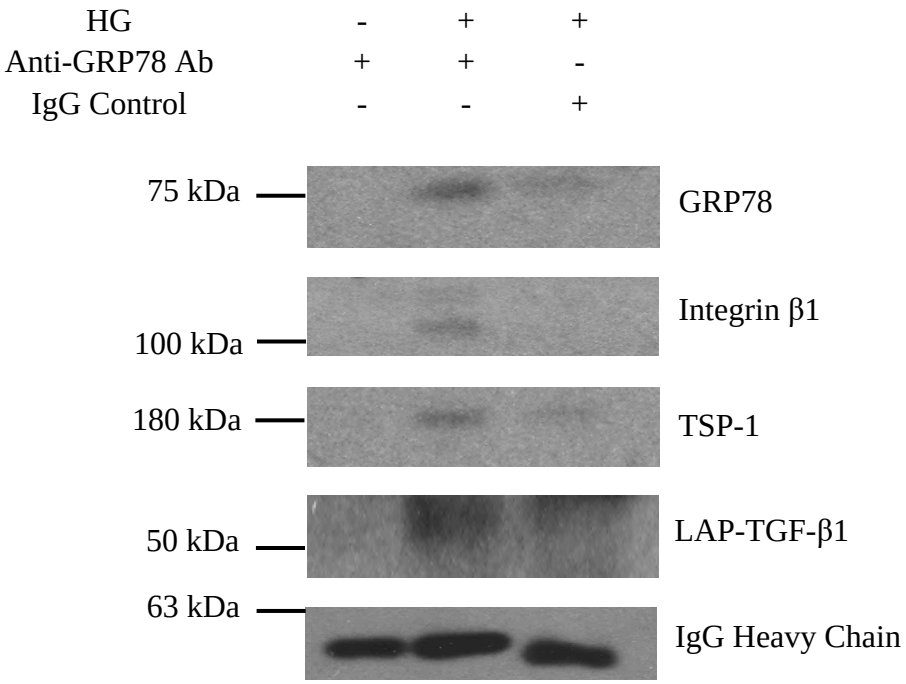


Figure 8. csGRP78 interacted with integrin β1, TSP-1, and LAP-TGF-β1 in the context of high glucose.

MCs were treated with 30 mM glucose (HG) for 24 hours. csGRP78 was labelled by incubating live cells with 5 μg/ml N88 antibody. IgG was used as a control. After immunoprecipitation, samples were assessed by immunoblotting. Cell surface GRP78 was found to associate with integrinβ1, TSP-1 and LAP. IgG heavy chain was used as a loading control (n=4).

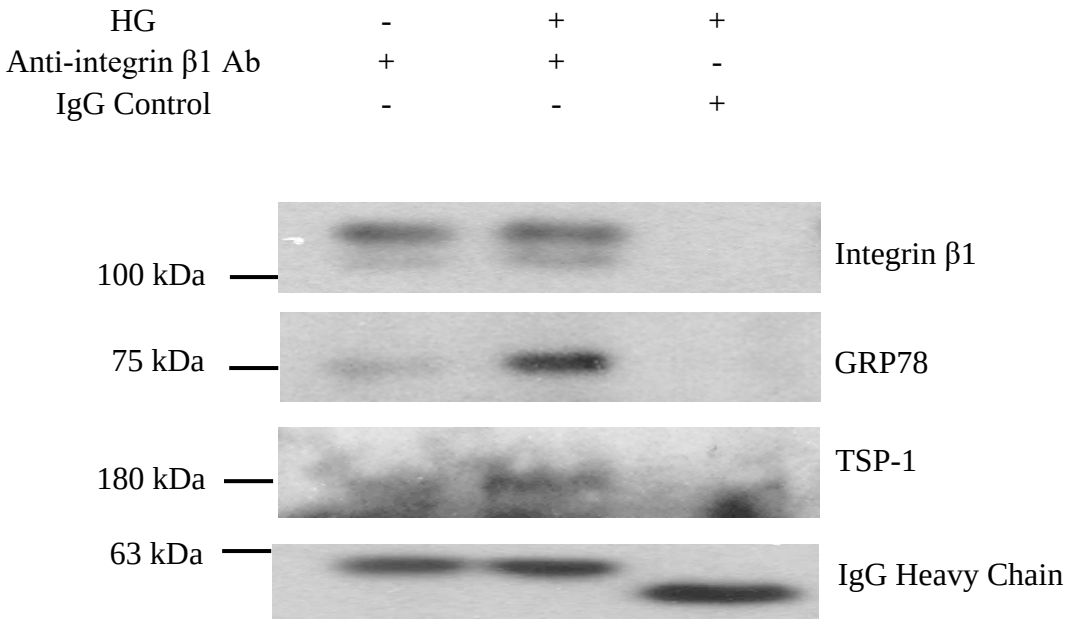


Figure 9. Surface integrin β1 IP confirmed the interaction of csGRP78 with integrin β1 and TSP-1 in the context of high glucose.

MCs were treated with 30mM glucose (HG) for 24 hours. Cell surface integrin β1 was labelled by incubating live cells with 5μg/ml anti-integrin β1, with nonspecific IgG antibodies used as control. Integrin β1 was seen to associate with GRP78 and TSP-1 at the cell surface. IgG heavy chain was used as a loading control (n=2).

3.4 The impact of csGRP78 inhibition on high glucose-induced TGF-β1 synthesis

The association between csGRP78 upregulation and increased active TGF-β1 secretion could also be explained by the involvement of csGRP78 in TGF-β1 synthesis. Therefore, we examined how anti-GRP78 antibodies influenced TGF-β1 promoter activity and mRNA expression in the context of high glucose. As shown in Fig. 10, a TGF-β1 promoter-luciferase reporter construct was used to monitor TGF-β1 transcription. Neither the GRP78 C38 nor N88 antibodies, which bind to the C- or N-terminus of GRP78 as a competitive inhibitor, respectively, had any effect on high-glucose induced TGF-β1 promoter activity. Similarly, TGF-β1 mRNA expression measured by qRT-PCR was higher in mesangial cells under high glucose treatment (Fig. 11). The neutralization of GRP78 by C38 antibody did not appear to attenuate TGF-β1 mRNA expression.

On the contrary, csGRP78 neutralization had a significant impact on the TGF-β1 protein level. As shown in Fig. 12, high glucose resulted in a significant elevation in the intracellular expression level of latent LAP-TGF-β1 complex, which was abolished by the C38 antibody. This indicated that csGRP78 had a potential effect on TGF-β1 protein synthesis or stability.

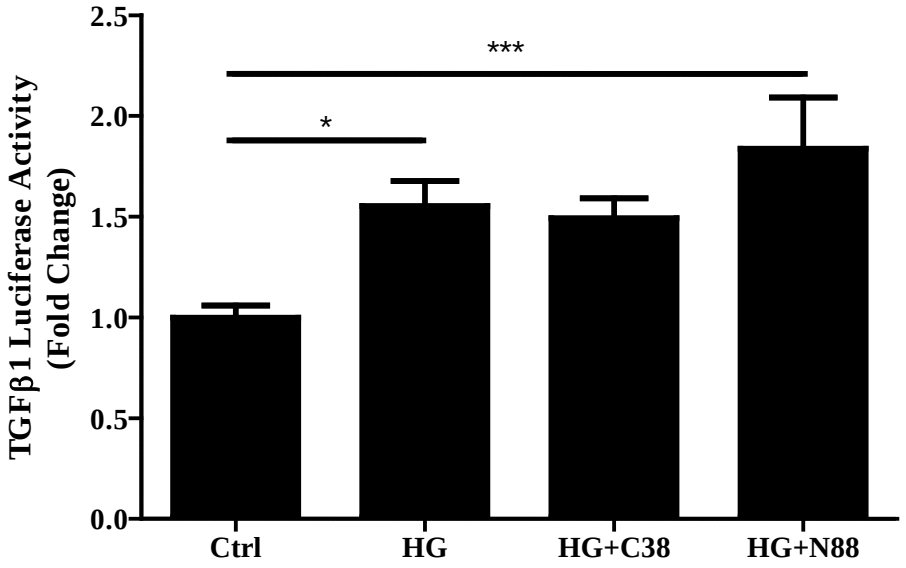


Figure 10. GRP78 neutralization did not affect high-glucose induced increase in TGF-β1 promoter activity.

MCs transfected with a TGF-β1-luciferase reporter construct were treated with 30mM glucose (HG) alone or with 10 μg/ml C38, N88 antibodies for 48 hours. Measured luciferase activity was normalized to β-Gal activity. The average fold change of each treatment group compared to the control was calculated and illustrated in the figure (n=15, n=12 for HG+N88) (*P<0.05; ***P<0.005).

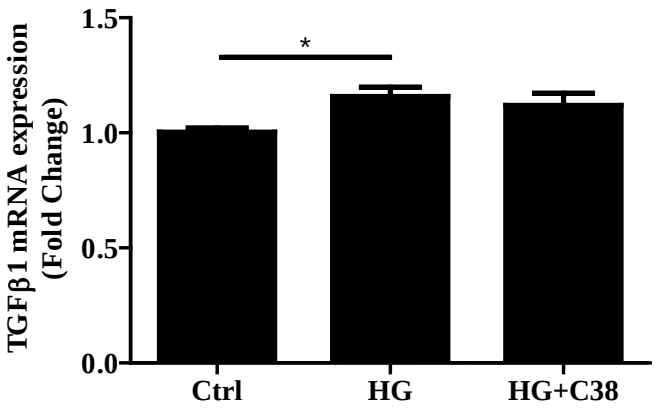


Figure 11. GRP78 neutralization did not affect high-glucose induced increase in TGF-β1 mRNA expression.

TGF-β1 mRNA expression was measured with qRT-PCR in MCs treated with 30mM high glucose (HG) alone or with 10 μg/ml C38 antibody for 48 hours. The average fold change of each treatment group compared to the control was calculated and illustrated in the figure (n=6; 4 replicates done by Renzhong Li) (*P<0.05).

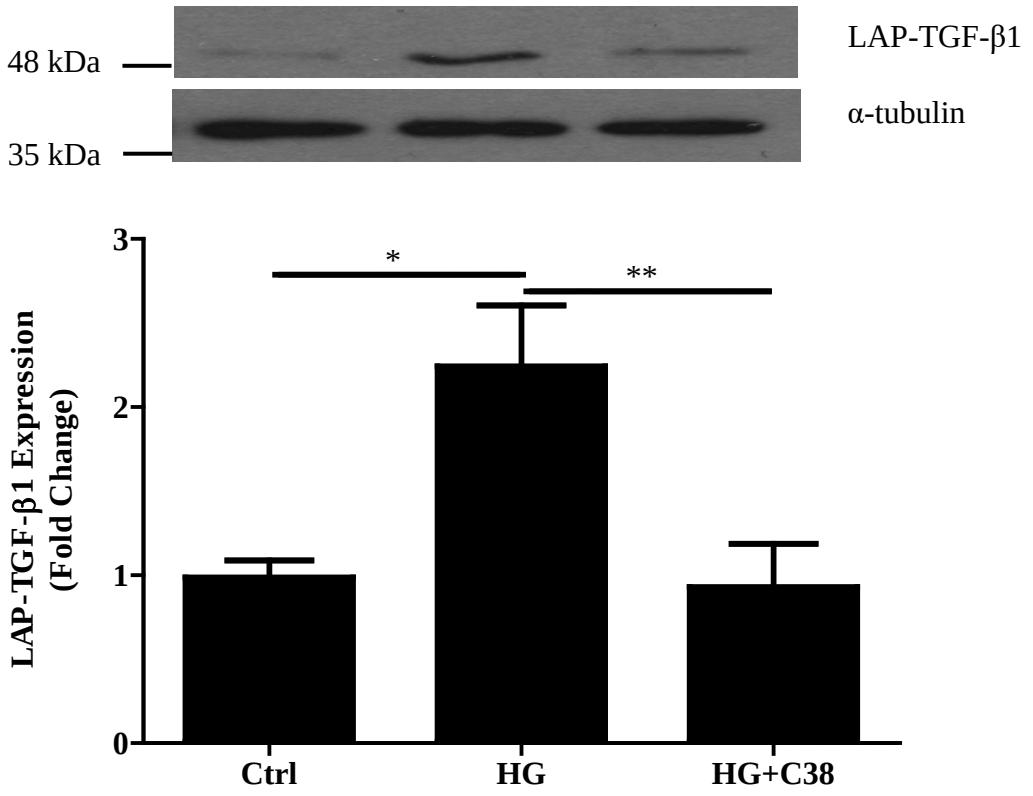


Figure 12. High glucose-induced increase in LAP-TGF-β1 expression was abolished by GRP78 neutralization.

MCs were treated with 30mM high glucose (HG) alone or with 10 μg/ml anti-GRP78 C38 antibody for 48 hours (n=6). LAP-TGF-β1 expression was measured in the whole cell lysate with western blotting and normalized to α-tubulin expression. The average fold change of each treatment group compared to the control was calculated and illustrated in the figure (*P<0.05, **P<0.01).

3.5 csGRP78 inhibition prevented high glucose-induced TGF-β1 signaling

A functional assay was employed to assess TGF-β1 signaling activity by measuring the phosphorylation level of downstream transcription factor Smad3. The subtilase cytotoxin (SubAB) is a member of the AB5 toxin family which is produced by virulent strains of Shiga toxigenic *Escherichia coli* (STEC) (Ray et al., 2012). Its catalytic A subunit (SubA) has been demonstrated to cleave GRP78 between the Leu^{416} and Leu^{417} residues of the hinge region, producing a 28-kDa fragment of the COOH-terminal domain (Ray et al., 2012). It was previously shown that SubA alone cannot penetrate the plasma membrane; therefore it can only target the GRP78 proteins expressed on the cell surface (Ray et al., 2012). Our results using HepG2 cells which have a constantly high level of csGRP78 showed that the cleaved C-terminal and N-terminal segments were observed in conditioned media after treatment with both 0.025 μg/ml and 0.1 μg/ml SubA. The segments were, however, observed in whole cell lysates with the 0.1 μg/ml treatment, suggesting that SubA can enter the cell at a higher dosage (Fig. 13). Therefore, a SubA dosage of 0.025 μg/ml was selected to inhibit GRP78 activity on the cell surface only.

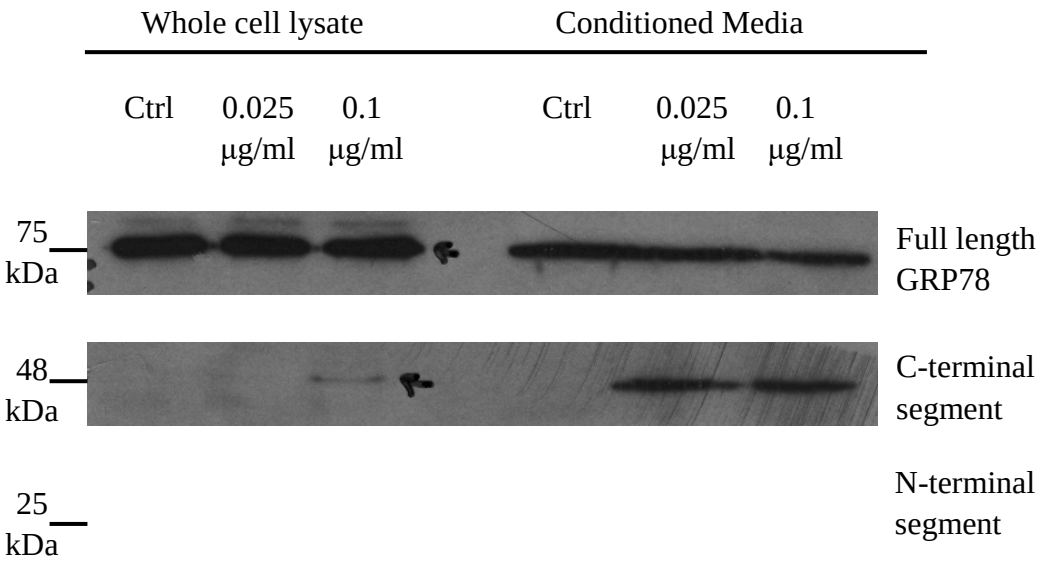


Figure 13. At low doses, SubA cleaved GRP78 on the cell surface without penetrating the plasma membrane.

Whole cell lysate and conditioned media were collected from HEPG2 cells after treatment with 0.025 μg/ml or 0.1 μg/ml SubA and assessed with western blotting (n=3). The C-terminal and N-terminal segments of GRP78 were detected respectively with C20 and N20 anti-GRP78 antibodies. The full length protein can be detected by both antibodies. The cleaved segments of GRP78 were seen in conditioned media, but not whole cell lysate, at the dosage of 0.025 μg/ml.

As shown in Fig. 14, we demonstrated that Smad3 phosphorylation was significantly increased by high glucose treatment and the response was inhibited by the addition of SubA. Moreover, the C38 neutralizing antibody was also able to abolish high glucose-induced Smad3 phosphorylation (Fig. 15), which further supported the effect of csGRP78 on TGF-β1 signaling activity. We further examined the impact of csGRP78 neutralization on the expression of α-SMA, a downstream target of TGF-β1, and TSP-1 (Fig. 16; Fig. 17). A similar trend was observed for the expression of both proteins in response to high glucose and csGRP78 neutralization.

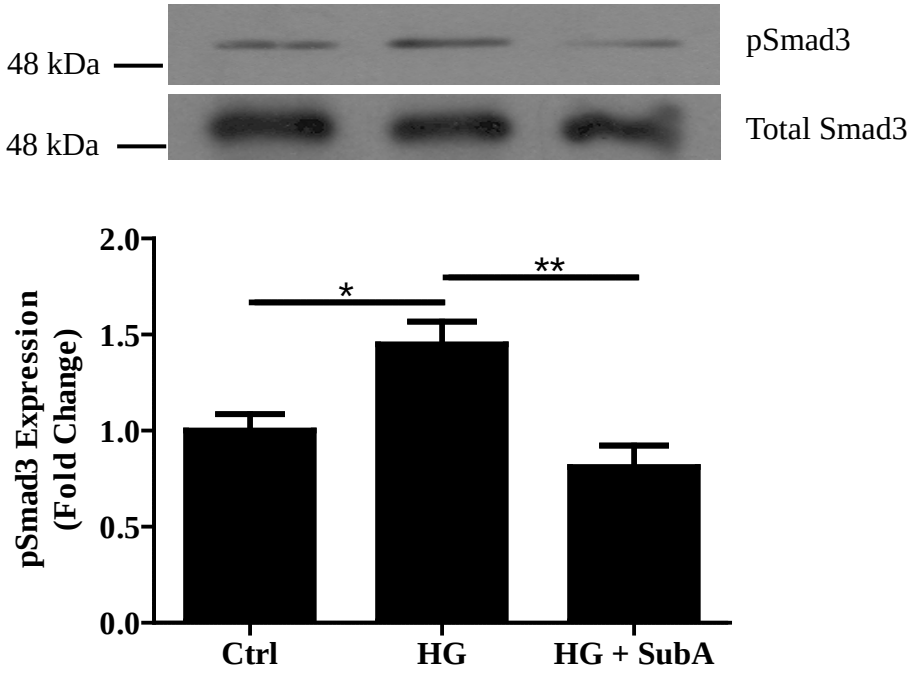


Figure 14. The high glucose-induced increase in Smad3 phosphorylation is abolished by the GRP78-cleaving peptide SubA.

MCs were treated with 30mM high glucose (HG) alone or with 0.025 μg/ml SubA for 24 hours. pSmad3 expression in the whole cell lysate was assessed with western blotting and normalized to total Smad3 expression. The average fold change of each treatment group compared to the control was calculated and illustrated in the figure (n=6) (*P<0.05,**P<0.01).

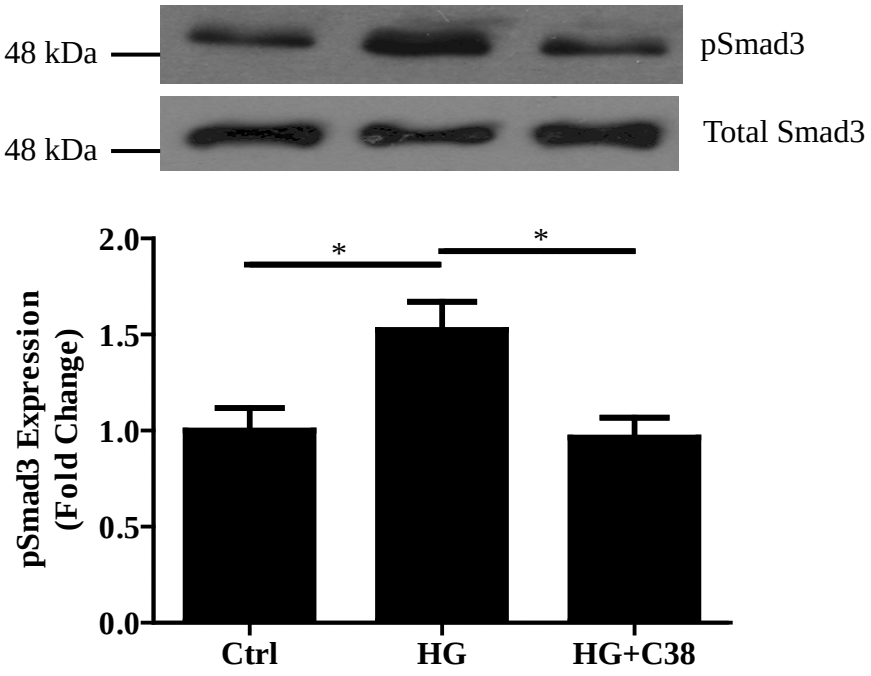


Figure 15. High glucose-induced increase in Smad3 phosphorylation is abolished by GRP78 neutralization.

MCs were treated with 30mM high glucose (HG) alone or with 10 μg/ml anti-GRP78 C38 antibody for 48 hours. pSmad3 expression was assessed in the whole cell lysate with western blotting and normalized to total Smad3 expression. The average fold change of each treatment group compared to the control was calculated and illustrated in the figure (n=8) (*P<0.05).

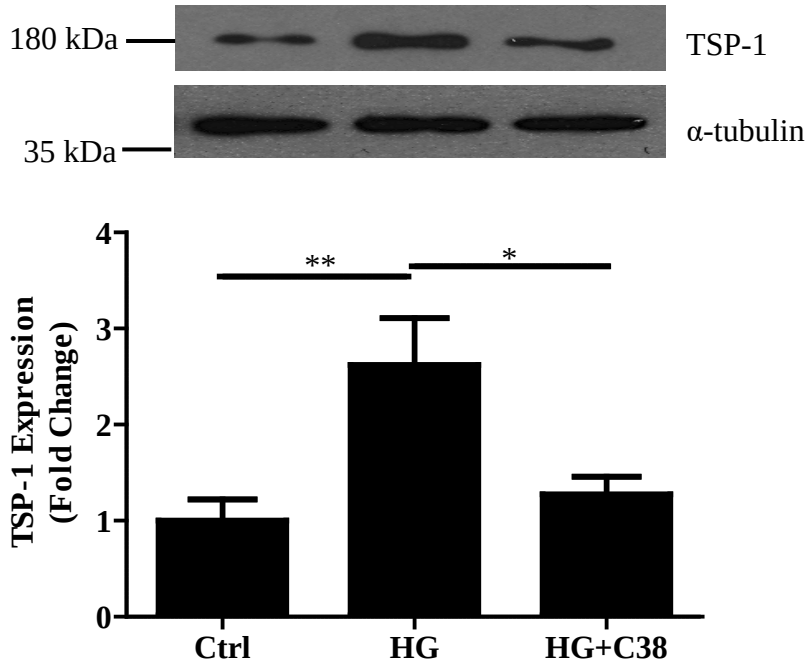


Figure 16. The high glucose-induced increase in TSP-1 expression is abolished by GRP78 neutralization.

MCs were treated with 30mM high glucose (HG) alone or with 10 μg/ml anti-GRP78 C38 antibody for 48 hours. TSP-1 expression was assessed in the whole cell lysate with western blotting and normalized to α-tubulin. The average fold change of each treatment group compared to the control was calculated and illustrated in the figure (n=7; n=6 for HG+C38) (*P<0.05, **P<0.01).

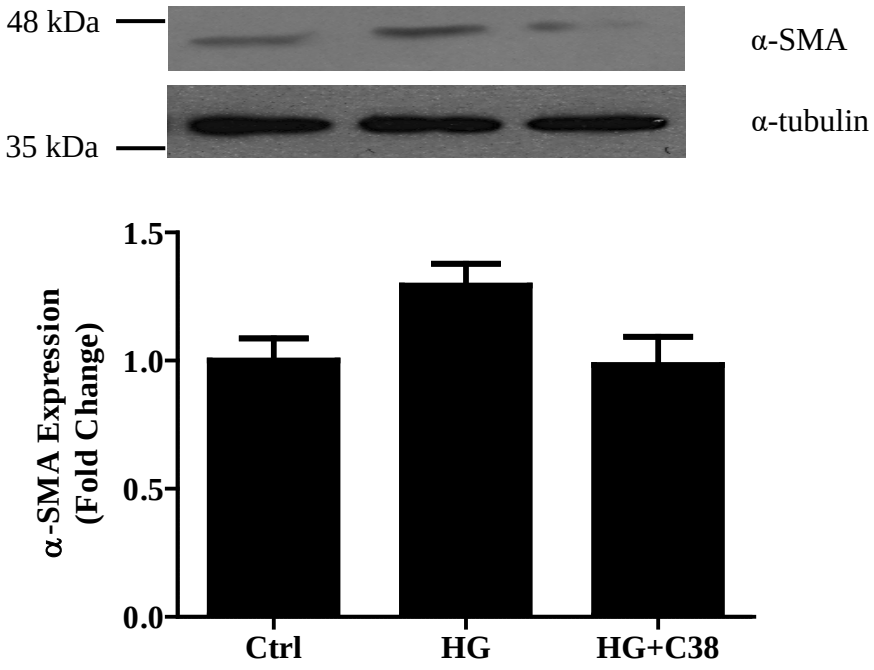


Figure 17. The high glucose-induced increase in α-SMA expression is attenuated by GRP78 neutralization.

MCs were treated with 30mM high glucose (HG) alone or with 10 μg/ml anti-GRP78 C38 antibody for 48 hours. α-SMA expression was assessed in the whole cell lysate with western blotting and normalized with α-tubulin expression. The average fold change of each treatment group compared to the control was calculated and illustrated in the figure (n=7) (*P*=0.0558).

TGF-β1 signaling activity was further assessed using the CAGA-12-luciferase reporter construct, which is an indicator of Smad3 transcriptional activity (Suenaga et al., 2008). Luciferase activity was slightly elevated by high glucose after 24-hour and 48-hour treatment, while the increase reached statistical significance after 72-hour treatment (Fig. 18). The increase in CAGA-12-luciferase activity was alleviated by SubA with the same dosage as previously used (Fig. 19). Together, the results suggested that csGRP78 was essential to the increase in TGF-β signaling in response to high glucose.

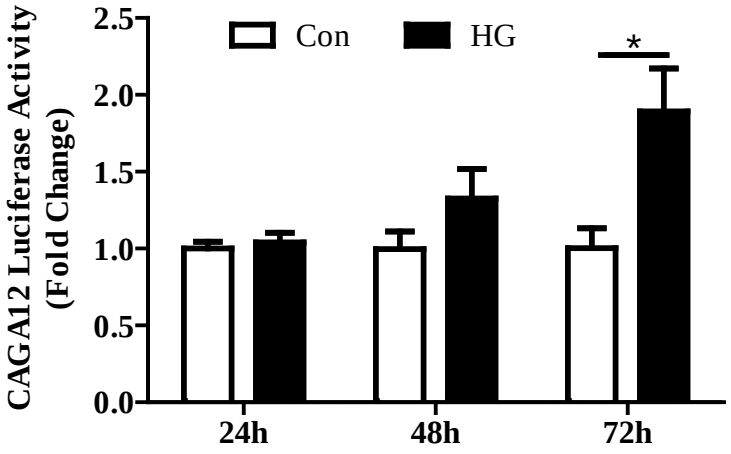


Figure 18. CAGA12 luciferase activity was significantly elevated after 72-hour high glucose treatment, but not after 24-hour or 48-hour treatment.

MCs were transfected with the CAGA-12-luciferase reporter construct to measure Smad3 transcriptional activity. Cells were then treated with 30 mM high glucose (HG) for 24 hours, 48 hours or 72 hours respectively. Luciferase activity was measured and normalized to β-Gal activity. The average fold change of each treatment group compared to the control was calculated and illustrated in the figure (n=4) (*P<0.05).

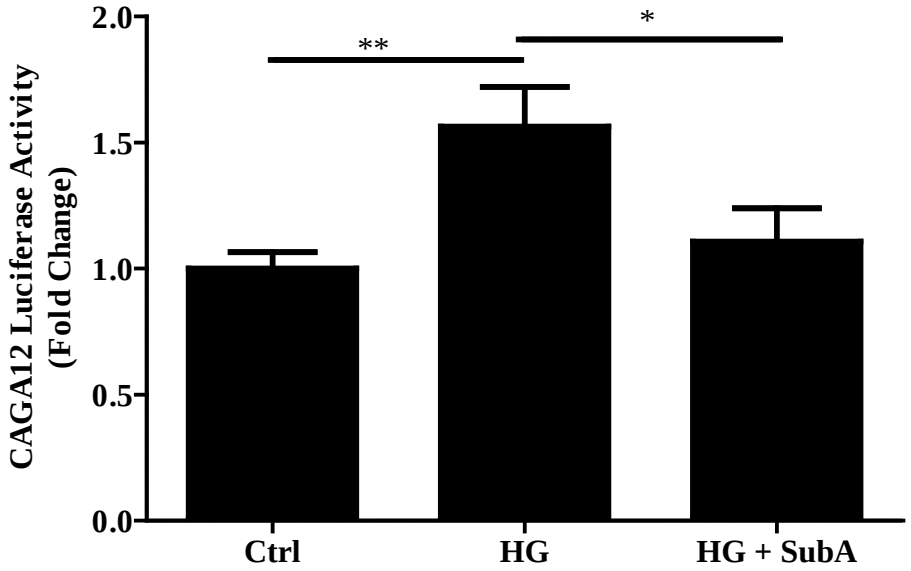


Figure 19. The elevated CAGA12 activity by high glucose was inhibited by SubA.

MCs were transfected with 1 μg CAGA-12-luciferase reporter construct and 0.05 μg pCMV β-Gal. Cells were then treated with 30 mM high glucose (HG) and 0.025 ug/ml SubA for 72 hours. Luciferase activity were measured and normalized with β-Gal activity. The average fold change of each treatment group compared to the control was calculated and illustrated in the figure (n=11 for Ctrl; n=10 for HG; n=7 for HG + SubA) (*P<0.05, **P<0.01).

4 Discussion

Extensive studies have shown that csGRP78 acts as a multifunctional receptor on the cell surface and plays a role in various diseases, including cancer, autoimmune diseases, and viral infections (Quinones, de Ridder, & Pizzo, 2008). Its cell surface expression is proposed to be an adaptive strategy in multiple cell types in response to stress, but the mechanism of re-localization is still unknown (Tsai et al., 2015). Although csGRP78 seems to be a promising candidate for a new therapeutic target in cancer, there is no evidence showing its relevance in kidney diseases.

As an important structural cell type comprising one-third of the cell population in the glomerulus, mesangial cells are intimately involved in the generation and progression of glomerulosclerosis (Abboud, 2012). The expansion of mesangial matrix due to the overproduction of ECM proteins and mesangial cell proliferation is primarily responsible for tissue scarring and loss of renal function (Abboud, 2012). Our results demonstrated for the first time that csGRP78 expression was elevated in glomerular mesangial cells exposed to high glucose. Based on the participation of csGRP78 in the signaling pathways and pathological process of various diseases, the examination of its role in glomerulosclerosis may help us understand the progression of DN and discover potential novel therapeutic targets (Gonzalez-Gronow et al., 2009). Fig. 20 summarizes the proposed mechanism of how GRP78 translocates to the cell surface and influences TGF-β1 signaling.

Different mechanisms are potentially involved in mesangial expansion and glomerulosclerosis, but TGF-β1 appears to be the key factor leading to excessive ECM production through its Smad and non-Smad signaling pathways (Ziyadeh, 2004). The correlation of csGRP78 with TGF-β1 signaling activity has been demonstrated in cancer cells. csGRP78 is suggested to provide an additional localization mechanism for the latent TGF-β1 complex on the plasma membrane, as GRP78 knockdown in mouse myeloma cells reduces surface LAP/TGF-β levels (Oida & Weiner, 2010). csGRP78 is also reported to complex with integrin β1 on the plasma membrane, which relates to cell-matrix adhesion and interaction (Z. Li et al., 2013). Integrins bind to the RGDLXX(I/L) motif of pro-TGF-β1 and facilitate the secretion of active TGF-β1 primarily through mechanical force (Dong et al., 2017). The interaction between csGRP78 and integrin β1 indicates a potential mechanism by which csGRP78 may regulate TGF-β1 activation.

Moreover, our results showed that csGRP78 interacts with TSP-1, a 450 kDa extracellular matrix protein (Bornstein, 1995). TSP-1 is known to play an essential role in TGF-β1 activation by interacting with the latent complex (Schultz-Cherry et al., 1995). The interaction between csGRP78 and TSP-1 may facilitate this mechanical process of TGF-β1 activation, which leads to increased TGF-β1 signaling in mesangial cells exposed to high glucose.

There has been evidence that TSP-1 is upregulated in glomerular mesangial cells by high glucose (Yevdokimova et al., 2001). Our data support this, showing that high

glucose increased TSP-1 expression. Interestingly, this increase in TSP-1 expression was inhibited by csGRP78 neutralization, suggesting that decreased abundance of this extracellular glycoprotein may also contribute to the attenuation of high glucose-induced active TGF-β1 by csGRP78 inhibition. The effects of csGRP78 inhibition on TSP-1 upregulation may be mediated by regulation of Akt signaling by csGRP78, which has been demonstrated in cancer cells (Abdalla et al., 2015). It has previously been shown that the inhibition of Akt attenuates TGF-β1-induced pulmonary fibrosis through the regulation of TSP-1 synthesis (Abdalla et al., 2015). Furthermore, in rat glomerular MCs, TSP-1 is upregulated by sublytic complement C5b-9 via the PI3K/Akt pathway, which is associated with elevated TGF-β1 secretion and cell proliferation in the context of human mesangioproliferative glomerulonephritis (Gao et al., 2006). Additionally, upon binding to α_2M, csGRP78 was shown to enhance Akt kinase activity and thus promote cell proliferation and unfolded protein response in cancer cells (Misra, Deedwania, & Pizzo, 2006). Further study needs to be performed to determine whether the same signaling occurs in glomerular mesangial cells in response to high glucose to upregulate TSP-1 and increase TGF-β1 activation.

Not only do our results support the csGRP78 regulation of TGF-β1 activation, but they also suggest that csGRP78 may be involved in the regulation of TGF-β1 synthesis at the protein level. Although no significant effect was seen on high glucose-induced TGF-β1 promoter activity and mRNA expression when csGRP78

was neutralized, the protein expression of LAP-TGF-β1 latent complex was significantly lowered. A plausible explanation for this finding is that the intracellular signaling pathway triggered by csGRP78 stimulates translation of the LAP-TGF-β1 protein. Although many studies do not differentiate the regulation of TGF-β1 at the transcription and translation stages, Xiao *et al.* reported that TGF-β1 transcription is controlled by p38 MAPK, ERK, and JNK, whereas the activation of Rho GTPase, PI3K, Akt signaling was essential for TGF-β1 translation process (Xiao et al., 2008). Future studies examining the interaction between csGRP78 and these signaling molecules would help to better describe the importance of csGRP78 in TGF-β1 synthesis. It is also worthwhile to compare the magnitude of effects of csGRP78 inhibition on both latent and active TGF-β1 in conditioned media. A greater change in active TGF-β1 concentration would suggest that csGRP78 is involved predominantly in TGF-β1 activation in contrast to synthesis. However, both processes are essential in the disease progression and may be practically difficult to differentiate.

We demonstrated that csGRP78 regulated downstream TGF-β signaling in mesangial cells in response to high glucose. The cleavage and neutralization of csGRP78 inhibited canonical Smad3 activation as assessed by its C-terminus phosphorylation and transcriptional activity. The impact of csGRP78 neutralization on the Smad3-responsive α-smooth muscle actin (α-SMA) was thus tested, which is considered the hallmark phenotypic alteration in activated and pro-fibrotic mesangial

cells (S. Li et al., 2017). These data support a potential and novel pro-fibrotic role of csGRP78.

A recent study showed that csGRP78 had an inhibitory impact on TGF-β1 signaling in cancer cells upon its binding to CD109, a GPI-anchored protein on the plasma membrane (Tsai et al., 2018). Their results suggested that csGRP78 exerted its effect by promoting the degradation of TGFβRI through caveolar endocytosis. This opposing data on TGF-β1 regulation by csGRP78 is likely attributable to the cell type studied. Indeed, in cancer cells csGRP78 plays a pro-apoptotic role, which is opposite that of its proliferative and pro-fibrotic role in glomerular MCs (Tsai et al., 2018).

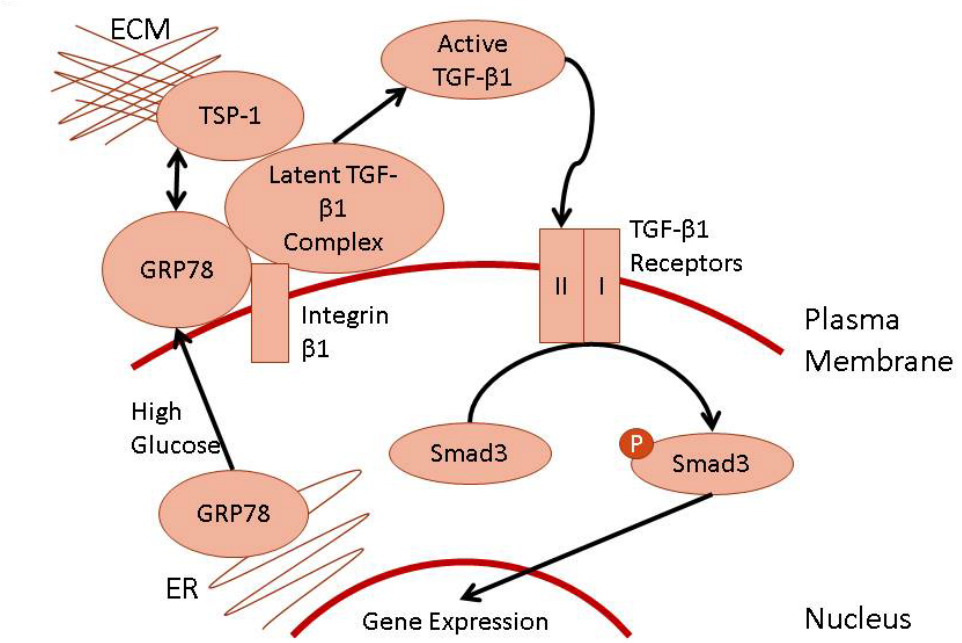


Figure 20. A proposed mechanism for csGRP78 modulation of TGF-β1 signaling.

In hyperglycemic conditions, GRP78 translocates from ER to the plasma membrane as a peripheral protein and interacts with integrin β1 and TSP-1. This interaction potentially results in the elevated secretion of active TGF-β1, potentially through mechanical activation, which then binds to its receptors. The receptor activation leads to increased Smad3 phosphorylation and responsive gene expression. The effect of csGRP78 on TGF-β1 synthesis is not as yet fully understood, and is not shown in the figure.

Current treatment strategies for DN involve the tight control of hyperglycemia, dyslipidemia, and hypertension, as well as lifestyle advice and weight management (Bennett & Aditya, 2015). Since TGF-β1 is recognized as a key mediator in disease progression, attempts have been made to develop TGF-β1 antagonists as a potential therapeutic measure, but were later proved unsuccessful due to their adverse effects (Murphy-Ullrich & Suto, 2017). In addition to its pro-fibrotic role, TGF-β1 is also intimately associated with development, tissue homeostasis and regeneration processes (Murphy-Ullrich & Suto, 2017). Current TGF-β1 inhibitors used in animal and clinical studies, such as monoclonal anti-TGF-β1 antibodies and receptor antagonists, are not capable of differentiating homeostatic and disease-promoting TGF-β1 activity, potentially leading to severe adverse events (Murphy-Ullrich & Suto, 2017). Moreover, global TGF-β1 inhibitors failed to show many therapeutic benefits in clinical trials (Murphy-Ullrich & Suto, 2017). The translocation of GRP78 to the plasma membrane provides a possible mechanism of distinguishing cells affected by hyperglycemia, suggesting the possibility of a more therapeutically effective and safe therapy.

Future studies are needed to further define the involvement of csGRP78 in TGF-β1 activation and synthesis. Both SubA and C38 antibody, which were used as inhibitors of GRP78, target its COOH-terminal domain. Since it was suggested that the COOH- and NH_2-terminal domains of GRP78 could lead to different or even opposite downstream effects, it would be worthwhile to examine the effect of

NH_2-terminal domain inhibitors, such as N20 and N88 antibodies, on TGF-β1 activation and signaling activity (Gonzalez-Gronow et al., 2009).

Additionally, the relationship between csGRP78 and other signaling molecules induced by TGF-β1 should be examined in glomerular mesangial cells. The effect of csGRP78 on canonical TGF-β1 signaling can be further examined via the phosphorylation of Smad2, and the examination of non-canonical pathways such as PI3K/Akt signaling may provide new information on the regulation of TGF-β1 signaling by csGRP78.

Another strategy to examine the importance of csGRP78 is to target its transportation to the cell surface. One potential target is MTJ-1, which was found to be essential for csGRP78 translocation (Misra et al., 2005). Silencing of MTJ-1 gene expression via siRNA transfection can be an alternative measure of csGRP78 inhibition, and its effect on TGF-β1 signaling would be specific to the cell surface fraction of GRP78 without affecting the intracellular fraction. The role of ER stress in csGRP78 translocation has been previously described in cancer cells and thus should also be further examined in MCs due to its nature as another important mediator of DN progression induced by high glucose (Mooradian & Haas, 2011; Tsai et al., 2018). It would also be important to understand the duration of GRP78 cell surface presence. Furthermore, whether csGRP78 is secreted into the ECM or internalized and recycled to the ER can potentially provide insights on its signaling mechanism in mesangial cells, as well as its association with TGF-β1 production and activation processes.

The therapeutic potential of anti-GRP78 antibodies has been tested in cancer models. Monoclonal anti-GRP78 antibody MAB159 has been tested in murine xenograft models with colon cancer, small cell lung carcinoma, lung adenocarcinoma, breast adenocarcinoma and others (Liu et al., 2013). It was observed that MAB159 only located in the tumor but not in normal organs, and it showed a beneficial effect by suppressing tumor growth and metastasis (Liu et al., 2013). A similar approach may be adapted in diabetic mouse models. The expression of csGRP78 should also be examined in other renal cell types to obtain a more comprehensive understanding of its role in overall phenotypic changes in glomerulosclerosis and DN progression, which can be critical to explain any therapeutic benefits or adverse effects observed in the animal research. Ultimately, the research on csGRP78 may be extended to other organs affected by diabetes as a potential target to treat other complications.

5 Conclusion

In conclusion, our results suggested that high glucose exposure can induce the expression of csGRP78 in glomerular mesangial cells, which may play a critical role in the progression of DN by increasing TGF-β1 synthesis and activation, leading to its elevated signaling activity. We propose that csGRP78 interacts directly with the latent TGF-β1 complex and associated proteins to induce the release of active TGF-β1, and also regulates TGF-β1 protein level through intracellular signaling. Future studies on how csGRP78 mediates its pro-fibrotic effect may help understand the disease progression and develop novel therapeutic agents.

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