CELL SURFACE GRP78 IS REQUIRED FOR THE UPREGULATION OF TSP-1 BY HIGH GLUCOSE IN KIDNEY MESANGIAL CELLS

CELL SURFACE GRP78 IS REQUIRED FOR THE UPREGULATION OF TSP-1 BY HIGH GLUCOSE IN KIDNEY MESANGIAL CELLS

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Master of Science

Descriptive Note

McMaster University MASTER OF SCIENCE (2020) Hamilton, Ontario (Medical Science)

TITLE: Cell surface GRP78 is required for the upregulation of TSP-1 by high glucose in kidney mesangial cells

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NUMBER OF PAGES:

Lay Abstract

Diabetic nephropathy is a condition that is associated with a gradual loss of kidney function as well as the presence of protein in the urine. As the name implies, diabetic nephropathy occurs as a result of diabetes mellitus. The disease causes the mesangial cells in the kidney to produce excess extracellular matrix leading to scarring in the kidney, a process called fibrosis. One of the key fibrotic proteins is called transforming growth factor beta-1 (TGF-β1), stored in a latent form. A major activator of TGF-β1 is thrombospondin-1 (TSP-1). Our results demonstrate that the cell surface localization of glucose regulated protein 78 (GRP78) is required for the upregulation of TSP-1 in a high glucose environment, leading to activation of profibrotic pathways that are well known to perpetuate the fibrotic phenotype seen in diabetic nephropathy.

Abstract

Diabetic nephropathy (DN) is a complication associated with diabetes and is characterized by proteinuria and a progressive loss of kidney function. The disease morphologically manifests as an increase in the extracellular matrix (ECM) produced by kidney cells including specialized mesangial cells found in the kidney glomeruli. The mesangial cells undergo increased proliferation and hypertrophy, produce ECM components at an elevated rate and in turn the ECM itself is broken down at a reduced rate. This leads to fibrosis, or the scarring of the glomeruli. The process of fibrosis is known to be promoted by pro-fibrotic factors such as transforming growth factor beta-1 (TGF-β1), which is activated by various proteins including thrombospondin-1 (TSP-1). Both of these proteins are known to have an increased rate of expression and activation in a high glucose environment and in the kidneys of diabetic patients. Glucose-regulate protein 78 (GRP78) is another protein altered by high glucose, as it is translocated to cell surface in DN (cell surface GRP78, csGRP78). In this study, we investigate the role csGRP78 has in the regulation of TSP-1 and downstream signaling by high glucose, using primary rat mesangial cell cultures. Our results confirm that TSP-1 protein levels are increased in the cell lysate and in the ECM of cells treated with high glucose. We further show that inhibitors of csGRP78 and downstream PI3K/Akt reduce the high glucose-induced increase in TSP1 at both protein and transcript levels, and attenuate TGF-β1 signaling.

Acknowledgements

First, I would like to thank my supervisor Dr. Joan Krepinsky for her invaluable support over the past two years. She offered words of wisdom and guidance during tough times, and always maintained optimism in my abilities and my work. She was also enthusiastic and encouraging when things were going well, always pushing me to do my best and succeed. Her hands-on approach, as well as all the advice, both scientific and otherwise are lessons I will not soon forget and hope to carry forward in all my future endeavors.

I would also like to thank my supervisory committee, Dr. Mark Inman and Dr. Peter Margetts. They offered keen scientific insight when I seemed to be stuck and were always ready and willing to offer their expertise whenever it was required. I would not have been able to succeed had it not been for their support.

I would also like to thank my parents, Baber Ahmed and Fatima Baber for their unconditional love and moral support. They fostered my interest and love for learning and science from a young age and always encouraged me to keep striving for success.

Finally, I would like to thank colleagues and friends. My colleagues in the lab were always there to offer moral and technical support, going out of their way to teach and train me in the procedures of the lab and ensuring we all succeed together. My friends outside the lab were always there to offer unseen support and motivate me when things were difficult and encourage me when things were good. They would listen to me and always ensure I felt heard and appreciated. I could not have done it without them, and I thank them every day for it.

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List of Abbreviations

DN- Diabetic Nephropathy

ESRD- End stage Renal Disease

ECM- Extracellular Matrix

TGF-β1- Transforming Growth Factor Beta 1

LAP- Latency Associated Peptide

LTBP- Latent TGF-β1 Binding Peptide

MMP 2- Metalloproteinase 2

TSP-1- Thrombospondin-1

GRP78/csGRP78- Glucose Regulated Protein 78/Cell Surface Glucose regulated Protein 78

SD- Sprague Dawley

PBS- Phosphate-Buffered Saline

Co-IP- Co-immunoprecipitation

PSB- Protein Solubilization Buffer

SDS-PAGE- Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis

TBST- tris-buffered saline with 0.1% Triton X-100

HG- High Glucose

WCL- Whole cell lysate

LY- LY294002

Wort- Wortmannin

Akt-in- Akt Inhibitor VIII

PI3K- Phosphoinositide 3-kinase

YAP- Yes-associated Protein

RTEC- Renal Tubular Epithelial Cells

CKD- Chronic Kidney Disease

pSMAD3- Phosphor-SMAD3

MTJ-1- DnaJ-Like protein 1

Declaration of Academic Achievement

I, Usman Ahmed, declare all the work within this thesis to be my own. This work has not been submitted or published by any other institution. My supervisor, Dr. Joan Krepinsky as well as my supervisory committee, Dr. Mark Inman and Dr. Peter Margetts supported and offered guidance to me for the duration of this thesis.

There are no conflicts of interest to declare.

1.0 Introduction

1.1 Kidney Anatomy and Physiology

The renal system is one of several organ systems responsible for sustaining life in many different kinds of organisms. In mammals, the renal system is responsible for a variety of tasks crucial to survival, including waste elimination, maintenance of pH, blood pressure, and blood volume. Central to the renal system are the kidneys which are the major organs of the system. The kidneys are highly vascularized, receiving blood from the renal arteries, and are responsible for the filtration of incoming blood and the removal of waste products from the filtered blood[1]. The kidneys themselves are composed of millions of nephrons, which are the smallest functional unit of the kidney [1]. The nephrons are composed of a tubule portion which collects the filtrate from the blood, and the corpuscle portion which consists of the vasculature that is responsible for selectively allowing certain components within the blood to pass through [1]. The tiny capillaries that make up the corpuscle form a structure known as the glomerulus, which is surrounded by Bowman's capsule. The blood entering the kidneys eventually passes through the glomeruli of each nephron, and upon doing so some of the blood's components are filtered into the tubule portion of the nephron. The glomerulus is composed of a variety of cell types, including podocytes, epithelial cells and mesangial cells, the latter of which are the focus of this study [2]. The mesangial cells of the glomeruli are primarily responsible for providing structural support to the capillary network via the production and secretion of the mesangial matrix [2]. The matrix itself is composed of a wide variety of proteins, including fibronectin and collagen [3].

1.2 Diabetes and Diabetic Nephropathy

Diabetes mellitus is a metabolic disorder that is characterized by elevated levels of the molecule glucose within the circulation. Diabetes can be divided into two main types, aptly named Type I and Type II diabetes. Type I diabetes has an early age of onset, due to the fact that it is believed to be caused by genetic and environmental factors [4]. It involves the destruction of the pancreas' beta cells by the individual's own immune system. These beta cells are responsible for the production of the hormone insulin [4]. In comparison, Type II diabetes is primarily the result of lifestyle factors such as diet, obesity and level of physical activity [5]. It is characterized by a decrease in insulin production from the beta cells, in combination with insulin resistance of the body's cells. Type II diabetes has a later age of onset than Type I, and accounts for the vast majority of diabetes cases, with upwards of 90 percent of all diabetes mellitus cases being Type II [5]. Both types of diabetes ultimately result in the same detrimental excess of glucose molecules within the vasculature of individuals. The excess glucose has the potential to cause micro- and macrovascular complications in diabetic patients and this risk increases the longer a patient has diabetes [6]. One such complication is diabetic nephropathy (DN), also known as diabetic kidney disease. DN is characterized by the chronic loss of kidney function, resulting in high mortality rates, and also increased risk of patients developing end stage renal disease (ESRD) [7]. It is also usually characterized by a persistent increase in the amount of the protein albumin being excreted in the urine [8]. Large proteins such as albumin are not typically found within the urine, thus albumin's presence in the urine of DN patients is indicative of kidney dysfunction, usually early DN. Eventual loss of kidney function can be seen as a reduction in the glomerular filtration rate (GFR), which may ultimately lead to ESRD [7]. In fact, DN is the leading cause of ESRD and is estimated to affect upwards of 15 to 40% of type I diabetes patients and 5- 20% of type II

diabetes patients[8]. Thus, the cost of DN in terms of human life and health care resources cannot be understated.

DN is characterized by specific pathologic changes in the kidneys (figure 1). The glomeruli of affected kidneys show glomerulosclerosis, which is the end result of the process by which the glomeruli undergo scarring, referred to as fibrosis, and manifests as the formation of non-functional scar tissue. In normal physiological conditions, the process of fibrosis is an essential part of tissue repair and wound healing [9]. The mesangial cells of the kidney are major contributors to the pathogenesis of DN as they undergo early proliferation and hypertrophy in patients with DN [10]. They are also responsible for producing the extracellular matrix (ECM) that provides structural support to the delicate capillaries that make up the glomeruli [2]. However, in DN states, the mesangial cells produce and secrete the proteins that make up this matrix at an elevated rate, which is also then broken down at a decreased rate. The combined effects of an increase in matrix production, and decrease in its breakdown, also lead to the thickening of the glomerular basement membrane, impeding the filtration abilities of the glomeruli [10]. The high glucose environment in the glomeruli of diabetic patients is thought to be a major factor in the development and progression of DN [11].

1.3 TGF-β1 and TSP-1

The excess glucose within the circulation of DN patients causes an increase in profibrotic factors [12]. A key profibrotic protein is the protein Transforming Growth Factor β1 (TGF-β1). TGF-β1 plays an integral role in the process of fibrosis and its expression has been shown to increase in DN [13] [14]. It is part of the transforming growth factor beta superfamily of cytokines, which also includes members such as bone morphogenetic proteins, growth differentiation factors, as well as activins and inhibins [14]. Previous studies have established that elevated

expression and activation levels of TGF-β1 contributes to the expansion of the extracellular matrix that is characteristic of DN by activating downstream pro-fibrotic pathways, including Smad-dependent and non-Smad dependent pathways [9]. A study by Guo and colleagues investigated the effect TGF-β1 inhibition had on the progression and development of glomerulosclerosis. The researchers found that TGF-β1 inhibition using siRNA that specifically targeted TGF-β1 resulted in inhibition of the synthesis of collagen proteins induced by high glucose in mouse mesangial cells [15]. In addition, Benigni et al demonstrated that the inhibition of TGF-β1 using an antibody was able to arrest the progression of DN in rats that were also being treated with ACE inhibitors, the current standard of care treatment. The pancreatic β-cell toxin streptozotocin was used by the researchers to induce diabetes in the rats[16].

TGF-β1 typically exists in an inactivated form, during which it is bound to an amino acid sequence called the latency associated peptide (LAP) which renders the protein inactive [17]. The LAP renders certain receptor binding sites on TGF-β1 inaccessible, preventing it from binding to its receptors to induce downstream signaling [14]. The disruption of the interaction between the LAP and TGF-β1 is thus crucial to converting the latent TGF-β1 to its active form. During intracellular processing, the LAP is proteolytically cleaved, subsequently noncovalently associating with TGF-β1 and facilitating the recruitment of latent TGF-β1 binding protein (LTBP). Binding of LTBP to TGF-β1 forms an inactive complex, that can then be cleaved to release active TGF-β1 by several proteases such as plasmin, metalloproteinase 2 (MMP 2) and MMP9 or by mechanical force[17].

One protein found to be capable of activating latent TGF-β1 is Thrombospondin-1 (TSP-1). TSP-1 is a member of the thrombospondin family of glycoproteins with many functions which is present in the ECM. [14]. For example, under normal physiological conditions, TSP-1 was

shown to be upregulated in response to tissue injury [14]. It has multiple domains that are capable of binding to various receptors and partners, one of which is TGF-β1. The structure of TSP-1 includes a sequence known as the KRFK sequence, which has been shown to bind to the LAP domain of latent TGF-β1 [14]. Upon doing so, TSP-1 non-proteolytically renders the latent TGF-β1 active. In other words, TSP-1 activates TGF-β1 without cleaving off the LAP from the latent form of the protein. [14]. By binding to the LAP, TSP-1 causes a conformational change in TGF-β1 that causes its receptor binding sites to no longer be sequestered by the LAP domain [14]. It has been previously shown that the factors that cause an increase in TGF-β1 levels in DN also similarly cause an increase in TSP-1 levels, possibly suggesting a correlation between the two proteins [13]. Previous studies have also established that TSP-1 is elevated by high glucose and in mesangial areas of diabetic mice with DN [18]. Daniel and colleagues demonstrated in 2007 that mice deficient in TSP-1 are conferred a multifactorial protection from the detrimental changes associated with DN induced by streptozotocin. For example, TSP-1 deficient diabetic mice had a reduced amount of ECM accumulation, inflammatory cell accumulation and glomerular cell proliferation [18]. They also had improved kidney function and an 8-fold reduction in proteinuria 20 weeks after the induction of diabetes.

1.4 GRP78

Glucose regulated protein 78 (GRP78) is a protein that is present in the endoplasmic reticulum (ER) of cells under normal physiological conditions [19]. It is responsible for assisting in the folding of newly synthesized proteins that are making their way through the cell [19]. The protein is also involved in the ER's response to certain stressful stimuli, a response designated as the ER stress response [20]. Experiments previously conducted in our lab show that GRP78 is translocated to the cell surface in high glucose environments[21]. In this state, the protein is

designated csGRP78 (cell surface GRP78). The appearance of GRP78 on the cell surface is not unique to only high glucose environments, as studies conducted by other researchers indicate that csGRP78 can also be found in certain types of cancer cells [19].

1.5 GRP78 and TSP-1

Previous studies have attempted to characterize possible associations between csGRP78 and TGF-β1 within the context of certain cancer cell lines [22]. For example, a protein called Cripto-1, which is a GPI-anchored protein, has been shown to interact with the cell surface form of GRP78 in cancer. The interaction between Cripto-1 and csGRP78 causes the inhibition of TGF-β1 signaling pathways, promoting the survival of the cancer cells, as typically TGF-β1 promotes apoptosis in malignant cells [22]. This functionality of TGF-β1 contrasts with its functionality in the mesangial cells of the glomeruli. In this context, as previously mentioned, the elevated TGF-β1 levels cause mesangial cell proliferation, and also causes them to produce excess components of the ECM [9]. Our lab has previously demonstrated that csGRP78 is responsible for excess ECM production in the context of high glucose, a process for which TGF-β1 is known to be required[21]. Thus, in this study, we sought to investigate whether csGRP78 was involved in the activation of TGF-β1 through the regulation of one of its activators, TSP-1.

2.0 Hypothesis and Aims

It is hypothesized that in a high glucose environment, the translocation of GRP78 to the cell surface enables increased expression of TSP-1 which leads to the activation of TGF-β1, stimulating the pro-fibrotic response that contributes to the pathological phenotype seen in DN (Figure 2).

2.1 Aim 1: To establish if csGRP78 increases TSP-1 present in mesangial cells in response to high glucose.

2.2 Aim 2: To define how csGRP78 regulates high glucose-induced increases in TSP1.

3.0 Materials and Methods

3.1 Cell Culture Systems

Primary mesangial cells from the glomeruli of Sprague Dawley (SD) rats were used. Glomeruli were obtained using magnetic beads, which are grown in selective media to remove other cell types and leave only mesangial cells. The SD cells were given growth media (Dulbucco's Modified Eagle Media from Sigma-Aldrich) with added 20 percent fetal bovine serum (if passage 11 or younger) or 16 percent fetal bovine serum (if passage 12 and older). The growth media also contained penicillin in a concentration of 100 µg/mL. Cells were incubated at 37°C, with 95% air and 5% carbon dioxide, and allowed to grow to 95% confluence before being plated for experiments. Cells subjected to high glucose were treated using glucose at a concentration of 24.4 mmol/L, resulting in a final concentration of glucose of 30 mmol/L. Treatment with glucose served as a model for DN.

3.2 Antibodies, Proteins and Reagents

For western blot experiments, the following list of primary antibodies were used: Thrombospondin-1 (1:1000, R&D Systems), GAPDH (1:1000, Millipore), Tubulin (1:1000, Sigma-Aldrich),. The following secondary antibodies were also used in western blot experiments: donkey anti-goat secondary antibody (Bio-Rad), goat anti-mouse secondary antibody (Bio-Rad) and goat anti-rabbit secondary antibody (Bio-Rad). The secondary antibodies are conjugated to horseradish peroxidase. The following reagents were used for various

treatments: Vaspin (Cedarlane), LY294002 (Sigma-Aldrich), Akt-Inhibitor VIII (Millipore Sigma), GRP78 C38 (IgG2b) (Dr. Rupa Ray), mouse IgG (Cedarlane/Millipore).

3.3 Protein Extraction

Cell medium was removed from cell culture plates and the cells were washed with cold PBS three times. Care was taken to ensure all PBS was removed from the plates. Next, cold cell lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% Glycerol, 1 mM NaF. 1 mM β-Glycerol phosphate, 01 mM Na_3VO_4 containing the appropriate proteases was added to the plates and left on ice for ten minutes. The cells were then scraped, and lysate was collected and placed into 1.5 mL Eppendorf tubes, then centrifuged at 4°C at a speed of 14,000 RPM for ten minutes. Following centrifugation, the aqueous phase present in the tubes was collected and transferred to new Eppendorf tubes. 2 µL of the sample was then mixed with 1000 µL of protein assay reagent in order to measure the optical density (595) of the samples and determine the concentration of the samples. The samples volumes were then equalized using supplementary lysis buffer, and 5x PSB was added to each sample. The samples were then boiled for 5 minutes, and quickly spun down before being stored at -80°C.

3.4 ECM isolation

To extract the ECM laid down by cells, the cells were first cultured on 60 mm culture plates containing 3 mL total of growth media. After treatment, the media in each plate was removed and the cells were washed 3 times in cold 1x PBS. The cells were then lysed using 1mL of DOC lysis buffer that contained added protease inhibitors. The DOC lysis buffer is prepared using the following: 0.5% DOC, 50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet P-40. Once added to each plate, the cells were left on ice for 10 minutes. Following this, the DOC lysis

buffer was aspirated, and fresh DOC lysis buffer was added again. This was followed by another 10-minute incubation period on ice. Next, the plates were washed, twice with DOC lysis buffer, and three times with 1x PBS. Following this, co-IP lysis buffer (containing 1 mM DTT, 360 mM N-Octyl glucopyranoside and protease inhibitors) was added in 150 µL amounts to each plate. Once added to each plate, the co-IP lysis buffer was pipetted up and down to ensure the entirety of the plate's surface was covered. The plates were then scraped, and the buffer collected in fresh Eppendorf tubes. 48 µL of each sample was collected into new tubes, and 12 µL of 5X PSB was then added to each sample before finally being boiled for ten minutes. The entirety of the sample volume was used for western blotting. To ensure that ECM isolation was not contaminated by the contents of the cellular layer, cell lysate was also loaded into the gels together with the ECM isolates. The cell lysate served as a positive control and was distinguished from the ECM samples by probing for GAPDH, which is expected to be seen in the cell lysate sample, but not in the ECM isolate samples.

3.5 Western Blotting

The protein samples collected from either cell lysate or ECM isolation were subjected to western blotting in order to visualize and quantify protein amounts. First the proteins were separated using SDS-PAGE. The samples were run on a 7.5 percent gel, at 100V for 2 hours. The separated proteins were then transferred from the gel to a membrane, by transferring at 260 mA for 2 hours. The transfer was conducted on ice. Next, the membrane was washed in 1x TBST for 6 minutes, three times, before being blocked in 10 mL of 5% milk in TBST for one hour. Following this, the membrane was washed again in 1x TBST for 6 minutes, three times. The membrane was then incubated with the necessary primary antibody overnight at 4°C. After overnight incubation, the membranes were washed and incubated with 2 µL of the necessary

secondary antibody conjugated to horseradish peroxidase in 10 mL of 5% milk in TBST. The membranes were then washed a final time, before being treated with ECL solutions, and exposed to film, which was then developed.

3.6 Luciferase Assay

Cells were plated on a 12-well plate and transfected at 50 percent confluence with 0.5 µg of TSP1-Luciferase reporter construct (plasmid 12409, Addgene)[23] and 0.1 µg of Beta-galactosidase plasmid using Qiagen Effectene transfection kit. Cells were lysed following treatment with 1X Reporter Lysis Buffer (Promega) and the lysate was collected. Luciferase activity was measured using a luminometer while Beta-Gal activity was measured using a 96-well microplate spectrophotometer measuring 420 nanometer wavelengths.

3.7 Statistical Analysis

Unpaired two-tailed t-test was used in order to compare the means of the two groups of data. One-way ANOVA was also conducted to compare the means of more than two groups. Figures contain error bars reporting the SEM, and $p < 0.05$ was considered significant.

4.0 Results

4.1 TSP-1 protein is increased upon high glucose treatment

To begin our investigation, first we had to establish that TSP-1 protein levels are increased in high glucose (HG) environments. This has previously been demonstrated in our lab and by other researchers[18]. However, repeating these results was required to establish the experimental system. Thus, SD rat mesangial cells were treated with HG (30mM) for 48 hours.

Compared with control, the results demonstrate that high glucose increases TSP-1 protein present in whole cell lysate (Figure 3). These results are consistent with previous data.

4.2 TSP-1 protein amounts in the ECM are elevated upon high glucose treatment

Proteins within the thrombospondin family have been shown to be present in the ECM and have been shown to be bound by proteins typically associated with the ECM (such as collagen) in order to form the extensive interconnected network structure that characterizes the ECM [25]. As a result, we sought to investigate if the increase in protein expression evident in cell lysate also manifests in the ECM laid down by cells in culture. ECM isolation involves the lysis and subsequent removal of the overlaying cell layer so that the ECM layer upon which the cells exist can be accessed. Thus, ECM isolation was conducted as stated above, focusing first on the removal of cellular layers and associated debris. Our results show that HG treated cells feature an ECM that contains a higher amount of TSP-1 when compared to untreated controls (Figure 4).

4.3 High glucose-induced increase in TSP1 protein levels is attenuated by inhibition of csGRP78 with the protein Vaspin in WCL and with the C38 antibody in WCL and ECM

We first sought to test the effects of the protein vaspin, as well as the anti-csGRP78 antibody named C38, on the HG-induced increase in TSP1, in WCL. Vaspin is a member of serine protease inhibitors that has been shown to be capable of binding to csGRP78[26] and inhibiting its function. Meanwhile, C38 is an antibody that targets the C-terminus of csGRP78, binding to it and inhibiting it from carrying out many of its known functions[27]. SD cells were treated for 48H with or without 100 ng vaspin (Figure 5A) or 2ug of C38 or isotype IgG (Figure 5B). Control groups were untreated. IgG antibody was used as a negative control, as it should not interfere with csGRP78. The whole cell lysate from these cells was collected and a western blot

analysis was conducted. The HG-induced increase in TSP1 expression was significantly attenuated by vaspin. (figure 5A). In addition, the HG-induced increase in TSP1 expression was also significantly attenuated by treatment with the C38 antibody. The IgG antibody did not have any significant effect (figure 5B). The results of figure 5B strongly suggests that C38 attenuates the increase in TSP1 seen upon high glucose treatment, at least in WCL. The results of this previous experiment raised the question as to what effect, if any, C38 would have on the amount of TSP1 protein present in the extracellular matrix (ECM) of cells, as TSP1 is secreted when produced by the cells. SD cells were plated on a 6-well plate and treated with high glucose and C38 antibody as per figure 5B, with a treatment time of 48H. Instead of WCL being collected, however, the ECM was isolated and subjected to western blotting. It can clearly be seen that TSP1 is elevated upon high glucose treatment and attenuated by C38. This result supports the results seen in WCL. The same experiment was conducted again, this time with IgG instead of C38 as a negative control. The results were similar to what was seen in WCL; IgG does not affect TSP1 protein amounts in the ECM (figure 5C).

4.4 High glucose-induced TSP increase in protein was attenuated by PI3K inhibitors LY294002 and wortmannin and Akt Inhibitor VIII in WCL

For these experiments, we chose three drugs that were known to exert similar effects to one another, while targeting different protein. LY294002 (LY) is a chemical compound that functions as a potent phosphoinositide 3-kinase (PI3K) inhibitor[28] Wortmannin (wort), like LY is a strong inhibitor of PI3K[29]. Meanwhile, Akt Inhibitor VIII (akt-in) is, as the name implies, an inhibitor of Akt proteins. SD cells were plated on 6-well plates and treated for 48H with HG with or without 20 µg of LY (figure 6A), 100 ng of wort or 10 µg of Akt-in (Figure 6B). Control groups were untreated. The whole lysate was collected from these cells and a western blot was

conducted. The results firstly, again, demonstrate the HG-induced increase in TSP1 protein levels in both 6A and 6B. LY treatment significantly attenuated the increase in TSP1 seen upon HG treatments (Figure 6A). In addition, Wort treatment significantly reduces the amount of TSP1 protein present. Similarly, Akt-in treatment also attenuates the HG response, demonstrating a significant decrease in the amount of TSP1 protein (figure 6B).

4.5 High glucose-induced increase in TSP1 promotor activity was attenuated upon treatment with Vaspin and the anti-GRP78 Antibody C38

The results of 4.4 demonstrate that both vaspin and C38 are capable of attenuating the HG induced increase seen in TSP1 protein amounts, in WCL as well as ECM for C38. Thus, we sought to investigate the effect each of these treatments would have on TSP1 gene transcription. To this end, we conducted a luciferase assay using a luciferase construct in which the TSP1 promotor drives the expression of a luciferase reporter gene. SD cells were plated on two 12 well plates with 3 wells for each of the four conditions across two experiments. Cells were either untreated (control) or HG treated with or without vaspin orC38. The dosages of the treatments were as in 4.4. The results demonstrated first that TSP1 promoter expression is increased upon HG treatment. This HG induced increase is significantly attenuated by vaspin (Figure 7A). In addition, the HG induction is also attenuated by C38 (Figure 7B).

4.6 High glucose-induced increase in TSP1 promoter activity was attenuated by inhibition of PI3K/Akt

As above, this experiment was conducted in order to investigate TSP1 gene transcription activity using a luciferase construct. The results in 4.5 show that LY, wort and Akt-in attenuate high glucose-induced increases in TSP1 at least on the level of the WCL. The effect of these

compounds was tested again, except this time on the level of promoter activity. SD cells were plated on a 12-well plate, with three wells for each condition. The cells were treated for 24H with HG with or without LY, wort or Akt-in Akt with dosages as per 4.5. The results demonstrate that LY significantly attenuated the HG-induced increase in TSP1 promoter expression. LY by itself also reduced the expression of the TSP1 promoter significantly (Figure 8A). The results also illustrate that the increase in TSP1 gene expression is very significantly attenuated by wort and Akt-in treatment as well. Wort treatment caused a reduction below control levels. Meanwhile, Akt-in demonstrated an even more potent effect, further attenuating the HG induced increase. When cells were treated with both inhibitors alone, wort did not have significant effect on TSP1 promoter activity when compared to control, however Akt-in by itself significantly reduced the amount of TSP1 promoter activity when compared to control (Figure 8B). Thus, both Akt-in and LY both exerted their powerful inhibitory effects with or without the presence of high glucose.

5.0 Discussion

Thrombospondin-1 is a protein responsible for the activation of TGF-β1, a key profibrotic cytokine[14]. Both TSP1 and TGF-β1 have been shown to be elevated in high glucose conditions and in diabetic kidneys in both animal models as well as humans.[13] The elevation of TGF-β1 is thought to contribute to pathological changes that occur in DN, including hypertrophy and proliferation of mesangial cells, and increase in the ECM deposition by these same cells. Strategies that attempt to inhibit TGF-β1 directly have been unsuccessful due to TGF-β1's wide range of effects in a variety of cellular pathways. For example, antibodies that blocked TGF-β1 could not be administered in humans in therapeutic quantities due to the off target toxic effect

observed upon treatment[30]. Thus, a novel treatment target for DN is necessary. TSP-1 appears to be a promising target for DN treatments, however its regulation in DN is not fully understood.

In this study, we attempted to elucidate the mechanism by which TSP-1 is upregulated by csGRP78 in DN in order to then activate TGF-β1. GRP78 is a protein that has previously been shown to exist on the surface of cells during certain contexts, including certain cancers and DN[19–21]. Under normal physiological conditions, it is responsible for proper protein folding in the ER of cells, but on the cell surface, it adopts novel functions and can participate in different signaling pathways. For example, at the cell surface, csGRP78 has two exposed domains, the C terminus and the N terminus. The binding of alpha 2 macroglobulin to the N terminus of csGRP78 has been shown to promote the activation of Akt in certain cancers[31]. PI3K is an enzyme which plays a role in mechanisms such as cellular growth, proliferation, and differentiation[28]. It has previously been shown that the PI3K/Akt pathway is capable of upregulating TSP-1 in glomerulonephritis[32]. It has also been shown that GRP78 is similarly capable of upregulating the PI3K/Akt pathway, and is itself also upregulated in DN. Thus, during this study, we investigated the possibility of csGRP78 playing a role in TSP-1 upregulation via the PI3K/Akt pathway in high glucose by using the treatments of LY294002, Wortmannin and Akt-inhibitor VIII.

First, the investigation into TSP-1 levels in high glucose carried out in this project show that TSP-1 levels are increased in high glucose. This is in agreement with data previously collected in our lab, as well as research conducted by others [18]. TSP-1 is intimately involved in the activation of TGF-β1. Its elevation in high glucose is to be expected, as the presence of excess glucose in the cell's environment leads to a number of consequences. For example, oxidative stress in high glucose environments has been shown to increase the expression of TSP-

1 in mesangial cells [24]. This is due to a decrease in the protein nitric oxide-protein kinase G, which is responsible for the transcriptional repression of TSP-1 encoding genes [24]. In addition, in podocytes, the glucose molecule has been shown to cause an increase in TSP-1 expression [33]. It has also previously been shown by other researchers that the resulting increase in TSP-1 amounts in high glucose contexts also causes an increase in the amount of active TGF-β1 found within the cells [13].

Vaspin is capable of binding to csGRP78 and upon doing so prevents interactions between csGRP78 and various other proteins[26]. Similarly, C38 operates the same way, binding specifically to the C terminus of csGRP78 and inhibiting it from carrying out its functions[27]. The results conducted in this study appear to support this and suggest that both vaspin and C38 treatment attenuate high glucose induced increases in TSP-1. It has been shown previously that csGRP78 is activated upon activated α2M binding. Once activated, csGRP78 has been shown to increase the activity of Akt kinases.[21]. As csGRP78 activates proteins involved in the PI3K/Akt pathway, its inhibition by either vaspin or C38 may cause a reduction in this activation of the PI3K/Akt pathway. It has previously been shown in cancer cells that autoantibodies, as well as alpha-2-macroglobulin, that recognize csGRP78, bind to the N terminus of csGRP78[34]. These interactions promote the activation of the Akt pathway. Conversely, autoantibodies towards the C terminus of csGRP78 act to inhibit Akt activation upon binding to csGRP78[35]. In mesangial cells however, previous data from our lab demonstrated that both the binding of N20 antibodies to the N terminus of csGRP78, as well as binding of C20 antibodies to the C terminus of csGRP78, attenuated the activation of the Akt pathway in high glucose contexts[21]. Given these previous results, we speculated at the time that both the N and C terminus domains of csGRP78 are necessary for Akt signaling in high glucose contexts[21]. The results from the current study

support this, as this result demonstrated that treating mesangial cells with C38 antibody attenuated the high glucose induced increase in TSP-1 protein amounts seen in whole cell lysate. As previously mentioned, upregulation of the PI3K/Akt pathway has been shown to upregulate TSP-1 expression in certain contexts. Given this result and the data previously obtained in our lab, it would appear that csGRP78 may be upregulating TSP-1 expression via the Akt pathway, and that C38 antibody causes attenuation of this upregulation, by preventing csGRP78 from activating the Akt pathway. To further test this mechanism of action, we conducted further experiments in this study involving various inhibitors of the PI3K/Akt pathway.

LY294002, wortmannin and Akt-inhibitor VIII are all responsible for inhibiting the PI3K/Akt pathway. When cells were treated with each of these inhibitors, the high glucose induced increase in TSP-1 was attenuated, both on the level of the protein in whole cell lysate, and on the gene expression level. These results suggest that TSP-1 upregulation in high glucose contexts is at least in part regulated by components of the PI3K/Akt pathway. It is possible that csGRP78 is signaling via this pathway in high glucose contexts, as previously stated. As mentioned, TSP-1 upregulation via the PI3K/Akt pathway has been shown in different contexts before. For example, the PI3K/Akt pathway has been shown to be responsible for the sublytic complement C5B-9 induced upregulation of TSP1 within the context of glomerulonephritis[32]. If csGRP78 is signaling via the PI3K/Akt pathway to upregulate TSP-1, then inhibiting both csGRP78 from signaling or inhibiting the PI3K/Akt pathway itself will result in an attenuation of TSP-1 upregulation.

csGRP78 is not the only cell surface protein that TSP-1 has been shown to interact with. As an extracellular matrix protein, TSP-1 interacts with a wide variety of cell surface receptors, including cluster differentiation and integrin proteins, in order to carry out various biological

pathways. For example, Yamashiro and colleagues demonstrated that in rat vascular smooth muscle cells that have undergone mechanical stretch, TSP-1 causes the nuclear shuttling of Yes-associated protein (YAP) via interactions with the cell surface receptor integrin αvβ1[36]. They reported that upon stretching, cells would produce and secrete increased amounts of TSP-1 which would bind to integrin αvβ1 on the cell surface. This would cause a signal cascade that culminated in YAP being sent to the cell nucleus in order to cause gene expression activity that ultimately allowed for the cells to repair and protect against stretch associated damage [36].

In another study, Julovi and colleagues investigated TSP-1's high affinity for the integral membrane receptor CD47, and how this interaction promotes the fibrosis seen in chronic kidney injury. Using a mouse model of chronic ischemic kidney injury, the authors investigated the possibility of limiting TSP-1-CD47 interactions in order to attenuate fibrosis[37]. They also investigated the interaction in renal tubular epithelial cells (RTEC) and human patient serum. Wild type mice treated with anti-CD47 antibodies, as well as CD47 knock out mice demonstrated reduced expression of both TSP-1 and pro-fibrotic markers. Similarly, RTEC from WT mice demonstrated upregulation of pro-fibrotic markers upon exogenous TSP-1 treatment that was attenuated in RTEC from the knockout mice[37]. Immunohistochemistry of kidney tissue from CKD patients showed TSP-1 localization in the tubule and glomerular matrix, and patient sera was analyzed and showed a positive correlation between TSP-1 amounts and worsening glomerular filtration rate. The same group of authors previously demonstrated that TSP-1 interactions with CD47 also promotes acute kidney injury and its accompanying fibrosis[37]. The two discussed studies, in addition to others, demonstrate that there is a clear precedent for TSP-1 interacting with cell surface proteins like csGRP78, and that these interactions that occur on the cell surface can have wide ranging consequences both inside and outside the cells.

Future studies are needed in order to fully elucidate the nature of TSP-1's interactions with csGRP78 and the corresponding downstream effects. For example, the chaperone protein responsible for the translocation of GRP78 to the cell surface is known as MTJ-1. Previously in our lab, MTJ-1 has been knocked down in cells using siRNA that specifically targets MTJ-1[21]. This method of MTJ-1 knock-down essentially serves as method to inhibit csGRP78, as its translocation to the cell surface will be impeded. It would be interesting to investigate what effect this inhibition technique would have on the expression of TSP-1 in whole cell lysate. As demonstrated above, inhibiting csGRP78 with C38 antibody resulted in decreased amounts of TSP-1 in whole cell lysate. If this siRNA knock-down of MTJ-1 effectively inhibits csGRP78, it can be expected it would have a similar affect to C38 on TSP-1 protein amounts. Another aspect of investigation involves co-immunoprecipitation (co-IP) experiments. Co-IP experiments allow for the detection of direct and indirect protein to protein interactions. Previous data in our lab suggest that when co-IP experiments are performed on csGRP78, one of the proteins that can be detected via immunoblotting is TSP-1. One future experiment could investigate the reciprocal, whether TSP-1 when co-IPd from the ECM of cells, allows for the detection of csGRP78 via immunoblotting. Additionally, future studies should also investigate the effect the various treatments used in this study have on proteins more downstream of csGRP78 in its signaling pathway, such as TGF-β1 and pSMAD3. Various treatments in this study demonstrated an ability to reduce TSP-1 protein and gene expression levels. If TSP-1 levels are being reduced, it is likely that TGF-β1 and, consequently, pSMAD3 levels are also being reduced. This is because TSP-1 is a potent activator of TGF-β1, which induces pro-fibrotic effects via pSMAD3 signaling, among other pathways[14]. Future investigation is required, however.

6.0 Conclusion

In conclusion, this study demonstrated that cell surface GRP78 is required for the upregulation of thrombospondin-1 in high glucose conditions, and the inhibition of either csGRP78 itself, or the PI3K/Akt pathway it signals through results in a decrease in TSP-1 amounts both at the protein and gene expression level (Figure 9). This decrease in TSP-1 may potentially result in a reduction in pro-fibrotic factors that contribute to the phenotype seen in diabetic nephropathy. Future studies should focus on the effects clarifying that nature of TSP-1's interactions with csGRP78 and also on the effects the inhibitors used in this study have on pro-fibrotic proteins downstream of TSP-1 in the signaling pathway.

7.0 Figures

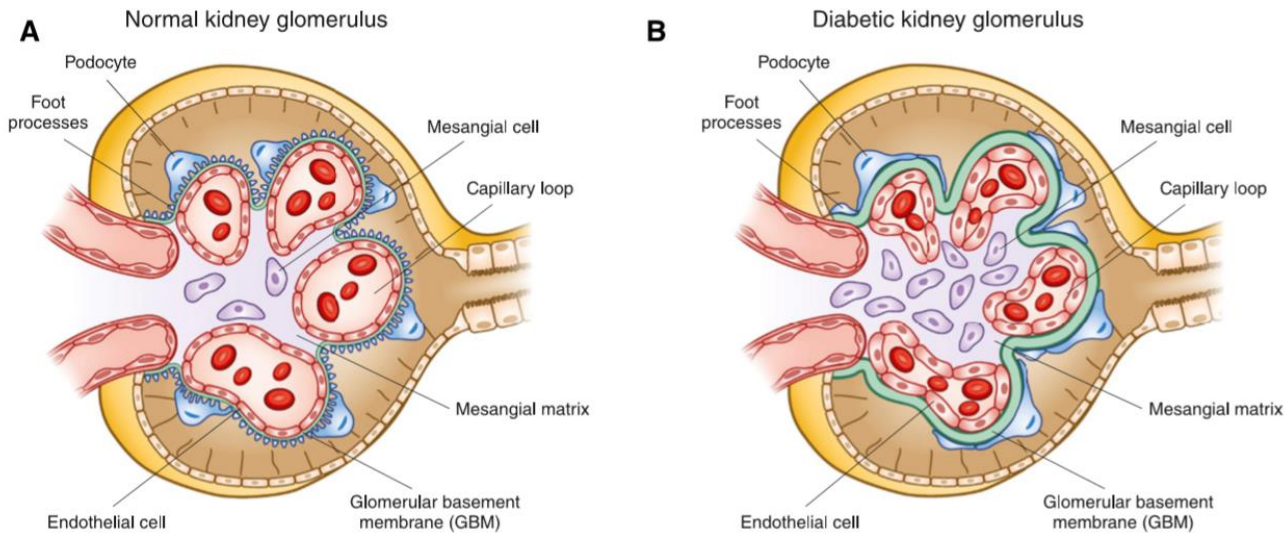

Figure 1: Schematic diagram of the glomerulus of the kidney, displaying the structural alterations that occur as a result of diabetes [2]. A: A normal kidney glomeruli. B: A diabetic kidney. Notice the thickening of the basement membrane, an increase in the number of mesangial cells, hypertrophy of the endothelial cells.

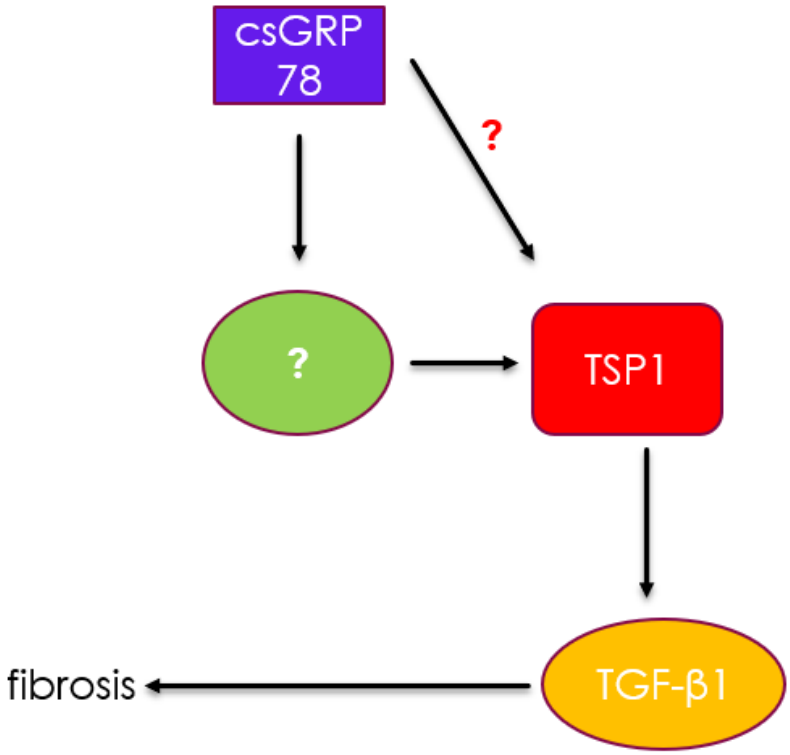

Figure 2: Schematic diagram of the hypothesized pathway: High glucose causes an increase in the amount of GRP78 present at the cell surface (csGRP78), which then, via unknown mechanisms, causes an increase in the production and secretion of TSP-1, leading to increased TGF-β1 activation and subsequent fibrosis. My thesis investigates whether csGRP78 regulates the increased expression of TSP-1, and which pathway might be involved.

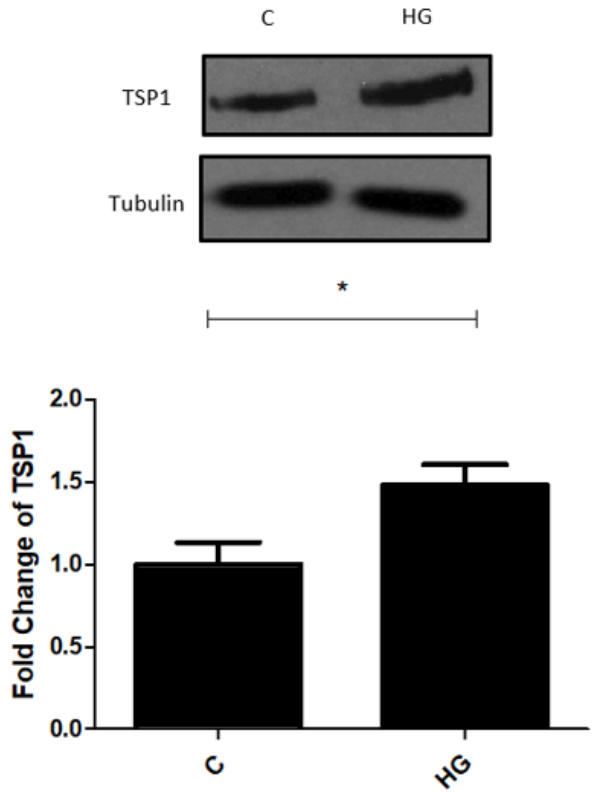

Figure 3: TSP-1 Protein amounts in control and high glucose treated (48h) cell lysate. The high glucose treated cell lysate display a significant 1.5-fold increase in TSP-1 levels ($p = 0.024$, $n = 6$)

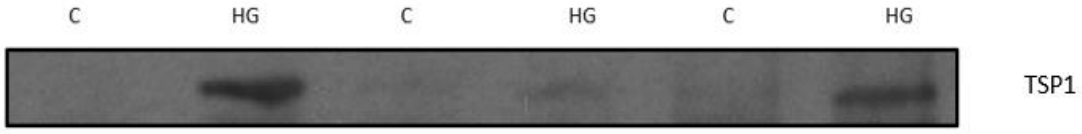

Figure 4: TSP-1 protein levels in ECM in control and high glucose treated (48h) cell cultures. The amount of TSP1 protein in the ECM increases in the high glucose treated cell cultures when compared to controls.

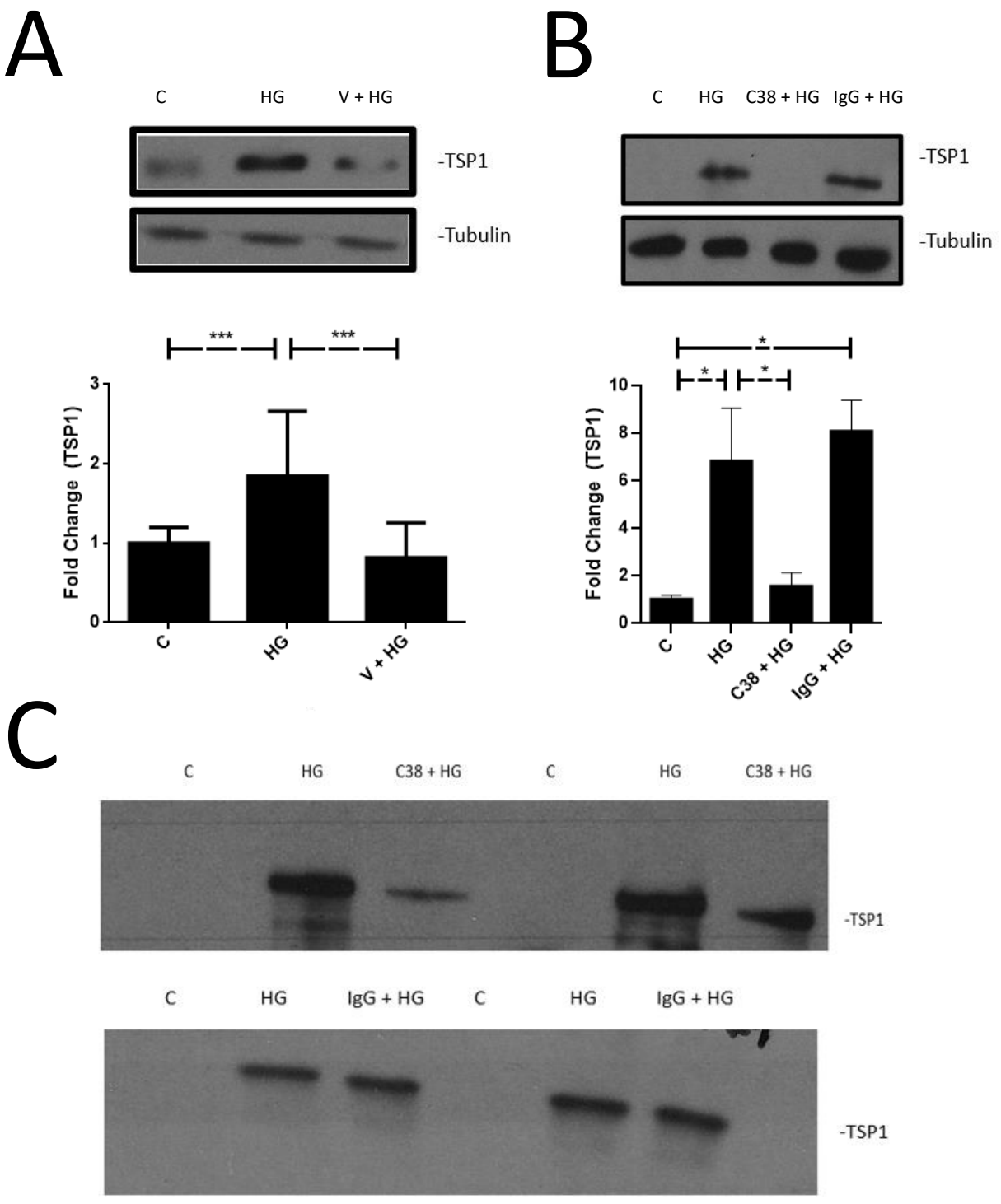

Figure 5: **A:** SD cells were treated with 30 mM of HG alone or HG with 100 ng of vaspin for 48H. The amount of TSP-1 protein was increased upon high glucose treatment when compared to control, while vaspin attenuated this high glucose induced increase in TSP-1 protein levels. *** = $P < 0.01$, n=10. **B:** SD cells were treated with 30 of HG alone or with 2 µg of C38 anti-GRP78 antibody or IgG antibody for 48H. High glucose treatment resulted in an increase in the amount of TSP-1 protein compared to control, while C38 treatment inhibited the TSP-1 high glucose response. * = $P < 0.05$, n = 8. **C:** SD cells were treated with 30 mM of HG alone or with 2 µg of C38 anti-GRP78 antibody or IgG antibody. High glucose treatment resulted in an increase in TSP1 protein levels within the ECM. C38 antibody treatment attenuated the increase in TSP1 levels seen in the ECM of the treated cells

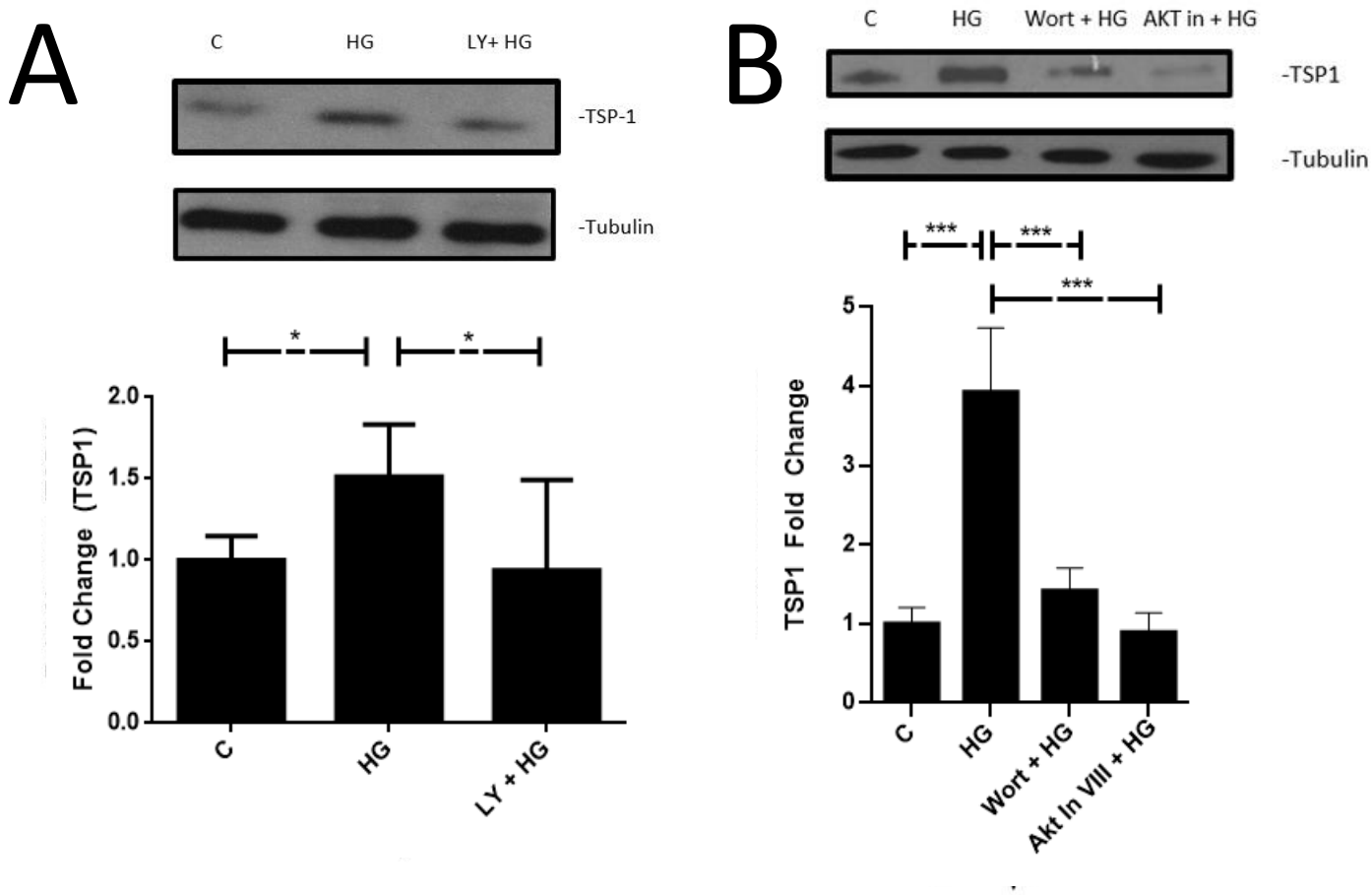

Figure 6: A: SD cells were treated with 30 mM of HG alone or HG 20 µg of LY294002 for 48H. The amount of TSP-1 protein was increased upon high glucose treatment when compared to control, while LY294002 attenuated this high glucose induced increase in TSP-1 protein levels. * = $P < 0.05$, n = 10. **B:** SD cells were treated with 30 mM of HG alone or HG with 100 ng of Wortmannin and 10 µM of Akt inhibitor VIII for 48H. High glucose treatment resulted in an increase in the amount of TSP-1 protein when compared to control. Both wortmannin and Akt inhibitor VIII attenuate the high glucose-induced increase in TSP-1 protein levels. *** = $P < 0.01$, n=8

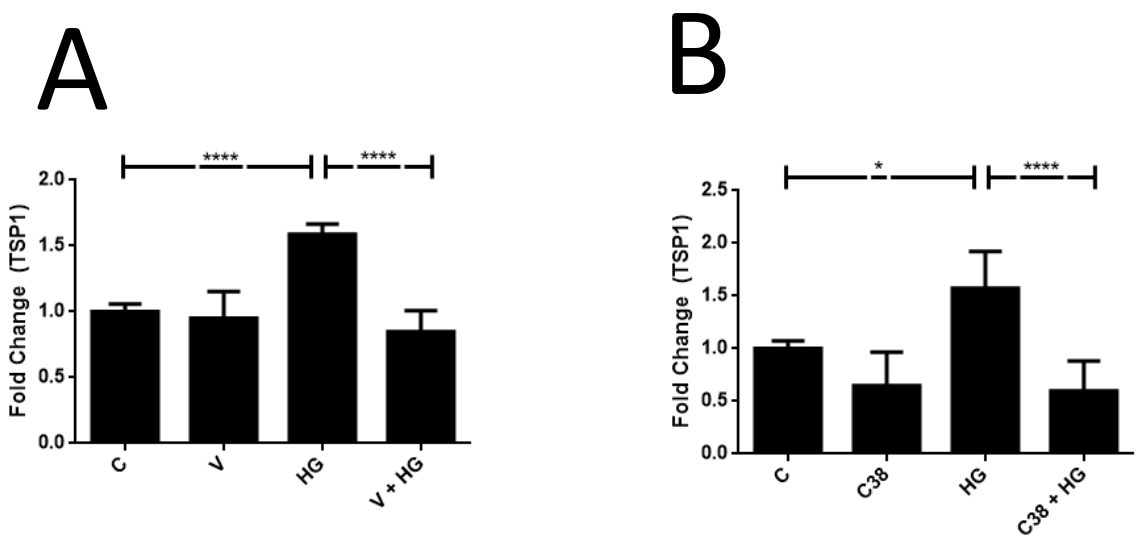

Figure 7: **A:** SD cells were treated with 30 mM of HG alone or HG with 100 ng of vaspin for 24H. Gene expression levels were then measured using a luciferase assay. HG treatment resulted in an increase in TSP-1 gene expression while vaspin attenuated this HG-induced increase. **B:** SD cells were treated with 30 mM of HG alone or with 2 µg of C38 antibody. High glucose treatment resulted in an increase in TSP-1 gene expression that was attenuated with C38 treatment. * = $P < 0.05$, **** = $P < 0.0001$, n = 6.

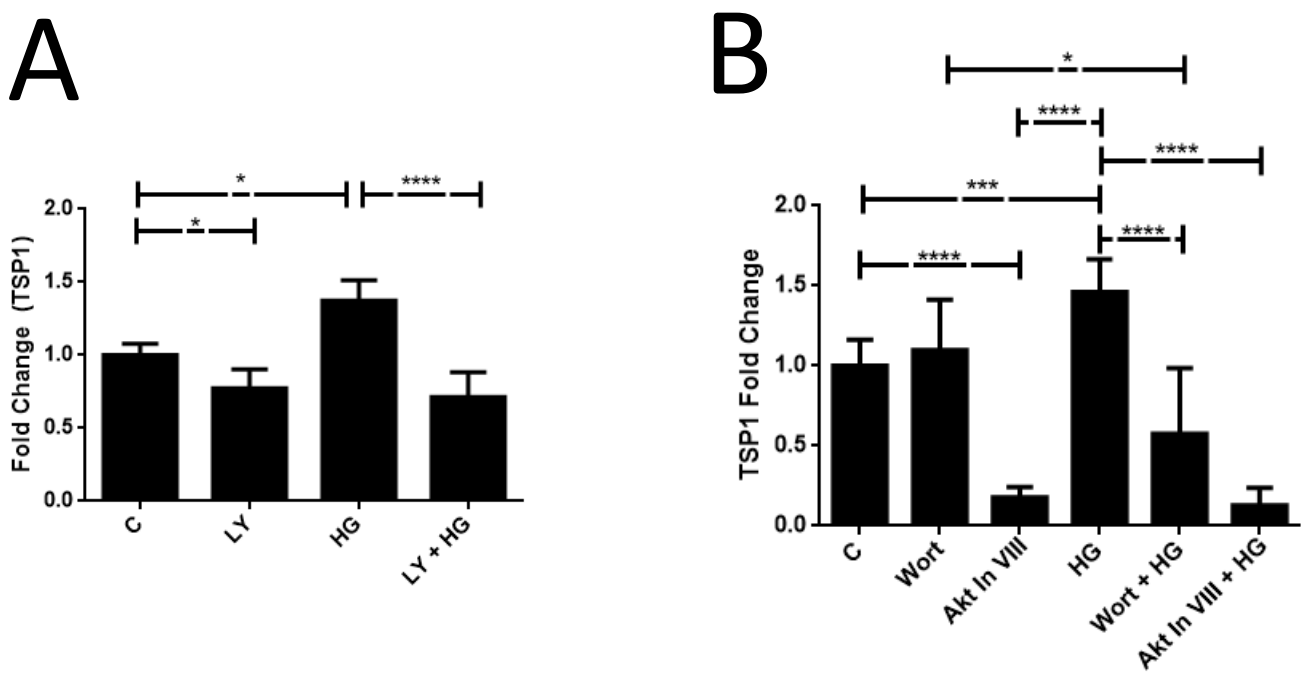

Figure 8: **A:** SD cells were treated with 30 mM of HG alone or with 20 µg of LY294002. A luciferase assay was then performed to quantify gene expression activity. HG treatment induced an increase in TSP-1 gene expression levels, while LY294002 attenuated this HG induced increase. **B:** SD cells were treated with 30 mM of HG alone or HG with 100 ng of Wortmannin and 10 µM of Akt inhibitor VIII for 24H. High glucose treatment resulted in an increase in TSP1 gene expression. This was reduced by both wortmannin and Akt inhibitor VIII treatment. * = P < 0.05, *** = p < 0.01 **** = P < 0.0001, n = 6.

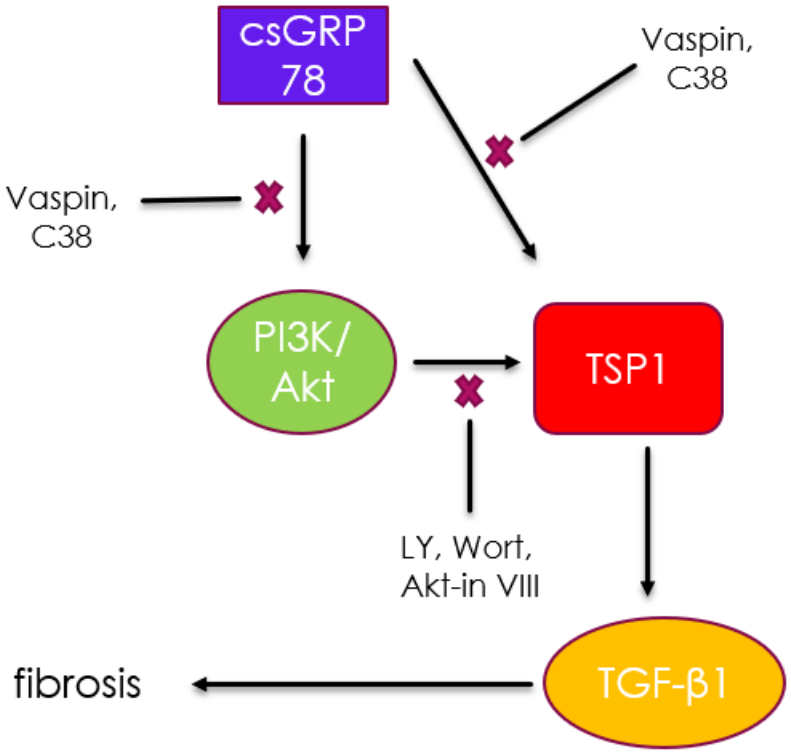

Figure 9: Schematic diagram of the proposed pathway through which csGRP78 regulates TSP-1 expression, including the proposed mechanism of action of the treatments used in this study. High glucose increases GRP78 at the cell surface (shown previously), leading to an upregulation of TSP-1 by csGRP78 either directly, or indirectly via the PI3K/Akt pathway. The two inhibitors of csGRP78, vaspin and C38, act to either inhibit direct interactions between csGRP78 and TSP-1, and/or prevent it from activating downstream signaling of the PI3K/Akt pathway. The PI3K inhibitors, LY294002 and Wortmannin, as well as the Akt inhibitor, Akt inhibitor VIII, all inhibit the PI3K/Akt pathway that is signaled through by csGRP78, thereby preventing the PI3K/Akt- induced activation of TSP-1. Both csGRP78 inhibitors as well as PI3K/Akt inhibitors all ultimately attenuate the increased expression of TSP-1 seen in response to high glucose.

8.0 References

1. Kriz W, Kaissling B. Structural Organization of the Mammalian Kidney. In: *Seldin and Geibisch's The Kidney*. ; 2013. doi:10.1016/B978-0-12-381462-3.00020-3

2. Alicic RZ, Rooney MT, Tuttle KR. Diabetic kidney disease: Challenges, progress, and possibilities. *Clin J Am Soc Nephrol*. Published online 2017. doi:10.2215/CJN.11491116

3. Hinz B. The extracellular matrix and transforming growth factor-β1: Tale of a strained relationship. *Matrix Biol*. Published online 2015. doi:10.1016/j.matbio.2015.05.006

4. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. *Lancet*. 2014;383(9911):69-82. doi:10.1016/S0140-6736(13)60591-7

5. Chatterjee S, Khunti K, Davies MJ. Type 2 diabetes. *Lancet (London, England)*. 2017;389(10085):2239-2251. doi:10.1016/S0140-6736(17)30058-2

6. Nathan DM, DCCT/EDIC Research Group for the DR. The diabetes control and complications trial/epidemiology of diabetes interventions and complications study at 30 years: overview. *Diabetes Care*. 2014;37(1):9-16. doi:10.2337/dc13-2112

7. Reidy K, Kang HM, Hostetter T, Susztak K. Molecular mechanisms of diabetic kidney disease. *J Clin Invest*. 2014;124(6):2333-2340. doi:10.1172/JCI72271

8. Bennett K, Aditya BS. An overview of diabetic nephropathy: Epidemiology , pathophysiology and treatment. *J Diabetes Nurs*. 2015;19(2):61-67.

9. Schiller M, Javelaud D, Mauviel A. TGF-β-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. *J Dermatol Sci*. 2004;35(2):83-92. doi:10.1016/J.JDERMSCI.2003.12.006

10. Abboud HE. Mesangial cell biology. *Exp Cell Res*. 2012;318(9):979-985. doi:10.1016/J.YEXCR.2012.02.025

11. Paulini J, Higuti E, Bastos RMC, Gomes SA, Rangel ÉB. Mesenchymal Stem Cells as Therapeutic Candidates for Halting the Progression of Diabetic Nephropathy. *Stem Cells Int*. 2016;2016:1-16. doi:10.1155/2016/9521629

12. Fuad N. Ziyadeh. Mediators of DIabetic Renal Disease: The Case for TGF-β as the Major Mediator. *J Am Soc Nephrol*. 2004;15(1). doi:10.1097/01.asn.0000093460.24823.5b

13. Yevdokimova N, Wahab NA, Mason RM. Thrombospondin-1 Is the Key Activator of TGF-beta1 in Human Mesangial Cells Exposed to High Glucose. *J Am Soc Nephrol*. Published online 2001.

14. Murphy-Ullrich JE, Suto MJ. Thrombospondin-1 regulation of latent TGF-β activation: A therapeutic target for fibrotic disease. *Matrix Biol*. Published online 2018. doi:10.1016/j.matbio.2017.12.009

15. Han DC, Hoffman BB, Hong SW, Guo J, Ziyadeh FN. Therapy with antisense TGF-β1 oligodeoxynucleotides reduces kidney weight and matrix mRNAs in diabetic mice. *Am J Physiol Physiol*. 2000;278(4):F628-F634. doi:10.1152/ajprenal.2000.278.4.F628

16. Benigni A, Zoja C, Campana M, et al. Beneficial Effect of TGFβ Antagonism in Treating Diabetic Nephropathy Depends on When Treatment Is Started. *Nephron Exp Nephrol*. 2006;104(4):e158-e168. doi:10.1159/000094967

17. Meng XM, Nikolic-Paterson DJ, Lan HY. TGF-β: The master regulator of fibrosis. *Nat Rev Nephrol*. Published online 2016. doi:10.1038/nrneph.2016.48

18. Daniel C, Schaub K, Amann K, Lawler J, Hugo C. Thrombospondin-1 Is an Endogenous Activator of TGF- □ in Experimental Diabetic Nephropathy In Vivo. 2007;56(December). doi:10.2337/db07-0551.LAP

19. Ni M, Zhang Y, Lee AS. Beyond the endoplasmic reticulum: atypical GRP78 in cell viability, signalling and therapeutic targeting. *Biochem J*. 2011;434(2):181-188. doi:10.1042/BJ20101569

20. Zhang Y, Liu R, Ni M, Gill P, Lee AS. Cell surface relocalization of the endoplasmic reticulum chaperone and unfolded protein response regulator GRP78/BiP. *J Biol Chem*. 2010;285(20):15065-15075. doi:10.1074/jbc.M109.087445

21. Krieken R Van, Mehta N, Wang T, et al. Cell surface expression of 78 kDa glucose regulated protein (GRP78) mediates diabetic nephropathy From the 1 Division of Nephrology , McMaster University and St . Joseph ' s Healthcare , Hamilton , ON Canada ; 2 Department of Medicine , Firestone Instit. *J Biol Chem*. Published online 2019. doi:10.1074/jbc.RA118.006939

22. Gray PC, Vale W. Cripto/GRP78 modulation of the TGF-β pathway in development and oncogenesis. *FEBS Lett*. 2012;586(14):1836-1845. doi:10.1016/J.FEBSLET.2012.01.051

23. Shinguso T, Bornsteinmll P. Overlapping Egr-1 and Spl Sites Function in the Regulation of Transcription of the Mouse Thrombospondin 1 Gene *. *J Biol Chem*. 1994;269(51):32551-32557.

24. Wang S, Shiva S, Poczatek MH, Darley-Usmar V, Murphy-Ullrich JE. Nitric oxide and cGMP-dependent protein kinase regulation of glucose-mediated thrombospondin 1-dependent transforming growth factor-β activation in mesangial cells. *J Biol Chem*.

Published online 2002. doi:10.1074/jbc.M108360200

25. Posey KL, Hankenson K, Veerisetty AC, Bornstein P, Lawler J, Hecht JT. Skeletal Abnormalities in Mice Lacking Extracellular Matrix Proteins , Thrombospondin-1 , Thrombospondin-3 , Thrombospondin-5 , and Type IX Collagen. *Am J Pathol*. 2008;172(6):1664-1674. doi:10.2353/ajpath.2008.071094

26. Nakatsuka A, Wada J, Iseda I, et al. Vaspin Is an Adipokine Ameliorating ER Stress in Obesity. *Diabetes*. 2012;61(November):2823-2832. doi:10.2337/db12-0232

27. Ridder GG De, Ray R, Pizzo S V. A murine monoclonal antibody directed against the carboxyl-terminal domain of GRP78 suppresses melanoma growth in mice. *Melanoma Res*. 2012;22:225-235. doi:10.1097/CMR.0b013e32835312fd

28. Gharbi SI, Zvelebil MJ, Shuttleworth SJ, et al. Exploring the specificity of the PI3K family inhibitor LY294002. *Biochem J*. 2007;21:15-21. doi:10.1042/BJ20061489

29. Powis G, Berggren MM, Gallegos A, et al. Wortmannin , a Potent and Selective Inhibitor of Phosphatidylinositol-3-kinase ’. *Cancer Res*. 1994;54:2419-2424.

30. Voelker J, Berg PH, Sheetz M, et al. Anti-TGF-b1 antibody therapy in patients with diabetic nephropathy. *J Am Soc Nephrol*. 2017;28(3):953-962. doi:10.1681/ASN.2015111230

31. Abdalla M, Sabbineni H, Prakash R, Ergul A. The Akt inhibitor , triciribine , ameliorates chronic hypoxia-induced vascular pruning and TGF β -induced pulmonary fibrosis. *Br J Pharmacol*. 2015;172:4173-4188. doi:10.1111/bph.13203

32. Gao L, Qiu W, Wang Y, Xu W, Xu J, Tong J. Sublytic complement C 5 b- 9 complexes

induce thrombospondin- 1 production in rat glomerular mesangial cells via PI 3 -k / Akt : association with activation of latent transforming growth factor- b 1. *Clin Exp Immunol*. 2006;144:326-334. doi:10.1111/j.1365-2249.2006.03069.x

33. Han SH, Yang S, Jung DS, et al. Gene expression patterns in glucose-stimulated podocytes. *Biochem Biophys Res Commun*. Published online 2008. doi:10.1016/j.bbrc.2008.03.121

34. Misra UK, Deedwania R, Pizzo SV. Activation and cross-talk between Akt, NF-κB, and unfolded protein response signaling in 1-LN prostate cancer cells consequent to ligation of cell surface-associated GRP78. *J Biol Chem*. 2006;281(19):13694-13707. doi:10.1074/jbc.M511694200

35. Misra UK, Pizzo S V. Ligation of cell surface GRP78 with antibody directed against the COOH-terminal domain of GRP78 suppresses Ras/MAPK and PI 3-kinase/AKT signaling while promoting caspase activation in human prostate cancer cells. *Cancer Biol Ther*. 2010;9(2). doi:10.4161/cbt.9.2.10422

36. Yamashiro Y, Thang BQ, Ramirez K, et al. Matrix mechanotransduction mediated by thrombospondin-1/integrin/YAP in the vascular remodeling. *Proc Natl Acad Sci U S A*. 2020;117(18):9896-9905. doi:10.1073/pnas.1919702117

37. Julovi SM, Sanganeria B, Minhas N, Ghimire K, Nankivell B, Rogers NM. Blocking thrombospondin-1 signaling via CD47 mitigates renal interstitial fibrosis. *Lab Investig*. Published online 2020. doi:10.1038/s41374-020-0434-3