Growth Hormone Induces TGF-β1 Signaling in Podocytes and Contributes to Nephropathy

A Thesis Submitted for the Award of Doctor of Philosophy

by

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CERTIFICATE

This is to certify that this thesis entitled "**Growth hormone induces TGF-β1 signaling in podocytes and contributes to nephropathy**" submitted by **Mr. Dhanunjay Mukhi**, bearing registration number **15LBPH09** in partial fulfillment of the requirements for the award of Doctor of Philosophy in the Department of Biochemistry, School of Life Sciences, is a bonafide work carried out by him under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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BC 803	Lab Work & Seminar	5	Pass

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DECLARATION

I, Dhanunjay Mukhi, hereby declare that this thesis entitled **"Growth hormone induces TGF-β1 signaling in podocytes and contributes to nephropathy"** submitted by me under the guidance and supervision of **Dr. Anil Kumar Pasupulati**, is original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Date Signature of the Student

Signature of the Supervisor

This is just the beginning of my research scientist career.

After all the years of hard work and focused studies, finally, the day had come true. I am taking charge as a full-time research scientist. I am the winner and I will be a winner ever. The environment and the association that I had throughout my journey have made this day come true. Now it is time to express my deep gratitude to everyone who made this thesis possible.

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Abbreviations

ADH: Anti diuretic hormone

GFB: Glomerular filtration barrier

GBM: Glomerular basement membrane

Igs: Immunoglobulins

FPs: Foot processes

SD: Slit-diaphragm

GFR: Glomerular filtration rate

UCAR: Urine creatinine albumin ratio

VEGF: Vascular endothelial growth factor

FPE: Foot process effacement

CKD: Chronic kidney disease

ESRD: End-stage renal disease

CD2AP: CD2 associated protein

JAM-A: Junction adhesion molecule-A

ECM: Extracellular matrix

DM: Diabetes mellitus

DN: Diabetic nephropathy

IL: Interleukin

GH: Growth hormone

GHR: Growth hormone receptor

TGF-β: Transforming growth factor-β

TGF-βR: Transforming growth factor-β receptor

EMT: Epithelial to mesenchymal transition

MET: Mesenchymal to epithelial transition

ZEB2: Zinc finger E-box binding homeobox2

MMPs: Matrix metalloproteases

TIMPs: Tissue inhibitory metalloproteases

LAP: Latency associated peptide

LTBP: Latent TGF-β binding protein

SLC: Small latent complex

LLC: Large latent complex

SMAD: Mothers against decapentaplegic

R-SMADs: Reactive SMADs

I-SMADs: Inhibitory SMADs

BMP: Bone morphogenic protein

TNF: Tumor necrosis factor

TRAF6: TNF-associated factor

MCP-1: Monocyte chemo attractant protein1

UUO: Unilateral ureteral obstruction

HEK: Human embryonic kidney

CM: Conditioned medium

GFP: Green fluorescent protein

TEM: Transmission electron microscope

FPKM: Fragments per kilo base transcripts of million mapped reads

KEGG: Kyoto Encyclopedia of Gene and Genomes

SEAP: Secreted embryonic alkaline phosphatase

H&E: Hematoxylin and Eosin

PAS: Periodic Acid Schiff's

ELISA: Enzyme linked immunosorbent assay

CXCL: CXC-ligand

Introduction

Introduction

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Introduction

1.0.1. Kidney structure and function:

Kidneys are vital organs and play an instrumental role in maintaining body homeostasis. Kidneys are localized to the retroperitoneum on both sides of the vertebra. Each kidney is surrounded by a renal capsule, adipose capsule, and fascia. A large number of nephrons constitute each kidney. A cross-sectional view of the kidney defines two regions: Cortex and Medulla. The renal cortex primarily comprises the glomerular part whereas renal medulla comprises the tubular part of the nephron. The glomerulus is indispensable for the filtration of blood plasma and allowing the passage of small molecules such as glucose and electrolytes to be a part of glomerular filtrate. The tubular part of nephron ensures the absorption of water, selective electrolytes, and glucose from the glomerular filtrate. Thus, glomerulus and tubule together dictate the final composition of the ultra-filtrated urine. The renal tubule is divided into proximal and distal convoluted tubules, which are connected by the Henle's loop and finally merged into a collecting duct. Several renal tubules and collecting ducts together form pyramid shapes in the kidney called Bartolini pyramids. The base of each pyramid merges into the renal hilum through which urine is collected into the urinary bladder.

The renal system ensures filtration of plasma, regulates pH of body fluids, concentration of electrolytes, and water balance thus maintain homeostasis. Other hand, kidneys also secrete hormones important for regulating blood pressure and erythropoiesis. Renin, angiotensin, antidiuretic hormone (ADH), calcitriol, and erythropoietin are major biologically active components secreted from the kidney [1].

1.0.2. Glomerulus:

Ultrafiltration of glomerular filtrate, selective reabsorption, and formation of urine is an overall effort of approximately a million nephrons. More precisely, the glomerular filtration barrier (GFB) (composed of endothelium, glomerular basement membrane, and podocytes) regulates by and large the composition of urine (Fig. 1). Glomerular endothelium (70-100 nm) is fenestrated and allows the passage of small molecules across capillaries. Basement membrane (250-300 nm) is made up of fibrotic components such as collagen, fibronectin, and heparan sulfate proteoglycans. Podocytes are the only epithelial cells of the glomerulus and are highly

differentiated. Podocytes adhere to basement membrane firmly, and offers coverage to the outer lining of glomerular capillaries [2, 3].

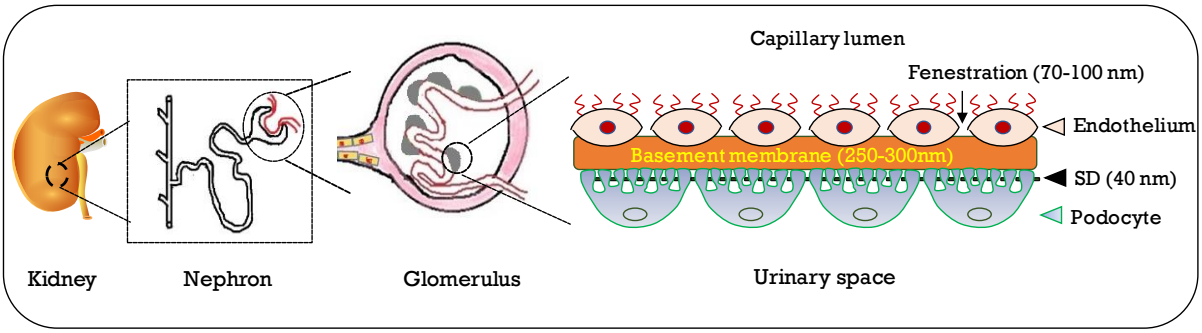

Figure 1. *Architecture of glomerular filtration barrier:* Endothelial cells have fenestrations with 70-100 nm range. Basement membrane is an extracellular matrix material, which is 250-300 nm range. Glomerular podocytes with their foot processes attached to the basement membrane and in between foot processes slit diaphragm (SD) was synthesized by podocytes. These podocytes and SD face towards urinary space. Source: Mukhi.D *et.al.* 2017.

The three-layered glomerular filtration barrier (GFB) serves as a molecular sieve, and under normal conditions restrict the passage of negatively charged macromolecules including proteins into the urine. GFB allows the filtration of water, electrolytes, metabolites, amino acids, and small solutes with less as 20 kDa [4]. It is noteworthy that a large amount of proteins are reabsorbed by renal tubule, and under normal conditions, only trace amounts of protein get excreted. Owing to aberrations in the glomerular filtration and reabsorption, several proteins including serum albumin excreted into the urine. Therefore, albuminuria is a direct measure of impaired renal function and it can be determined by measuring albumin in mean 24h urine collection [5].

The pathophysiologic mechanisms of proteinuria were classified as glomerular, tubular, and overflow proteinuria. Impaired glomerular filtration or tubular reabsorption results in the glomerular or tubular proteinuria, respectively. Overflow proteinuria occurs in the case of pathological conditions such as multiple myeloma (presented with overproduction of Igs) when small molecular weight proteins overwhelm the ability of the proximal tubules to reabsorb proteins. The average protein loss in the urine in mean 24h urine collection in the case of tubular proteinuria and overflow proteinuria, is usually, less than 2g. But the glomerular proteinuria is a severe cause of pathologic proteinuria that accounts for a huge loss of higher molecular weight

proteins (>4g per 24h). Glomerular proteinuria arises because of increased permeability of GFB to plasma proteins and in the case of diabetic nephropathy (DN) proteinuria often indicates a high likelihood of renal loss. Being GFB regulates the composition of glomerular filtrate, the appearance of high molecular weight proteins in the urine may suggest an injury to the filtration barrier. It should be noted that podocytes are principal components of the GFB to offer permselectivity and podocyte injury often associate with proteinuria.

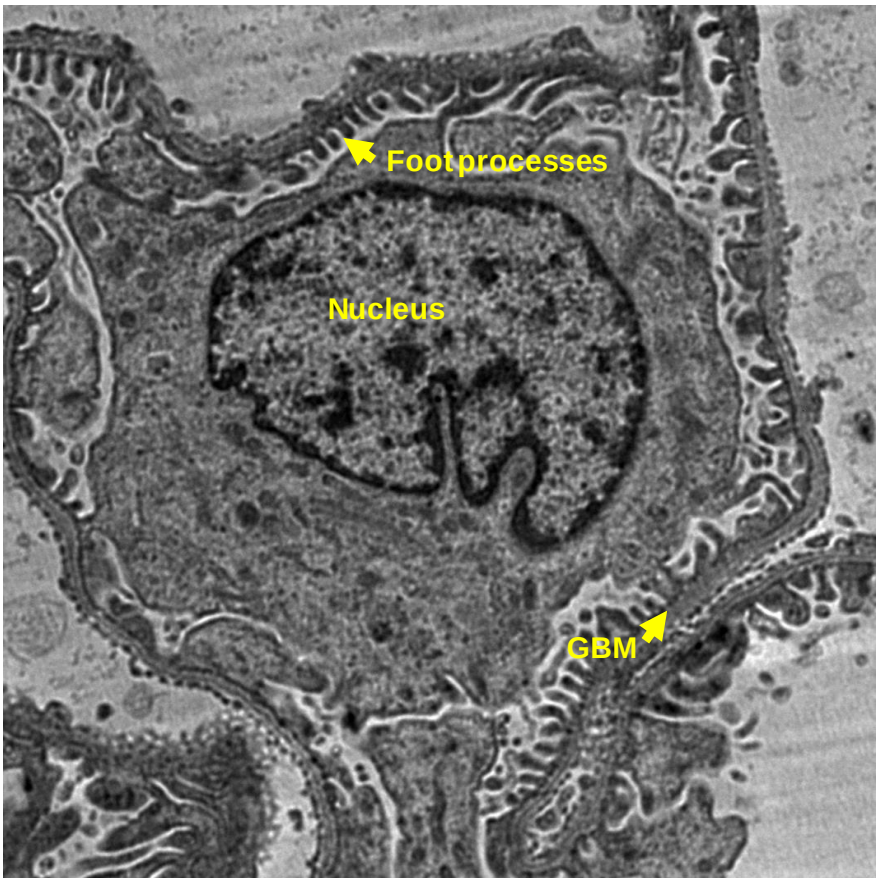

Figure 2. *Transmission electron microscopic image of podocyte:* Podocyte contains a huge nucleus in the center and the key features of podocyte are highlighted with arrowheads.

1.0.3. Podocytes:

Terminally differentiated glomerular visceral epithelial cells are popularly known as podocytes. Podocytes are structurally and functionally distinct from other type of cells in the glomerulus owing to their large size and unique shape. Podocyte consists of a cell body with foot-processes (FPs) (Fig. 2). The primary FPs further divides into secondary FPs, which cover the capillaries and ensure glomerular filtration by offering hydrostatic pressure [6]. The regularly

spaced neighboring secondary FPs interdigitate to form a zip-like structure known as the slit-diaphragm (SD). Several proteins that constitute the SD have been reported to play a significant role in the maintenance of proper structure of the podocytes by interacting with the podocyte cytoskeleton. The podocyte actin cytoskeleton provides support to the glomerular capillary wall and provides hydrostatic pressure required for filtration. Podocytes are relied upon the cytoskeleton to stabilize the glomerular capillaries. The podocyte actin cytoskeleton consists of α-actinin, actin, myosin, talin, vinculin, and paxillin that act as a podocyte backbone and involved in the maintenance of shape that is critical for its function [7]. Podocytes are slightly motile, and it is important for shedding contaminants that were stuck in the filtration slits during the filtration process.

The basic functions of the podocyte are (a) to stabilize the glomerular architecture by opposing the distensions of the glomerular basement membrane (GBM), (b) to maintain a great filtration surface across the SD, (c) to regulate the size and charge, characteristic features of the GFB, and (d) also involved in the regulation of glomerular filtration rate (GFR). Podocytes serve as size and charge-selective barrier, counteract intraglomerular pressure, secretion of vascular endothelial growth factor (VEGF) to maintain the integrity of fenestrated endothelial cells, and maintenance of GBM. They are also involved in the maintenance of the capillary wall and capillary loop tension. Podocytes function as an integrated filtration unit with an effective cross-talk between endothelial-mesangial cells with GBM. Injury to the podocytes leads to the blunting of the lamellipodial extensions and focal adhesions that eventually results in retraction of FPs, called foot processes effacement (FPE) [8]. Previous studies proposed that during injury, podocyte cytoskeleton reorganization is the most common pathway resulting in FPE. Under pathological or disease conditions, the highly ordered contractile actin filaments in FPs become disordered that results in proteinuria [8].

Reduction in podocyte density or altered architecture of SD is considered as a pathological feature in glomerular diseases. A decline in podocyte number results in the progressive loss of renal function. Reduced number of podocytes provokes residual podocytes to compensate for the glomerular filtration. These sequelae of events ultimately result in impaired glomerular filtration and proteinuria. Though, previous reports suggest that the depletion of podocytes is a key early event of chronic kidney disease (CKD), pathological mechanism and molecular pathways involved are yet to be revealed.

Proteinuria often referred to as albuminuria, is indexed by the amount of albumin is presented in the urine collected in 24 hrs. Based on the amount of albumin excreted, albuminuria is classified into several groups. In the case of microalbuminuria, 30 to 300 mg of albumin get excreted for 24h. Macroalbuminuria ranges about ≥300mg in 24h urine collected. Often, macroalbuminuria develops into overt proteinuria that is a clear signature of establishing end-stage renal disease (ESRD) and it is driven by a variety of factors such as hyperglycemia, hypertension, smoking, stroke, and sleep apnea.

1.0.4. Slit-diaphragm (SD):

The SD is ~ 40 nm aperture located between neighboring FPs and it is permeable for water and small molecules. The SD is a modified adherent cell-cell junction. SD serves as size-selective, shape, and charge-dependent barrier thus regulates the process of glomerular filtration and composition of urine. SD offers integrity to the glomerular capillaries and helps maintain the permselectivity of GFB. Podocytes synthesize and secrete the SD proteins that are including nephrin, podocin, CD2 associated protein (CD2AP), P-cadherin, catenin, Nck, and FAT. SD also constitutes tight junction proteins including zonula occluding (ZO-1), cingulin, occludin, junction adhesion molecule-A (JAM-A). However, JAM-A is particularly localized to podocyte FPs whereas occludin is localized to SD. SD proteins including nephrin and Neph1 participate in the signaling pathways that are essential in maintaining the integrity of podocytes. Mutations in podocyte SD proteins such as nephrin, podocin, and CD2AP results in congenital nephrotic syndrome, a heavy proteinuric disease [8].

The integrity of the SD complex is strictly connected to the podocyte internal scaffolding network of the cytoskeleton. The podocyte cytoskeleton plays a crucial role in regulating the morphology and establishing anchorage to the SD complex through a network of proteins. The cytoskeleton offers structural and functional support, which provides the framework for the podocyte and its interaction with the extracellular matrix (ECM). The cytoskeleton remodeling regulates cell shape, cell physiology, adhesion, and motility.

1.0.5. Risk factors for diabetic nephropathy (DN):

Risk factors that contribute to morbidity and mortality in diabetic settings are hyperglycemia, obesity, hypertension, and inflammation. Other etiological factors that contribute to chronic kidney diseases include chronic renal infections, smoke, sleep apnea, stroke, and cardiovascular disease. Several signaling molecules including transforming growth factor-β (TGF-β), interleukins (IL) such as IL-17, and elevated growth hormone (GH) levels deregulated during diabetes mellitus (DM). Our laboratory longed an interest in understanding the effect of elevated GH levels in the pathological mechanisms of DN. Mice that are transgenic for GH developed glomerulosclerosis with increased podocyte cell size whereas, all other glomerular cell types have undergone hyperplasia [9]. Since podocytes account for more than 30% of the total glomerular volume, an increase in podocyte size could be the physiological basis for glomerular hypertrophy [10]. Similarly, mice that express a mutated GH receptor (GHR) preserved renal function by ameliorating glomerular hypertrophy [11].

TGF-β is a prosclerotic molecule and is considered as the master regulator of various components of ECM [12, 13]. Elevated expression of TGF-β or its receptor (TGF-βR), TGF-βR2 levels in the glomerular and tubulointerstitial compartments is evidenced in experimental DM [14, 15]. Mice transgenic for TGF-β displayed apoptosis of podocytes and established glomerulosclerosis [16, 17]. Experimental diabetic animals injected with neutralizing antibodies for TGF-β1 or TGF-βR2 prevents the development of DN [18-20]. TGF-β activates SMAD3 and mice knockout for SMAD3 are protected from diabetic renal injury without affecting albuminuria [12, 21]. Targeting TGF-β or downstream signaling under the settings of diabetes ameliorated renal hypertrophy and improves renal function [21, 22].

DN is involved with the infiltration of non-innate renal cells into the glomerular region. Inflammatory molecules have a significant role in the pathogenesis of DN. A recent study revealed that diabetic kidneys express a high amount of macrophage markers such as F4/80, CD11b, CD45, CD40L, and monocyte chemoattractant protein1 (MCP1) [23]. The expression of podocyte-specific B7 ligand (CD80) is associated with human lupus nephritis. Similarly, induction of B7 ligand in podocytes in mice exposed to LPS induced a nephrotic range of proteinuria whereas knockout of B7 protected from LPS-induced proteinuria [24]. Recently, IL-17A has emerged as a key molecule that is triggered by the innate immune system in both renal

disease and non-renal diseases including multiple sclerosis, rheumatoid arthritis, psoriasis, and diabetes.

1.0.6. Pathogenesis of diabetic nephropathy:

Diabetes is characterized by hyperglycemia due to defects in insulin secretion and/or insulin action. Diabetic nephropathy (DN) is a secondary complication that develops in 20-40% of patients with type I (T1DM) or type II (T2DM) diabetes. Prominent early changes in the kidney during DN include hyperfiltration, hypertrophy, and microalbuminuria (Fig. 3).

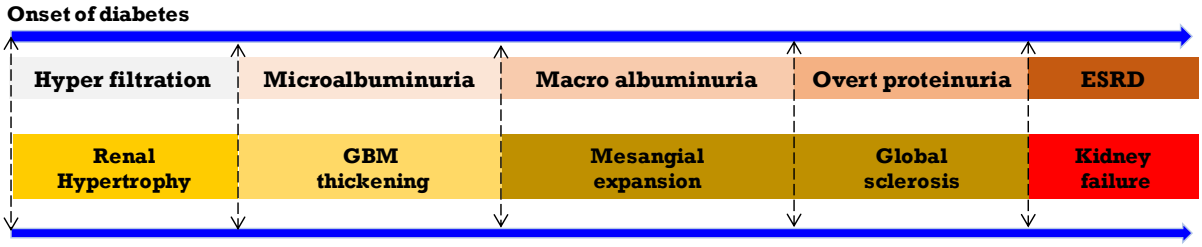

Figure 3. *Predominant clinical and morphological changes in the course of DN:* The onset of DN is evidenced by hyperfiltration and various degreed of proteinuria. The structural changes during the progression of DN such as renal hypertrophy, widening of glomerular basement membrane (GBM), mesangial cell expansion, global sclerosis and these events culminate in kidney failure.

Diabetic kidney disease accounts for more than 50% of new ESRD cases in the United States. Besides podocytes, glomerulus also harbors mesangial cells, which were considered as major culprits in the pathogenesis of diabetic renal disease because the expansion of mesangial matrix lowers the capillary surface area available for filtration. Reduced glomerular capillary surface area manifests in progressive loss of renal function [25-27]. Although the expansion of mesangial matrix is considered as a main characteristic feature of renal injury in diabetes mellitus [28], the incidence of proteinuria is not readily explained by such an expansion of mesangium in the diabetic milieu. Therefore, it is largely considered that injury to GFB; particularly podocytes results in proteinuria in patients with diabetes thus, mesangiocentric dogma is replaced with podocyte-centric dogma.

Since GFB tightly regulates the composition of the urine and there is much debate as to the role of each of the (three) components of the GFB in the pathophysiology of proteinuria in diabetic kidney disease. It was proposed that endothelial dysfunction (microangiopathy) is a causal factor in the pathogenesis of proteinuria [29]. Alternatively, accumulation of collagen and thickening of GBM thus loss of charge selectivity have also been proposed partly to explain the basis for proteinuria [30, 31]. However, a decrease in negatively charged proteoglycans of GBM occurs later in the course of DN, sometimes much later than the appearance of microalbuminuria, suggesting a role for the other components of the GFB in the pathogenesis of proteinuria in DN [32].

1.0.7. Growth hormone (GH) signaling:

Somatotrophs, specialized cells of the anterior pituitary lobe secrete GH. GH promotes post-natal growth, adipogenesis, bone growth, a proliferation of various cell types, and regeneration. The human GH family is comprised of five genes and is located on 17q23.3 chromosome. GH levels are under the control of GH-releasing hormone (GHRH) and somatostatin, where the first one induces GH secretion and the second one inhibits GH secretion. The rate of GH secretion declines with age with a peak at ~150µg/kg during puberty to ~25µg/kg by 55 age and in relation to declining in body mass index with age [33, 34]. GH secretion is induced by GHRH via G-protein coupled receptor-cyclic-AMP (GPCR/c-AMP) pathway and the half-life of GH in serum is ~14 minutes [33]. GH exerts actions at the molecular level via interacting with the GHR. Binding of GH to the GHR elicits activation of Janus kinase 2 (JAK2). Autophosphorylation of JAK2 upon ligand interaction with GHR facilitates the activation of downstream signaling intermediates called signal transducers and activators of transcription (STATs), MAPK, insulin receptor substrate (IRS-1&2), and phosphatidylinositol 3-kinase (PI3K). Upon activation, STATs translocate to the nucleus and transcribe a set of GH-regulated genes. The JAK2/STAT signaling cascade is further regulated by suppressor of cytokine signaling (SOCS) proteins.

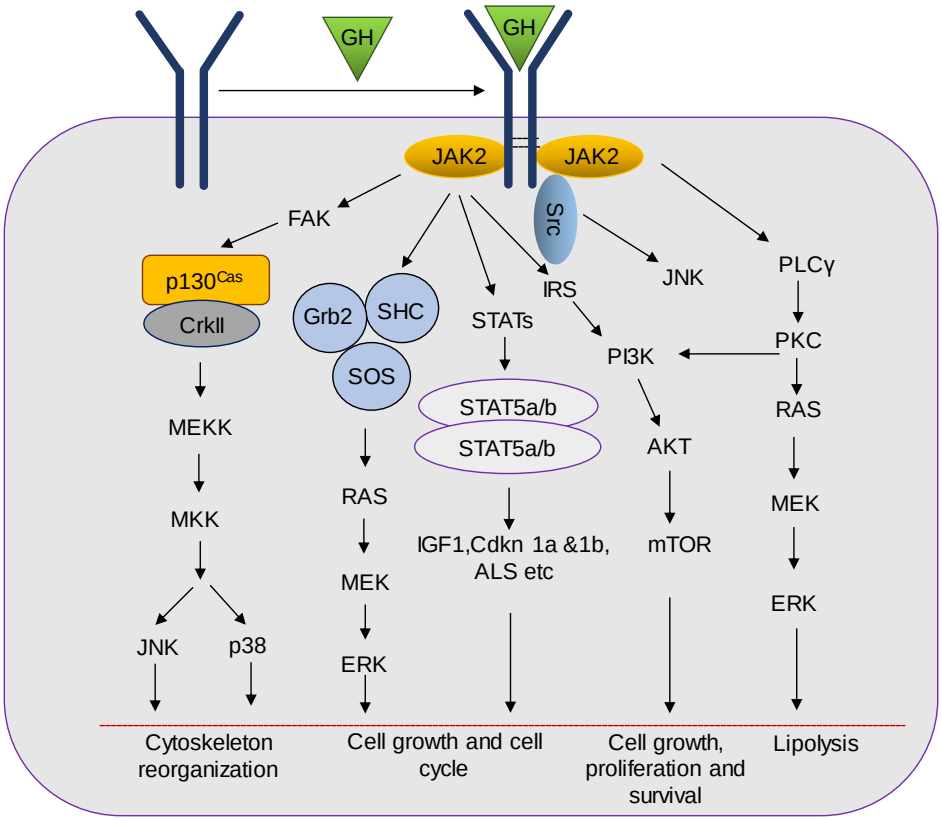

Figure 4. *Cellular mediators of GH signaling:* GH activates both canonical and non-canonical signaling intermediates. JAK2, STAT5 are canonical intermediates. Grb2-SHC-SOS, IRS-PI3K-mTOR and ERK-JNK- p38 are non-canonical intermediates. This image adapted from Nishad.RK *et.al.* 2018.

The association of Grb2-SOS (Growth factor Receptor-Bound 2-Son of Sevenless) with JAK2 is critical for the activation of the non-canonical signaling of GH including MAPK pathway. Typical signaling of GH represented in (Fig. 4). GH exerts its actions on target cells directly and/or indirectly by stimulating IGF-1 production, which serves as a surrogate marker of GH action. An intact JAK2-STAT5a/b signaling pathway is essential for stimulating the production of IGF-1. Serum levels of IGF-1 regulate the pituitary secretion of GH by negative feedback control. Though GH induces hepatic IGF-1 secretion, the concentration of free IGF-1 is largely determined by the rate of IGF-1 clearance and levels of IGF-binding proteins (IGFBPs). The composition of GH/GHR axis, including GHR, IGF-1, IGF-1 receptor (IGFR) and IGFBPs are expressed in the kidney under normal conditions with spatial expression across the segments of the nephron. GH and IGF-1 have significant effects on renal function by regulating hyperplasia, hypertrophy, intra-renal blood flow and tubular reabsorption. GH signaling

regulates the intrarenal hemodynamics and glomerular arteriolar vasodilation by inducing cyclooxygenase activity and generation of nitrous oxide. Each principal component of the GH signaling cascade is indispensable in normal health yet dispensable in disease states.

1.0.8. GHR and components of the JAK-STAT axis:

GHR is a 638 amino acid long polypeptide and is the prototype of the class I cytokine receptor family that includes receptors for the ligands, prolactin, erythropoietin, and thrombopoietin [35]. GHR was the first cytokine receptor that has the crystal structure solved for the extracellular domain (ECD). The crystal structure of GH bound to the GHR ECD showed that one GH molecule bound to two GHR molecules, but the biophysical studies demonstrate that GH binds to one GHR molecule that facilitates GHR dimerization that ultimately leads to activation of GH-signaling [35].

GHR does not possess the protein tyrosine kinase activity and therefore it relies on associating non-receptor protein tyrosine kinases (Janus kinase) for their signal transduction [36]. Janus kinase (JAK) family members are ranging from JAK1, 2, and 3 and TYK2 that can bind to specific receptors. Most of the JAK family members are expressed in many cell types, except JAK3 whose expression is restricted to the hematopoietic lineage. JAK1 and JAK2 are involved in various physiological processes such as development, and growth. JAK3 and TYK2 help in maintaining the homeostasis of the immune system. In the case of GHR, JAK2 is the only member that binds to the receptor [35, 37]. GHR also activates STAT1 and 3 via JAK2; however, these events do not require activated receptor (phospho-receptor).

JAKs are high molecular weight proteins that are ranging from 120 to 140 kDa. The C-terminal of JAK proteins comprise residues for the pseudo-kinase followed by kinase activity. The pseudo kinase activity is known to regulate kinase activity. Loss of pseudo kinase activity increases the basal kinase activity of JAK. Furthermore, a recent study has shown that the pseudo kinase of JAK2 exhibits autocatalytic activity by phosphorylating Ser 523 and Tyr 570, which are important for the kinase activity [38].

STATs are the intracellular intermediates of GH, prolactin, epidermal growth factor, and many other cytokines mediated signaling. GH has been shown to activate STAT1, 2,3,5a, and 5b. However, the predominant transcription factor that is involved in the pulsatile action of GH is STAT5a/b [39]. STAT5 is also called a mammary gland factor (MGF) as it was first identified in

prolactin signaling. JAK2 activation followed by STAT5a/b phosphorylation is an important event for STAT dependent gene expression, but current studies reveal that specific tyrosine phosphorylation in the GHR is important for STAT mediated gene expression [40].

1.0.9. Deregulation of GH/GHR signaling in diabetes mellitus:

GH/GHR axis has shown to be altered in T1DM and diabetic kidney diseases. Circulatory levels of GH are elevated in poorly controlled T1DM. Increased GH levels in DM can be explained by two inter-related mechanisms. In diabetes settings, decreased hepatic GHR expression results in GH resistance and consequent attenuation of hepatic production of IGF-1 (Fig. 5). Resultant low circulating IGF-1 levels stimulate GH secretion by a feedback mechanism [41, 42]. Additionally, hypoinsulinemia in T1DM results in increased hepatic production of IGFBPs.

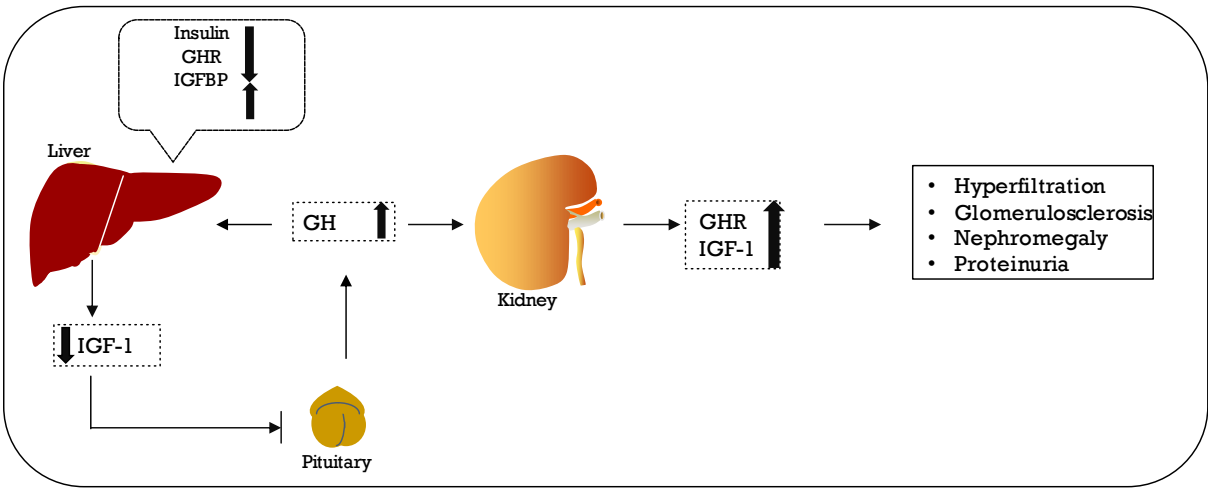

Figure 5. *GH/IGF1 axis in the DM:* No insulin or insulin action in diabetic condition reduces hepatic expression of GHR and increases serum IGF-1 binding proteins. Hepatic production of IGF-1 is the major source that act on pituitary to control the secretion of GH. Low levels of IGF-1 from the liver is a consequence of decreased hepatic expression of GHR. As a consequence, GH levels are elevated in the diabteic patients which is implicated in compormised renal function. Source: Mukhi.D *et.al.* 2017.

The increase in serum IGFBPs, particularly IGFBP-1 limits the IGF-1 action at the cellular level and thus stimulates GH hypersecretion by the pituitary somatotrophs [41]. However, GHR expression in the kidney is either unaltered or increased during diabetes, thus transducing the effect of excess GH levels. Accumulated evidence suggests a strong correlation

between hyperactivity of the GH/GHR axis and adverse renal outcome [9, 43-45].

Administration of GH to healthy individuals for a week manifested in elevated GFR[44, 46]. Interestingly, administration of Octreotide (a somatostatin analog) and somatulin (GH inhibitor) attenuated the aberrant increase in GFR, hyperfiltration and kidney size in diabetic nephropathy patients [47, 48]. Acromegaly cohorts demonstrated the adverse effect of GH on renal biology [49]. Acromegaly patients were in general presented with microalbuminuria. Surgical removal of pituitary-adenoma results in decreased GH levels vis-a-vis modulated both GFR and kidney size [45]. The increase in renal plasma flow rate could be a reason for increased GFR in GH-treated rats [50]. Elevated GH levels in GH-transgenic mice has been linked to diabetic renal complications [51, 52]. It is noteworthy that the expression of dominant-negative GHR improved glomerular function and hypertrophy [53]. Nevertheless, accumulated evidence highlights the adverse renal outcome in GH/GHR hyperactive cases, the actual role of GH remains to be enigmatic until a direct action of GH was demonstrated in podocytes [54].

1.1.0. Identification of GHR in podocytes:

Several studies have reported the direct association between hyperactivity of GH/GHR axis and elevated expression of components of the GH-signaling with hyperfiltration, glomerular hypertrophy, and proteinuria [55-57]. Nevertheless, it was not delineated whether glomerular dysfunction during DN is a cause of direct action of GH on glomerular cells or due to hemodynamic changes (blood pressure and vascular tone) that are affected by elevated GH levels. However, identification of a functional GHR on podocytes remained a breakthrough in the area of GH and podocyte biology. This study demonstrated that mouse and human podocytes exposed to GH activated the canonical JAK/STAT signaling and MAP kinase pathway [54]. It was found that GHR co-localized with both synaptopodin and WT1 (Wilm's tumor1), key markers for mature podocytes. GH induced redistribution of SH2-Bβ, a JAK2 adapter protein in podocytes. Further, GH also resulted in the activation of focal adhesion kinase, and remodeling of podocyte actin cytoskeleton [54]. Besides podocytes, GHR expression was documented in the other cell types including mesangial cells and the proximal tubule cells. The expression of GHR is spatially distributed in a descending gradient from the outer cortex to the inner renal medulla [58]. The GHR expression levels were ~10-20 times higher in the proximal tubular cells compared with other segments of renal tubule [59]. GHR expression is very weak during early

gestation, as there exist immature glomeruli, but its expression is not observed in later developmental stages [60]. Nevertheless, transgenic mice overexpressing human or bovine GH develop progressive glomerulosclerosis, suggesting that aberrant expression of GH elicits glomerular injury [55].

1.2.0. Mechanisms of podocyte injury:

An array of noxious stimuli elicits podocyte injury in the diabetic milieu [61]. Various modes of podocyte injury include hypertrophy, detachment of podocytes from GBM, epithelial to mesenchymal transition (EMT) of podocytes, and apoptosis (Fig. 6).

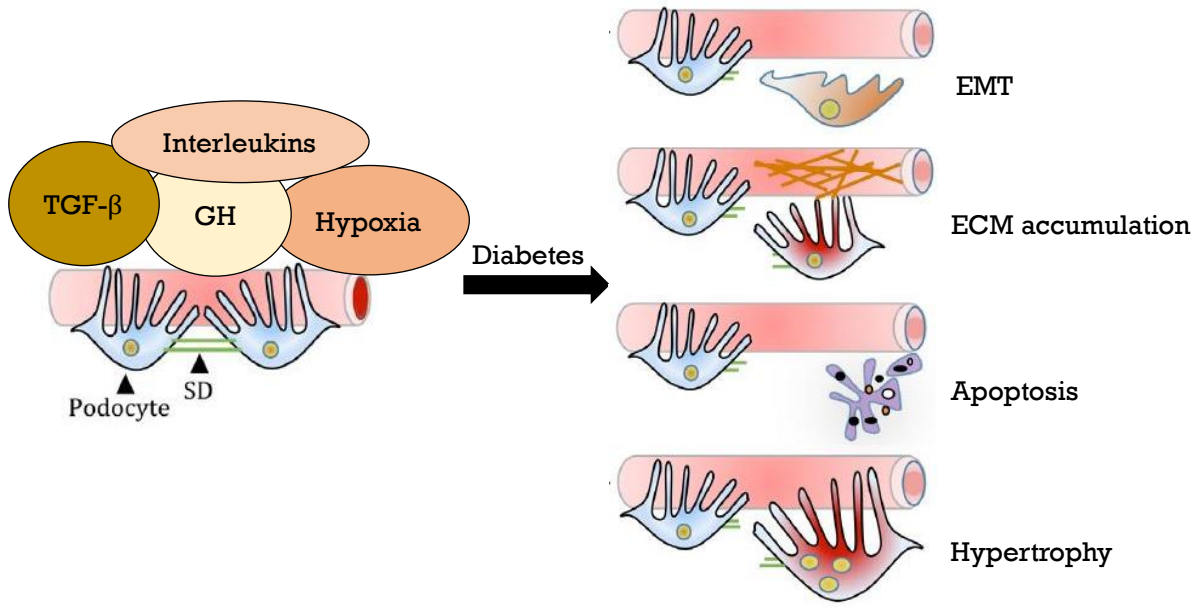

Figure 6. *Mechanisms of podocyte injury:* Various factors stimulate podocyte injury in the diabetic milieu by four different possibilities that could results in various we have to write better word than pathways epithelial to mesenchymal transition (EMT), extracellular matrix (ECM) accumulation, apoptosis and hypertrophy. TGF-β (transforming growth factor-β), GH (growth hormone), interleukins and hypoxia are some of the risk factors have been studied extensively in past years. Source: Mukhi.D *et.al.* 2017.

It was proposed that altered diabetic milieu implicated in the development of glomerular hypertrophy and sclerosis [62, 63]. Diabetic subjects are at increased risk of developing CKD and hyperglycemia-induced renal complications that are associated with high mortality and morbidity.

1.2.1. De-differentiation of podocytes:

Besides diabetic nephropathy, podocyte loss has also reported in IgA nephropathy and HIV-associated nephropathy. Urine sediments from subjects with glomerular diseases consist of viable podocytes, suggesting that podocytes detach from GBM as a consequence of exposure to noxious stimuli [64]. It was reported that patients with glomerular diseases shed viable podocytes in the urine. Interestingly, these podocytes isolated from the urine of the patients with glomerular disease can be cultured under suitable conditions [64]. These viable podocytes isolated from urine from patients with diabetic kidney diseases can be cultured in vitro [65].

Columnar epithelial cells (CEPs) of the metanephric mesenchyme differentiate into podocytes via mesenchymal-epithelial transition (MET) during kidney development [66, 67]. Apical tight junctions and adherent junctions that present in CPEs transform into slit-diaphragm during morphogenesis of podocytes from CEPs [68, 69]. It is reasonable to assume that podocytes in patients with glomerular diseases undergo de-differentiation that could mimic the reversal of MET. Epithelial cells adhere firmly to the GBM and they predominantly express E-cadherin, epithelial cell adhesion molecule (EpCAM), and tight junction proteins such as occludins and junction adhesion molecules [70]. Mesenchymal cells are invasive and express vimentin, snail, slug, and N-cadherin [70-72]. Mesenchymal cells are motile, unlike epithelial cells. Upon de-differentiation, epithelial cells could compromise cell-cell contacts and cytoskeletal networks, owing to which de-differentiated cells detach from GBM and become invasive [72]. De-differentiation of podocytes is further evidenced by altered epithelial polarity; reduce expression of cell-cell junctions, and elevated expression of mesenchymal markers. The podocyte de-differentiation represents EMT, that also occurs during embryonic development and wound healing.

Recent studies from our laboratory reported that in both in vivo (animal models) and in vitro (cultured podocytes) GH induces the expression of ZEB2 (zinc finger E-box binding homeobox2) [73]. ZEB2 transduces de-differentiation of epithelial cells by promoting cadherin switch wherein E-cadherin expression attenuated while and N-cadherin expression upregulated [74].

1.2.2. Apoptosis of podocytes:

One of the hallmark pathological features of DN is reduced podocyte count compared with non-diabetic subjects. Apoptosis of podocytes is claimed as one of the reasons for decreased podocyte population in diabetic kidney disease or glomerular diseased [75]. It should be noted that podocytes are highly differentiated and post-mitotically resting cells. It was reported that podocytes are arrested at G2/M phase of the cell cycle [76]. Apoptosis of the podocytes is documented at an early stage in the course of progressive glomerulosclerosis. It is noteworthy that podocyte loss occurs earlier than mesangial expansion. Podocyte viability is supported by nutrients, cytokines. and growth factors. Insulin and IGF-1 are critical for podocyte survival, whereas high glucose and TGF-β are known to induce podocyte apoptosis [77-79]. In diabetic settings reduced insulin levels predispose podocytes to apoptosis. Earlier reports from our laboratory showed that podocytes exposed to supraphysiological concentrations of GH undergone apoptosis [80]. GH-induced reactive oxygen species are a major factor that could provoke podocyte death by apoptosis. Quenching of free-radicals by N-acetyl cysteine prevented GH-mediated apoptosis of podocytes [54].

1.2.3. Hypertrophy of podocytes:

Various modes of cellular adaptation to physiological or pathological stimuli include dysplasia, atrophy, and hypertrophy. Hypertrophy refers to an increase in cell size and it could be due to an increase in intracellular protein rather than cytosol or by abrupt karyokinesis and polyploidy [79]. If enough cells undergo hypertrophy, it reflects in its organ size. Kidneys have increased susceptibility to hypertrophy, and enlarged kidney is a prominent feature in the course of DN [81, 82]. Hypertrophic cues may be triggered by mechanical signals such as stretch or by trophic signals such as growth factors. Podocyte hypertrophy is another histopathological change in relation to increased kidney size during DN. Podocyte hypertrophy in diabetic kidney is preceded by GBM thickening, glomerular hypertrophy and sclerosis [83]. It was reported in a study that enlargement of the glomerulus is owing to associated podocyte hypertrophy rather than hyperplasia [84]. Podocyte hypertrophy could be a compensatory stimulus for the neighboring podocytes loss either by apoptosis or by de-differentiation so as to meet an increase in functional demand. Altogether, podocyte hypertrophy could be an adaptive stimulus that might result in the detachment of podocytes to escape in the urine [84]. Culturing of viable

podocytes isolated from the urine of patients with diabetic kidney disease argues that shedding of podocytes by either EMT or consequence of hypertrophy [84, 85]. It is interesting that ~10-50% of podocytes recovered from the urinary sediments are multi-nucleated [65, 85]. Impaired stress response or mitotic catastrophe could explain the reason for the presence of multinucleated podocytes in urine from diabetic subjects.

It was speculated that elevated GH levels in diabetic conditions; implicated in renal hypertrophy [9]. However, the cellular mechanism regulating podocyte hypertrophy remains unknown. GH-transgenic mice showed both podocyte hypertrophy and mesangial hypertrophy, whereas these mice were also presented with overt proteinuria [9, 86]. In the case of GHR knockout diabetic mice, a substantial reduction in glomerular hypertrophy was noticed and importantly these mice were protected from complications of nephropathy [53]. Together all these studies argue that glomerular hypertrophy in diabetes is at least partially contributed by GH.

1.2.4. Accumulation of ECM and detachment of podocytes:

Three major components of the extracellular matrix (ECM) include structural proteins, polysaccharides, and interlinking proteins. The major function of ECM is to provide mechanical strength to the cells. ECM also provides signals for differentiation and morphogenesis. The abundant polysaccharides of ECM are glycosaminoglycans, hyaluronan, and heparan sulfates. These matrix polysaccharides aggregate with linker proteins to form large proteoglycan complexes. The negative charge of GBM and in turn to GFB is due to large proteoglycans including agrin and perlecan. The negative charge of these proteoglycans helps in offering electrostatic repulsion to anionic molecules by GFB, thus impeding the flow of them into the glomerular filtrate and to retain them in the plasma [87]. Predominant matrix structural proteins of GBM include collagen (type IV, and type XVII) and nidogen [87]. Matrix interlinking proteins (laminin and fibronectin) interact with both proteoglycans and collagen.

While matrix metalloproteases (MMPs) degrade ECM proteins, tissue inhibitors of metalloproteases (TIPMs) inhibitors of MMPs. TIPMs get upregulated in the early course of diabetic kidney disease [88]. MMPs and TIPMs together regulate homeostasis of ECM. The condition of glomerulosclerosis is presented with both excess synthesis of ECM components and impaired degradation of the same. Accumulation of ECM occurs in the glomerular mesangium,

tubulointerstitium, and GBM in the case of diabetic kidney diseases. It should be noted that glomerular endothelial cells and podocytes are doing synthesis of ECM components, thus these both cell types located on either side of GBM regulate the composition of GBM. Co-culturing of endothelial cells with podocytes revealed that these cells contribute about 50% of ECM proteins and rests of ECM components are provided by podocytes and mesangial cells [89]. Although increased expression of ECM proteins during DN is well documented, the mechanism is poorly understood. GH transgenic mice displayed increased production of ECM proteins and contribute to glomerulosclerosis [51]. A large body of evidence from in vivo studies (transgenic mice and GH injected animals) and in vitro studies (cultured podocytes) revealed that excess synthesis and impaired degradation of ECM occurs with GH treatment either directly or indirectly [51, 80, 90, 91]. Rats administered with GH displayed GBM thickening and mesangial sclerosis [91]. These studies strongly suggest that GH alters ECM turnover. It was proposed that podocytes fail to adhere to the thickened GBM and eventually shed in the urine. It is noteworthy that TGF-β1 plays a decisive role in regulating ECM molecules and also overexpress in diabetic kidney diseases.

1.3.0. TGF-β superfamily:

TGF-β was discovered in the 1980s as it could transform 3T3 cells into non-neoplastic fibroblasts. TGF-β superfamily contains a cluster of proteins ranging from ligands to receptors to intracellular effectors such as SMADs. More than 68 members have been included in the TGF-β superfamily. Among them, many are multifunctional cytokines including TGF-β and bone morphogenic proteins (BMPs) [92]. There are three TGF-β isoforms have been identified in mammals that are TGF-β1, TGF-β2, and TGF-β3. All three isoforms of TGF-β are secreted as latent complex proteins that are associated with latent TGF-β binding proteins (LTBPs). Latent complexes of TGF-β are inactive and are active upon the action of proteases such as plasmins and furin.

1.3.1 Canonical TGF-β/SMAD signaling:

TGF-β is produced by most of the cell types in the body. TGF-β is translated as a pro-peptide that is predominantly inactive form. Proteolytic action on pro-peptide yields a latency-associated protein (LAP) that is noncovalently attached to the mature TGF-β and this whole complex is called Small Latent Complex (SLC). The bioactive form of TGF-β is a 25 kDa dimer and is covalently linked by a disulfide bond [93].

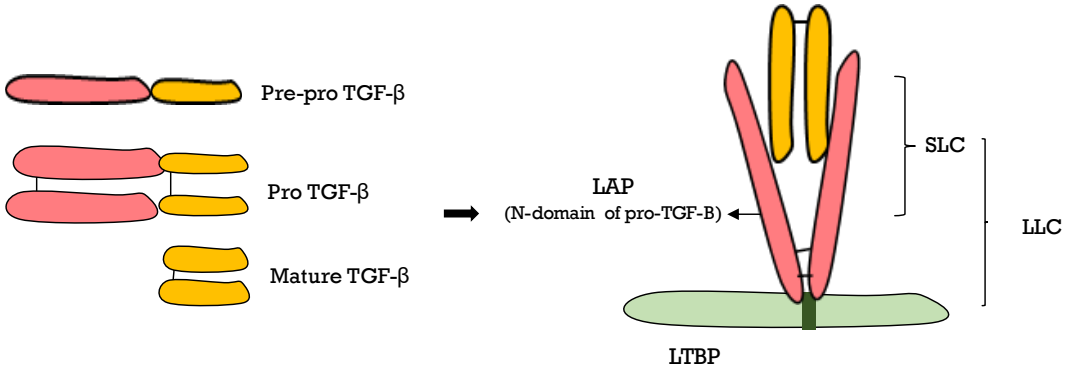

Figure 7. *Scheme of TGF-β synthesis and maturation:* TGF-β synthesized as pro-TGF-β, which consists of latency associated peptide (LAP) at N-terminus. Upon action of proteases, LAP is still associated with mature TGF-β by non-covalent interactions to form small latent complex (SLC). SLC binds with latent binding protein (LTBP) to form large latent complex (LLC), which will be secreted into the extracellular space.

If TGF-β is associated with LAP, it cannot bind to its receptors, and its bioavailability is restricted further by binding of LTBP, together called Large Latent Complex (LLC) (Fig. 7). LTBP binds the ECM and sequesters LAP-TGF-β *in vivo*. Bioactive TGF-β can be bound to various non-receptor cell surface proteins such as decorin, and beta glycan, that are also serve as ligand traps. Thus, multiple mechanisms control the bioactivity of TGF-β *in vivo* [94].

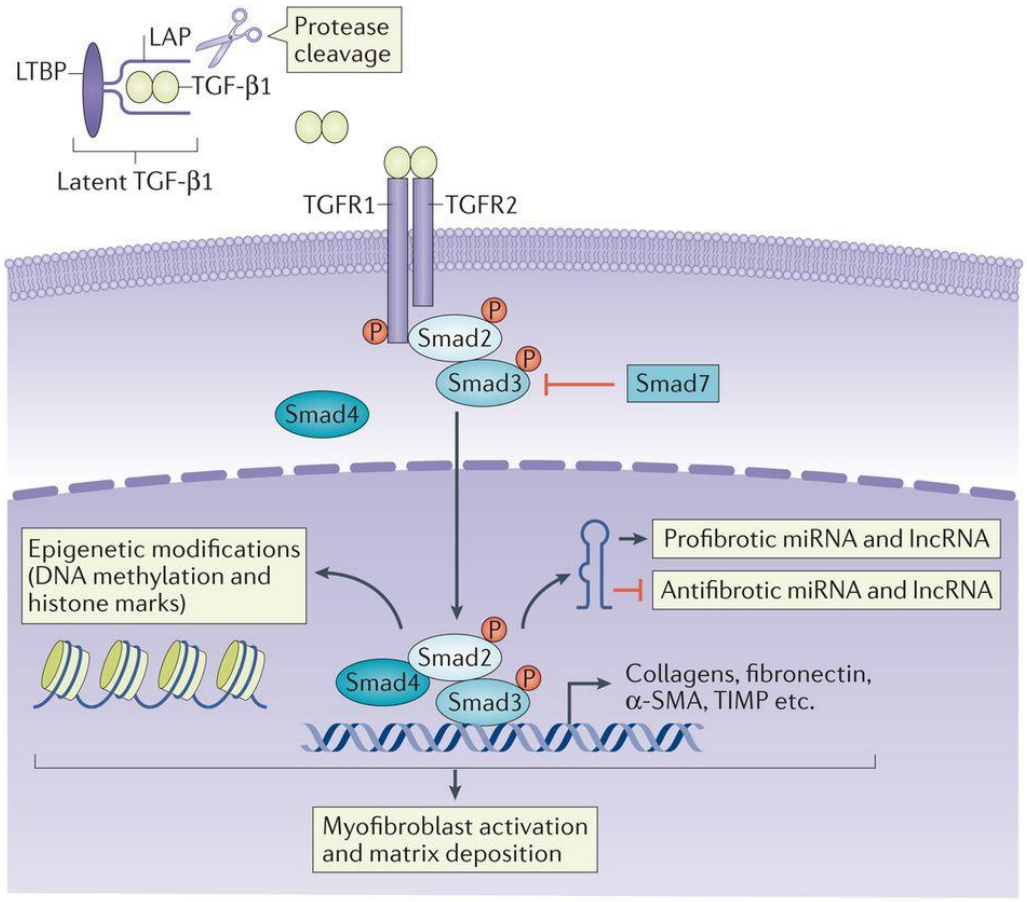

Figure 8. *TGF-β signaling pathway:* Latent complex of TGF-β limits TGF-β availability: Upon action of proteases mature TGF-β is released from latent complex, which binds on the TGF-βR. Once the receptors phosphorylated which phosphorylates SMAD2&3 proteins at c-terminal domain. Together with SMAD4, these activated SMADs translocate into nucleus and induces pro-fibrotic genes expression and suppress anti-fibrotic genes expression. Source: Xiao-ming-Meng *et.al*. 2016.

TGF-β signaling is stimulated by binding of a ligand to the homodimer of TGF-βR2 that stimulates autophosphorylation, which in turn phosphorylates the TGF-βR1 homodimer (Fig. 8). Thus, stimulated TGF-βR1 phosphorylates regulated SMADs (R-SMADs) at the carboxy-terminal serine residues. These R-SMADs in the case of TGF-β are SMAD2 (Serine-465,467) and SMAD3 (Serine-423,425) and in the case of BMP signaling R-SMADs are SMAD1 (Serine-463) and SMAD5 (Serine-465) [94]. Either of the phosphorylated R-SMADs forms a complex with the common SMAD (SMAD4) in both TGF-β and BMP signaling translocate to the nucleus to elicit transcriptional responses. It was recently identified that SMAD4 is also undergoing

phosphorylation in response to TGF-β1 at Thr-276 by ERK which enhances the nuclear accumulation of SMAD4 [95]. There are inhibitory SMADs (I-SMADs), SMAD6 and SMAD7, that are always in competition with the R-SMADs for binding to the activated TGF-β receptors.

1.3.2. TGF-β/SMAD axis in glomerular disease:

A prominent cytokine in the altered diabetic milieu is TGF-β1 and is produced by most of the cell types including epithelial cells. TGF-β1 plays a central role in regulating the expression of ECM molecules and cellular hypertrophy that is evident in both glomerular and tubular injury during DN [96-99]. Most of the cell types including podocytes respond to TGF-β by increasing the expression of ECM proteins including fibronectin, collagens, proteoglycans, laminin, and integrins. Several studies indicate that TGF-β and its receptor TGF-βR2 upregulated in glomerular and tubular regions under the settings of DN. As a result, the TGF-β internal signaling system, the SMAD pathway is triggered in such conditions and promotes pathogenicity. In addition, TGF-β induces a hypertrophic effect on some renal cells as evidenced by cells treated with anti-TGF-β antibodies cultured in high glucose condition prevented cellular hypertrophy [100].

Previous studies indicate that neutralizing antibodies against TGF-β1 prevented glomerular lesions in the experimental DN settings by downregulating the expression of collagen4 and fibronectin [100]. Interestingly, soluble human TGF-βR2 treatment ameliorated the development of DN in the 12-week injection of STZ rats. Mice that are heterozygous knockout for TGF-βR2 are impervious to the progression of STZ induced DN [101]. These results suggest that the strong association of the TGF-β/TGF-βR2 axis in the origin and progression of DN. Furthermore, podocytes exposed to TGF-β1 decreases tight junction and adherent junction proteins (ZO-1 and P-cadherin) and increasing desmin and snail expression, suggesting that TGF-β induces podocyte de-differentiation [102]. Similarly, podocytes treated with TGF-β1 increased permeability to albumin protein, suggesting that the de-differentiation of podocytes could be the mechanism of proteinuria during DN [102]. Bone morphogenic protein 7 (BMP7) is a negative regulator of TGF-β1. In BMP7 transgenic mice shedding of podocytes into the urine was ameliorated and is concurrent with reduced levels of TGF-β1 [103]. This data suggests that TGF-β1 has a central role in podocyte dehiscence from GBM during DN.

In addition, TGF-β1 induces a hypertrophic effect on renal cells as evidenced by cells treated with anti-TGF-β antibodies cultured in high glucose condition prevented cellular hypertrophy particularly, mesangial cells [104]. However, podocytes also undergo hypertrophy when treated with TGF-β1 by increasing cell cycle arresting protein, $p27^{Kip1}$ [104]. Both GH and TGF-β1 share certain similarities in contributing to the progression of DN. However, the spatial or temporal relation between TGF-β1 and GH in the pathogenicity of diabetic kidney disease had never established. TGF-β1 is induced in the renal system under the stimulus of high glucose, angiotensin-II, and hypoxia [105, 106].

Recently, DN is considered as inflammatory renal disease. Studies have demonstrated that interleukins play a significant role in the establishment and progression of DN [23, 107]. The role of IL-6, IL-17, IL-18, and IL-23 have already been established in the pathogenesis of diabetic kidney disease [107]. Although IL-17 induces TGF-β expression in other immune cell types, there are studies demonstrating that TGF-β induces differentiation of Th-17 cells, a major source of IL-17A secretion [108-110]. In the present study, we have investigated whether GH induces TGF-β and IL-17.

1.4.0. IL-17 family:

IL-17 family comprises of six cytokines (IL-17A-F) that share ~50% of homology among them. However, the magnitude of activity is different from one to another. For instance, the magnitude of action of IL-17A is 10 to 30-fold higher than IL-17F. IL-17A, IL-17E, and IL-17F are considered as proinflammatory cytokines and the other three cytokines' role is yet to be understood. There are five receptors that have been identified for six ligands of the family and are IL-17RA (also called IL-17R), IL-17RB, IL-17RC, IL-17RD, and IL-17E. IL-17 family members and their receptors function as homo and/or heterodimers but the specificity depends on the first receptor bound to the ligand and modulates the homodimer or heterodimer complex formation [111, 112].

IL-17A (also called IL-17) is the prototype of the IL-17 family. IL-17A is a pleiotropic cytokine that participates in inflammation and immunity by inducing the expression of chemokines and proinflammatory cytokines. IL-17A is produced by a subset of the T-helper (Th) cell population called, Th-17cells, which also secretes many other cytokines, including IL-6, IL-17F, IL-21, and IL-22. In addition to Th-17 cells, many other immune cells produce IL-17A that

include neutrophils, natural killer cells, macrophages, dendritic cells, and mast cells. IL-17A production is regulated by various cytokines that influence Th-17 cell differentiation and subsequent production is including by IL-1, IL-6, TGF-β and IL-21 [108, 113].

1.4.1. IL-17 signaling cascade:

The bona fide signaling components of the IL-17 pathway have not been fully elucidated. However, IL-17 has been shown to activate many common signaling intermediates that are NF-kB, JNK, P38, ERK1/2, PI3K, and JAK/STAT [111]. Recent studies identified some of the important signaling intermediates including tumor-necrosis factor receptor-associated factor 6 (TRAF6), Act1, TGF-β activated kinase (TAK1) and CCAAT/enhancer-binding proteins (C/EBPs). C/EBP is an important regulator of the IL-17 pathway that is activated by IL-17. The TRAF6 is identified as an adaptor signaling component for IL-17-mediated activation of NF-kB and JNK pathway [114]. Act1 is identified as the first adaptor signaling molecule recruited upon IL-17R activation. Act1 is an E3-ubiquitin ligase and it is essential for TRAF6 mediated TAK1 activation flowed by NF-kB signaling [111, 115]. Negative regulation of IL-17 signaling involves two distinct phosphorylation events that occur on C/EBP-β by ERK1/2. During signaling IL-17 activates ERK1/2, which phosphorylates C/EBP-β at Thr-188 that allows GSK-3β to phosphorylate C/EBP-β at Thr-179 that leads to shut down of IL-17 transcriptional response [116].

1.4.2. IL-17 signaling in glomerular disease:

IL-17 signaling is associated with inflammatory diseases such as psoriasis, rheumatoid arthritis, multiple sclerosis inflammatory bowel disease, lupus nephritis, renal allograft rejection, obesity, and diabetes [117]. In the diabetic kidney, the factors promote differentiation of Th-17 cells and production of IL-17A are associated with the presence of immune cell infiltration and upregulation of pro-inflammatory molecules such as TGF-β and MCP-1 [118, 119]. Current research in the DN has revealed that attenuation of kidney injury by targeting Th-17 cells by mycophenolate mofetil, an immunosuppressant [120]. Renal biopsies from patients with early and chronic nephropathy revealed that CD4+ IL-17+ cells are infiltrated, and it is associated with a progressive decline in GFR [121]. Elevated IL-17A levels in diabetic mice induce podocytopenia in the glomerulus [122]. IL-17A neutralizing antibodies prevented podocytopenia in accelerated-diabetic nephropathy mouse models [122]. IL-17 promotes renal fibrosis by

infiltrating neutrophils in the athymic rats after recovery from ischemic reperfusion injury and that compensates the role of natural killer cells [123]. However, there are contradictory studies demonstrating blocking or deficiency of IL-17A do not prevent renal fibrosis in the ischemia-reperfusion injury model [124]. Other hands, IL-17A knockout mice reduce renal fibrosis by preventing the infiltration of leukocytes in the unilateral ureteral obstruction model (UUO) [125].

1.5.0. Overarching Hypothesis:

Since, diabetes, particularly in the early course of T1DM presented with elevated GH levels and hyperactive GH/GHR axis is associated with adverse renal function. We hypothesize that GH elicits its action on glomerular cells and contributes to the pathogenicity of diabetic kidney disease. Furthermore, our hypothesis is supported by both identifications of GHR on podocytes activation of canonical JAK/STAT cascade upon exposure to GH.

Hypertrophy of glomeruli is an early feature of diabetic nephropathy. Nevertheless, the contribution of podocyte hypertrophy towards glomerular hypertrophy is not known. In this study, we would like to assess whether elevated GH contributes to glomerular hypertrophy via its action on podocytes.

Objectives: We have listed three possibilities for glomerular enlargement. 1) Accumulation of extracellular matrix. Podocyte contributes significantly to the glomerular extracellular matrix. 2) Hypertrophy of individual podocytes, which collectively contributes to glomerular hypertrophy. 3) Hyperplasia or multiplication of podocytes (Fig. 9). The third possibility is ruled out because podocytes are terminally differentiated cells, and they less likely to undergo proliferation. Therefore, we addressed the first two possibilities for glomerular hypertrophy:

1. Does GH induce podocyte to secrete excess extracellular matrix?
2. Does GH provoke podocyte hypertrophy i.e increase in cell size?

In my doctoral study, we have demonstrated that GH alters ECM turnover by inducing TGF-β/SMAD signaling and also by inducing BIGH3, which stabilizes TGF-β. Alternatively, GH also induced IL-17 signaling in podocytes, which might contribute to inflammation and fibrosis. My self and earlier members of our group have demonstrated that GH directly acts on podocytes. Since podocytes account for more than 30% of the total glomerular volume and the key component of the GFB, any alterations in podocytes could bring up severe renal

complications. The experimental results presented in this thesis addressed whether GH alters ECM remodeling and podocyte hypertrophy thus contributing to glomerular hypertrophy. Further, we have presented the data for the role of IL-17 that is secreted upon the treating of podocytes with GH.

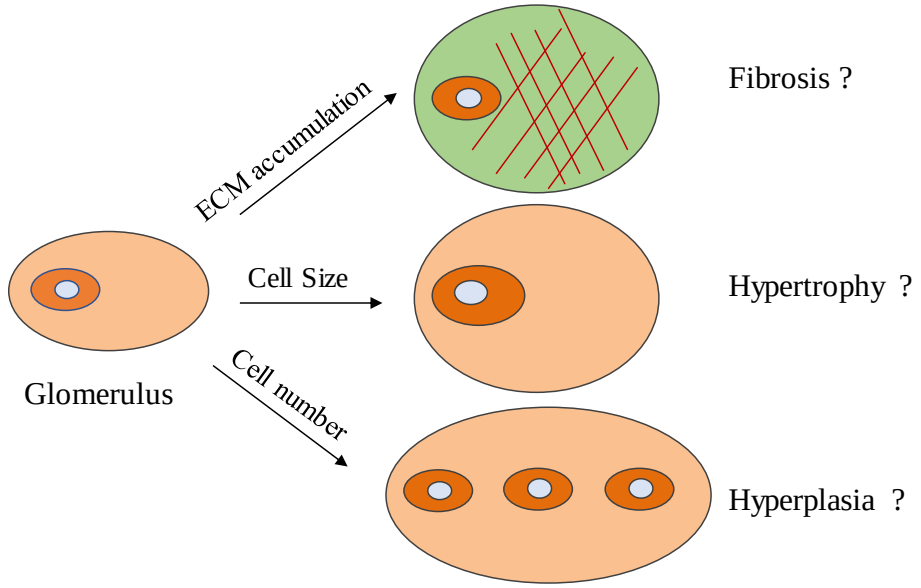

Figure 9. P*ossible modes of tissue enlargement:* Three possible modes that are critical for tissue growth. Hyperplasia is important for growth of an organ during development. Hypertrophy is an important event regulates hyperplasia; that is cell growth preceeded by proliferation. ECM integrates homo/heterogenous cell population to become tissue. Hypertrophy and ECM accumulation both are required for postnatal growth for terminal deifferentiated cells.

Materials & Methods

Materials & Methods

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2.3.2. HEK-Blue IL-17A reporter assay

2.3.3. Migration assay

2.3.4. Statistical analysis

Materials & Methods

2.1.0. Materials:

Human growth hormone (Genotropin) was purchased from Pfizer, Newyork, USA. TGF-β1 antibody (#NBP1-45891) and SMAD pathway antibodies pSMAD2&3 and SMAD2&3 were purchased from (#NBP2-54771, NBP1-77836, AF3797, NBP2-67372) Novus Biologics, Centennial, USA. Recombinant TGF-β1 (#240-B-002) and ELISA kit for TGF-β1 (#DB100B) were procured from R&D systems, Minneapolis, USA. All secondary antibodies (#111-035-144, 115-035-062) used in this study for western blotting were from Jackson Laboratories, Maine, USA and fluorescent-tagged secondary antibodies (#DI-1094, CY-2500) from Vector Labs, Burlingame, USA. PCR-array kit for ECM and Cell adhesion molecules (#PAHS-013Z) was purchased from Qiagen, Germany. cDNA synthesis kit (#G236) was purchased from ABM, Richmond, Canada. Primers used in these studies were procured for Integrated DNA Technologies, California, USA. Cell culture media RPMI1640 was purchased from Sigma. Fetal bovine serum and antibiotic-antimycotic solution were purchased from Gibco, Massachusetts, USA. TGF-βR1 inhibitor (SB431542) (#1614) was purchased from Tocris Bioscience, Bristol, UK. Lipofectamine2000 (#11668019) was purchased from Invitrogen, Carlsbad, USA. Cignal SMAD4 GFP-reporter kit (#336841) was purchased from Qiagen (SA Biosciences), Hilden, Germany. SYBR Green Master Mix and Western blotting developing reagents (ECL^{TM}) were obtained from Bio-Rad Laboratories Hercules, CA, USA. Phalloidin-TRITC (#P1951) purchased from Sigma, Missouri, USA. Prolong Diamond Antifade with DAPI (#P36962) was purchased from Molecular Probes, Oregon, USA. Inulin FIT-GFR kit was purchased from BioPal, Worcester, MA, USA. Urine albumin (#COD11573) and creatinine (#COD11502) kits were purchased from Biosystems, Barcelona, Spain. All other chemicals used in the study were purchased from Sisco Research Laboratories, Hyderabad, India.

2.1.1. Culturing of human podocytes:

Human podocytes were cultured according to the published literature [73]. Human podocytes were obtained from the University of Bristol, UK. Human podocytes were cultured in 10% serum-containing RPMI 1640 with 1% antibiotic-antimycotic at 33ºC under 5% CO2 for proliferation. At 70% of confluence, cells were transferred to 37ºC and maintained for 8 days for

differentiation. Media was changed every alternative day to maintain healthy cultures. The percentage of differentiation was verified by assessing the markers expressed by differentiated podocytes. Every time when we treat cells with GH or TGF-β, we serum-starved cells for 16-20h in serum and glucose free-medium. Then, cells were treated with recombinant human GH (GH) or recombinant human TGF-β (TGF-β) for specified time points.

2.1.2. GH and other treatment procedures:

Differentiated podocytes were treated with GH (100, 250, 500ng/ml) and TGF-β protein (2.5ng/ml). For the experiments involved with treating of SB43154 (inhibitor for TGF-βR1), a similar procedure was followed. However, cells were preincubated with SB431542 in the starvation medium prior to treating with GH or TGF-β and continued till the experiment terminated. Similarly, for albumin influx assay, podocyte monolayers treated with SB431542 prior to the GH or TGF-β for 20h. At the time of BSA incubation, the spent media was replaced with fresh media containing SB431542. 10μM of SB431542 was used as a final concentration in all *in vitro* experiments according to the published articles [126].

2.1.3. RNA isolation and cDNA synthesis:

Total RNA was isolated by the Trizol method according to the standard protocol [127]. After all treatments, cells were washed with PBS and collected in 1ml of Trizol. Then, cells were added with 0.2mL of chloroform and mixed vigorously for 15 seconds. Samples were incubated at RT for 2min and centrifugation was performed at 12,000 rpm for 15min at 4ºC. The upper transparent layer was collected into a fresh RNase-free tube. Total RNA was precipitated by adding 0.5ml of Isopropanol to the collected upper layer and inverted to tubes for several times gently. Samples were then centrifuged at 10,000 rpm for 10min at 4ºC. The RNA pellet was washed in 70% ethanol and then, the pellet was dried at 37ºC for 20min. The resulting RNA pellet was finally dissolved in DEPC-water and RNA concentration was determined.

cDNA synthesis was performed according to the company's protocol (ABM #G236). A total 1μg of RNA was used as an input for cDNA synthesis. Briefly, RNA was incubated in reverse transcriptase buffer supplemented with dNTPs, enzyme, and random hexamers. The resulting reaction was placed in a thermal cycler at 42ºC for 1h. This cDNA was diluted for real-time PCR experiments according to the gene of interest.

2.1.4. PCR-array for ECM components:

PCR-array kit for ECM and Cell Adhesion Molecules was purchased from Qiagen (#PAHS-013Z) and performed PCR-array according to the published article [80]. This kit contains pre-coated primers for 84 genes that code for ECM and cell adhesion molecules. Podocytes were treated with GH for 24h and extracted RNA and cDNA was prepared. Then, cDNA was loaded on to the plate and performed quantitative PCR according to the kit protocol. Data were analyzed in the RT^2-Profiler software according to the manufacturer.

2.1.5. SDS-PAGE and Western Blotting:

10% Denatured-Poly Acrylamide Gels were prepared using sodium dodecyl sulfate (SDS). Podocyte lysates were prepared in RIPA lysis buffer and protein estimation was done using the Lowry method. A total of 50µg of protein was prepared for loading by adding a sample loading buffer. Protein was loaded on 10% gel and polyacrylamide gel electrophoresis (PAGE) was performed at 100 V for 2h or until the dye was released. Followed by PAGE, proteins were transferred (western blotting) onto a nitrocellulose membrane at 70V for 1hr. Blots were directly placed in 5% non-fat skim milk powder for an hour to block the non-specific binding of the antibody. Blots were incubated with the respective primary antibody for overnight at 4^oC before being incubated with respective secondary antibody for 1hr at room temperature. Blots were developed using the ECL substrate employing Bio-Rad Versa Doc 5000 MFP and results were analyzed in Image Lab. Band intensities were quantified in Image J (NIH, Bethesda, USA).

2.1.6. Paracellular albumin influx assay:

Podocyte *in vitro*-functional assay was performed to assess permselectivity to albumin as per methods published earlier [80, 127]. $1.2x10^5$ cells per well were seeded on to the top chamber of a 6-well insert. The wells of the six-well plate were filled with 6ml of RPMI complete medium and incubated under permissive conditions for 24h. These podocytes were shifted to 37^oC and incubated for 8 days for differentiation. Podocytes were serum-starved for 24h and the indicated treatments applied to the cells. Following treatment, podocytes were washed with PBS containing 1mM $MgCl_2$ and 1mM $CaCl_2$ to preserve cell-adherent junctions. Then, six-well plates were filled with 6ml of 40mg/ml BSA containing serum-free RPMI medium and the top chamber filled only with 0.7ml of RPMI. After incubation, a small aliquot of a medium from the top chamber was collected to measure BSA efflux from the bottom chamber

to the upper chamber and concentration was assessed by the BCA protein assay.

2.1.7. Enzyme-Linked Immunosorbent Assay (ELISA):

Differentiated human podocytes were serum-starved in glucose-free RMPI 1640 medium for 20h under standard culture conditions. Cells were treated with GH in various concentrations in the serum-free media (not supplement with glucose) for 30min. Briefly, dead cells were removed by passing the culture media through 0.2µ membrane filters. The resultant supernatants were directly subjected to the TGF-β1 activation procedures as the manufacturer's protocol. Activated samples were assayed by an indirect sandwich Elisa kit according to the manufacturer's protocol (R&D systems # DB100B) and the TGF-β levels were determined based on the standards run along with the samples.

2.1.8. Preparation of conditioned media:

Conditioned media was prepared according to the published article with slight modification [128]. Differentiated podocytes were thoroughly washed with PBS before treating podocytes with or without GH (500ng/ml) for 30min. Subsequently, the media that was collected 30min later (Conditioned media) was processed for subsequent experiments. The conditioned media from GH-treated (CM-GH) and untreated (CM-Con) podocytes were centrifuged at 6000xg for 5min at 4^{O}C to remove cell debris and the resulting supernatant was further passed through a 0.2µ membrane filters. The resulting CM was then added to naïve podocytes accordingly and treated for 15 to 30min to assess activation of SMAD signaling or treated for 12h for assessing morphological parameters. A fraction of CM was also run on 10% gels to identify TGF-β protein in CM.

2.1.9. Immunofluorescence:

Human podocytes were seeded in 6-well plates on coverslips. After achieving 70% of confluence, cells were briefly washed in PBS and fixed with 4% PFA (paraformaldehyde) for 10 min at RT. Podocytes were permeabilized with 0.5% Triton X-100 in PBS for 15min at RT. Blocking was performed in 5% BSA prepared in PBS for 1hr and probed with primary antibody (1:100 dilution) for overnight 4^{o}C. Cells were then incubated with a secondary antibody conjugated with a fluorescent tag for an hour at RT. Cells were briefly washed with PBS and coverslips were mounted with DAPI containing antifade. Images were acquired in Leica

Microsystems trinocular microscopy.

2.2.0. Transient transfection & SMAD4-GFP reporter assay:

Transfection was performed in HEK293T cells. $6x10^6$ cells were seeded in a 10cm culture dish prior to the day of transfection. Then the cells were transfected using lipofectamine 2000, Invitrogen. Post 6hr of transection, cells were briefly washed with PBS and incubated in 10% serum-containing media for 16h in culture conditions followed by which all treatments were performed in serum-free medium.

SMAD4-GFP assay plasmids were a kind gift by Dr. K. Arun Kumar, Hyderabad, India. SMAD4-GFP reporter assay performed in HEK293T cells according to the kit's protocol with minor modifications [129]. In a 96 well plate, $6x10^3$ cells/well were seeded prior to the day of transfection. Cells were transfected either with SMAD4-GFP, positive control and negative control vector in triplicates for 6h. After transfection, cells were replaced with 10% FBS containing DMEM medium. Cells, were left untreated in complete medium for 16h, followed by serum starvation was performed for 12h and then cells were treated with TGFβ, CM-GH or GH in serum-free medium. After 16h of treatment, images were taken in Olympus 5000 fluorescence microscope and the fluorescence emission spectrum from 510-520nm was recorded by exciting at 488nm. Fluorescence values were plotted after normalizing with mock control values.

2.2.1. Phalloidin staining:

The phalloidin staining solution was prepared according to the earlier literature[130]. Phalloidin staining was performed to assess the podocyte morphology and size. Phalloidin-TRITC was purchased from Sigma, Missouri, USA. Podocytes were seeded on a coverslip and treated with or without GH or TGF-β as mentioned in treatment procedures. A stock solution of 0.1mg/ml concentration of phalloidin-stain was prepared in DMSO. A final concentration 0.1mM of phalloidin was used for the staining actin cytoskeleton. Briefly, cells were washed with PBS after treatments, and then cells were fixed with 3.7% formaldehyde solution in PBS for 5min. Followed by extensive washes with PBS podocytes were permeabilized with 0.1% Triton X-100 in PBS for 5-10min at RT and briefly washed with PBS. Then, podocytes were incubated with 50µg/ml phalloidin-TRITC conjugate for 2h at room RT. Cells were washed with PBS for 4-5 times to remove excess Phalloidin-TRITC conjugate. Finally, slides were mounted with DAPI containing antifade medium and left overnight at RT.

2.2.2. LC-MS/MS analysis of conditioned medium:

Mass spectrometry analysis was performed based on standard protocols [131]. Briefly, podocytes treated with GH for 30min and media were passed through 0.2µm filters. The resulting flow-through was precipitated by using 20% trichloroacetic acid (TCA) at -20^{o}C for 3h. 100µg of the pellet was dissolved in 50mM ammonium bicarbonate (NH_4HCO_3) solution and then treated with 100mM DTT for 1h at 95^{o}C. Followed by samples were treated with 250mM IDA for 45min at RT under dark conditions. Samples were then subjected to trypsin digestion overnight at 37^{O}C. The resulting samples were vacuum dried and dissolved in 20µL of 0.1% formic acid in water and centrifuged at 10000g for 5min and the supernatant collected. 10µL of the sample was injected into the BEH C18 UPLC column for separating peptides. The separated peptides were directed to MS and MS/MS analysis in the Synapt G2 Q-TOF instrument. The raw data was processed in MassLynx 4.1 Waters. Individual spectra were matched to the peptide database Uniprot Podocyte in PLGS software. We set the following parameters a minimum number of fragments match for the peptide is 2 and the minimum number of fragments match for protein is 5 and the minimum number of peptides match for protein is 2. Peptide tolerance and fragment tolerance 50ppm and 100ppm respectively. We used carbamidomethyl (C), oxidation (M) modification for peptides during analysis.

2.2.3. Animal experiments:

Swiss-albino mice were purchased from the National Center for Laboratory Animal Sciences (NCLAS, NIN, Hyderabad). All experimental group animals were maintained under the 12h day and night cycle throughout the experimental procedure. After acclimatization animals randomly grouped into control, GH, GH+SB. On day fifth of acclimatization, the GH group received intraperitoneal injections of GH (1.5mg/kg body weight/day) and the GH+SB group received intraperitoneal injections of SB431542 (1mg/kg body weight/day) an hour prior to the GH treatment [132]. The control groups received a similar volume of saline throughout the experimental procedures. Before scarifying animals, we collected urine samples for albuminuria analysis. All animals were sacrificed on day 14th of post GH treatment and perfused with PBS. Internal body fixation was done using 4% PFA. The kidneys were collected, and one kidney was used for histological analysis and the other kidney was used for molecular signaling studies. Institutional Animal Ethics Committee of University of Hyderabad approved animal protocols.

The podocyte GHR knockout (pGHRKO) mice (genetic background, C57BL/6J) with targeted deletion of the GHR in podocytes were generated as described before. Diabetes was induced in these mice using streptozotocin (2mg/kg) and maintained for 8 weeks. We collected urine for analysis before scarifying the animals. A whole kidney was processed for molecular analysis by western blotting and RNA analysis. A portion of the kidney was fixed in 10% PB-formalin for overnight and processed for embedding. Another portion was immediately fixed in Tissue-Tek optimum cutting temperature (OCT) compound for frozen sections by cryo-sectioning. The University of Michigan Animal Care and Use Committee approved all animal protocols pertinent to GHR KO animals.

2.2.4. Isolation of glomerular podocytes:

Isolation of the glomerulus was performed according to the earlier literature [133]. The glomeruli from the kidneys from mice treated with GH were isolated by a series of stainless sieves. Under aseptic conditions, decapsulated kidneys were sliced and placed on a sieve with a size of 200μm and gently grounded with a sterile syringe knob and washed in Hank's solution. The filtered kidney homogenate was then passed through a sieve (150μ pore size) and as a final step, the glomeruli were then collected as a suspension on a 75μ sieve. After 10min, the suspensions were centrifuged at 1,000 rpm for 5min. The supernatant was discarded, and the purified glomerular pellet was resuspended in PBS. Glomeruli images were taken in Olympus 5000 microscope.

2.2.5. Estimation of GFR and Urine Albumin Creatinine Ratio:

Glomerular filtration rate (GFR) was performed in mice using FIT-GFR Kit for Inulin according to the manufacturer's instructions and earlier publications [127]. At 7 weeks of age, mice were injected with 5 mg/kg inulin i.p followed by serial saphenous bleeds at 30, 60, and 90 minutes. Next, serum isolation was done and quantified on the inulin ELISA kit. Serum inulin clearance estimation was performed by the nonlinear regression method using a one-phase exponential decay formula ($y = Be{-}bx$), and GFR was calculated (GFR = ((I)/(B/b))/KW, where I is the amount of inulin delivered by the bolus injection, B is y intercept, b is the decay constant, x is time, and KW is kilo weight of the animal).

Albumin and creatinine levels were estimated individually using a kit from Biosystems according to the manufacturer's protocol. levels estimated using available assay kits (Biosystems, Barcelona, Spain).

2.2.6. Silver staining of SDS-PAGE gels:

Urine samples were thawed from -80ºC on ice. Urine samples from both control and GH treated mice subjected to PAGE at 100V for 2h. Gels were washed in distilled water twice and placed in a fixation solution (50% ethanol, 12% acetic acid, 50ul formaldehyde) for 1h at room temperature (RT), Followed by gels were washed three times with 50% ethanol for 20min each. Then, gels were sensitized with sodium thiosulfate (Na_2SO_4) for 1min. Gels were placed immediately in distilled water, followed by gels were incubated under dark conditions in a solution containing 0.2% silver nitrate (AgNo3) solution and 0.037% formaldehyde for 30min at RT. Finally, gels were developed in 6% sodium carbonate solution containing 0.037% formaldehyde. Immediately, upon significant visualization of bands developing was terminated by adding 5% acetic acid. Gels were imaged in the Bio-Rad Touch imaging system.

2.2.7. Transmission Electron Microscopy (TEM):

TEM sectioning and imaging was followed from the earlier protocols [133]. After animals were sacrificed and kidneys were perfused with PBS; tissue samples were fixed in 2.5% glutaraldehyde in 10mM phosphate buffer for 24hr at 4ºC. Samples were washed with 100mM cacodylate buffer for three times each 15min. Followed by kidneys were fixed with 1% osmium tetroxide (OsO4) in 0.1M cacodylate buffer for 1hr at 4ºC, then washed in sterile water three times for 15 mins each. Then, kidneys were stained with 3% uranyl acetate (UO2(CH3COO)2) in water for overnight and dehydrated the samples using alcohol gradients three times each 15mins, then kidneys were placed propylene oxide. Kidneys were infiltrated with a 1:1 ratio of propylene oxide: Araldite CY212 mix for 2h at RT. Secondly, the samples were infiltrated with a resin mix two times for 5h each. Then, kidneys were embedded in fresh resin and allowed for polymerization at 60-70ºC for 24h. 3µm thin sections were cut with the ultra-microtome, and sections were collected on to grids. Grids were allowed to air-dry overnight. Grids were stained with uranyl acetate for 15-30min, followed by stained with lead citrate for 15min. Imaging was performed using JEM-1400 TEM with Gatan Ultrascan CCD camera at CCMB, Hyderabad.

2.2.8. H&E and Periodic acid-Schiff's staining:

6µm paraffin-embedded sections were cut using Leica Microtome. Briefly, sections were deparaffinized in xylene, then rehydrated with ethanol gradients from 100% to 50%. Sections were stained with hematoxylin for 2min, then rinsed with sterile water and PBS followed by stained with eosin for 30seconds. Finally, slides were washed several times with PBS and dehydrated and mounted with DPX. Imaging was done in Trinocular microscope. The glomerular area was measured in Image J (NIH, Bethesda, USA).

For PAS staining, sections were processed similarly as H&E staining with minor changes. After removing wax and rehydrated, sections were incubated with 0.5% periodic acid for 5 mins, rinsed with distilled water. Sections were then added with Schiff's reagent for 10 min at RT. Slides were washed for 5min under running tap water followed by counterstained with hematoxylin for 1min. Finally, slides were and dehydrated and mounted with DPX.

2.2.9. Immunohistochemistry:

A similar procedure was followed with slight modifications from earlier published literature [127, 133]. Paraffin-embedded tissues were processed into 6µm thin sections using a Leica systems microtome. Sections were processed to deparaffinization and rehydrated with ethanol in gradients from 100% to 50%. Peroxidase blocking and permeabilization were performed in 30% H_2O_2 for 20min at RT. Antigen retrieval was done in Tris-EDTA-Tween buffer by heating sections in the micro oven with regular cooling intervals (three cycles of five minutes with intervals of two minutes). Blocking was performed in 5% BSA in PBS for 1hr at RT. Primary antibody was prepared in 5% BSA and added on to the sections and incubated for two hours at RT or overnight at $4^{O}C$. Followed by sections were washed twice with PBS and incubated with secondary antibody either with fluorescent-tagged or HRP (horseradish peroxidase) conjugate prepared in 5% BSA. Sections were developed in DAB staining solution and counterstained with hematoxylin. Finally, sections were washed with PBS four times and dehydrated and mounted with DPX. For the slides probed with fluorescent conjugated secondary antibody, no dehydration steps were followed, and slides were mounted with DAPI antifade. Images were acquired in Leica Trinocular Microscope.

2.3.0. RNA sequencing and analysis:

RNA sequencing performed according to the available literature [134]. Human podocytes were treated with GH for 30min and RNA isolated from duplicates of GH treated and untreated podocytes. The quality of the isolated RNA was verified in two methods by running an RNA gel and run on Bioanalyzer. High-quality RNA libraries were constructed using mRNA templates, random hexamers, oligo-dT primers, and Illumina second strand synthesis buffer. RNA sequencing was performed on Illumina 2500 Hi-Seq devise by performing paired-end reads according to the protocol by Nucleome Informatics, Hyderabad, India. Resulting raw reads were sent for removing the adapter sequences and low-quality reads based on the Q score <5. We performed sequencing in a way that the sequencing depth has crossed a threshold provided in the literature and our sequencing reads were crossed over fifty-three million reads. The mapping of reads was performed on TopHat2 software and the percentage of total mapping is more than 80%. Mismatch parameters were set to two. Multiple mapped reads or fragments were obtained no more than 10%. Gene expression quantitation was done using fragments per kilobase of transcript per million mapped reads (FPKM) method which tells the direct relative transcript expression based on the proportion of cDNA fragments originated from it. In general, 0.1 to 1 FPKM value as a threshold to consider whether a gene is expressed. Above 1 is considered as upregulated and -1 considered as downregulated expression. Differentially expressed genes were determined using the edgeR pipeline. A total of 30 genes were identified as significantly, and differentially regulated genes based on p value 0.01 as the cut-off.

2.3.1. Genes ontology (GO) terms analysis:

GO terms such as GO-biological process (GO-BP), molecular function (GO-MF) and cellular component (GO-CC), for differentially expressed genes were performed in GOrilla software which is web-based software accepts all formats for representing gene names. We represented gene names in Ensemble IDs. KEGG (Kyoto Encyclopedia of Gene and Genomes) pathway analysis was performed for differentially expressed genes. KEGG utilizes all published literature including data files, metabolomics, and metagenomics to predict significantly enriched pathways. Cluster analysis performed to identify genes that are expressed in a similar pattern in various conditions. RNA seq data was further validated in our laboratory to test the analysis is reliable. We also validated GO-MF and GO-BP.

2.3.2. IL-17 HEK-Blue reporter assay:

IL-17 HEK-blue cells were a gift by Dr. Srikanth Talluri, Dana Farber Cancer Institute, Harvard University. IL-17 reporter assay was performed according to the manufacturer's protocol, Invivogen, USA (# hkb-il17). Briefly, HEK cells expressing IL-17RA/C plasmid, Act1 adapter protein-expressing plasmid and plasmid that also express the gene encodes SEAP (secreted embryonic alkaline phosphatase) protein under five repeats of NF-kB and AP1 binding elements. These cells were maintained under tight selective antibiotics that preserves these plasmids not expelled out from the cells. HEK-Blue cells were cultured in 6-well plates and treated with GH (500ng) and TGF-β (2.5ng) for 30min in serum-free medium. SEAP degrades Quanti-blue a dye which turns blue upon SEAP action and the color change recorded at 655nm from the time the dye added, 15min of incubation to 16h of incubation with Quanti-blue.

2.3.3. Migration assay:

Migration assay performed according to the previous publications [127]. J774 macrophages were seeded in 6-well plates prior to the experiment. After making the scratch, cells were washed with PBS and CM-GH from podocytes was prepared after treating for 30min. J774 macrophages were directly treated with CM-GH for 0 to 12h and observed migration efficacy of CM-GH. Images were taken in Olympus 5000 microscope.

2.3.4. Statistical analysis:

Data throughout my thesis were presented as mean ± standard error (SEM) unless otherwise indicated. Graphpad Prism 8 software was used to analyze statistical differences between the distributions of two by unpaired non-parametric Mann Whitney test or multiple independent samples by Tukey's multiple comparison test, respectively. $p \leq 0.05$ was considered as significant.

Results

Results

Abstract

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4.5. Identification of Biological Process, Molecular Function, and Cellular Component

4.6. Validating RNA sequencing analysis by quantitative real-time PCR

4.7. KEGG pathway enrichment analysis identified IL-17 pathway activation in podocytes

4.8. Conditioned medium from GH treated podocytes induced J774 migration

4.9. GH induces IL-17 pathway activation

Results

Abstract:

The glomerular podocytes are specialized visceral epithelial cells and are the ultimate barrier to curb protein loss into the urine. Podocytes provide epithelial coverage to the glomerular capillaries and contribute extracellular matrix (ECM) components to the basement membrane. Elevated growth hormone (GH) levels are implicated in the pathogenesis of nephropathy in patients with T1DM. We have previously shown that podocytes express GH receptor and GH elicits a direct action on podocytes. Nevertheless, the role of GH on podocyte biology remains to be explored. To elucidate the effect of GH on podocyte biology, we performed PCR-array for the extracellular matrix and cell-adhesion molecules. Our studies showed that GH increased the expression of BIGH3. BIGH3 is a latent complex of TGF-β1 and controls cell-matrix interaction and cell migration. BIGH3 is also called as transforming growth factor-β induced protein. Therefore, we studied whether GH has any effect on TGF-β1 expression in podocytes. Interestingly, we found that GH induces TGF-β1, which can act in a both autocrine and paracrine manner. GH induces podocyte hypertrophy and expression of mesenchymal markers. Administration of GH to mice developed early renal changes that are associated with elevated expression of TGF-β/SMAD signaling. We also observed that GH promotes the migration of non-resident immune cells into the glomerular region. RNA-sequencing analysis revealed that podocytes secrete IL-17 in response to GH treatment, which elicits chemotaxis of macrophages. Exposure of podocytes either with GH, BIGH3 or TGF-β1 resulted in altered podocyte permeability to albumin across podocyte monolayer. Therefore, we report that the GH activates TGF-β1 signaling in podocytes that in turn might activate BIGH3 and IL-17 signaling. Hyperactivity of the GH/GHR axis results in (a) altered composition of podocyte ECM components, (b) elicits enhanced TGF-β1/SMAD signaling, and (c) induce IL-17 expression, thus promote an inflammatory response in podocytes. Altogether, GH contributes to the pathogenicity of diabetic kidney disease at least by inducing TGF-β1, a prosclerotic molecule. Targeting GH/GHR axis in podocytes could be a therapeutic strategy to combat podocyte injury in patients with diabetic nephropathy.

3.1. Results:

Diabetic nephropathy (DN) is characterized by increased glomerular size, GBM thickening, and glomerulosclerosis, which clinically manifests in proteinuria. Proteinuria indicates damage to the GFB. The three layers of GFB are fenestrated endothelium, glomerular basement membrane (GBM) and the podocyte layer. Podocytes have a unique architecture that is critical for glomerular permselectivity and GBM composition. The composition of GBM is highly affecting podocyte integrity, adequacy of podocytes thus maintains healthy renal filtration [67]. Podocyte depletion is considered as the main mechanism in several glomerular diseases including DN. Podocyte depletion could be due to apoptosis and/or detachment from the GBM [75]. Experimental animal models of nephropathy showed decreased expression of podocyte-specific proteins including podocin [135, 136]. Although several factors considered to provoke podocyte damage, a precise molecular mechanism for podocyte loss in diabetes is remains unexplored.

The pathogenesis of DN involves the deregulation of an array of factors that elicit podocyte injury. One of the key factors that deregulate during diabetes is GH/IGF-1 axis. GH is a diabetogenic hormone and it antagonizes insulin actions. Evidence for the causal role of GH in nephropathy has come from studies in transgenic mice wherein high levels of GH is associated with significant renal structural and functional modifications [43]. Animals with hyperactive GH/GHR axis exhibited podocyte injury with the poor renal outcome. Children with renal complications were documented with an increased secretory burst of GH compared to healthy children. Our previous studies demonstrate that podocyte expresses a functional GH receptor and exposure to GH, podocytes induced ZEB2 expression that resulted in diminished expression of P- and E-cadherins [73].

One of the abnormalities in patients with DN is the loss of podocytes in the urine. Urine sediments from patients with proteinuric disease consist of podocytes. The composition of ECM components is significantly altered in the case of DN [137, 138]. In this study, we analyzed whether GH has any effect on the expression of ECM and cell-adhesion components that determine podocyte-GBM interactions. Further, we have also investigated whether GH mediates ECM remodeling via inducing TGF-β, a central molecule of ECM expression and turnover.

3.2. GH induces BIGH3 expression in podocytes:

We have employed a PCR array to investigate whether GH can elicit the changes in the expression profile of podocyte genes encoding ECM molecules. Curiously, our data revealed that GH induces the increased expression of *BIGH3*, which is also known as transforming growth factor-β induced protein *TGFBI*. In addition to BIGH3, GH also altered the expression of several ECM and cell-adhesion molecules including EpCAM, collagen 2a1, integrin E, and MMP3 (Fig. 1A).

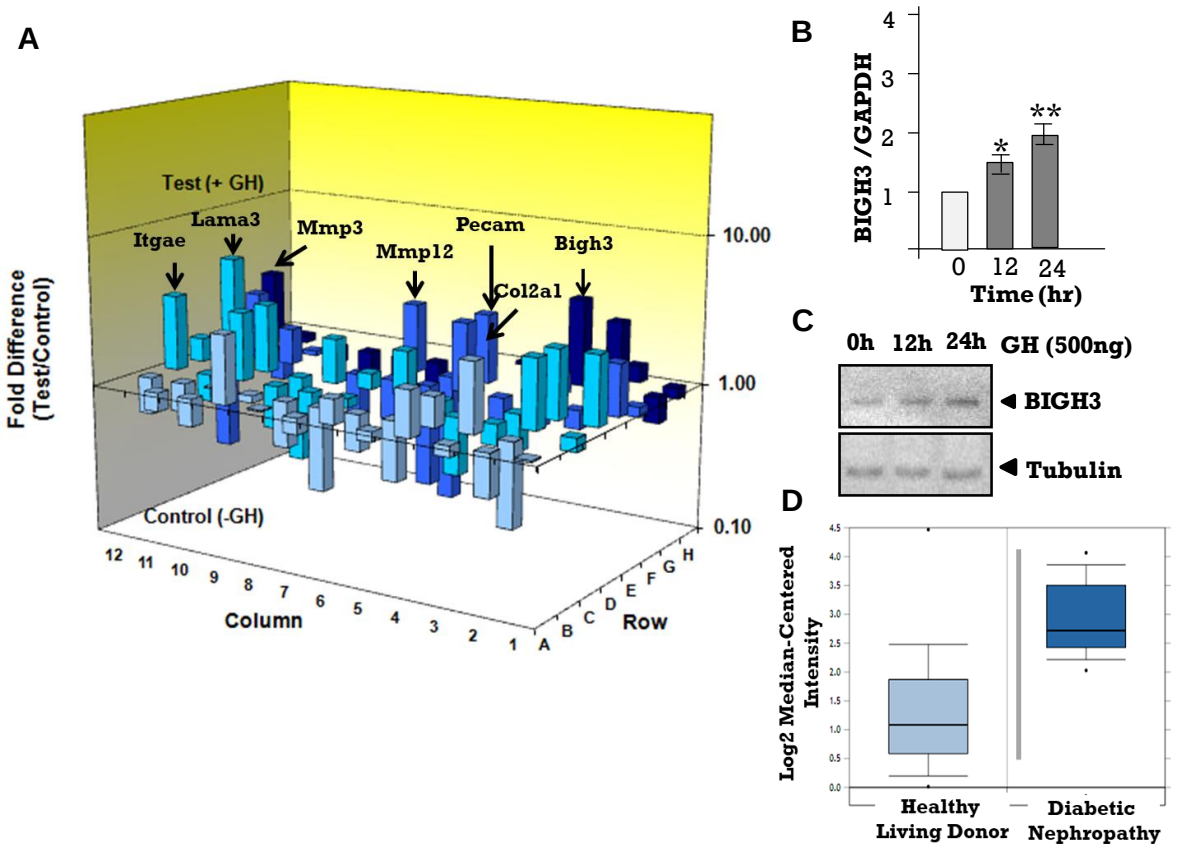

Figure 1. *GH induces BIGH3 expression in podocytes:* A) PCR-array data generated in podocytes treated with GH for 24h. B) Quantitative PCR for BIGH3 mRNA in podocytes exposed to GH (500ng/ml). C) Western blotting analysis of BIGH3 in podocytes treated with GH in time dependent manner. D) BIGH3 mRNA quantification in diabetic patients retrieved from Nephroseq database, University of Michigan.

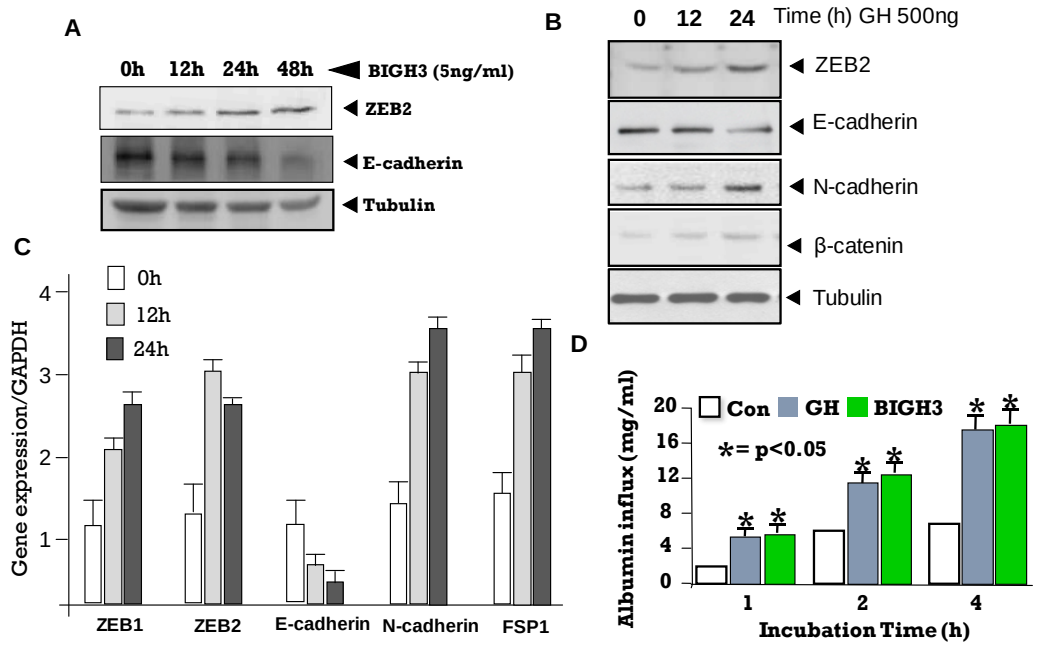

Figure 2. *BIGH3 induces EMT markers in podocytes and altered podocyte permeability:* A) Western blotting analysis of ZEB2 and E-cadherin in podocytes treated with BIGH3 5ng/ml for different time points. B) Western blotting analysis of EMT markers in GH treated podocytes. C) Quantitative PCR analysis of EMT markers in podocytes treated with GH in podocytes at various time points. D) Albumin influx assay in podocytes treated with GH or BIGH3 individually for 24h and measured albumin concentrations at various time points.

BIGH3 encodes for BIGH3-protein (TGF-βIp) and is also called as keratoepithelin. Results obtained from PCR array were further confirmed with qRT-PCR. For this purpose, we exposed podocytes to GH for varying time points, and mRNA was isolated. These experiments revealed that BIGH3 mRNA was increased in time-dependent manner from 1.5 folds to 2.1-folds by 24h (Fig.1B). Western blotting of for BIGH3 expression also revealed that GH induces BIGH3 in time dependent manner (Fig. 1C). Further, we analyzed the nephromine database (www.nephromine.org), a repository of global gene expression and found that expression of BIGH3 in the patients with DN (Fig. 1D).

3.3. GH induces epithelial to mesenchymal transition (EMT) in podocytes:

Past studies showed that podocyte de-differentiation and EMT could be the potential mechanism for podocyte dysfunction in glomerular diseases [75]. BIGH3 promotes tumor cell invasion via EMT. Therefore, we assessed the expression of EMT markers with BIGH3

treatment in podocytes and observed an increased expression of ZEB2 and decreased expression of E-cadherin an event represents EMT phenomenon (Fig. 2A). Further, we found that a decreased expression of E-cadherin and increased expression of ZEB2 and N-cadherin in GH treated podocytes suggesting BIGH3 playing a role in GH mediated EMT (Fig. 2B). mRNA analysis confirmed the immunoblotting results for EMT markers (Fig. 2C).

3.4. GH and BIGH3 alter the filtration barrier function:

To address the functional repercussions of GH and BIGH3 we employed paracellular albumin permeability assay that measures the albumin flux across the podocyte monolayer as mentioned detailed in the methods section. Podocytes were cultured on transwell filters as a monolayer and exposed to either GH or BIGH3 for 24h and the flux of BSA across the podocyte monolayer was measured up to 4h. Both GH and BIGH3 increased albumin flux across the podocyte monolayer (Fig. 2D) suggesting that podocyte integrity got compromised upon exposure to GH or BIGH3.

Since we observed that GH elicits expression of BIGH3, a component of TGF-β superfamily, we asked a question of whether GH also induces TGF-β? It is noteworthy that, TGF-β is prosclerotic molecule and it is a master regulator of ECM turnover that is secreted from most cell types in the body. Three TGF-β isoforms have been identified among which isoform 1 (TGF-β1) has been associated with the pathophysiology of DN. Earlier studies implicated TGF-β1 as a central molecule responsible for the excess deposition of ECM proteins in diabetes. Both mRNA and protein levels of TGF-β1 are significantly elevated in DN patients. Studies from experimental diabetic animals further substantiated the role of TGF-β1 in the pathogenesis of DN. Neutralizing TGF-β1 with anti-TGF-β1 antibodies reversed the established type II diabetic renal injury. Therefore, in the present study, we investigated whether GH induces TGF-β in podocytes and contributes to glomerular injury.

3.5. GH induces TGF-β1 and the cognate TGF-β-SMAD pathway in human podocytes:

Considering both the central role of podocytes in glomerular permselectivity established the role of TGF-β1 in increasing podocyte permeability, we investigated the direct action of GH on the TGF-β/SMAD pathway in human podocytes. We observed that GH (100-500 ng/ml) induces TGF-β1 protein levels in human podocytes as analyzed by immunoblotting (Fig.

3A&B). TGF-β is synthesized as pro-TGF-β in the ER and secreted into the matrix as a large latent complex that is activated after protease action or acid activation[93]. Next, we estimated mature TGF-β1 concentration in conditioned medium from GH treated podocytes (CM-GH). GH increased TGF-β1 concentration in both a dose (250 and 500 ng/ml) and time (0-60 min) dependent manner (Fig. 3C&D). Furthermore, GH also increased phosphorylation of SMAD2&3 in podocytes (Fig. 3E&F). TGF-β has three isoforms, to identify which isoform is induced with GH in podocytes, we employed liquid chromatography-mass spectrometry (LC-MS/MS) analysis and identified that GH induces TGF-β isoform 1 (TGF-β1) (Fig. 3G). These results indicate that GH induces expression of TGF-β1 and activation of TGF-β1 down-stream signaling in podocytes.

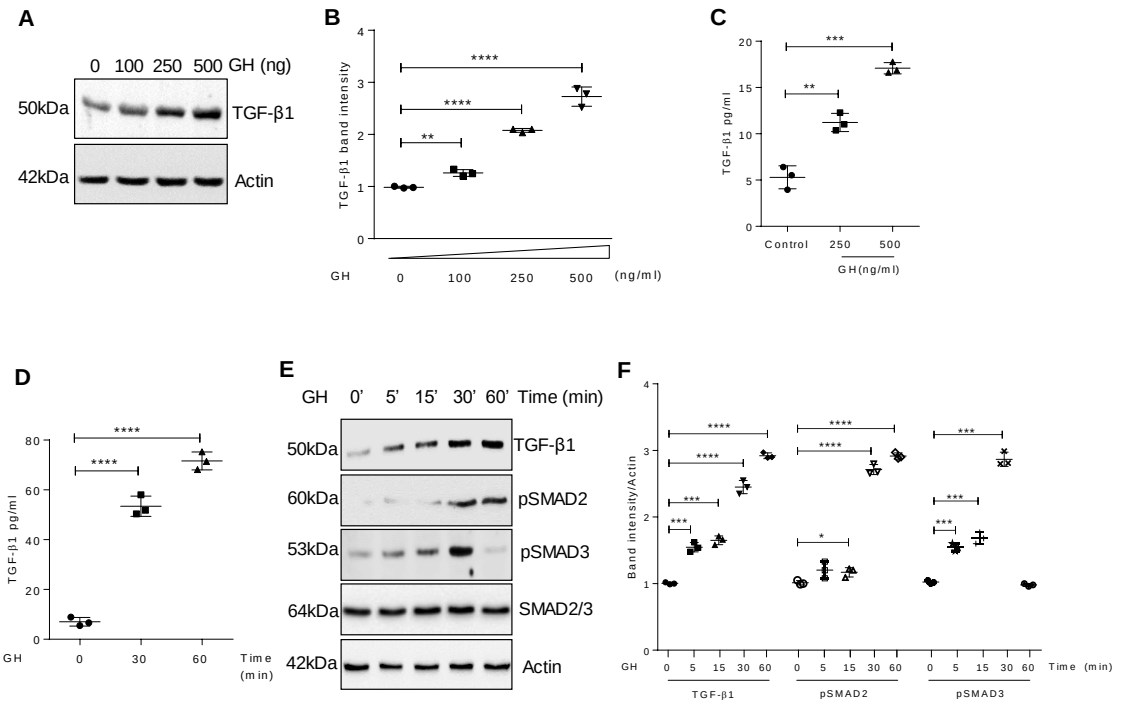

Figure 3. *GH induces TGF-β in podocytes:* A) Western blotting analysis of TGF-β in podocytes treated with GH for various concentrations. B) Representative band intensities quantified in image J. C) Elisa analysis for TGF-β in culture media from podocytes treated with GH various concentrations. D) Elisa analysis for TGF-β in culture media from podocytes treated with GH 500ng/ml for various time points. E) Western blotting analysis of TGF-β and SMAD pathway activation at various time points. F) Representative band intensities quantified in image J, NIH. Representative images of at least three experiments.

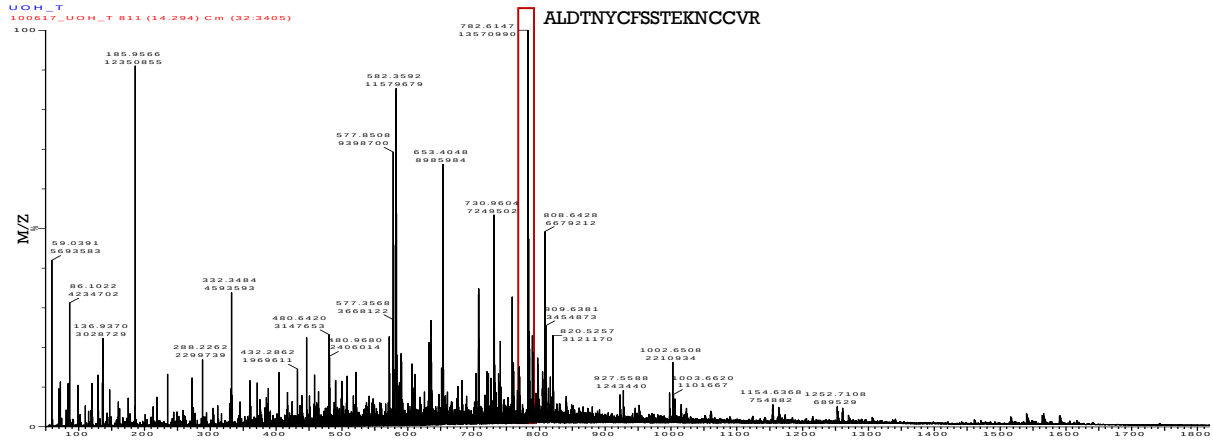

Figure 3G. *Mass spectrum analysis of TGF-β1 in CM-GH from podocytes:* The base peak with the peptide has highest abundance in the CM-GH podocytes.

3.6. GH induced TGF-β in podocytes acts in both autocrine and paracrine manner:

TGF-β1 is known to act in a both autocrine and paracrine manner. To elucidate the paracrine action of GH induced TGF-β1, we treated podocytes with GH and collected conditioned media. Conditioned media from GH-treated podocytes induced phosphorylation of SMAD2&3 in podocytes naïve to GH (Fig. 4A).

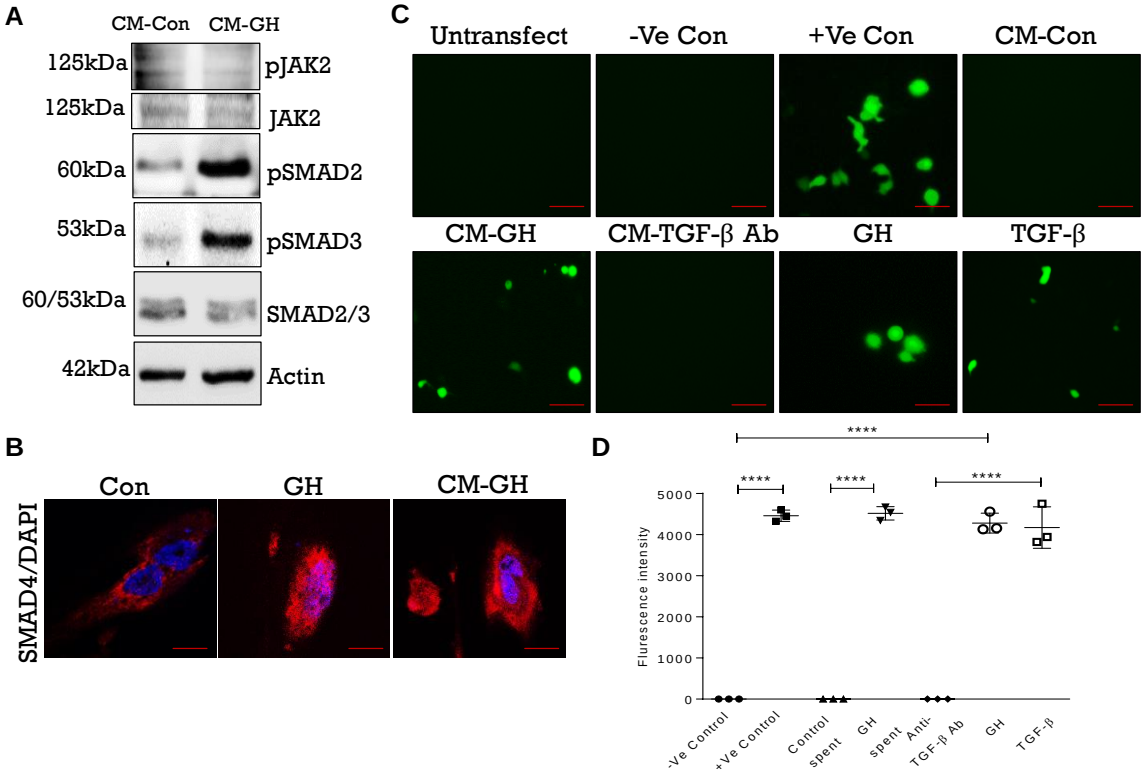

Figure 4. *GH induced TGF-β1 is active in paracrine manner:* A) Western blotting analysis of SMADs in podocyte lysates treated for 30min with conditioned media from GH (CM-GH) treated podocytes for 30min. B) Immunofluorescence analysis performed in podocytes treated with CM-GH for SMAD4 nuclear accumulation. C) SMAD reporter assay performed in podocytes treated with GH, CM-GH, CM-con, TGF-β1 and conditioned media neutralized with TGF-β1 antibody. D) Fluorescence intensities recorded and plotted against un-transfected cells. Each experiment repeated three times and $p<0.05$.

The data suggests the possibility of GH-dependent secretion of TGF-β1 into the conditioned media, which then elicited activation of SMAD2&3. TGF-β dependent regulation of its target genes requires the interaction of SMAD4 with SMAD2&3 and the localization of the

SMAD2/3/4 complex to the nucleus. We observed increased accumulation of SMAD4 in the nucleus following treatment with GH or conditioned media from GH-treated podocytes (Fig. 4B). We next performed TGF-β neutralization studies with podocytes transfected with a GFP expression plasmid under control of the SMAD binding element. Both, GH and conditioned media from GH treated podocytes induced GFP expression in this model (Fig. 4C). However, conditioned media from GH treated podocytes that were pre-incubated with anti-TGF-β1 antibody failed to induce GFP expression (Fig. 4C&D). Together, this data suggests that TGF-β1 is induced by GH that is active in a both autocrine and paracrine manner.

3.7. SB431542 prevents GH induced TGF-β mediated SMAD activation:

Targeting TGF-β mediated SMAD activation have shown to improve renal function [21]. SB431542 has proven to inhibit renal fibrosis and other complications mediated by TGF-β [139]. SB is a small molecule that targets the TGF-β signaling pathway by competing for ATP binding domains in the ALK5 (TGF-βR1) receptor [140].

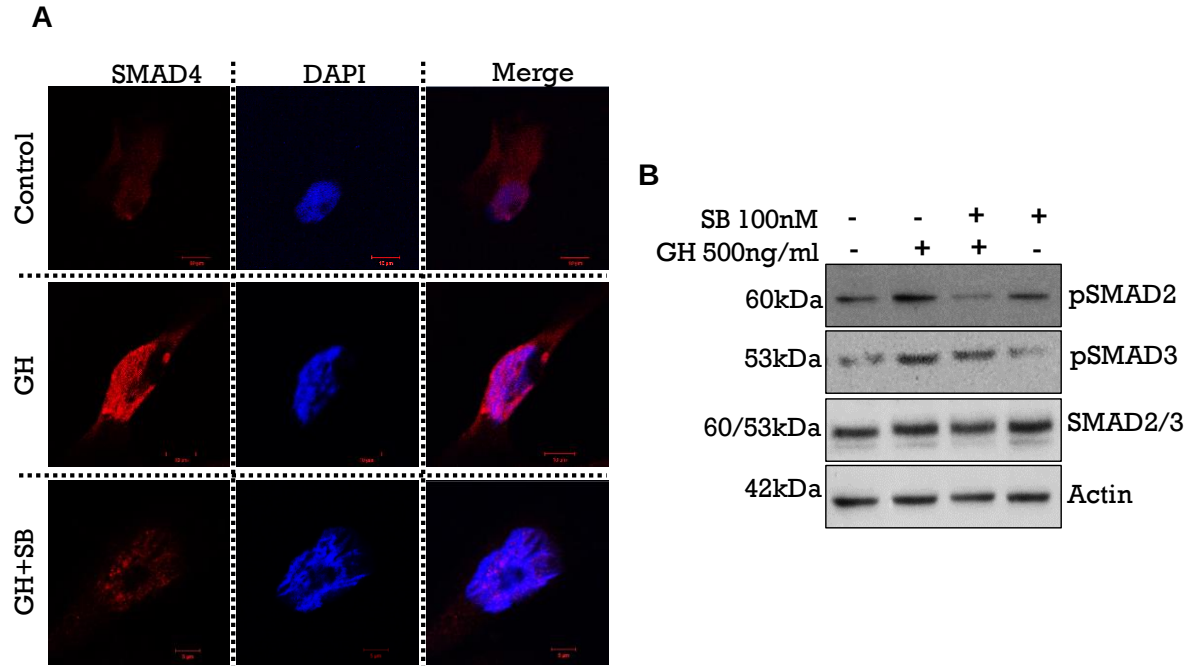

Figure 5. *SB431542 prevents GH induced TGF-β1 mediated SMAD activation in podocytes:* A) Immunofluorescence analysis of SMAD4 in GH for 12h treated in the podocytes pre-treated with SB431542. B) Western blotting analysis of SMAD2&3 in podocytes pretreated with SB431542 for 24h and GH for 30min.

To verify that observed effects of GH on activation of SMADs are mediated via GH-dependent secretion of TGF-β1 in podocytes; we employed the TGF-βR1 inhibitor, SB431542 (hereafter named as SB). SB treatment inhibited SMAD4 nuclear accumulation in both direct treatment with GH and CM-GH (Fig. 5A). SB pre-treatment to podocytes treated either with GH or CM-GH, we observed reduced phosphorylation of SMAD2&3 in the western blotting analysis (Fig. 5B).

3.8. GH alters podocyte morphology and regulates expression of ECM components in podocytes:

Podocytes have a unique cell-architecture. Alterations in podocyte morphology alter filtration properties of GFB that is also depend on ECM composition. Changes in the structural organization of the actin cytoskeleton usually accompany changes in cell size. Profiling of F-actin polymerization in podocytes by Phalloidin staining revealed that both TGF-β1 and GH increased F-actin polymerization in podocytes (Fig. 6A). TGF-βR1 inhibitor (SB) attenuated GH induced increase in podocyte cell size (Fig. 6A). Cell morphology is also influenced by its interactions with ECM proteins and TGF-β1 is known to play a critical role in ECM turnover. Next, we found increased expression of extracellular matrix molecules in podocytes exposed to CM-GH compared to controls (Fig. 6B). Alteration in the podocyte cytoskeleton alters the morphology of the podocytes that results in proteinuria [141]. Podocytes treated with GH or TGF-β1 showed increased permeability to albumin (Fig. 6C). Interestingly, SB pre-treatment prevented the effect of these molecules on podocytes. These results suggest that GH increases podocyte size that in turn alters podocyte permeability.

3.9. Activation of TGF-β/SMAD pathway in GH injected mice:

The overactive TGF-β/SMAD pathway is evident in experimental nephropathy models and in patients with DN. Our *in vitro* data suggest that GH induces TGF-β1 in podocytes and to delineate the *in vivo* effect of GH on TGF-β in podocytes we administered GH to mice for two weeks. We observed an increased glomerular size in GH injected mice compared to controls (Fig. 7A-C & Fig. 8A&B). Histological analysis of kidney from GH injected mice revealed mild to moderate renal fibrosis mesangial expansion, and synechiae formation between bowman's space and glomerular tufts (Fig. 7B-C). There was also a significant decrease in GFR in GH injected animals (Fig. 7D).

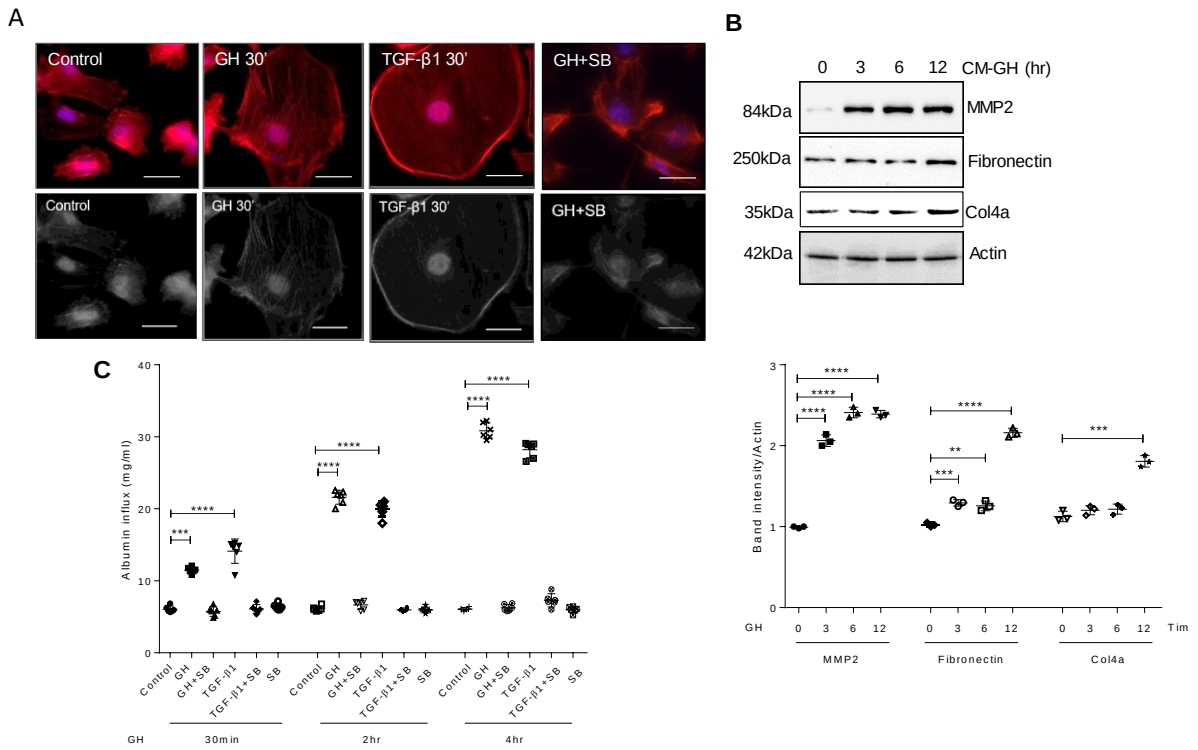

Figure 6. *GH alters podocyte morphology and permeability:* A) Phalloidin staining images of podocytes treated with GH and TGF-β1 for 30min and the representative bright field images of same cells. B) Western blotting analysis of ECM molecules in CM-GH podocyte lysates at varying time points. Representative band intensities were quantified in image J. C) Albumin influx assay in podocytes pre-exposed to SB341542 in the presence of GH and TGF-β1. Asterisk * indicates p value <0.05.

Since TGF-β is a prosclerotic molecule and master regulator of fibrosis, we conducted further analysis to understand the involvement of TGF-β/SMAD signaling for the glomerular hypertrophy and fibrosis in the mice administered with GH. TGF-β1 levels were elevated in platelet-poor plasma preparations from GH injected mice (Fig. 7E). Furthermore, glomerular lysates from GH injected mice revealed an increased abundance of TGF-β1 protein (Fig. 7F) and increased phosphorylation of SMAD2&3 was also observed. Immunohistochemistry analysis demonstrated increased expression of TGF-β1 in both glomerulus and renal tubule (Fig. 7G). Next, we analyzed podocyte architecture by transmission electron microscopy and found that there was effacement of podocyte foot-processes and thickening of GBM (Fig. 8A&C). Urine from GH administered mice have higher albumin to creatinine ratio (UCAR, Fig. 8D) and more

abundance of high molecular weight proteins (Fig. 8E) compared with control mice These results suggest that TGF-β1 could mediate the effect of GH on the glomerular podocytes.

4.0. TGF-βR1 inhibitor attenuates GH induced renal anomalies:

In order to ascertain that the observed GH-dependent glomerular injury is specifically due to TGF-β1, we employed the TGF-βR1 inhibitor (SB) in our further studies. SB treatment alleviated the GH-induced glomerular hypertrophy and increases in glomerular tuft area (Fig.8A&B).

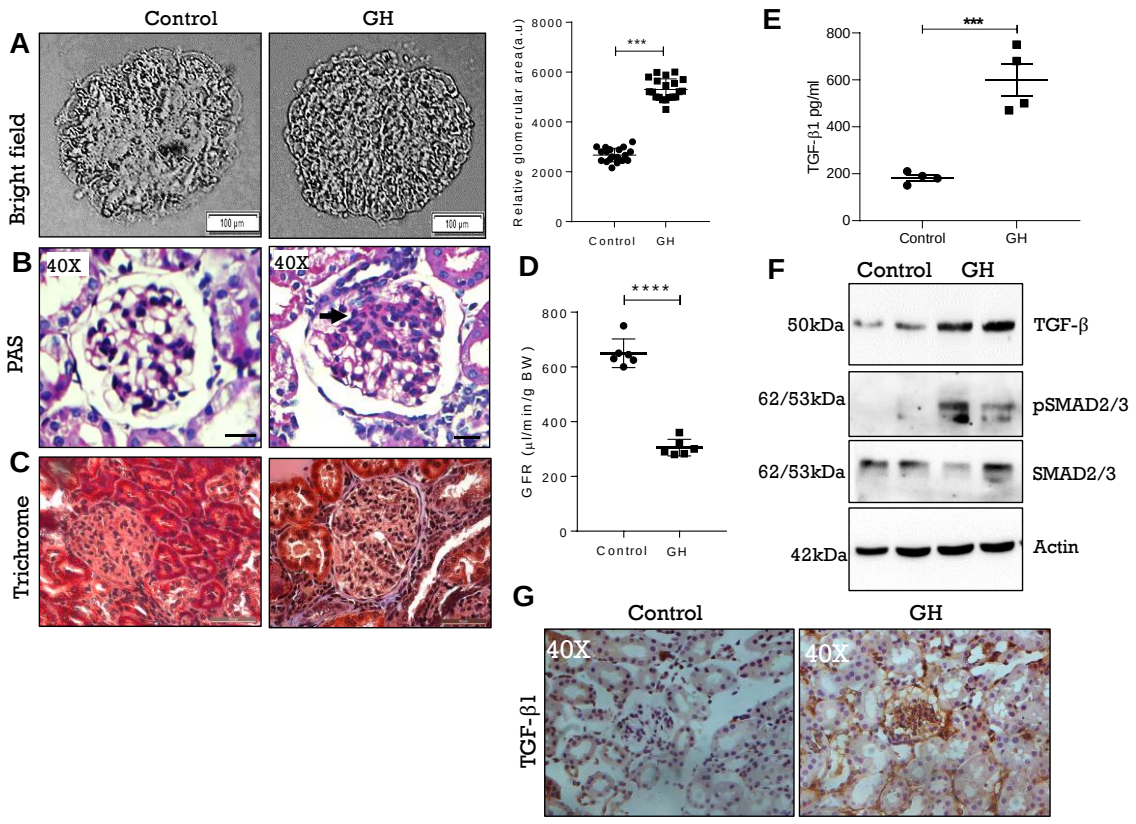

Figure 7. *GH administration induced glomerular hypertrophy and fibrosis is associated with overactive TGF-β signaling:* A-C) Histological analysis of glomeruli from GH injected mice by various staining techniques. Glomerular area was quantified in GH injected mice and presented data was analyzed for 3 glomeruli from each mice n=5. D) Glomerular filtration rate (GFR) estimated in the urine samples from GH injected mice. E) Elisa analysis of TGF-β1 in the plate poor plasma samples of GH injected mice. Data from n=6 per group. **** indicate $p<0.0001$. F) Western blotting analysis of TGF-β/SMAD signaling in glomerular lysates from GH injected mice. G) Immunohistochemical analysis of TGF-β1 in kidney cortical sections.

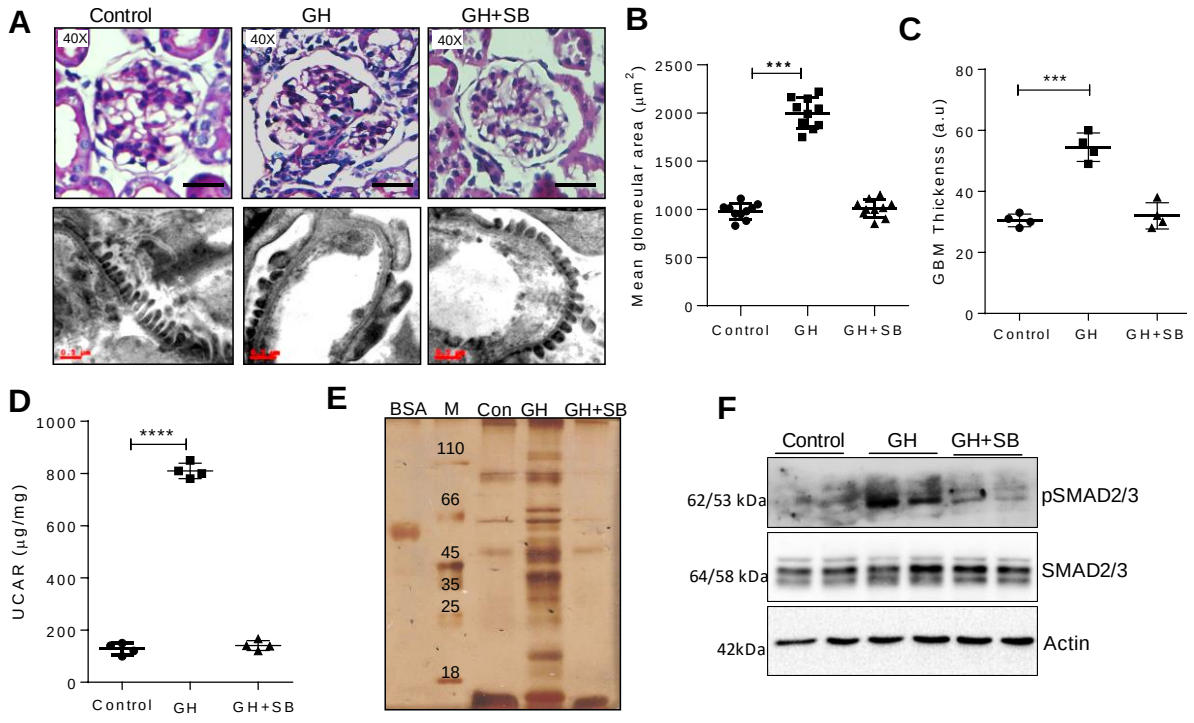

Figure 8. *SB431542 administration inhibits GH mediated renal aberrations and preserved podocyte architecture:* A) PAS staining in SB injected mice and representative quantification of glomerular area. TEM images of podocyte architecture in GH-injected mice and representative quantification of GBM and foot processes of podocytes. B) Western blotting SMAD2&e protein analysis of glomerular lysates from SB treated mice. C) Urine albumin creatinine analysis to assess the high-molecular proteins. Images presented here are the mean of n=6, *** p>0.001.

Moreover, SB treatment preserved podocyte foot processes comparable to control mice and attenuated the thickness of GBM (Fig. 8A&C). Urine analysis revealed that SB treatment could prevent loss of high molecular weight proteins in GH injected mice (Fig. 8D&E). SB treatment also resulted in decreased activation of SMAD2&3 (Fig. 8F). These results demonstrate that GH induces glomerular hypertrophy, alterations in podocyte morphology, and impairment of renal function as evidenced by increased UCAR and decreased GFR and inhibition of TGF-βR1 could prevent certain GH mediated adverse effects on the podocyte and the glomerulus.

4.1. Targeting GHR in podocytes ameliorated diabetic renal complications:

Elevated GH levels in the T1DM accelerated the development of DN. Studies also showed that mice expressing non-functional GHR ameliorated the development of DN. GH transgenic mice developed glomerular hypertrophy with increased podocyte size. To study that targeting GHR in podocytes could preserve the glomerular size and SMAD pathway activation (pSMAD3), we created a conditional GHR knockout mouse (pGHR$^{-/-}$) specific to the podocytes using Cre-lox P system (Fig. 9A). We induced diabetes in these mice by injecting streptozotocin 2mg/kg body weight/ day for 2weeks and observed for early changes in the renal morphology. These mice upon the setting of diabetes showed enlarged kidneys (Fig. 9B).

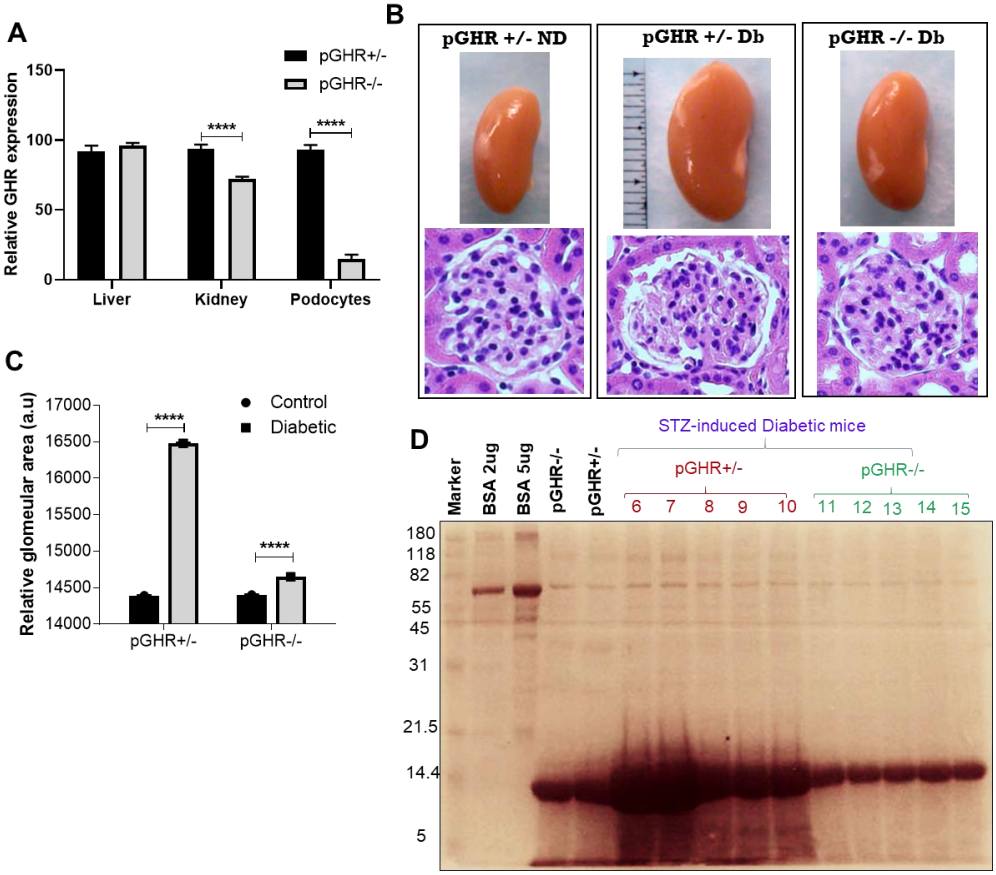

Figure 9. *Conditional deletion of GHR in podocytes preserve glomerular function:* A) mRNA analysis of GHR expression in conditional knockout mice. B) Kidney images from conditional hetero and homozygous knockout for GHR in podocytes with and without streptozotocin (STZ) injection. Representative glomerular morphology by H&E staining. C) Relative glomerular size was estimated by image J. D) Silver staining analysis of urine collected from these mice and run along with BSA standards. Images represent n=6 per group and **** indicate p<0.0001.

When we assessed for glomerular hypertrophy by H&E staining, we found that these mice had glomerular hypertrophy (Fig. 9B&C). We next studied the proteinuria in these mice under diabetic settings and we observed increased amounts of albumin in the urine collected from the heterozygous mice for GHR (Fig. 9D). While the proteinuria was ameliorated in pGHR$^{-/-}$ mice, We, next investigated whether SMAD pathway is inhibited in these mice. We stained for activated SMAD3 (pSer423&425) and found that SMAD3 levels were increased in GHR heterozygous mice (pGHR$^{+/-}$) compared with pGHR$^{-/-}$ mice (Fig. 10). These results strongly suggest that direct action of GH on podocytes could be enough for renal hypertrophy and proteinuria that could be possibly mediated by TGF-β/SMAD signaling in podocytes.

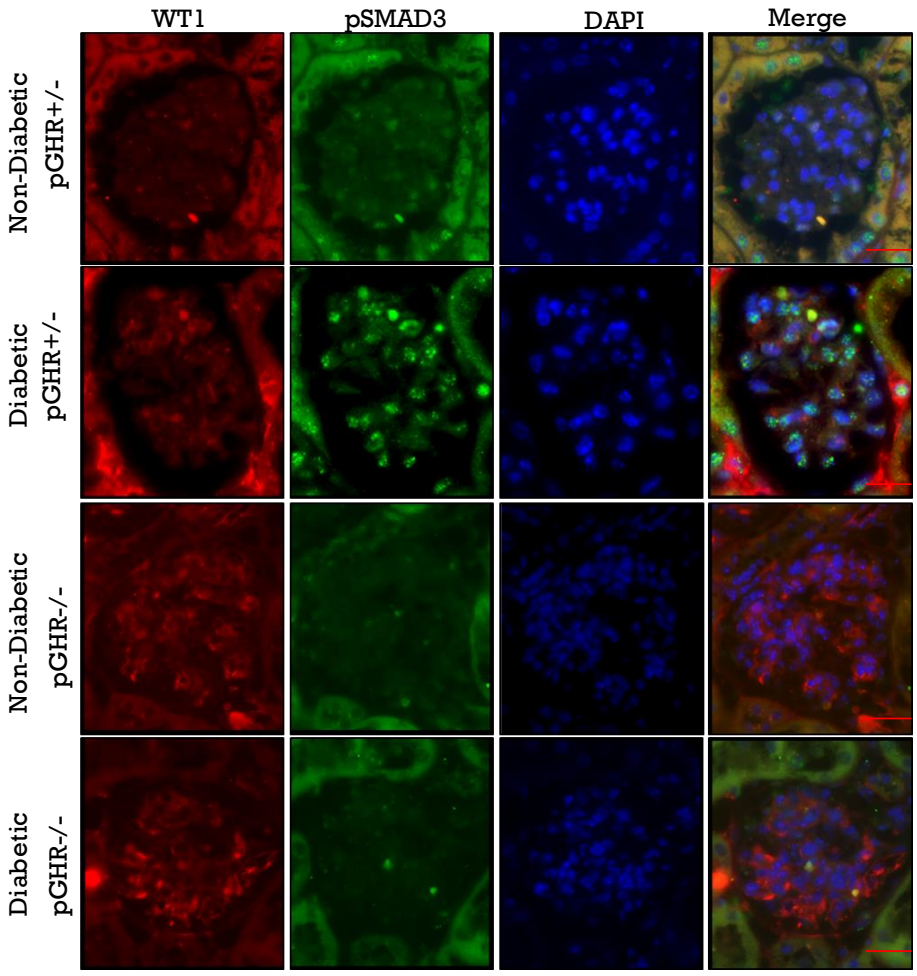

Figure 10. *Targeting GHR in podocytes diminishes SMAD activation:* Immunofluorescence analysis of pSMAD3 in glomerular sections from pGHR$^{+/-}$ and pGHR$^{-/-}$ mice with and without diabetes. Images represent n=6 per group.

4.2. HIPK2 mediates the GH induced TGF-β1 signaling in podocytes:

Homeodomain interacting protein kinase2 (HIPK2) is an upstream regulator of cell death and repair mechanisms. Recently, HIPK2 was shown to regulate hypertrophy and proliferation [142]. To study whether HIPK2 transduces GH induced TGF-β1 expression**,** we investigated the HIPK2 expression with GH in podocytes. We found increased HIPK2 expression in podocytes in time dependent manner (Fig. 11A). Our microarray data (GSE21327) also revealed that GH induces HIPK2 in podocytes (Fig. 11B). We analyzed the expression of TGF-β1 in the HIPK2 knockdown conditions. To study this, we transfected HIPK2 shRNA into the HEK293T cells. GH fails to induce TGF-β1 expression in HIPK2 knockdown cells suggesting that GH induced TGF-β1 expression is at least partially mediated via HIPK2 (Fig. 11C). These results demonstrate the essential role of HIPK2 in GH induced TGF-β1 expression.

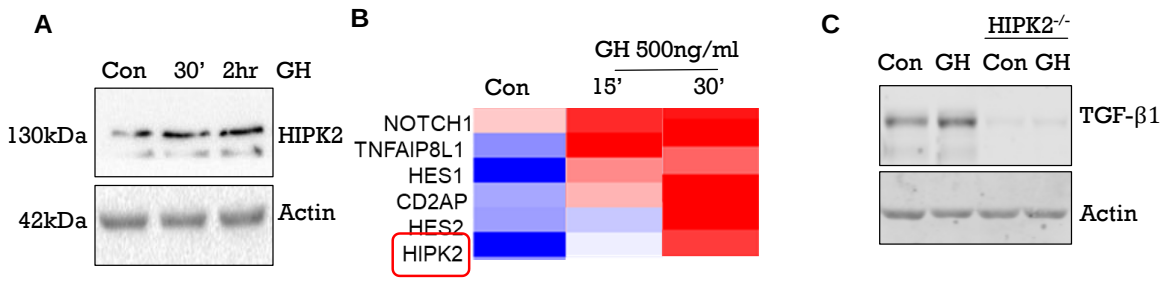

Figure 11. *GH induces HIPK2 which mediates TGF-β expression:* A) Western blotting analysis of HIPK2 in podocytes treated with GH (500ng) for various time points. B) Microarray data in podocytes exposed to GH for 15min to 30min. C) western blotting analysis of TGF-β in HEK cells transfected with shRNA for HIPK2 in the presence or absence of GH (500ng). Immunohistochemical analysis of HIPK2 in glomerular sections form diabetic patients.

Infiltration of inflammatory cells and activation of innate immunity pathways have now been recognized as adverse events during the course of DN [23, 117, 121]. Available literature suggests a controversial role of GH in the inflammation. Genome-wide analysis of the GH-transgenic mice revealed elevated inflammatory cytokines in the kidney [143]. These transgenic mice also showed elevated expression of IL-6 and TNF-α in the adipose tissue [144]. Another study indicates that long-term treatment of GH in GH deficient patients showed reduced levels of inflammatory cytokines and c-reactive protein [145, 146]. Our studies with GH injected mice exhibited infiltration of non-native glomerular cells into the glomerular region. It was reported

that podocytes undergo EMT secrete certain chemokines, however, the detailed cytokine profile is not known (reference is needed here as you said). Therefore, we investigated the inflammatory role of podocytes with GH treatment.

4.3. RNA sequencing of podocytes treated with GH yielded limited number of transcripts:

To identify the early transcriptomic response of GH and to investigate the cytokine profile, we treated differentiated human podocytes with GH for 30min and total RNA was isolated for RNA-sequencing.

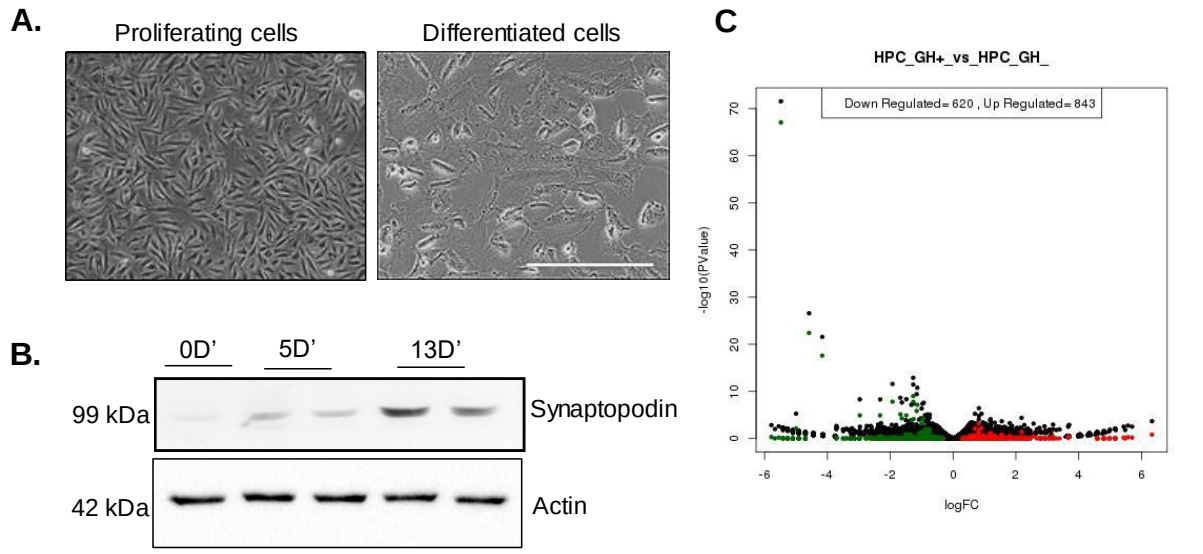

Figure 12. *Differentially expressed genes in podocytes treated with GH:* A) Images represent morphological changes in the differentiated podocytes. B) Western blotting analysis of synaptopodin, a differentiation marker expressed by podocytes. C) Volcano plot demonstrating differentially expressed genes in GH treated podocytes.

Sample name	AKP HPC_-GH1	AKP HPC_-GH2	AKP HPC_+GH1	AKP HPC_+GH2
Total reads	60624674	67392900	57087000	61772430
Total mapped	56381584 (93.0011%)	62083328 (92.1215%	53693828 (94.0561%	55320993 (89.5561%)
Multiple mapped	1707734 (2.8169%)	1817807 (2.69733%)	1855968 (3.25112%)	1755077 (2.8412%)
Uniquely mapped	54673850 (90.1842%)	60265521 (89.4241%	51837860 (90.805%)	53565916 (86.7149%)
Read-1	27298410 (45.0285%)	30210770 (44.8278%	25918930 (45.4025%	26696091 (43.2168%)
Read-2	27375440 (45.1556%)	30054751 (44.5963%	25918930 (45.4025%	26869825 (43.4981%)
Reads map to '+'	27329428 (45.0797%)	30113591 (44.6836%	25881166 (45.3364%	26767459 (43.3324%)
Reads map to '-'	27344422(45.1045%)	30151930 (44.7405%	25956694(45.4686%	26798457(43.2168%)
Splice reads	22097227 (36.4492%)	23475489 (34.8338%	20457470 (35.8356%	19425389 (31.4467%)
Non-splice reads	32576623 (53.7349%)	36790032 (54.5904%	31380390 (54.9694%	34140527 (55.2682%)

Table1. *RNA sequencing mapping statistics:*

Prior submission of samples to the sequencing facility, we assessed differentiation percentage by cell morphology and differentiation markers such as synaptopodin (Fig. 12A-B). RNA-sequencing was performed on Illumina 2500 Hi-Seq platform and gene expression was quantified using 'fragments per kilobase per million reads' (FPKM) method. In our mapping statistics, 90% of total reads in control samples were uniquely mapped to the human reference genome, whereas 87% of the total reads were uniquely mapped in the case of GH treatment (Table 1).

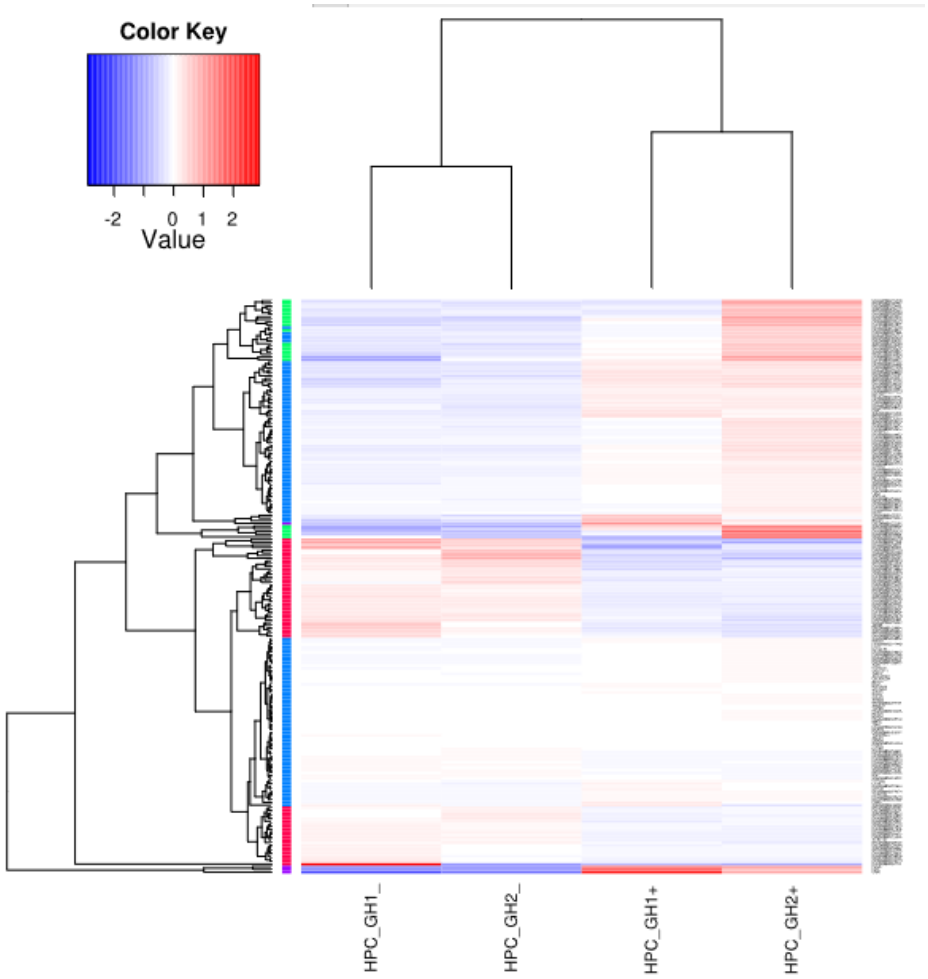

Figure 13. *Hierarchical clustering analysis of differentially expressed genes:* Color code from blue to red indicates downregulation to upregulation of a cluster of genes.

This indicates that our RNA-sequencing depth has crossed the threshold to detect minor expression levels or low abundant transcripts have also been sequenced [147]. At the sequencing depth of above 50 million reads, the percentage of more than 80% open reading frames covered

[147]. The datasets of duplicate samples are highly correlated from control to GH treated podocytes and were used to derive respective FPKM values. We considered genes with the FPKM value of minimum 1, as expressed and found a total of ~12580 genes expressed in control and ~12532 in GH treated podocytes.

4.4. GH signaling involves the expression of early target genes in podocytes:

While many of the genes reported previously in response to GH treatment in podocytes, this study we documented several novel early genes. For instance, GH induces Fos ligand, Egr1 and ATF3 expression in preadipocytes within 30min while other target genes including Igf1 expressed in 48h of GH treatment [148].

Hierarchical clustering and heatmap analysis to show the gene expression patterns between control vs GH treatment (Fig. 13). Using the edgeR -sequencing pipeline, we found 30 genes were differentially expressed and their significance was expressed as p value <0.05 or FDR value (Fig. 14A&B). Absolute fold change cutoff value of one in $\log_2$ scale was set up to determine the up and downregulated genes and found 25 genes were upregulated and five genes were downregulated with GH treatment. These upregulated genes are including Fos family and chemokine ligands.

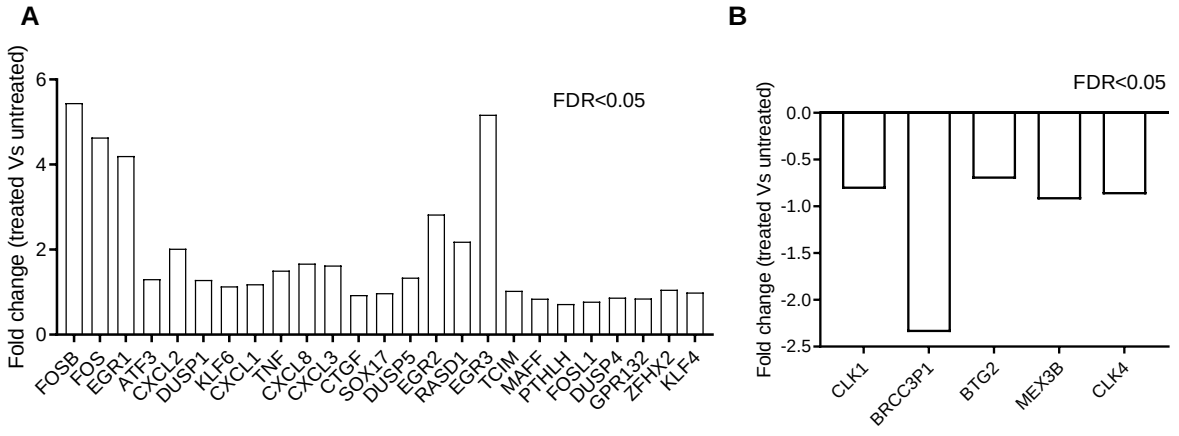

Figure 14. *Significantly expressed genes in GH treated podocytes:* A) Fold change of significantly upregulated genes in GH (500ng/ml) treated podocytes. B) Fold change of significantly downregulated genes in GH treated podocytes. Experiment performed in duplicates and the data presented in mean values after normalization and the FDR value is <0.05.

4.5. Identification of Biological Process, Molecular Function, and Cellular Component:

Gene Ontology (GO) analyses were carried out to determine molecular processes and biological pathways that are associated with differentially expressed genes in GH treated podocytes. The GO terms that are analyzed, biological process (BP), molecular function (MF) and cellular component (CC) for differentially expressed genes were performed in Gorilla software, which is web, based software. The GO-BP enriched terms are including cell-cell adhesion ($p<7.70E-07$), cell-cell signaling($p<0.00016$), negative regulation of ERK1/2, MAPK pathways and chemokine mediated signaling (Fig. 15A). GO-MF terms enriched are including chemokine activity, calcium ion binding, MAPK serine/threonine/tyrosine phosphatase activity and CXCR-chemokine receptor binding (Fig. 15B). GO-CC enriched terms are including integral components of the plasma membrane, extracellular region, neuron projection membrane and nucleosome (Table 2).

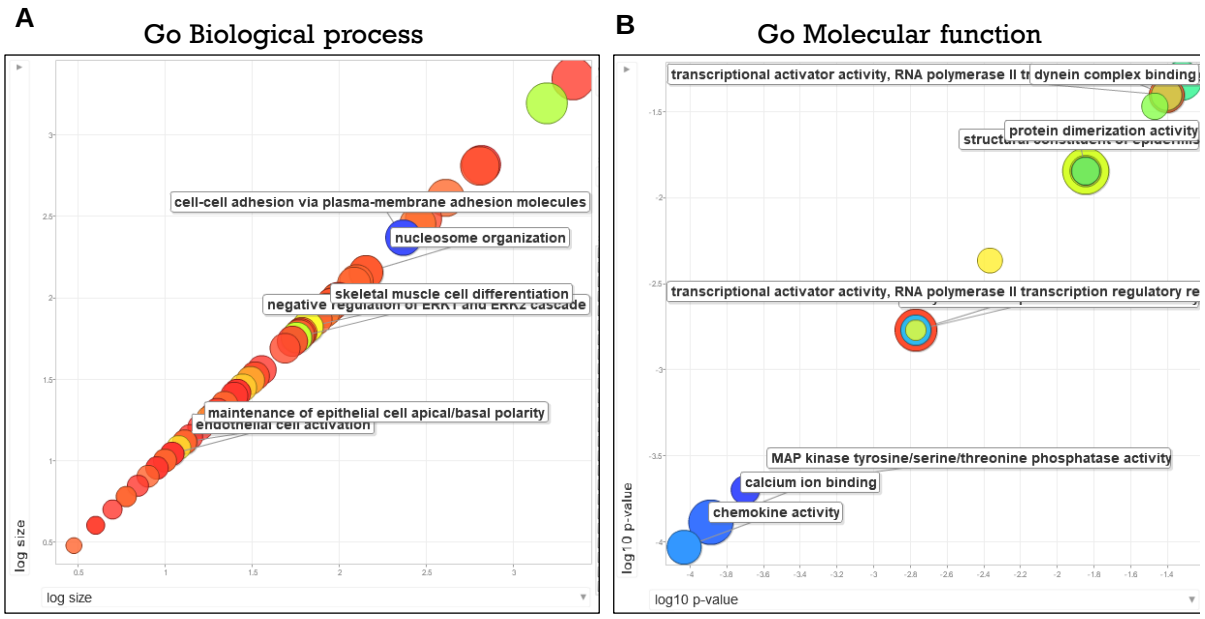

Figure 15. *Gene ontology terms, biological process and molecular function:* A) Gene ontology biological process (GO-BP) in GH treated podocytes. B) Gene ontology molecular function in podocytes treated with GH. Graphs were generated in dcGO web-based software.

goId	goName	No. of inp	Total annotated genes	p value
GO:0005887	integral component of plasma membrane	38	181	0.0004
GO:0005576	extracellular region	78	569	0.0027
GO:0032589	neuron projection membrane	4	8	0.0088
GO:0000786	nucleosome	6	17	0.0099
GO:0005796	Golgi lumen	5	13	0.0124
GO:0097060	synaptic membrane	11	46	0.0145
GO:0030315	T-tubule	4	11	0.0312
GO:0044304	main axon	4	11	0.0312
GO:0009897	external side of plasma membrane	6	22	0.036
GO:0014731	spectrin-associated cytoskeleton	2	3	0.0379
GO:0032809	neuronal cell body membrane	2	3	0.0379
GO:1904724	tertiary granule lumen	3	7	0.0389
GO:0043204	perikaryon	5	18	0.0505

Table 1. *GO-cellular component in GH treated podocytes:*

4.6. Validating RNA sequencing analysis by quantitative real-time PCR:

Differentially expressed genes were taken for further validation by qPCR. We made the cDNA from the same RNA which was used as input for RNA sequencing. Our validation experiments showed upregulation of Fos ligands and chemokine ligands (Fig. 16A-D**)**. While many independent studies demonstrated Fos ligands and ATF3 expression by GH but their role

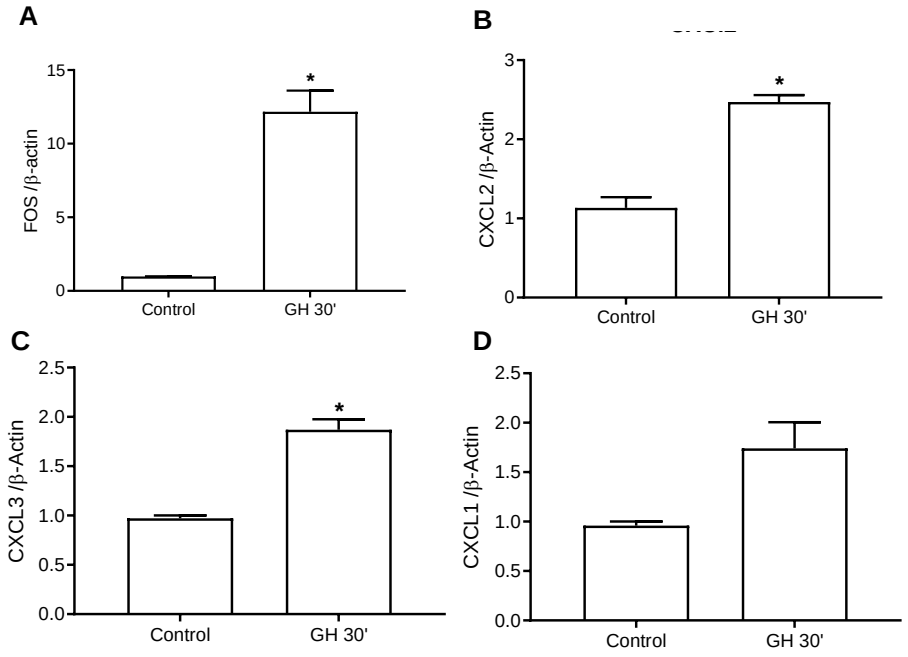

Figure 16. *Validation of RNA sequencing data:* Quantitative PCR analysis of FOS and chemokine ligands in RNA sequencing samples. A) FOS, B) CXCl2, C) CXCL3 and CXCL1. * indicates $p<0.05$.

in podocytes remained to be addressed.

4.7. KEGG pathway enrichment analysis identified IL-17 pathway activation in podocytes:

To study the pathways that are controlled by these differentially expressed genes we performed KEGG pathway analysis. KEGG utilizes all available literature, data files, metabolomics and metagenomics for predicting the significantly enriched biological pathways that are regulated by the differentially expressed genes. KEGG predicts pathways based on the number of genes that covers the total annotated genes in a biological pathway. Our analysis revealed that GH treatment activates IL-17 pathway in podocytes with the highest significance and number of genes covered (Fig. 17A&B).

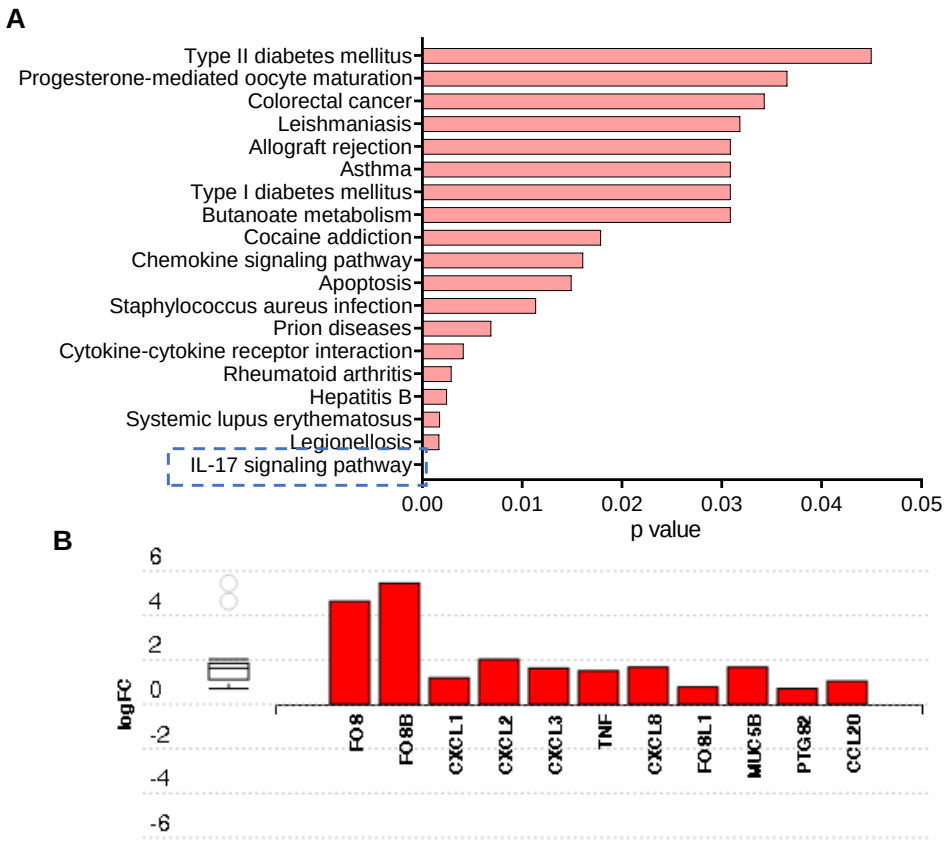

Figure 17. *KEGG pathway analysis of upregulated genes:* A) KEGG pathway analysis performed in GH treated podocytes. B) Transcripts plotted in a graph that are fell in IL-17 pathway in KEGG analysis.

IL-17 family is consisting of a subset of cytokines that are IL-17 A to F and their role is yet to be understood. IL-17A has a widely studied molecule among the other cytokines of this family. Recently studies begin to understand the role of IL-17A cytokine in podocytes. However, IL-17 is involved in the inflammatory pathway particularly, T-helper cells mediated auto-immune pathology.

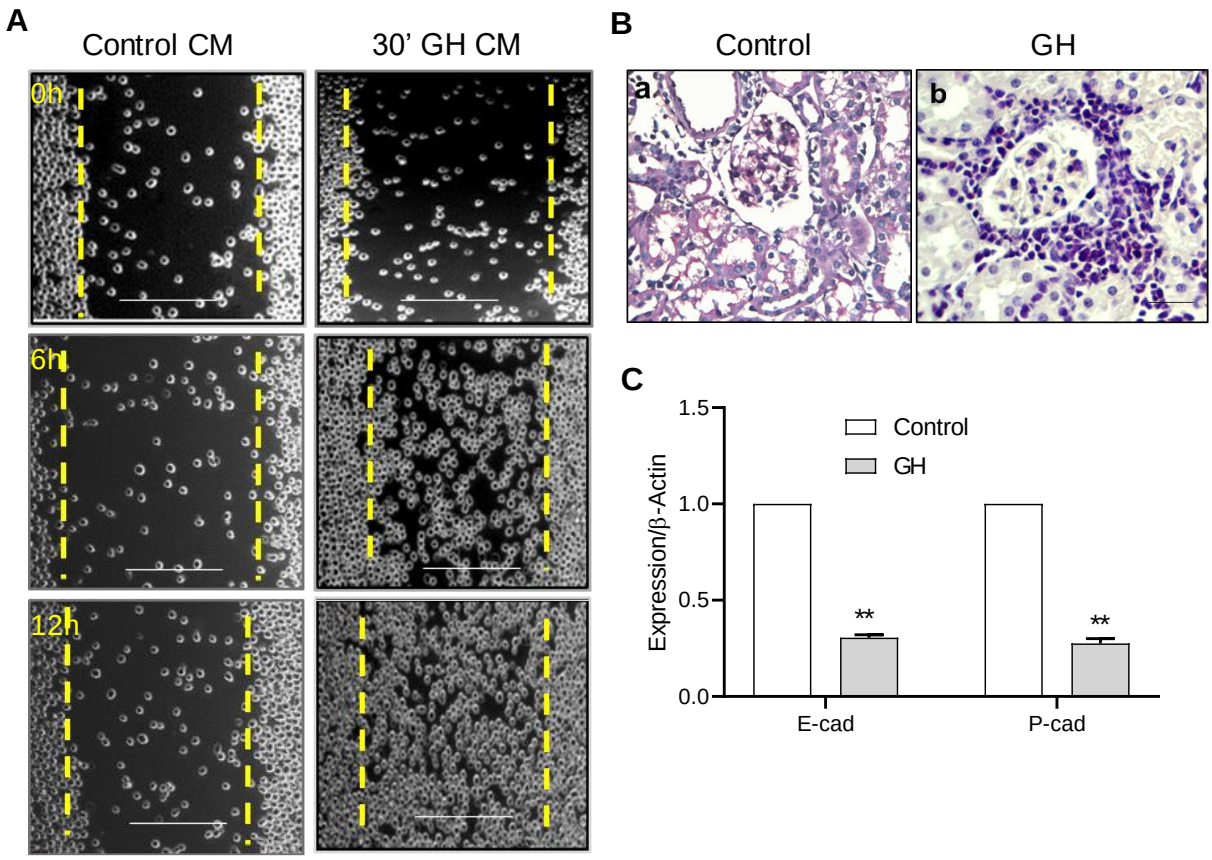

Figure 18. *Validation of gene ontology terms:* A) Migration assay performed in J774 cells with conditioned media from GH treated podocytes for 30min. Conditioned was prepared after 30min of GH treatment to podocytes and filtered through 0.2µm membranes. J774 cells were grown till 70-80% confluence and then made scratch with 0.1ml pipette tip. Cells were directly incubated with conditioned median for various time points and observed migration towards scratch. B) H&E analysis of glomerular sections from GH injected mice. C) Quantitative PCR analysis of E-cadherin and P-cadherin in podocytes treated with GH.

4.8. Conditioned medium from GH treated podocytes induced J774 migration:

To validate the GO-MF term chemokine activity, we treated podocytes with GH for 30min, and conditioned medium was prepared. Furthermore, we have grown J774 macrophages and treated with the conditioned medium prepared from podocytes treated with GH and observed for macrophage movement towards the scratch made (Fig. 18A). Additionally, we validated this phenomenon in animals injected with GH and showed massive cellularity in the glomerulus (Fig. 18B). To validate the GO-BP term cell-adhesion via plasma membrane by adhesion molecules, we treated podocytes with GH and found that the expression of cell adhesion molecules particularly, cadherin molecules (E-cadherin) decreased (Fig. 18C). We previously reported this observation in podocytes, where we have shown that GH induces ZEB2/ZEB2-NAT and decreased cadherin molecules particularly, E-cadherin and P-cadherin that lead to epithelial to mesenchymal transition [73]. Similarly, E-cadherin expression was decreased in glomerular lysates from GH injected mice (Fig. 2B).

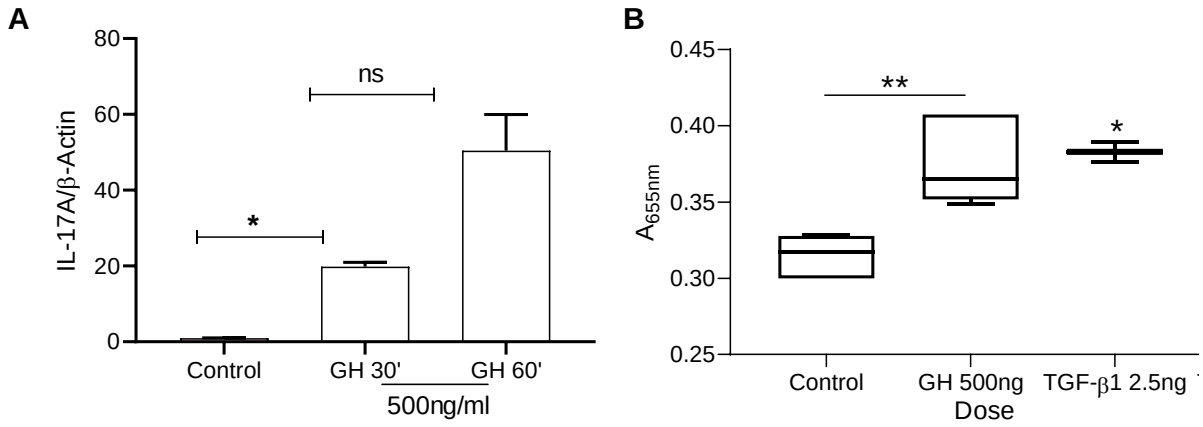

Figure 19. *GH induces activation of IL-17 pathway:* A) Quantitative PCR analysis of IL-17A expression in podocytes treated with GH for 30min and 60min. B) IL-17 reporter assay in GH treated for 30min in IL-17RA/RC-HEK Blue cells and the conditioned media was analyzed for the presence of secreted embryonic alkaline phosphatase (SEAP). Experiments repeated at least three times.

4.9. GH induces IL-17 pathway activation:

The role of IL-17A in the development and progression of DN has recently been started understanding. Past recent studies reiterate that IL-17 signaling plays a crucial role in recruiting inflammatory infiltrates into the glomerulus particularly DN. Our qRT-PCR analysis confirmed

that IL-17A is expressed in podocytes treated with GH in time dependent manner (Fig. 19A). Next, we studied whether GH included IL-17 is functionally active. To study this we used specialized cells called HEK-Blue which express three components that are important for IL-17 signaling, they are IL-17RA/RC heteromeric receptor, Act1 adapter protein and serum embryonic alkaline phosphatase (SEAP) that was cloned under five repeats of NF-kB and AP1 binding elements. We seeded these cells $5x10^3$ cells/well in 96-well plate prior to treatment with GH and TGF-β1. The following day we treated HEK-Blue with GH and TGF-β1 for 30min and collected 20ul of media from these cells and performed SEAP-reporter assay for the detection of IL-17. We observed that HEK-blue cells treated with GH showed IL-17 expression in 30min of GH or TGF-β1 treatment. This result delineates IL-17 expression, secretion and functional activity of IL-17 in both GH and TGF-β1 treatment conditions.

Summary

Summary

The kidney is a very complex organ and understanding the pathophysiology of kidney and exploring the modes of treating kidney ailments are difficult due to the inherent complexity of the organ. Podocytes are the key cell type that principally contributes to the renal permselectivity besides offering structural support to the filtration apparatus. Being, a crucial cell type and central to various functions of the glomerulus, podocytes gaining interest as a key regulator of the strict function of the glomerulus. It is noteworthy that podocytes vis-à-vis glomeruli are injured during diabetic kidney diseases. Hallmarks of diabetic kidney diseases are podocyte hypertrophy, in turn, glomerular hypertrophy, and glomerulosclerosis.

Diabetes mellitus, particularly T1DM is presented with elevated GH levels owing to the absence of a negative feedback loop by IGF-1 [75]. Several components of the GH system including GH and GHR are elevated in the diabetic kidney [43, 149]. Studies from our laboratory reported a direct action of GH on podocytes and activation of canonical JAK-STAT signaling [54]. Supraphysiological levels of GH are implicated in the glomerular hypertrophy. Administration of GH to mice elicited glomerular hypertrophy with a significant increase in the thickness of the glomerular basement membrane (GBM). GH transgenic mice showed significant podocyte hypertrophy and sclerosis rather than hyperplasia ruling out the contribution of podocyte proliferation towards glomerulomegaly.

Increasing evidence suggests that renal hypertrophy in diabetic subjects is associated with podocyte hypertrophy that is preceded by the thickness of the GBM [83]. Since podocytes adhere to the basement membrane, the altered composition of the basement membrane during diabetic milieu negotiates podocyte interaction, which may lead to impaired podocyte function and proteinuria. The composition of GBM is determined by podocytes and endothelial cells, which together contribute to more than 50% of the core GBM. Podocyte hypertrophy and enhanced secretion of ECM proteins in turn thickening of GBM could be one of the possibilities for glomerular hypertrophy in diabetic conditions.

Our laboratory longed an interest in understanding the molecular and cellular basis for podocyte hypertrophy and resultant glomerular hypertrophy in the early course of diabetic nephropathy. Since elevated GH levels are implicated in the pathogenesis of diabetic

nephropathy, and direct action of GH on podocytes, it is obvious to suspect the contribution of GH towards podocyte hypertrophy and/or glomerulosclerosis. Among the three possible modes of tissue growth by which GH may contribute to glomerular hypertrophy (as we hypothesized in the introduction chapter), we exclude the possibility of podocyte hyperplasia. We are unable to demonstrate that GH elicits podocyte proliferation both *in vitro* and *in vivo*. However, our data support that GH induces both podocyte hypertrophy and accumulation of extracellular matrix (ECM) and glomerulosclerosis (Fig. 20).

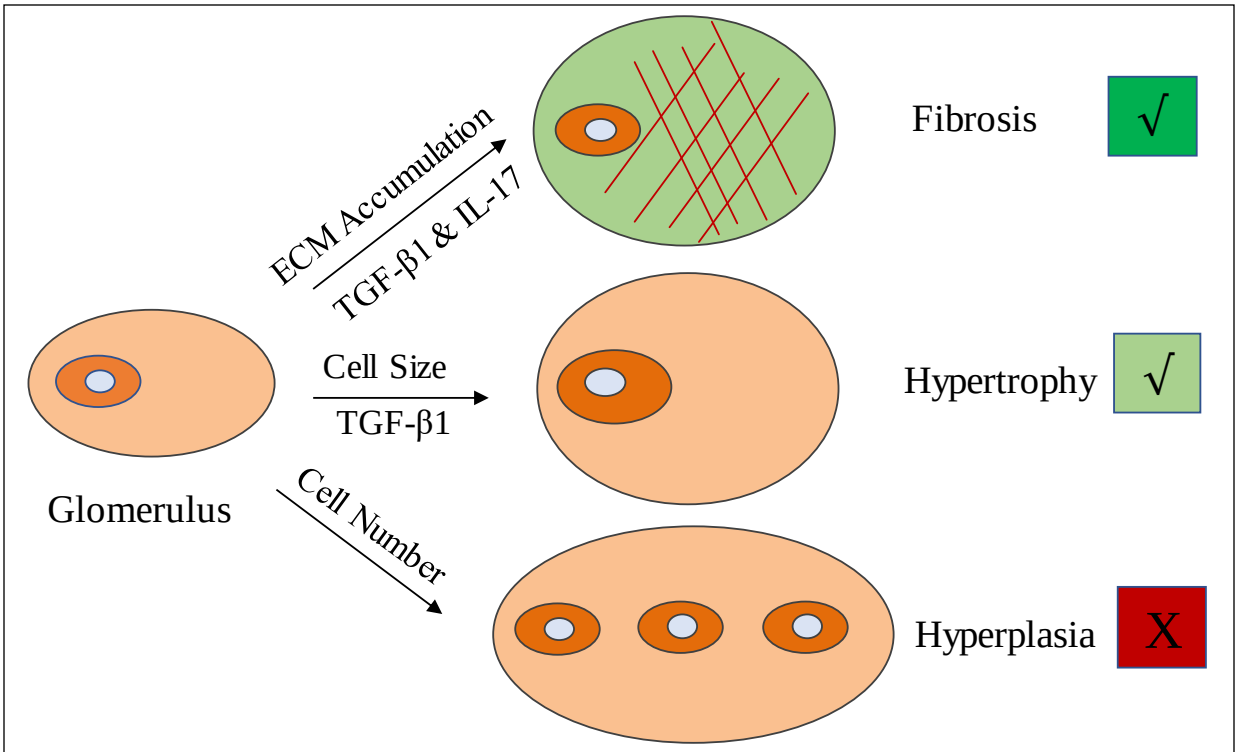

Figure 20. *Summary of the possible modes that GH elicits glomerulomegaly:* GH exposed to podocytes alter ECM turnover via inducing TGF-β1 expression. Podocyte hypertrophy is the prior event in the onset of DN and our study revealed that in part action of TGF-β podocytes undergo hypertrophy upon exposure to GH. IL-17 promotes renal fibrosis by activating pro-fibrotic pathways. However, the role of GH-induced IL-17 secretion need to be fully studied. We ruled out the possibility of hyperplasia of podocytes by various methods. Together, ECM accumulation and podocyte hypertrophy are elicited by GH that could be the possible mechanism for glomerulomegaly in the DN and GH administered mice.

The crux of my doctoral study is to demonstrate that GH induces TGF-β1, which could serve as a growth factor and pro-sclerotic molecule or both. Our study revealed that GH induces TGF-β1 in podocytes (*in vitro*) and contributes to podocyte hypertrophy and GH induced ECM accumulation and glomerulosclerosis (*in vivo*). Podocytes exposed to GH or TGF-β1 showed an increase in cell size and compromised their permselectivity. Mice exposed to GH or TGF-β1 showed proteinuria. Conditional knock-out mice for GHR in podocytes are protected from an increase in kidney size and proteinuria. Studies with GH transgenic mice revealed excess secretion of inflammatory cytokines and immune cell infiltration into the glomerulus [90, 143]. Similar to the earlier studies, we observed immune cell infiltration in GH injected mice. Fittingly, our RNA sequencing analysis revealed an elevated expression and secretion of IL-17 in podocytes exposed to GH. IL-17 is a chemotactic factor that elicits the migration of immune cells including macrophages.

The major observations in my doctoral studies are:

- GH induces BIGH3, a component of TGF-β latent complex: We determined that GH induces BIGH3 expression which stabilizes TGF-β1. Our study also revealed that GH induces several cell adhesion molecules and ECM components such as collagen type2α1, integrin E, laminin α3/β3, Pecam, MMP3, and MMP12 in human podocytes.
- GH induces TGF-β1/SMAD signaling in human podocytes: GH treatment in podocytes induces TGF-β1 in both dose and time-dependent manner. Furthermore, exposure to GH increased phosphorylation of SMAD2&3 in podocytes and nuclear localization of activated SMAD complex.
- GH induced TGF-β1 in podocytes acts in both autocrine and paracrine manner: Besides GH, conditioned media from GH-treated podocytes also induced phosphorylation of SMAD2&3 in podocytes naïve to GH. This suggests the possibility of GH-dependent secretion of TGF-β1 into the conditioned media, which then elicited activation of SMAD2&3. Both, GH and conditioned media from GH treated podocytes induced nuclear accumulation of active SMAD complex, suggesting that TGF-β1 that was induced in the GH-dependent manner can act in both autocrine and paracrine manner.

- GH impaired podocyte permselectivity: Podocytes treated with GH showed increased permeability to albumin across a podocyte monolayer. Interestingly, prior treatment of podocytes with SB431542 (TGF-βR1 inhibitor) abrogated the observed effect of GH on permeability.
- GH induced differential expression of EMT markers: An increased expression of EMT markers (ZEB1, ZEB2, FSP1, and N-cadherin) were observed in association with reduced expression of E-cadherin in podocytes exposed to GH.
- GH altered podocyte morphology and regulates expression of ECM components in podocytes: GH treated podocytes showed increased cell size. TGF-β1 also induced an increase in podocyte size. SB431542 attenuated GH induced increase in podocyte size. We found increased expression of extracellular matrix molecules in podocytes exposed to GH.
- HIPK2 mediates GH induced TGF-β1 secretion by podocytes: Our microarray analysis revealed that podocytes exposed to GH induced HIPK2 expression. Furthermore, GH treatment to the HIPK2 knockdown cells could not rescue the expression of TGF-β1.
- GH activated TGF-β/SMAD signaling in vivo: GH injected mice showed increased TGF-β1 levels in the serum and glomerular lysates. We also observed that elevated TGF-β expression activates SMAD signaling in GH injected mice.
- Administration of SB431542 preserved podocyte architecture in mice: GH injected mice exhibited severe complications in podocyte-GFB integrity. Administration of SB431542 could suppress these aberrations and preserved the integrity of podocyte-GFB which is evidenced by protein-free urine.
- Targeted deletion of GHR in podocytes attenuated glomerulomegaly and SMAD signaling: We observed that podocyte-specific GHR knockout mice showed normal glomerular architecture that was associated with diminished SMAD3 activation under the diabetic settings.
- TGF-β1 induced re-activation of Notch-signaling in podocytes: We observed that GH-induced TGF-β1 expression re-activated Notch1 signaling in podocytes that in turn activated de-differentiation of podocytes.

➢ Podocytes secrete IL-17 upon stimulation with GH: We found that GH-induces IL-17 expression in podocytes. Further, we have validated the IL-17A expression and secretion by GH. We speculate that IL-17 elicits enhanced migration of immune cells into the glomerulus and activate pro-sclerotic fibroblasts.

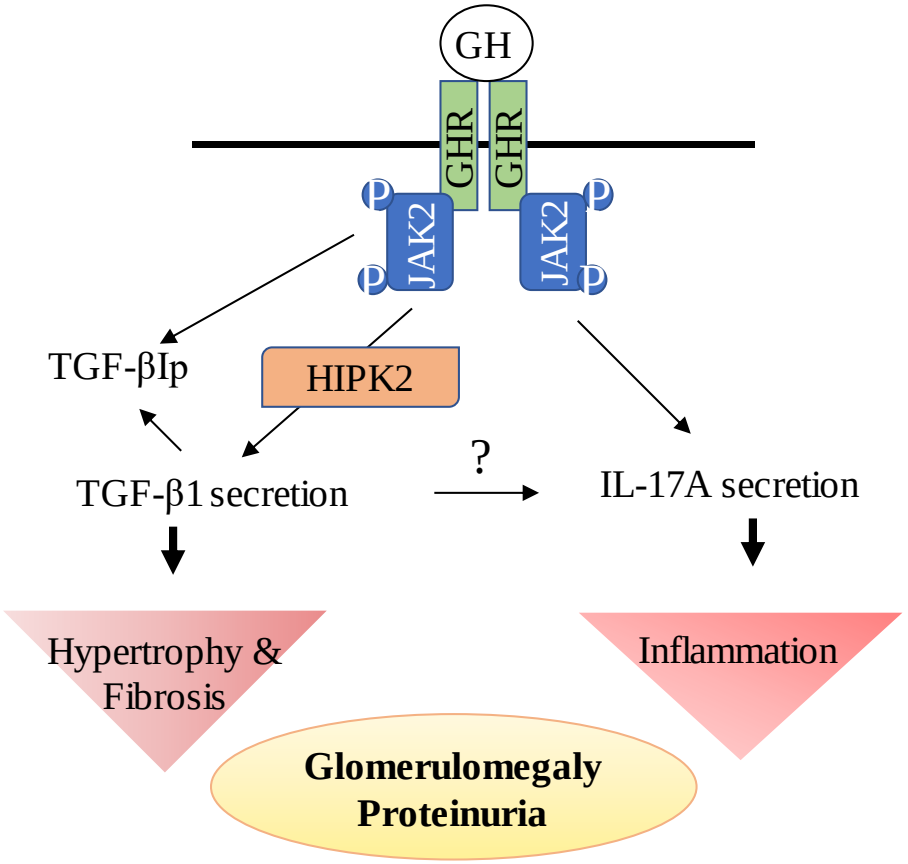

Figure 21. *Schematic diagram of GH mediated molecular and cellular events that cause podocyte injury:* GH binding activates JAK2 protein, which ultimately induces TGF-β1 secretion. HIPK2 expression is required for GH induced TGF-β1 expression. On the other axis, GH activates IL-17 signaling that is involved in immune cell infiltration into the glomerulus. GH elicited hypertrophy, ECM accumulation in podocytes and immune cell migration altogether contribute to glomerulomegaly and proteinuria. TGF-β also induced IL-17 secretion but in detailed study is required to elucidate the mechanistic link between GH induced TGF-β1 and IL-17 secretion.

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Conferences

1. 17th Annual Research Day, Department of Medicine. University of Pittsburgh, Pittsburgh (29th to 30th April 2019).
2. Annual Aging Institute Research Day, 2018. University of Pittsburgh, Pittsburgh (December 6, 2018).
3. 6th International Conference on Molecular Signaling 2018, University of Hyderabad (8th to 10th February 2018).
4. International Congress of Cell Biology 2018, CCMB, Hyderabad (27th to 31st January 2018).
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Growth hormone induces Notch1 signaling in podocytes and contributes to proteinuria in diabetic nephropathy

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Running title: *Role of Notch Signaling in GH-induced Proteinuria*

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Keywords: Growth hormone, gamma-secretase, notch intracellular domain 1, podocyte dysfunction, epithelial-mesenchymal transition, proteinuria, diabetic nephropathy, chronic kidney disease.

ABSTRACT

Growth hormone (GH) plays a significant role in normal renal function and overactive GH signaling has been implicated in proteinuria in diabetes and acromegaly. Previous results have shown that the glomerular podocytes, which play an essential role in renal filtration, express the GH receptor, suggesting the direct action of GH on these cells. However, the exact mechanism and the downstream pathways by which excess GH leads to diabetic nephropathy is not established. In the present manuscript, using immortalized human podocytes *in vitro* and using a mouse model *in vivo*, we show that excess GH activates Notch1 signaling in a γ-secretase-dependent manner. Pharmacological inhibition of Notch1 through the γ-secretase inhibitor DAPT (N-[N-(3,5-Difluorophenacetyl)-l-alanyl]-S-phenyl glycine t-butylester) abrogates GH-induced epithelial to mesenchymal transition (EMT) and is associated with a reduction in podocyte loss. More importantly, our results show that DAPT treatment blocks cytokine release and prevents glomerular fibrosis, all of which are induced by excess GH. Further, DAPT prevented glomerular basement membrane thickening and proteinuria induced by excess GH. Finally, using kidney biopsy sections from people with diabetic nephropathy, we show that Notch signaling is indeed upregulated in such settings. All these results confirm that excess GH induces Notch1 signaling in podocytes, which contributes to proteinuria through EMT as well as renal fibrosis. Our studies highlight the potential application of γ-secretase inhibitors as a therapeutic target in people with diabetic nephropathy.

Renal interstitial fibrosis is the hallmark of progressive chronic kidney disease, which correlates well with renal failure (1). Renal fibrosis is characterized by myofibroblast proliferation and activation,

Growth hormone induces TGF-β1 in glomerular podocytes: Implications in podocytopathy and proteinuria

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ORIGINAL RESEARCH ARTICLE

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Hypoxia induces ZEB2 in podocytes: Implications in the pathogenesis of proteinuria

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Abstract

The glomerular filtration barrier (GFB) plays a critical role in ensuing protein free urine. The integrity of the GFB is compromised during hypoxia that prevails during extreme physiological conditions. However, the mechanism by which glomerular permselectivity is compromised during hypoxia remains enigmatic. Rats exposed to hypoxia showed a decreased glomerular filtration rate, podocyte foot-processes effacement, and proteinuria. Accumulation of hypoxia-inducible factor-1α (HIF1α) in podocytes resulted in elevated expression of zinc finger E-box binding homeobox 2 (ZEB2) and decreased expression of E- and P-cadherin. We also demonstrated that HIF1α binds to hypoxia response element localized in the ZEB2 promoter. Furthermore, HIF1α also induced the expression of ZEB2-natural antisense transcript, which is known to increase the efficiency of ZEB2 translation. Ectopic expression of ZEB2 induced loss of E- and P-cadherin and is associated with enhanced motility of podocytes during hypoxic conditions. ZEB2 knockdown abrogated hypoxia-induced decrease in podocyte permselectivity. This study suggests that hypoxia leads to activation of HIF1α–ZEB2 axis, resulting in podocyte injury and poor renal outcome.

KEYWORDS
HIF1α, hypoxia, podocyte, proteinuria, ZEB2.

1 | INTRODUCTION

Oxygen is a vital element and supports most of the metabolic events in higher organisms. The continuous supply of adequate levels of oxygen is crucial for normal functioning of the human body. However, human physiology is challenged with extreme environmental conditions resulting in hypoxia, a state of deficiency of oxygen in the blood and tissues. Hypoxia affects the homeostasis and functioning of various organs including kidneys. Kidneys possess low-resistance microvasculature that is exposed to both high volume and continuous perfusion. The kidneys have a high oxygen demand, so as to facilitate energy dependent basic renal functions such as active salt absorption (Hansell, Welch, Blantz, & Palm, 2013). The constraints of low oxygen supply, dictated by both renal architecture

Abbreviations: CKD, chronic kidney disease; ESRD, end-stage renal disease; GFB, glomerular filtration barrier; GFR, glomerular filtration rate; HIF1α, hypoxia-inducible factor-1α; HRE, hypoxia response element; NAT, natural antisense transcript; ZEB2, zinc finger E-box binding homeobox 2.

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REVIEW
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Novel Actions of Growth Hormone in Podocytes: Implications for Diabetic Nephropathy

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The kidney regulates water, electrolyte, and acid-base balance and thus maintains body homeostasis. The kidney's potential to ensure ultrafiltered and almost protein-free urine is compromised in various metabolic and hormonal disorders such as diabetes mellitus (DM). Diabetic nephropathy (DN) accounts for ~20–40% of mortality in DM. Proteinuria, a hallmark of renal glomerular diseases, indicates injury to the glomerular filtration barrier (GFB). The GFB is composed of glomerular endothelium, basement membrane, and podocytes. Podocytes are terminally differentiated epithelial cells with limited ability to replicate. Podocyte shape and number are both critical for the integrity and function of the GFB. Podocytes are vulnerable to various noxious stimuli prevalent in a diabetic milieu that could provoke podocytes to undergo changes to their unique architecture and function. Effacement of podocyte foot process is a typical morphological alteration associated with proteinuria. The dedifferentiation of podocytes from epithelial-to-mesenchymal phenotype and consequential loss results in proteinuria. Poorly controlled type 1 DM is associated with elevated levels of circulating growth hormone (GH), which is implicated in the pathophysiology of various diabetic complications including DN. Recent studies demonstrate that functional GH receptors are expressed in podocytes and that GH may exert detrimental effects on the podocyte. In this review, we summarize recent advances that shed light on actions of GH on the podocyte that could play a role in the pathogenesis of DN.

Keywords: growth hormone, podocytes, diabetic nephropathy, zinc finger E-box binding homeobox2, dedifferentiation, hypertrophy

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INTRODUCTION

The vertebrate kidney plays an essential role in filtration of blood, regulation of water, electrolyte, and acid-base balance, and thereby maintenance of overall body homeostasis. The function of the kidney to ensure almost protein-free ultrafiltered urine depends on the collective action of millions of nephrons (1). A nephron comprises two highly coordinated units: glomerulus and renal tubule. The glomerulus filters plasma to prevent protein loss into the glomerular filtrate. The renal tubule reabsorbs water and electrolytes in addition to contributing selective salts and Tamm–Horsfall proteins to the glomerular filtrate. The contribution of renal tubular absorption and secretion notwithstanding, the final composition of urine is largely determined by the integrity of glomerular filtration barrier (GFB, **Figure 1A**). The GFB consists of three critical components—endothelium,

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RESEARCH ARTICLE

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Stabilization of hypoxia-inducible factor 1α by cobalt chloride impairs podocyte morphology and slit-diaphragm function

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Abstract

Glomerular podocytes are the major components of the renal filtration barrier, and altered podocyte permselectivity is a key event in the pathogenesis of proteinuric conditions. Clinical conditions such as ischemia and sleep apnea and extreme physiological conditions such as high-altitude sickness are presented with renal hypoxia and are associated with significant proteinuria. Hypoxia is considered as an etiological factor in the progression of acute renal injury. A sustained increase in hypoxia-inducible factor 1α (HIF1α) is a major adaptive stimulus to the hypoxic conditions. Although the temporal association between hypoxia and proteinuria is known, the mechanism by which hypoxia elicits proteinuria remains to be investigated. Furthermore, stabilization of HIF1α is being considered as a therapeutic option to treat anemia in patients with chronic kidney disease. Therefore, in this study, we induced stabilization of HIF1α in glomerular regions in vivo and in podocytes in vitro upon exposure to cobalt chloride. The elevated HIF1α expression is concurrence with diminished expression of nephrin and podocin, podocyte foot-processes effacement, and significant proteinuria. Podocytes exposed to cobalt chloride lost their arborized morphology and cell-cell connections and also displayed cytoskeletal derangements. Elevation in expression of HIF1α is in concomitance with loss of nephrin and podocin in patients with diabetic nephropathy and chronic kidney disease. In summary, the current study suggests that HIF1α stabilization impairs podocyte function vis-à-vis glomerular permselectivity.

KEYWORDS

cobalt chloride, hypoxia-inducible factor 1α, podocytes, proteinuria, slit-diaphragm

1 | INTRODUCTION

Physiological systems including renal system are challenged with hypoxia during extreme physiological conditions (altitude sickness and hypoxemia) and pathological conditions (ischemia, sleep apnea, and preeclampsia). Kidneys are specialized organs that play a critical role in response to both systemic and local hypoxia. Kidneys have a high oxygen demand to facilitate energy-dependent active salt absorption. In contrast, kidneys possess low-resistance microvasculature that gets exposed to both high volume and continuous perfusion. Both high oxygen demand, as well as the limit in the oxygen availability, allows kidneys susceptible to

Growth Hormone and Metabolic Homeostasis

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Abstract

Pituitary growth hormone (GH) is a peptide hormone predominantly secreted by somatotrophs in the anterior pituitary under the tight control of the hypothalamic–pituitary axis and GH secretagogues. GH elicits its effects directly on target organs and cells interacting with GH receptors and through stimulation of insulin-like growth factor 1 production. GH plays critical roles in regulating somatic growth and the metabolism of carbohydrates, lipids, and protein. GH increases insulin secretion and glucose uptake. Conversely, a GH deficient state is characterised by enhanced insulin sensitivity. Diabetogenic actions of GH are evident in conditions of GH excess, such as acromegaly or poorly controlled Type 1 diabetes mellitus. In patients with GH deficiency, administration of GH resulted in impaired glucose tolerance and insulin sensitivity. Owing to its multiple and complex effects, the regulation of GH secretion and its function in normal health and metabolic diseases is a major research interest in the field of molecular endocrinology. This review provides an overview of the effects of GH on glucose, lipid, and protein metabolism, insulin resistance, and metabolic homeostasis.

INTRODUCTION

Hormones control several steps of intermediary metabolism, including glucose oxidation, glycogen metabolism, gluconeogenesis, and fatty acid oxidation. The importance of hormones from the anterior pituitary, the islets of Langerhans, adrenal glands, and the thyroid in intermediary metabolism is well recognised. Over recent years there has been a significant increase in the understanding of how these hormones regulate metabolic homeostasis. An array of hormones, including insulin, glucagon, adrenaline, cortisol, thyroxin, amylin, glucagon-like peptide-1, glucose-dependent insulinotropic peptide, and pituitary growth hormone (GH), play prominent roles in the maintenance of glucose metabolism and homeostasis. Impaired glucose homeostasis is evident in several clinical conditions

12th INTERNATIONAL PODOCYTE CONFERENCE

May, 30th – June 2nd 2018, Montreal, Canada

Presented by NephCure Kidney International and Nephrology Division, McGill University, Montreal, Canada

March 14, 2018

RE: Abstract submission

Dear Mr. Dhanunjay Mukhi

On behalf of the organizing committee for the 12th International Podocyte Conference, I am pleased to let you know that your abstract has been accepted for a poster presentation. The presentation time will be communicated to you at a later date. Please see the Poster instructions specifying the format at www.podocyteconf.org.

This year, will have an additional feature for the trainees at the conference. After the deadline for the abstract submission March 15th, 2018, the scientific committee will be choosing some abstracts to be presented as a 2 min presentation (1 slide) at the BLITZ session scheduled May 31th; this is in addition to the Poster presentation. We will communicate if your abstract has been chosen for the BLITZ presentation at a later date.

Awards will be given to the best poster and blitz presentations.

We have discussed your request for the registrations waiver. I am happy to let you know that your request has been approved, and the conference will cover this expense. We understand this covers only a part of the anticipated expenses associated with the attendance of the conference. We hope that this shell facilitate your participation.

We are looking forward welcoming you at the 12th International Podocyte Conference in Montreal! Sincerely,

Yours truly,

Elena Torban, PhD
Chair, 12th International Podocyte conference
Associate professor of Medicine,
McGill University, Montreal, Canada

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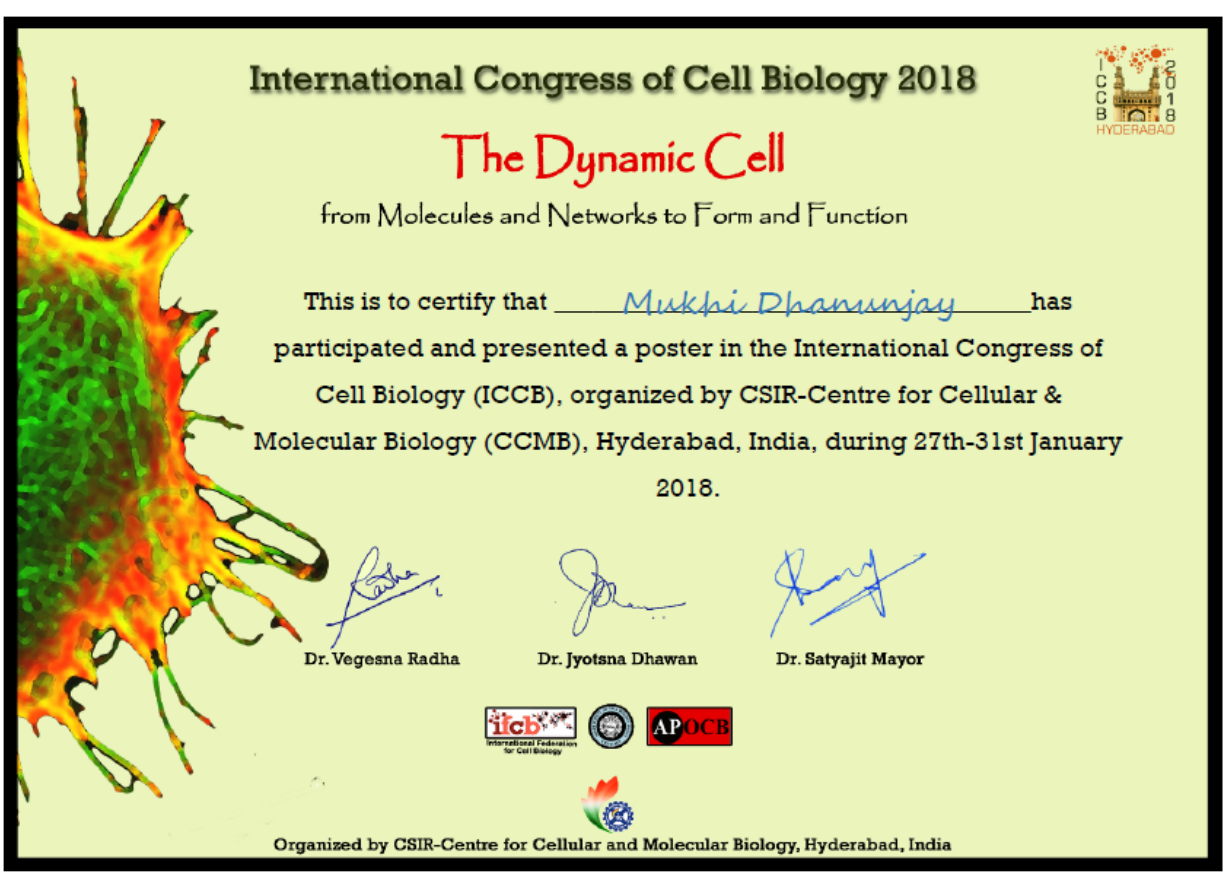

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This is to certify that Mukhi Dhananjay has participated and presented a poster in the International Congress of Cell Biology (ICCB), organized by CSIR-Centre for Cellular & Molecular Biology (CCMB), Hyderabad, India, during 27th-31st January 2018.

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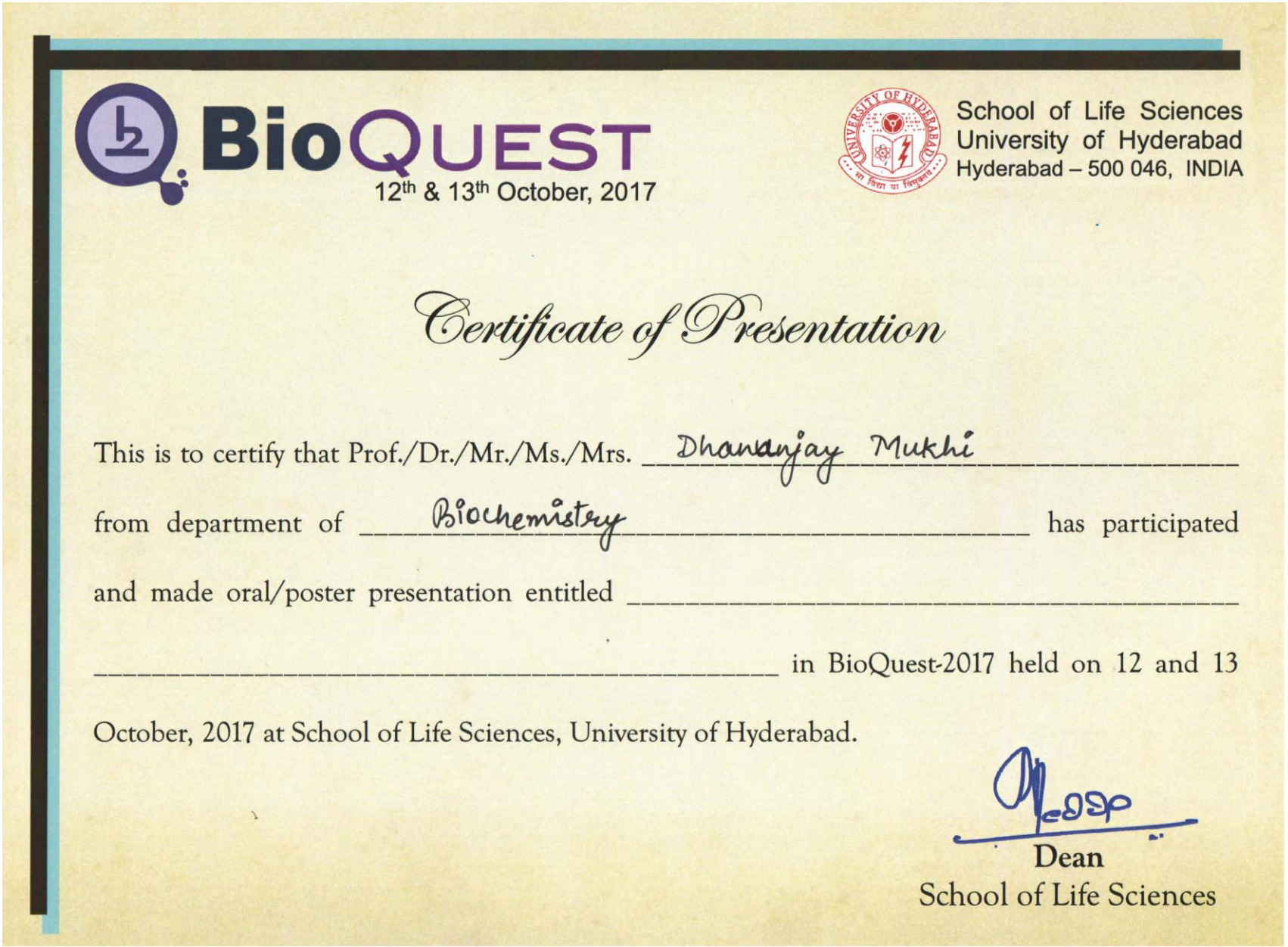
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Growth Hormone Induces TGF-β1 Signaling in Podocytes and Contributes to Nephropathy

by Dhanunjay Mukhi

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