Growth Hormone Induces Reactivation of Notch Signaling in Podocytes and Contributes to Nephropathy

A thesis submitted during 2020 to the University of Hyderabad
in partial fulfillment of the award of a Doctor of Philosophy in the Department of Biochemistry, School of Life Sciences

by

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CERTIFICATE
(for Ph.D. Dissertation)

This is to certify that this thesis entitled "**Growth Hormone Induces Reactivation of Notch Signaling in Podocytes and Contributes to Nephropathy**" submitted by **Mr. Rajkishor Nishad**,bearing registration number **15LBPH05** 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.

A. Publications:

1. **R.Nishad**,P.Meshram, AK. Singh, GB. Reddy, AK. Pasupulati.Activation of Notch1 signaling in podocytes by glucose-derived AGEs contributes to proteinuria.(2020). BMJ Open Diabetes Research and Care.
2. **R. Nishad**, D.Mukhi, SV.Tahaseen, SK.Mungamuri, AK.Pasupulati.Growth Hormone Induces Notch Signaling in Podocytes: Implications in Diabetic Nephropathy. (2019). JBiol Chem.
3. KM. Nakuluri*, **R. Nishad***, D.Mukhi, S. Kumar, V. P.Nakka, LP. Kolligundla, P.Narne, SSK. NP.Phanithi, A.K. Pasupulati. Cerebral ischemia induces TRPC6 in the glomerular podocytes.(2019). Scientific Reports.*Equal contribution.
4. KM. Nakuluri, D. Mukhi, **R.Nishad**, MA. Saleem, SK. Mungamuri, RK. Menon, AK. Pasupulati. Hypoxia induces ZEB2 in podocytes: Implications in the pathogenesis of proteinuria. (2018). Journal of Cellular Physiology.
5. **R. Nishad**, D.Mukhi, RK.Menon, AK. Pasupulati.Growth Hormone and Metabolic Homeostasis. (2018).EMJ. Diabetes.
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7. **R. Nishad**, KM. Nakuluri, M. Motrapu, AK. Pasupulati.Epithelial-mesenchymalTransition of Glomerular Podocytes: Implications in Proteinuria.(2017). MGMJMed Sci.
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B. Conferences:

[1]Oral presentation at Hy-Sci Biology 2019, organized by CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India. (Aug2019).**[2]**International Congress of Cell Biology-2018, organized by CSIR-Centre for Cellular and Molecular Biology Hyderabad, India. (Sept 2018).**[3]**7th International Conference on Stem Cells and Cancer (ICSCC-2016): Proliferation, Differentiation and Apoptosis, organized by International Center for Stem Cells, Cancer and Biotechnology (ICSCCB), Pune, India (Oct2016).**[4]**Best poster award in Bioquest-2015, University of Hyderabad, Hyderabad, Telangana, India.

Further, the student has passed the following courses towards fulfillingthe coursework requirements for a Ph.D. degree.

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BC 802	Research ethics, Data analysis, and Biostatistics	3	Pass
BC 803	Lab Work & Seminar	5	Pass

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DECLARATION

I, Rajkishor Nishad, hereby declare that this thesis entitled **"Growth hormone induces reactivation of Notch 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.

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Abbreviations:

ADAM proteases: A disintegrin and metalloproteinase

α-SMA: α-Smoot muscle actin

DAPT: N-[N-(3,5- Difluorophenacetyl)-l-alanyl]-S-phenyl glycine t-butylester

EMT: Epithelial to mesenchymal transition

E-CAD: E-cadherin

ELISA: Enzyme-linked immunosorbent assay

GH: Growth hormone

GHR: Growth hormone receptor

GBM: Glomerular basement membrane

GFR: estimated Glomerular filtration rate

Hes1: Hairy Enhancer Split 1

Hey1: Hairy/Enhancer-of-split related with YRPW motif protein 1

HPC: Human podocyte cells

H&E: Hematoxylin and eosin

IGF1: Isulin like growth factor 1

Jag1: Jagged 1

MPC: Mouse podocyte

NICD: Notch intracellular domain

N-CAD: N-cadherin

Pax2: Paired box gene 2

p53: Tumor protein 53

RBPjk: Retinol-binding protein-jk

Smad2/3: Mothers against decapentaplegic homolog 2/3

SNAIL1: SNAIL1(snail homolog 1(Drosophila))

SLUG: SNAIL2(snail homolog 2(Drosophila))

SIP1: Smad interacting protein 1

TGF-β: Transforming growth factor-β

TGFBIp: Transforming growth factor-beta-induced protein

UACR: Urinary Albumin and Creatinine Ratio

ZEB2: Zinc finger E-box binding homeobox2

AGEs: Advanced glycated end products

ADAM proteases: A disintegrin and metalloproteinase

BSA: Bovine serum albumin

Col IV: Collagen IV

DN: Diabetic nephropathy

EMT: Epithelial to mesenchymal transition

ESRD: End-stage renal disease

eGFR: estimated Glomerular filtration rate

WT1: Wilms tumor 1

E-CAD: E-cadherin

HES1: Hairy Enhancer Split 1

MMP: Master-mind-like protein

MT: Masson's trichrome

NICD1: Notch intracellular domain 1

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

N-CAD: N-cadherin

PAS: Periodic acid-Schiff

PDGF: Platelet-derived growth factor

RBPJ: Retinol-binding protein-j

RAGE: Receptor for advanced glycated end products

TNBS: 2,4,6-Trinitrobenzene sulfonic acid

TEM: Transmission electron microscopy

TGF: Transforming growth factor

TGF-β1: Transforming growth factor β1

TGFBR1: Transforming growth factor β receptor1

VEGF: Vascular endothelial growth factor

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Chapter 1: Introduction

1.1 Kidneys: Structure and Function:

Kidneys are the primary excretory organs located behind the peritoneum on both sides of the vertebral column. Each kidney consists of over 1 million nephrons known as the functional unit of the kidney, which contains a glomerular and the tubular region. The cross-sectional view of the kidney defines two regions: Cortex comprising of glomerular part and Medulla comprising of a tubular portion. The glomerulus consists of a filtration barrier that ensures the release of protein and cells-free ultrafiltrate. The tubular element is responsible for absorbing water, glucose, and few electrolytes from the glomerular filtrate. In this way, the glomerulus and the tubule together direct the prior arrangement of the ultra-filtrated urine. The renal tubule comprises of proximal (PCT) and distal tangled tubules (DCT), connected by the Henle's circle that at last converges into a collecting tube. Several renal tubules and collecting duct together form pyramid shapes structures in the kidney called Bartolini pyramids. The base of each pyramid consolidates with the renal hilum through which urine is gathered into the urinary bladder.

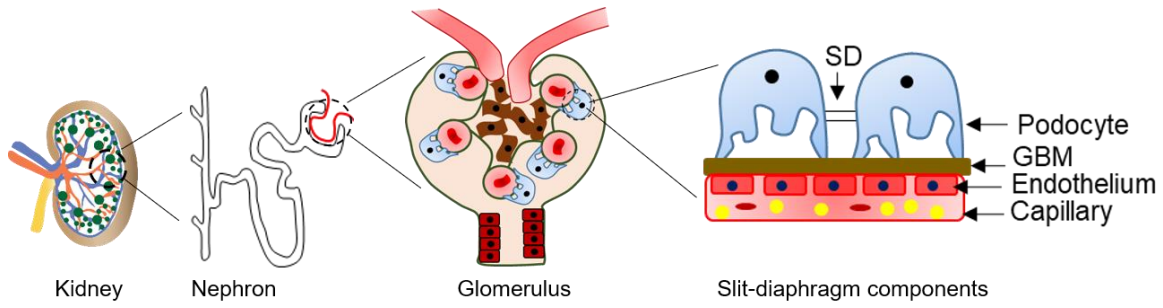

Figure 1*:* Kidney *s*tructure and glomerular filtration barrier: Each kidney consists a million nephrons and each nephron consists of glomerulus and tubules. Glomerulus is made of highly vascularized blood capillaries. The capillary *e*ndothelial cells have specialized fenestrations with the size of 70-100 nm. The glomerular basement membrane (GBM) is made up of various extracellular components and its thickness ranges from 250-300 nm. Podocytes cover the capillaries and their foot-processes is connected by slit-diaphragm. Together, podocytes, GBM, and endothelium constitute glomerular filtration barrier.

Kidneys play a vital role in maintaining body homeostasis by regulating blood pressure, ion balance, reabsorption of nutrients, and removal of metabolic by-products and toxins [1].

Further, kidneys also regulate the osmolarity of plasma by inflecting the number of solutes, electrolytes, and water in the blood. They are responsible for the production of erythropoietin, thereby triggering red blood cell production and maintain acid-base tension in the body. Kidneys secrete renin, which is involved in the regulating of blood pressure by its interaction with angiotensin known as the renin-angiotensin-aldosterone system (RAS). The conversion of vitamin D to the active form calcitriol is carried out by the kidneys [2].

In healthy conditions, kidneys assure ultrafiltrated urine with the stringently regulated concentration of protein [1]. The concentration of protein in urine generally measures the 24 h albumin level; hence albuminuria is considered as a known marker of detrimental renal condition. According to the American Diabetic Association (ADA) guidelines, albumin levels extent from 30 to 300 mg in urine collected in 24 h, denotes microalbuminuria. At the same time, proteinuria or macroalbuminuria is characterized by the presence of ≥300 mg albumin/24h [3].

1.2 Glomerulus:

The glomerulus comprises four different cell types; fenestrated endothelial cells, mesangial cells, parietal epithelial cells, and podocytes. The fenestrated endothelium is 70-100nm thick and allows only small molecules to pass across the capillaries. Owing to its fenestrations, endothelial cells cover up to 50% of the total glomerular surface area. Damage to these cells results in the loss of cell junctions [4]. Mesangial cells assist in maintaining homeostasis of the mesangial matrix and provide support to the capillary network. Activation of mesangial cells in a diseased condition results in hypertrophy, proliferation, and excessive deposition of the matrix, ultimately leading to glomerulosclerosis [4]. Podocyte is a descendant of the parietal epithelial cells (PECs). Damage to PECs leads to ECM deposition on GBM, resulting in glomerulosclerosis and glomerulonephritis. The glomerulus consists of a filtration barrier that ensures the release of protein-free and cells-free ultrafiltrate. The glomerular

filtration barrier (GFB) consists of three different cell types involved in the formation of ultrafiltrate, viz. the fenestrated endothelium, glomerular basement membrane (GBM), and the visceral epithelial podocyte cells [1]. The basement membrane of thickness 250-300nm is composed of fibronectin, collagen, and heparin sulfate proteoglycans. Podocytes are adherent to the basement membrane and offer epithelial coverage to glomerular capillaries [5].

GFB is mostly composed of endothelial and epithelial cells with the GBM interposed. The GFB acts like a molecular sieve and restricts the entry of macromolecules like proteins in the urine while allowing the filtration of electrolytes, amino acids, metabolites, small solutes (<20kDa), and water molecules [6]. In healthy conditions, the kidneys assure ultrafiltrated urine with the stringently regulated concentration of protein [1]. The aberrations and insults to the GFB and absorption result in the excretion of several proteins in the urine. The concentration of protein in urine is generally recorded by measuring the levels of albumin collected for 24 h, hence albuminuria is considered a known marker of detrimental renal condition. Proteinuria was categorized into different types based on its pathophysiology as tubular proteinuria (impaired tubular reabsorption), glomerular proteinuria (defect in glomerular filtration), and overflow proteinuria, in which the protein reabsorption ability of tubules is compromised due to small proteins. This condition generally occurs in pathologies such as multiple myeloma, where an overabundance of Igs is seen. The average loss of protein in overflow proteinuria and tubular proteinuria (2g/24h urine) is approximately half of that of protein loss in glomerular proteinuria (>4g/24h urine). The latter one is caused due to compromised GFB permeability to plasma proteins. In normal conditions, the filtration through GFB is tightly regulated; hence, the appearance of proteins in the urine suggests injury to the filtration barrier. Podocytes are significant components of GFB, and an insult to podocyte is often associated with proteinuria.

1.3 Podocytes:

Podocytes are terminally differentiated epithelial cells that give epithelial inclusion to the glomerular vessels. Podocytes, inferable from their one of a kind structure and limitation, control glomerular permselectivity, add to the GBM, and neutralize intracapillary hydrostatic weight. Accordingly, podocytes are viewed as instrumental in managing the typical capacity of the glomerulus and fundamental for the ultrafiltration of blood and the development of final urine [7]. Podocytes have an intricate design establishing a major cell body, primary and secondary FPs which fold over the glomerular vessels [8]. The essential FPs further partitions into auxiliary FPs, which cover the vessels and guarantee glomerular filtration by offering hydrostatic weight. Major processes are bound by the intermediate filaments and microtubeules whereas the FPs comprises of actin-based cytoskeleton [7]. The FPs of the adjacent podocytes are connected by SD, a modified adherent junction, forming a zipper-like structure. Several SD proteins interact with podocyte cytoskeleton (consisting of actin, α-actinin, myosin, vinculin, paxillin, and talin) and helps in its structure and function maintenance [9]. The glycocalyx in FPs provides a negative charge throughout the GFB surface, that helps in maintaining the cytoarchitecture of podocytes by keeping them physically separated [10, 11]. As the integrity of the SD is attributed as the necessary factor for the proper filtration ability of podocyte, any insult to podocyte eventually results in proteinuria [12].

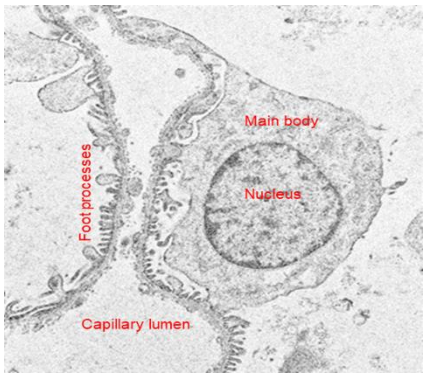

Figure 2: Ultrastructure of a podocyte: Transmission Electron Microscope (TEM) image of podocyte is representing a large main body and nucleus with their foot processes. Image is captured using ZOEL microscope at 2K × 2K resolution and 120 kV. Scale bar 5µm.

The general function of podocyte is (a) to maintain the glomerular structure by contending the expansion of the GBM, (b) to maintain a higher filtration surface across the SD, (c) to balance the size and maintain the charge, a characteristic feature of the GFB, and (d) the maintenance of glomerular filtration rate (GFR). Podocytes secrete vascular endothelial growth factor (VEGF), which is involved in maintaining GBM and endothelial cells' integrity. They also support the tension on the capillary wall and capillary loop. Podocytes, with active crosstalk between different glomerular cells and basement membrane, proves to be an integrated filtration unit. However, injury to the podocytes develop loss of the lamellipodial expansion and focal adhesions that ultimately leads to reversal of FPs, called as foot processes effacement (FPE). In diseased conditions, the podocyte cytoskeletal rearrangement occurs, resulting in proteinuria [13]. Also, disturbance in any domain of FP may result in foot process effacement, i.e., flattening of the FPs and proteinuria [14].

A decline in the density of podocytes and altered SD architecture represents as a pathology in glomerular disease. Also, the remaining podocytes have to compensate for the glomerular filtration. A reduced podocyte count leads to loss of renal function. All these events result in proteinuria. Proteinuria, frequently alluded to as albuminuria, is filed by the measure of albumin presented in 24 hr collected urine. In the event of microalbuminuria, 30 to 300 mg of egg whites get discharged for 24h. Macroalbuminuria runs about ≥300mg in 24h urine gathered. Regularly, macroalbuminuria forms into pronounced proteinuria that is an distant form of end stage renal disease (ESRD), and it is directed by an assortment of elements, for example, hyperglycemia, hypertension, smoking, stroke, and rest apnea.

1.4 Components of Slit-diaphragm (SD):

The SD attributes sieving property to the GFB. It is a modified adherent junction protein spanning 30 to 50 nm wide filtration slits, and its extracellular region comprises rod-like units [7]. These units are linked by a linear bar producing a zipper-like structure [15]. SD acts as the

shape, size, and charge-selective filtration barrier. It provides the structural integrity to the glomerular capillaries and helping in to maintain the GFB permselectivity. The SD functions as a primary sensor and regulator of FP length and shape adaptations [16], hence disruption in the SD structure or function is seen in most kidney diseases originating at the level of podocytes [17]. SD consists of proteins like Podocin, Nephrin, CD2 associated protein (CD2AP), Catenin, P-cadherin, FAT, etc. and tight junction proteins like Zonula Occludin-1 (ZO-1), Cingulin, Occludin, Junction Adhesion Molecule-A (JAM-A). Occludin is present in SD, whereas JAM-A is localized to the podocyte foot processes. Neph1 and Nephrin are involved in signaling pathways responsible for maintenance of the integrity of the podocytes. Genetic mutations in any of the SD proteins (podocin, nephrin, and CD2AP) results in nephrotic syndrome [13].

The structural stability of SD depends on the podocyte cytoskeleton scaffoldings. The podocyte cytoskeleton plays a crucial role in directing the morphology and setting up anchorage to the SD complex through a network of proteins. Further, cytoskeleton offers structural and functional support, which gives the system to the podocyte and its connections with the extracellular matrix (ECM). Also, the cytoskeleton remodeling essentially involved in cell physiology, cell shape, adhesion and motility.

1.5 Risk factors for diabetic nephropathy (DN):

Noxious factors involved in the morbidity and mortality in diabetic settings are hyperglycemia, hypertension, obesity, and inflammation. Also, chronic kidney diseases include chronic renal infections, sleep apnea, smoke, stroke and cardiovascular disease. There are several signaling pathways including transforming growth factor-β (TGF-β), Notch signaling, Interleukins and enhanced growth hormone (GH) levels get dysregulated in diabetes mellitus. Mice that are transgenic for GH showed severe glomerulosclerosis with decreased podocyte number and proteinuria [18]. Therefore, our laboratory longed an interest in understanding the effect of elevated GH levels on kidneys, specifically on podocytes.

TGF-β is a pro-sclerotic molecule and believed as the key regulator of various components of ECM [19, 20]. Enhanced level of TGF-β or its receptor (TGF-βR), TGF-βR 1-2 expression in the glomerular and tubulointerstitial compartments is confirmed in case of DM [21, 22]. Transgenic mice with TGF-β showed apoptosis of podocytes and set for glomerulosclerosis [23, 24]. Mice with diabetes injected with neutralizing antibodies for TGF-β1 or TGF-βR2 protected from the development of DN [25-27]. TGF-β activates SMAD3, and SMAD3 knockout mice for SMAD3 showed protection from diabetic renal injury without affecting albuminuria [19, 28]. Therefore, targeting TGF-β signaling or downstream signaling components under the settings of diabetes ameliorated podocyte loss and advances renal function [28, 29].

DN is associated with the invasion of immune cells into the glomerular area. Inflammatory molecules plays a significant aspect in the progression of DN. Recent findings revealed that diabetic kidneys express higher levels of macrophage markers, for example, F4/80, CD40L, CD11b, CD45, and monocyte chemoattractant protein1 (MCP1) [30]. Podocyte-specific B7 ligand (CD80) expression is associated with human lupus nephritis. Similarly, mice exposed to LPS showed correlation with induced B7 ligand in podocytes and nephrotic range of proteinuria, whereas removal of B7 prevented LPS-induced proteinuria [31].

1.6 Pathogenesis of DN:

Diabetes is characterized by hyperglycemia due to compromised insulin secretion and/or insulin action. It is believed that DN is a secondary complication, which develops in 20-40% of patients having type I (T1DM) or type II (T2DM) diabetes. Conspicuous early changes in the kidney with DN show hyperfiltration, hypertrophy, and microalbuminuria (Fig. 3).

Diabetic kidney disease represents more than 50% of ESRD cases in the United States. Besides podocytes, glomerulus also contains mesangial cells, which were considered a

significant role in the progression of diabetic kidney disease. Because the expansion of mesangial matrix leads in the decreased capillary surface area and results in compromised filtration [32-34].

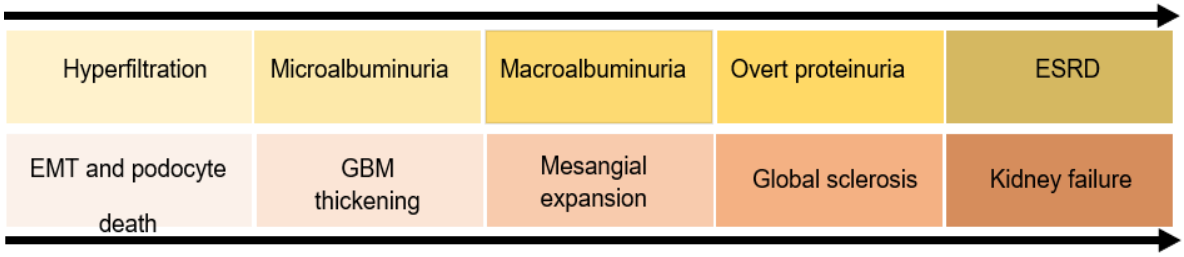

Figure 3: Clinical and morphological changes during the course of DN: DN is evidenced by hyperfiltration and various levels of presence of protein into the urine. The morphological changes during the progression of DN such as kidney hypertrophy, widening of GBM, mesangial expansion and sclerosis result in kidney failure.

Despite, the increased mesangial matrix is considered as a major feature of kidney injury in diabetes mellitus [35]. It is primarily believed that damage to GFB, particularly podocytes lead to the development of proteinuria in diabetic patients. Since GFB firmly manages the structure of the urine and there is a lot of discussion on the role of each of the (three) components of the GFB in the progression of proteinuria in diabetics kidney. It was suggested that endothelial damage (microangiopathy) is a causal factor in the advancement of proteinuria [36]. However, loss of proteoglycans from GBM takes place later in the case of DN, some of time a lot later than the presence of microalbuminuria, suggesting a functional component of the GFB in the progression DN [37].

1.7 Growth hormone signaling:

GH exerts its pleiotropic effects by binding to its cognate GH receptor (GHR). The predominant model is that GHR exists as an inactive dimer and a single molecule of GH binds to GHR dimer. The binding of the GH molecule to the extracellular domain of the GHR facilitates rotation of the two GHR molecules that results in the intracellular domain of each GHR molecules binding to a JAK2 molecule [38]. JAK2 contantaly remains to be associated

with the cytoplasmic domain of GHR, and in the unliganded states, the pseudokinase domain of JAK2 masks its catalytic domain. Binding of GH to the GHR reorientates and rotates the receptor subunits, which results in the transition from similar transmembrane domains to one where the transmembrane domains separate at the point of entry into the cytoplasm [39]. This arrangement facilitates the movement of the pseudokinase inhibitory domain of one JAK2 away from the kinase domain of the other JAK2 within the GHR–JAK2 complex. This results in the transactivation of JAK2. Activated JAK2 then phosphorylates the intracellular domain of the GHR, which then recruits several downstream proteins as depicted in Fig4. Activation

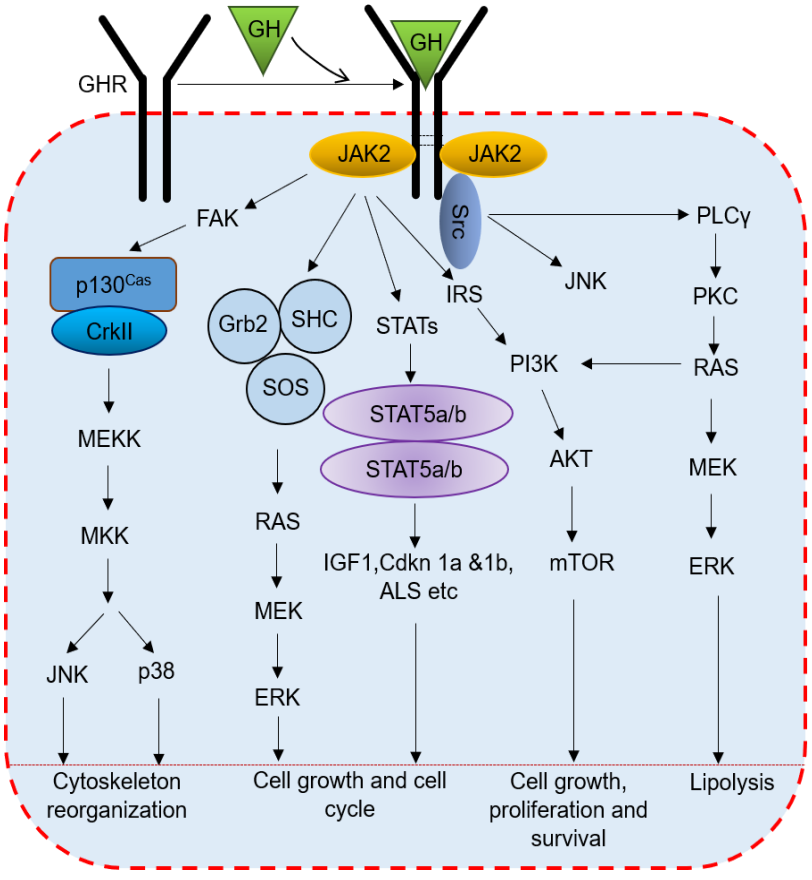

Figure 4: Cellular and molecular actions regulated by GH signaling. The binding of GH to its receptor GHR activates JAK2, which further trigger the downstream signaling cascades. The GH/GHR axis and its downstream components control various metabolic and cellular events including cell growth, cell proliferation, lipolysis and cytoskeleton reorganization. GH: growth hormone; GHR: growth hormone receptor; FAK: focal adhesion kinase; GRB2-SOS: growth factor receptor-bound 2-son of sevenless complex; IRS: insulin receptor substrate; PI3K: phosphatidylinositol 3-kinase; SH2-Bβ: src-homology 2 domain Bβ; STAT: signal transducer and activator of transcription.

of JAK2 is a critical step for triggering GHR signaling. The STAT family of transcription factors is recruited to the activated GHR– JAK2 complex. STATs are phosphorylated at a single tyrosine residue by JAK2 and then undergo either homo or hetero-dimerization and translocate to the nucleus where they act as transcription factors.

Among various STATs, STAT5b exerts the majority of the biological effects of GH. GHR can also activate MAPK, downstream of both JAK2 and Src kinase. GHR localization to the lipid raft preferentially activates MAPK while cytosolic GHRlocalisation activates STAT5. Insulin receptor substrate-1 (IRS-1) plays a critical role in GHR induced MAPK activation [40]. Furthermore, GHR signaling is also associated with the activation of the phosphatidylinositol-3 kinase (PI3K)/Akt pathway in a JAK2/IRS-1-dependent manner. Attenuation of GHR activation is arbitrated by the proteins of suppressor of cytokine signaling (SOCS) family, which includes SOCS-1–7, and cytokine-inducible SH2-domain-containing protein. It is noteworthy that SOCS family proteins are also induced by the JAK/STAT signaling cascade. GH itself has been shown to induce SOCS-2 and SOCS-3. SOCS proteins suppress GH signaling by inhibiting JAK2 activity and compete with STAT for binding with GHR or by inducing the proteasomal degradation of the GHR complex. Additionally, protein tyrosine phosphatases have also been implicated in terminating the GHR signal cascade [41]. JAK2 binding stabilizes and prevents GHR from degrading [42]; on the other hand, GH induces desensitization of the GHR via JAK2 kinase activity [43]; phosphatases have also been implicated in terminating the GHR signal cascade [41]. JAK2 binding stabilizes and prevents GHR from degradation [42]; on the other hand, GH induces desensitization of the GHR via JAK2 kinase activity [43].

1.8 GHR and components of the JAK-STAT axis:

GHR is a 638 amino acid long polypeptide and which is encoded by the GHR gene. GHR is classified as a prototype of the class I cytokine receptor family, which includes receptors for the ligands, erythropoietin, prolactin, and thrombopoietin [44]. GHR does not has tyrosine kinase properties and it depends on associating with non-receptor protein tyrosine kinases (Janus kinase) for their signal transduction via JAK-STAT pathway [45]. Janus kinase (JAK) is classified from JAK1, 2, and 3 and TYK2 that can interact to specific receptors. JAK1 and JAK2 both are participating in many physiological events such as growth and developmental processes. However, JAK3 and TYK2 help in maintaining the balance of the body immune system. JAK2 is the only member that directly interacts with GHR [44, 46].

STATs are the intracellular mediators of GH, epidermal growth factor, prolactin, and also for other cytokines mediated signaling. Studies showed that GH directly involves in the activation of STAT1, 2,3,5a, and 5b. However, the primary transcript synthesis generally found is in the pulsatile action of GH is STAT5a/b [47]. STAT5 is also termed as mammary gland factor (MGF) because it was first noticed in prolactin signaling. JAK2 activation subsequently leads to STAT5a/b phosphorylation is an essential event for STAT dependent gene expression. Still, the current studies reported that specific tyrosine phosphorylation at GHR is critical for STAT mediated gene expression [48].

1.9 Diabetes mellitus is presented with deregulation of GH/GHR signaling:

The compromised GH/GHR signaling is altered in T1DM and diabetic kidney diseases. Also, the circulatory levels of GH in poorly controlled T1DM has been reported at an elevated level. The reasons behind increased GH levels in DM can be discussed by two inter-related mechanisms. First, in diabetic conditions, decreased expression of GHR in hepatic cells results in GH resistance and consequently decreased IGF-1 synthesis in hepatic cells (Fig. 5). Finally, which leads to a low circulating IGF-1 levels, which stimulate GH secretion by a feedback

mechanism [49, 50]. Second, hypoinsulinemia in T1DM results in enhanced synthesis of IGFBPs in hepatic cells. The elevated serum IGFBPs, majorly IGFBP-1, prevent the IGF-1 action at the cellular level and leads to GH hypersecretion by the pituitary somatotrophs [49]. However, GHR expression in the kidney is either unaltered or enhanced in diabetic conditions, thus transducing the effect of elevated GH levels. Accumulating pieces of evidence suggests a strong relationship between hyperactivity of the GH/GHR axis and its role in adverse renal function outcome [18, 51-53].

GH injection to healthy individuals for a week resulted in increased GFR [52, 54]. Interestingly, injection of Octreotide (a somatostatin analog) and somatulin (GH inhibitor) maintain the normal GFR, hyperfiltration, and kidney size in DN patients [55, 56]. Acromegaly patients are presented with microalbuminuria [57]. Similarly, GH-transgenic mice was also presented with diabetic kidney complications [58, 59]. It is imperative that the expression of predominant negative GHR showed reversal from compromised glomerular function and hypertrophy [60]. Though all these evidence highlight the compromised kidney outcome in the hyperactive GH/GHR axis, the precise role of GH remains enigmatic till the demonstration of a direct action of GH on podocytes [61].

1.10 Identification of GHR in podocytes:

Studies from several groups have demonstrated the direct link between hyperactive GH/GHR axis and with kidney dysfunction, which includes hyperfiltration, dedifferentiation and proteinuria [62-64]. Nevertheless, it was not well described whether impaired glomerular function during DN is due to direct action of GH; on glomerular cells or due to hemodynamic changes. However, the discovery of GHR on podocytes remained a breakthrough in the area of GH vs. podocyte biology. Gaddameedi et al. demonstrated that podocytes from mouse and human with GH esposure activates the canonical JAK/STAT and MAP kinase pathway genes [61]. Interestingly, it was also found that GHR expression co-localized with both synaptopodin and WT1 (Wilm's tumor1), key markers for differentiated podocytes. GHR expression is very minimal at an early stage of gestation, as there exist immature glomeruli, but its expression is not observed at the adult stage of the kidney [65]. Nevertheless, overexpressing human or bovine GH in mice develop progressive glomerulosclerosis, suggesting that elevated expression of GH induces glomerular injury [62].

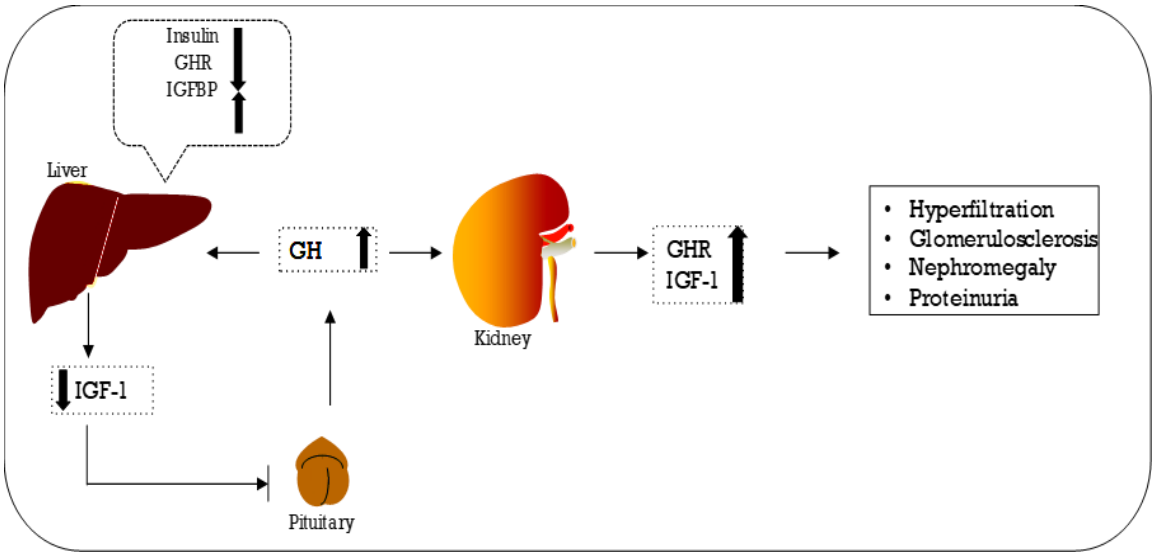

Figure 5: The signaling cascade of GH/IGF1 in the DM: The insulin deficiency and compromised insulin action in diabetic condition leads to decreased hepatic expression of GHR and increases serum IGF-1 binding proteins. Hepatic production of IGF-1 is the rich source that act on pituitary to regulate the secretion of GH. On the other hand decreaesd levels of IGF-1 synthesis leads to low levels of hepatic expression of GHR. Together, leads to elevated level of GH in the diabteic patients which is implicated in renal dysfunction. Mukhi.D and Nishad R. et.al. 2017.

1.11 Mechanisms of podocyte injury:

An array of noxious stimuli elicits podocyte injury in the diabetic milieu [66]. Various modes of podocyte injury include hypertrophy, detachment of podocytes from GBM, epithelial to mesenchymal transition (EMT) of podocytes, and apoptosis (Fig. 6). It was proposed that altered diabetic milieu implicated in the development of glomerular hypertrophy and sclerosis [67, 68]. Diabetic subjects are at increased risk of developing CKD and hyperglycemia-induced renal complications that are associated with high mortality and morbidity.

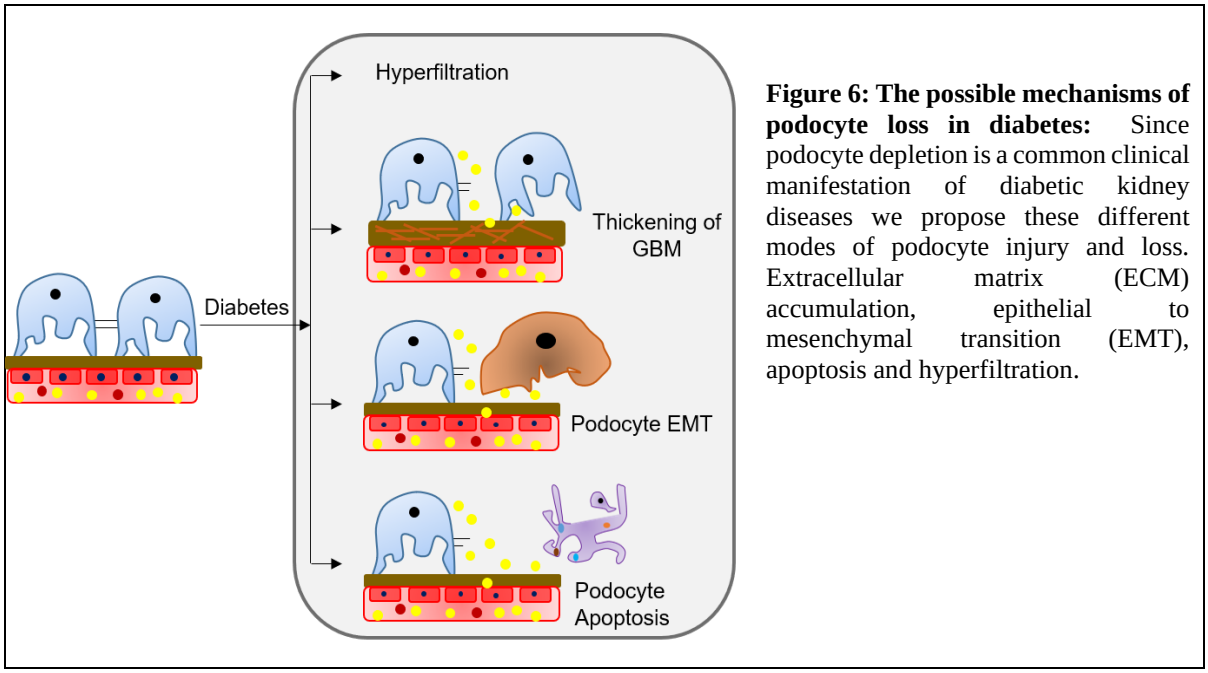

Figure 6: The possible mechanisms of podocyte loss in diabetes: Since podocyte depletion is a common clinical manifestation of diabetic kidney diseases we propose these different modes of podocyte injury and loss. Extracellular matrix (ECM) accumulation, epithelial to mesenchymal transition (EMT), apoptosis and hyperfiltration.

1.12 Accumulation of ECM and detachment of podocytes:

Proteins, polysaccharides, and interlinking proteins are the three major components of the extracellular matrix (ECM). ECM while providing mechanical strength also helps in signaling events required for differentiation and morphogenesis. Polysaccharides such as glycosaminoglycans, hyaluronan, and heparan sulfates of the ECM associate with linker proteins to form large proteoglycan complexes. These proteoglycan complex along with agrin and perlecan impart negative charge to the GBM and, in turn, to GFB. The negative amount imparted by the proteoglycans creates electrostatic repulsion towards anionic molecules thereby, retaining them in the plasma [87]; collagens (type IV and XVII) and nidogen are the

major structural components of the GBM [87]. Further, matrix interlinking proteins namely, laminin and fibronectin interact with both collagen and Proteoglycans.

Diabetic kidney diseases usually present with ECM accumulation in the glomerular mesangium, tubulointerstitium, and GBM due to the imbalanced expression of tissue inhibitors of metalloproteases (TIPMs) and matrix metalloproteases (MMPs). Under normal circumstances MMPs and TIMPs work in harmony to regulate the composition of ECM wherein, MMPs degrade the ECM proteins, and the TIPMs are the inhibitors of MMPs. However, in diabetic kidney disease, TIPMs get upregulated, causing the excess synthesis of ECM components and impaired degradation of ECM, leading to glomerulosclerosis [88, 51]. It is noteworthy that ECM components are contributed by glomerular endothelial cells and podocytes located on the either side 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 contributed by mesangial cells [89]. Although enhanced expression of ECM during DN is well documented, the mechanism is poorly understood.

ECM accumulation and its impaired degradations were also extensively noted in the pathophysiology of GH treated (direct/indirect) animals, transgenic mice, and in cultured podocytes [51, 80, 90, 91]. These reports strongly suggest that GH alters ECM turnover. Further, it was suggested that podocytes failed to adhere to the thickened GBM and eventually shed in the urine. Also, It is noteworthy that TGF-β1 also plays a pivotal role in the ECM turnover; it was observed to be overexpressed diabetic kidney diseases. It is noteworthy, that TGF-β1 plays a decisive role in regulating ECM components and overexpresses in diabetic kidney diseases.

1.13 Podocyte epithelial-mesenchymal transition:

Epithelial-mesenchymal transition (EMT) is a tightly regulated cellular event during which an epithelial cell with adherent properties undergoes a phenotypic switch and attains the characteristics of a mesenchymal cell with enhanced migratory capacity, invasiveness, and increased production of ECM. Now EMT is accepted as a pathological event in nephropathy, tissue fibrosis, and cancer metastasis. However, EMT is a fundamental process that takes place during embryonic developmental stages like mesoderm formation from embryonic epithelium and delamination of the neural crest.

The phenotypic switch between healthy and diseased podocytes partially resembles type II EMT only [69]. Podocytes possess epithelial features, such as apical-basal cell polarity and tight junctions. Epithelial markers expressed by podocytes include E- and P-cadherin, WT-1, and ZO-1. The decreased epithelial polarity, rearrangement of actin-cytoskeleton and, injury to SD are predominant features of EMT to podocytes. Podocytes EMT also represents a cadherin switch from E- and P-cadherin to N-cadherin. High levels of glucose exposure to podocytes, showed the increased synthesis of fibroblast-specific protein-1 (FSP-1), which indicate EMT [70]. The elevated level of collagen synthesis by injured podocytes also indicates that podocytes undergo EMT [71]. Although podocytes express vimentin, under exposure to TGF-β1, a strong stimulator for EMT, an additive effect in the vimentin expression was observed [71]. Injured podocytes express multiple transcriptional factors that ensue EMT, which include ZEB2, SNAIL, and SLUG. It was also reported that podocyte dedifferentiation is associated with loss of epithelial markers expressions, such as P-cadherin and ZO-1. Despite the fact that podocytes undergo EMT upon noxious stimuli that are prevalent during various pathophysiological conditions, it has been proposed that the EMT of podocytes satisfies the criteria of type II EMT partially [69]. Mature podocytes represented with a spindle-shaped and arborized cell body. Upon subjected to various noxious stimuli, podocytes lose their apical-

based polarity, and there is a loss of morphological features and function, as evidenced by redistribution of stress fibers occurs and also in permselectivity. Prevalent features such as redistribution of actin fibers and loss of cell polarity are unique to podocyte EMT.

1.14 Apoptosis of podocytes:

A decreased podocyte number in the subjects with DN is a hallmark of compromised kidney function. It is considered as apoptosis of podocytes is one of the primary reasons for decreased podocyte number in the population of DN [5]. It should be noted that podocytes are terminally differentiated resting cells. It was reported that podocytes are arrested at the G2/M phase of the cell cycle and do not undergo proliferation [72]. Nutrients, cytokines, and growth factors generally support podocyte survival. IGF-1 and Insulin are essential for podocyte survival, whereas a high dose of glucose and TGF-β promotes podocyte apoptosis [73-75]. In diabetic settings, reduced insulin levels predispose podocytes to apoptosis. Earlier reports from our laboratory reported that a high dose of GH induces podocytes apoptosis [76]. GH-induced reactive oxygen species are a significant factor that could provoke podocyte cell death by apoptosis. However, the quenching of free-radicals by N-acetyl cysteine prevented GH-mediated apoptosis of podocytes [61].

1.15 TGF-β superfamily:

TGF-β superfamily contains a cluster of proteins ranging from ligands to receptors to intracellular effectors. More than 68 members have been included in the TGF-β superfamily. Many are multifunctional cytokines, including TGF-β and bone morphogenic proteins (BMPs) [92]. Three TGF-β isoforms have been identified in mammals: TGF-β1, TGF-β2, and TGF-β3. These three isoforms are secreted as latent complex proteins and are associated with latent TGF-β binding proteins (LTBPs). Latent complexes of TGF-β are inactive and only active upon the cleaved by proteases such as plasmin and furin.

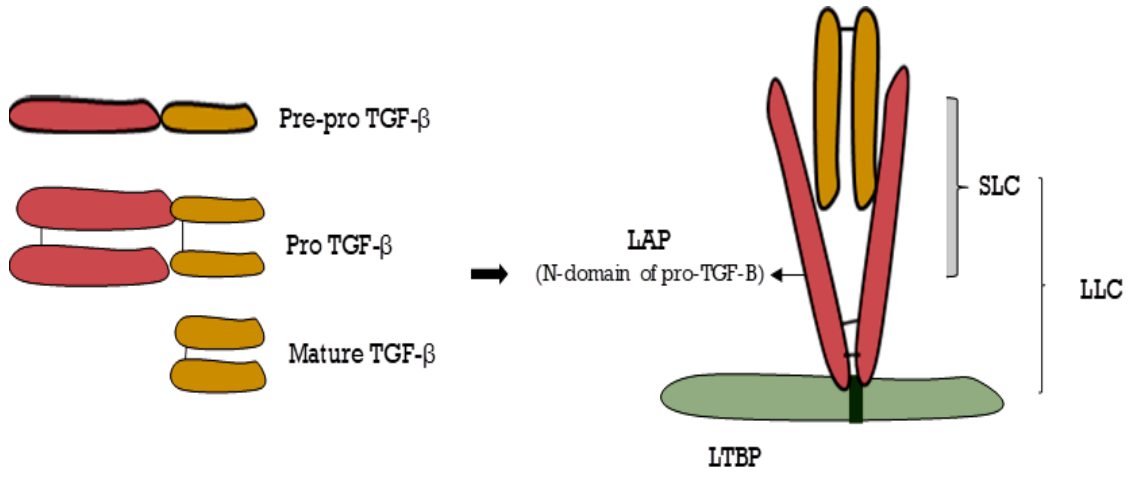

Figure 7: Schemetic representation of TGF-β synthesis and maturation: TGF-β is synthesized as pro-TGF-β with latency associated peptide (LAP) at N-terminus, which requires for proper folding and dimerization. After proteolysis of LAP it remain associated with mature TGF-β by non-covalent interactions to form small latent complex (SLC). Next SLC binds with latent binding protein (LTBP) to form large latent complex (LLC), which finally get secreted out into the extracellular space.

1.16 Canonical TGF-β/SMAD signaling:

TGF-β is produced by the majority of the cell types in the body. TGF-β is a pro-peptide, which is predominantly inactive. However, the 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) (Fig. 7). The bioactive form of TGF-β is a 25 kDa dimer composed of 12.5-kDa subunits, which is synthesized by various cell types [93]. 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). LTBP binds the ECM and sequesters LAP-TGF-β in vivo. Bioactive TGF-β can be attached to various cell-surface proteins such as decorin, and beta glycan, serving as ligand traps. Thus, multiple mechanisms control the bioavailability and activity of TGF-β [94].

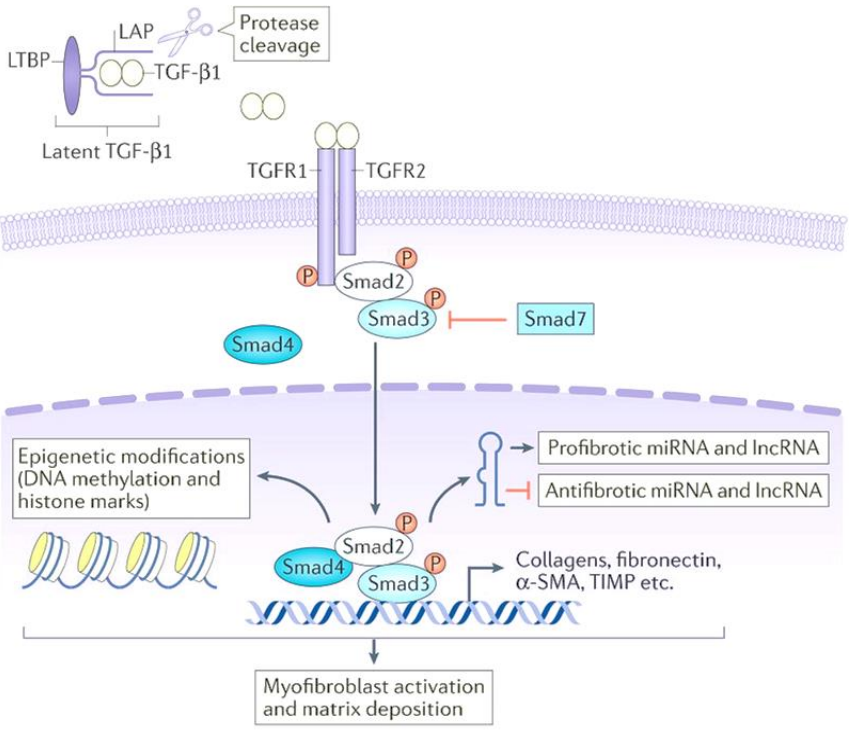

Figure 8: TGF-β signaling: Latent complex of TGF-β is inactive and it undergoes cleavage by proteases to form mature TGF-β, which binds on the TGF-βR. Binding of TGF-β induce receptor phosphorylation, which further phosphorylates SMAD2&3 proteins. SMAD4 helps SMAD2&3 to translocate into nucleus and regulate target gene expression. Image Source: Xiao-ming-Meng et.al. 2016.

TGF-β signaling is stimulated by binding of a ligand to the homodimer of TGF-βR2. The ligand-receptor association stimulates autophosphorylation of not only receptor TGF-βR2 and also phosphorylation of TGF-βR1 (Fig. 8). Activated 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]. Next, R-SMADs form a complex with SMAD4 and translocate to the nucleus, which elicits

downstream transcriptional responses, thus transduce either in TGF-β or BMP signaling. Studies have shown that SMAD4 undergoes phosphorylation in response to TGF-β1 at Thr-276 by ERK, enhancing 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.

1.17 TGF-β/SMAD signaling in glomerular disease:

TGF-β1 is a major cytokine that gets elevated in the diabetic milieu and is produced by many cell types, including epithelial cells. TGF-β1 acts as a master regulator in the turnover of ECM molecules and fibrosis evident in glomerular and tubular injury during DN [77-80]. Several cell types, including podocytes, which respond to TGF-β and increases the expression of ECM proteins, for example, collagens, fibronectin, laminin, proteoglycans, and integrins. Several studies showed that TGF-β and its receptor TGF-βR1 expression gets elevated in glomerulus under the settings of DN.

Previous studies showed neutralizing antibodies against TGF-β1 ameliorated glomerular lesions formation in the experimental DN [81]. Interestingly, antagonists to TGF-βR2 treatment protected from the development of DN in the 12-week of STZ induced diabetic rats. Also, the transgenic mice with heterozygous knockout for TGF-βR2 showed protection from the progression of STZ induced DN [82]. These data suggest that the significant association of TGF-β/TGF-βR2 axis in the initiation and progression of DN. The in vitro experimental data from TGF-β1 exposed podocytes showed decreased expression of tight and adherent junction proteins (ZO-1 and P-cadherin) and increased expression of desmin and snail proteins, suggesting that TGF-β stimulate podocyte de-differentiation [83]. Similarly, podocytes exposed with recombinant TGF-β1 shows increased leakage for albumin protein, suggesting that the de-differentiation of podocytes could be the reason behind DN induced proteinuria [83]. Bone morphogenic protein 7 (BMP7) is a well-known antagonist of TGF-β1 in the

biological system. Transgenic mice with BMP7 showed protection from the shedding of podocytes into the urine and concurrent with reduced serum TGF-β1 level [84]. This data revealed that TGF-β1 plays a vital role in podocyte detachment from GBM during DN. However, the spatial or temporal association between GH and TGF-β in the pathogenesis of diabetic kidney disease had never been established. There are several stimuli, for example, high glucose, angiotensin-II, and hypoxia can induce TGF-β1 in the renal system. [85, 86].

1.18 Notch signaling

Notch signaling is a type of juxtracrine signaling, i.e., physical interaction between where, one cell carries receptor, and the other one carries ligand. The transmembrane receptor of Notch interacts extracellularly with the transmembrane ligand on the neighboring cell. This interaction initiates proteolytic cleavage of the notch receptor by ADAM metalloproteases and the γ-secretase complex [87], which results in the release of the notch intracellular domain (NICD). Formation of NICD leads its nuclear translocation and activates the downstream target genes. After NICD translocate in the nucleus and it interacts with a CBF1/Suppressor of Hairless/LAG-1 (CSL) family DNA-binding protein known as RBPJ (recombination signal binding protein for immunoglobulin kappa J region) in mammals, Su(H) (Suppressor of Hairless) in flies and LAG1 (Longevity-assurance gene-1) in *C. elegans*.

The co-repressors bound to Rbpj are displaced, and a transcriptionally active complex of NICD, RBPJ, and Maml (Mastermind) assembles, thus leading to Notch target genes activation [87]. This is followed in the canonical signaling of Notch. While in the noncanonical pathway, cleavage of the Notch receptor may not be required. Also, in some forms CSL is not involved, which may affect the interactions with signaling pathways upstream of NICD-CSL [88]. Among different developmental signaling pathways, Notch pathway is known to be unique because the secretion of ligands (DSL) is not in the extracellular space; hence they cannot diffuse off and affect cells located in the vicinity, rather these ligands are attached to

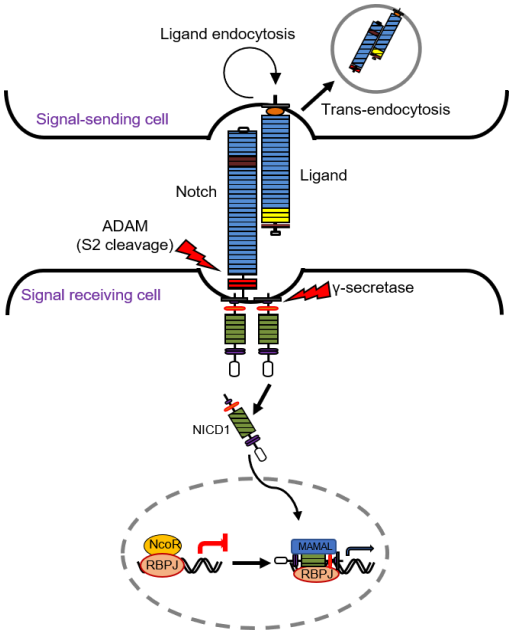

Figure 9: Mechanism of Notch signaling. The key components are Notch ligand and Notch receptor. Notch activation of a signal receving cell triggered by its interaction with ligand of a neighboring cells. This interaction triggers proteolytic cleavages of Notch at 2 sites (S2- and γ-secretase clevage). S2 clevage releases extracellular domain of Notch receptor and γ-secretase cleavage releases the Notch intracellular domain (NICD), which translocates to the nucleus. The Rbpj co-repressor complex is replaced by a co-activator complex containing NICD1 (green icons), which activates Notch target genes. In the absence of nuclear NICD1, Rbpj associates with a co-repressor complex (NcoR), which inhibit the transcription of Notch target genes.

the membrane of cells where they are produced [89]. Therefore, only the cells coming in close contact can bring about the ligand-receptor interaction, transmitting the notch signaling, hence is called juxtracrine signaling. The primary juxtracrine developmental signaling pathway, Notch extended to perform the fine-detail work of development, such as making the sharp boundaries between different tissue compartments, drives an uniform-spaced checkerboard pattern of gene expression over a field of cells, or a switch that drives the cell fate determination [90-93].

1.19 The role of Notch in glomerular disease:

Though Notch signaling was crucial in the developmental stages in the glomerulus, the healthy adult glomeruli entirely lacks Notch expression. Elevated expression of Notch pathway components (NICD & Jagged1) was observed in glomerular diseases [94]. Over-expression of Notch signaling components correlate with severity of kidney disease. The precise role of Notch in the specification of podocytes was demonstrated in Zebrafish. Once podocyte cell fate is determined, expression of Notch signaling components gradually decreased suggesting that both development and differentiation are tightly controlled by Notch signaling [95].

Additionally, it was shown that ectopic expression of NICD manifest in glomerulosclerosis and proteinuria in developing podocytes. Progression of glomerular injury was proportional with depletion of mature podocytes. Notch activity was shown to be high in case of renal progenitors [96]. Nevertheless, Notch activation in renal progenitors is crucial for their proliferation, while abrogation of Notch activity is essential for lineage development of progenitor cells towards podocytes. Strikingly, extended activation of Notch in renal progenitors induce podocyte depletion via mitotic catastrophe. Interestingly, inhibition of NICD accumulation (employing γ-secretase inhibitor) improved proteinuria by preserving podocyte number in mice treated with Adriamycin.

Studies with NICD transgenic mice revealed pathological role of sustained Notch activation [97]. Overexpression of transcriptionally active Notch component resulted in sudden onset of proteinuria [97]. However, increased NICD1 expression in podocytes paralleled with p53 expression. Though elevated expression of NICD and p53 provoked podocyte apoptosis, ablation of Rbpj protected mice from proteinuria and prevented podocyte depletion in T1DM mouse model. Furthermore, inhibition of Notch activation improved proteinuria in not only PAN model of kidney disease but also hyperglycemia induced glomerulosclerosis and proteinuria [98]. Mechanistic experimental studies showed that Notch components interact with both the VEGF and the TGF-β pathway in the glomerulus [98, 99]. Inhibition of Notch activation by γ-secretase inhibitor prevented glomerular injury in lupus nephritis model [100]. The accumulated evidence suggest that aberrant activation of Notch signaling is strongly associated with either podocyte injury or loss vis-a-vis pathogenesis of glomerular disorders, while inhibition of Notch signaling showed promising recovery from multiple glomerular disease models.

1.20 **Objectives:**

We propose three possibilities for decreased podocyte count in diabetic settings with elevated GH levels: [1]. Accumulation of extracellular matrix vis-à-vis thickening of basement membrane causes podocytes dehiscence. [2]. Phenotypic switch of podocytes from their innate epithelial form to adapted mesenchymal nature, thus loss into urine. [3]. Apoptosis of podocytes (Fig. 6). Therefore, we investigated the possible mechanism of GH induced podocytopathy with the following specific objectives.

I. Does GH induce reactivation of Notch signaling in differentiated podocytes and elicit podocyte injury?

II. Does GH induce mitotic catastrophe in quiescent podocytes?

Chapter 2: Growth hormone induces Notch1 signaling in podocytes

2.1 Introduction:

In the early glomerular development, particularly at the S-shaped body formation, podocyte fate determination is regulated by the highly conserved Notch signaling, which transduces short-range signals between neighboring cells [8, 101-103]. The components of Notch signaling include four ligands (Jag1-4) and 5 Notch receptors (Notch1-5). After ligand (Jag1-4) binding, Notch receptors undergo a series of cleavages catalyzed by the ADAM proteases and γ-secretase complex, which results in the release of the Notch intracellular domain (NICD) [104]. The resulting NICD translocates to the nucleus [105], wherein it forms a tertiary complex by associating with the DNA-binding protein, retinol-binding protein-jk (RBPjk), and the coactivator, Mastermind-like protein 1 (MAML-1). This tertiary complex activates the expression of cognate genes [106-109].

Vooijs et.al, demonstrated that Notch1 is highly active in the developing kidney; however, in the mature kidney, the detection of active Notch1 is minimal [110]. Consistence with this observation, Cheng et al. reported that inhibition of Notch signaling by the γ-secretase inhibitor, DAPT during early development of the mouse kidney, results in a severe deficiency of proximal tubules and glomerular podocytes, indicating the importance of Notch signaling during kidney development [103]. Interestingly, persistent activation of Notch signaling in the mature kidney leads to podocyte damage and subsequent kidney failure [111]. Further, it was also shown that ectopic Notch activation in terminally differentiated podocytes is correlated with diffuse mesangial sclerosis and focal segmental glomerulosclerosis (FSGS), which are associated with de novo Pax2 expression and p53-induced podocyte apoptosis, respectively [96, 111]. These data reveal that Notch signaling plays an essential role in the progression of fibrotic kidney diseases.

Despite unraveling the importance of Notch during renal development, the precise mechanism of Notch activation in adult kidney and its consequences on normal glomerular

function are remain to be elucidated. Although many studies show that the role of Notch1 signaling in glomerular diseases [112-114], the correlation between activated Notch1 signaling and diabetic proteinuria remains enigmatic. Elevated levels of circulating growth hormone (GH) are associated with the development of proteinuria in type1 diabetes and acromegaly [115, 116]. Conditions of high GH are typified by hyperfiltration, glomerulosclerosis, and albuminuria. On the other hand, both decreased GH secretion and action protect from glomerular complications. Earlier studies from our group established the role of GH on glomerular cells, particularly on podocytes [117, 118]. In the present study, we demonstrate that excess GH activates Notch signaling in adult human podocytes and the murine kidneys. Blocking the activated Notch signaling through pharmacological inhibition of γ-secretase reverted the GH-induced kidney fibrosis, interstitial infiltration of plasma-lymphocytic cells and proteinuria, hindered the glomerular basement (GBM) thickening with severe foot process effacement in vivo and EMT and fibrotic marker expression of podocytes in vitro. Further, our study demonstrates that an elevated level of circulatory GH at the adult stage may compromise kidney function by activating Notch signaling in podocytes, and it could be a potential therapeutic target.

2.2 Results:

2.2.1 GH induces Notch1 activation in immortalized human podocytes:

To identify the biochemical pathways downstream of GH signaling that is hyperactivated in podocytes in response to excess GH, we previously performed microarray analysis on immortalized HPC treated with GH and identified transcriptional activation of EMT regulator “ZEB2” [107]. By reanalyzing the same microarray data, we now identified that in response to GH treatment, Notch1 signaling is upregulated in human podocytes (Fig. 10A). To reconfirm the upregulation of Notch signaling by GH in podocytes, we also analyzed

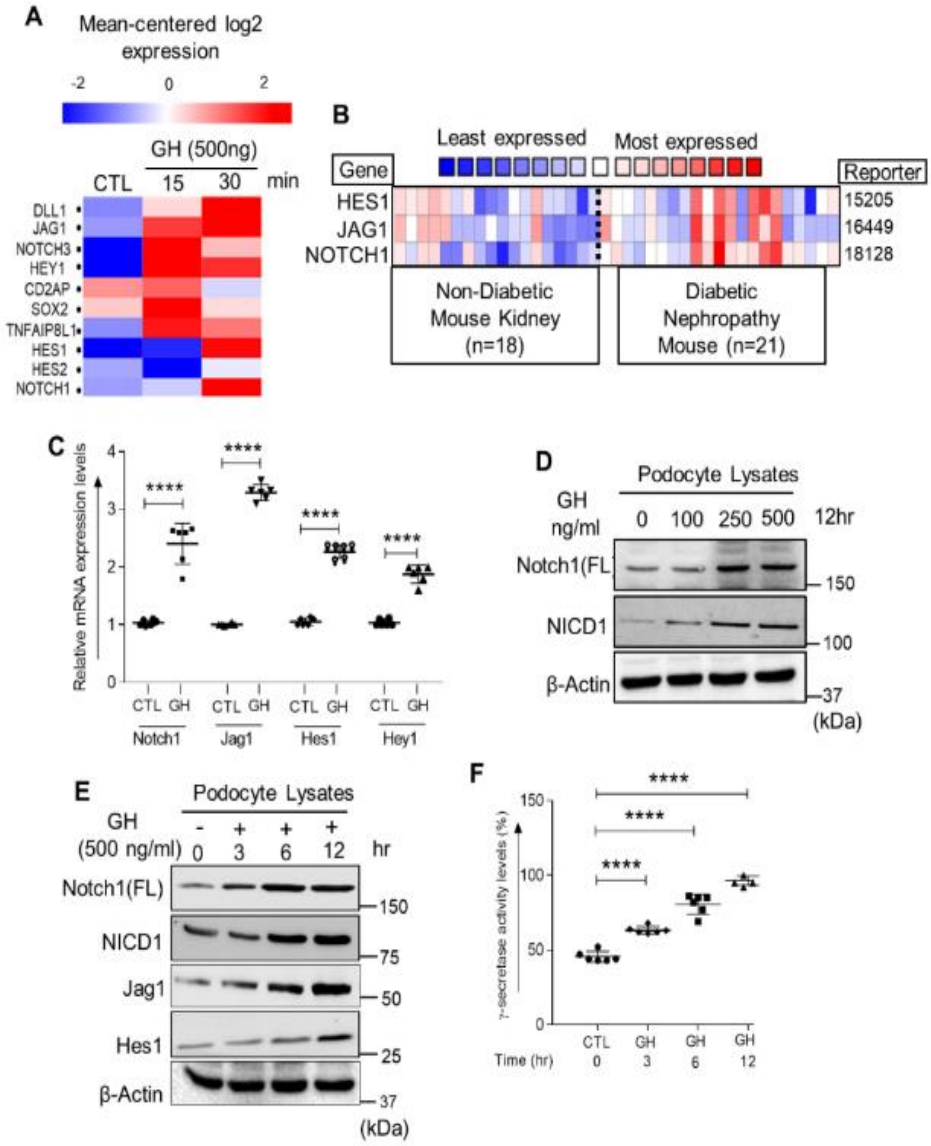

Figure 10: GH re-activates Notch1 signaling in matured podocytes. ***A.*** Heatmap of Notch signaling components in podocytes treated with or without GH for 15 and 30min. ***B.*** Nephroseq analysis comparing expression of Notch components from nondiabetic vs diabetic mouse kidney. ***C-E.*** qRT-PCR and immunoblotting study for expression of Notch componenet genes in with or without GH treatment for different concentration and time interval. ***F,*** γ-secretase activity in human podocytes treated with or without GH (500 ng/ml) for 0–12 h. ****, $p<0.0001$. Data represent the mean±S.D. Student's t test.

the expression of Notch1 and its targets Hes1 and Jag1 in non-diabetic mouse kidney (n=18) versus diabetic nephropathy mouse kidney (n=21) in Hodgin Diabetes Mouse Glomerulus data set available at Nephroseq (https://nephroseq.org) (Fig. 10B).

Next, to validate the data obtained in our microarray analysis as well as that from Nephroseq, we measured the mRNA levels of both Notch1 and its target genes by quantitative real-time PCR (qRT-PCR), and as expected, the levels of Notch1 and its targets were upregulated with GH treatment in the human podocytes (Fig. 10C). GH had induced the expression of activated Notch1 (NICD1) and its target proteins in both concentration (Fig. 10D) and time-dependent (Fig. 10E) manner. γ-Secretase is an intramembrane protease that cleaves many membrane proteins, including Notch1, which generates the NICD1 [119]. In response to GH treatment to an increase in Notch1-full length (FL) level in the podocytes (Figs. 10 C-E), we also observed a rise in NICD1 levels (Fig. 10D&E). Accordingly, we also observed a time-dependent increase in γ-secretase activity with GH treatment in human podocytes (Fig. 10F). All these data also corroborate that Notch1 signaling is upregulated in response to GH treatment in HPC.

2.2.2 γ-Secretase activity is required for GH-mediated Notch activation

To confirm the role of γ-secretase in GH-induced Notch1 activation in HPC, we next treated the HPC with GH in the absence or presence of well-established γ-secretase inhibitor, DAPT (N-[N-(3,5- Difluorophenacetyl-L-alanyl)]-(S)-phenyl glycine t-butyl ester). As expected, DAPT treatment to the GH exposed HPC has decreased the γ-secretase activity in a time-dependent manner (Fig. 11A). GH was not able to induce the expression of Notch1 or its target genes Hes1 and Hey1 in the presence of DAPT in HPC as measured as shown by qRT-PCR (Fig. 11B) and western blotting (Fig. 11C) as well as by immunofluorescence of the HPC (Fig. 11D). DAPT also inhibited the activation of NICD1 and induction of its target genes Hes1 and Hey1 in glomerular lysates from mice treated with GH, as measured at protein levels by both immunohistochemical staining (Fig. 11E-H) and western blot (Fig. 11I&J).

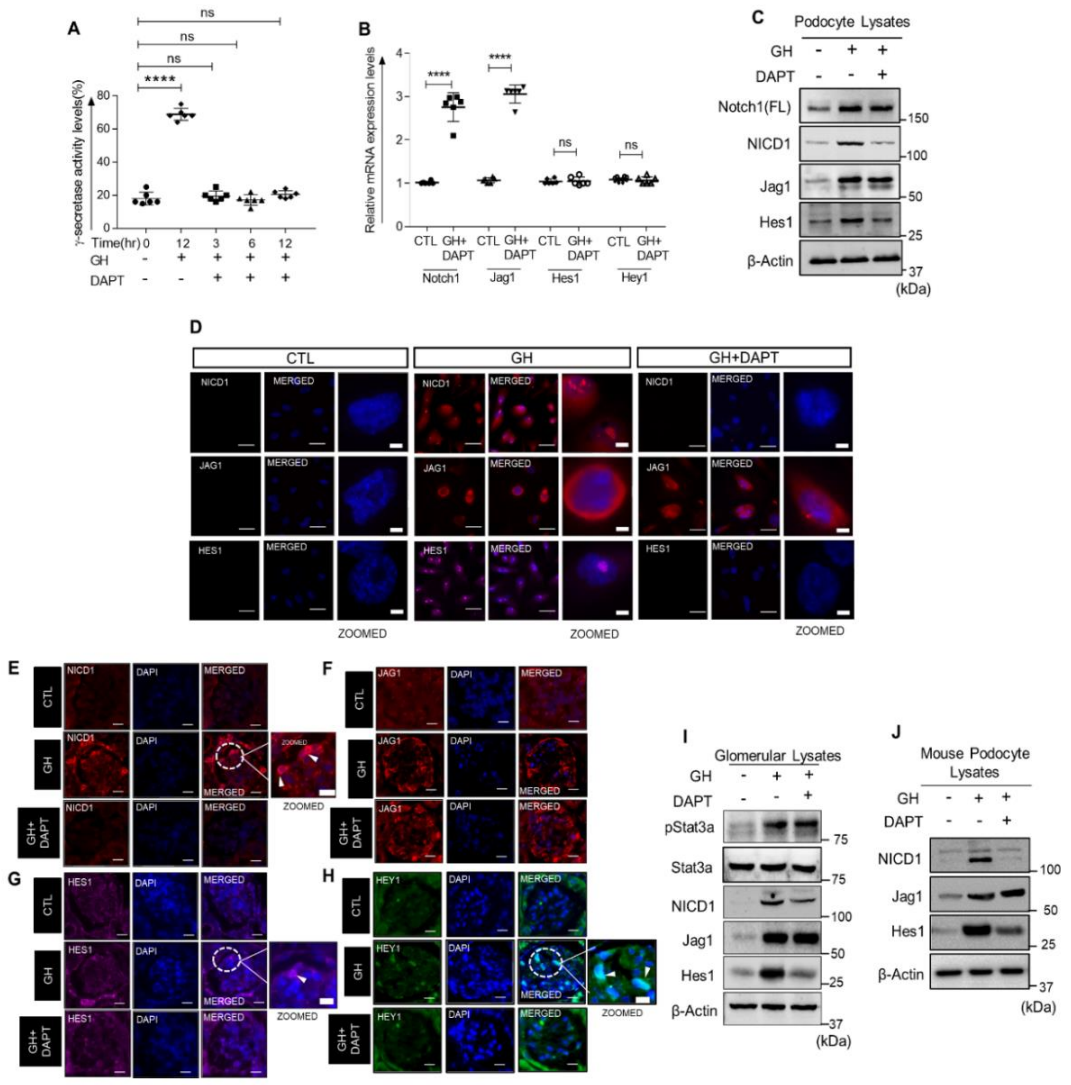

Figure 11: Inhibition of γ-secretase abolishes activation of Notch signaling in podocytes. ***A***, γ-secretase activity was measured in response to CTL, GH and GH+DAPT treated immortalized HPC with indicated time intervals (0,3,6 and 12hr). **p<0.0 1, ns (not significant). ***B,*** Relative mRNA expression was analyzed for NOTCH1, JAG1, HES1 and HEY1 in immortalized HPC with CTL and GH (500ng/ml) + DAPT (5μg/ml) conditions. β-Actin was used as an endogenous control. All the genes NOTCH1, JAG1, HES1and HEY1 mRNA levels were normalized to β-Actin levels and reported as fold change on y-axis. ***,p<0.001 and ns (not significant). ***C,*** Immunoblotting for Notch1(FL), NICD1, JAG1 and HES1 in response to CTL, GH and GH+DAPT in HPC. ***D,*** Immunofluorescence analysis for NICD1, JAG1 and HES1 in response to with or without GH and GH+DAPT in HPC. ***E-H***, Representative images of glomerulus showing immunostaining of NICD1 (active Notch1) (A), JAG1 (B), HES1 (C) and Hey1 (D) in with or without GH and GH+DAPT mice. ***I&J,*** Immunoblotting for pSTAT3a, total Stat3a, NICD1, JAG1, HES1 and β-Actin from HPC and mlouse podocyte in with or without GH and GH+DAPT. ***,p<0.001. ns= not significant. Student t-test**.** β-Actin as a internal control. Data are the mean ± SD .

However, the expression of Jag1 was unaffected from DAPT treatment (Fig 11B, C, D, F, I&J). All these data confirm that GH requires functional γ-secretase for it to activate Notch1 signaling in both mice kidneys and HPC.

2.2.3 Activated Notch signaling is required for GH induced EMT in podocytes

Previously, we had shown that excess GH in the podocytes leads to the induction of EMT [107]. Next, we tested the influence of Notch signaling in GH-induced EMT in podocytes. GH treatment induced the EMT markers (E-CADH, N-CADH, SANIL, SLUG, ZEB2, Vimentin, and α-SMA) in HPC and mouse glomerular lysates as shown at both mRNA

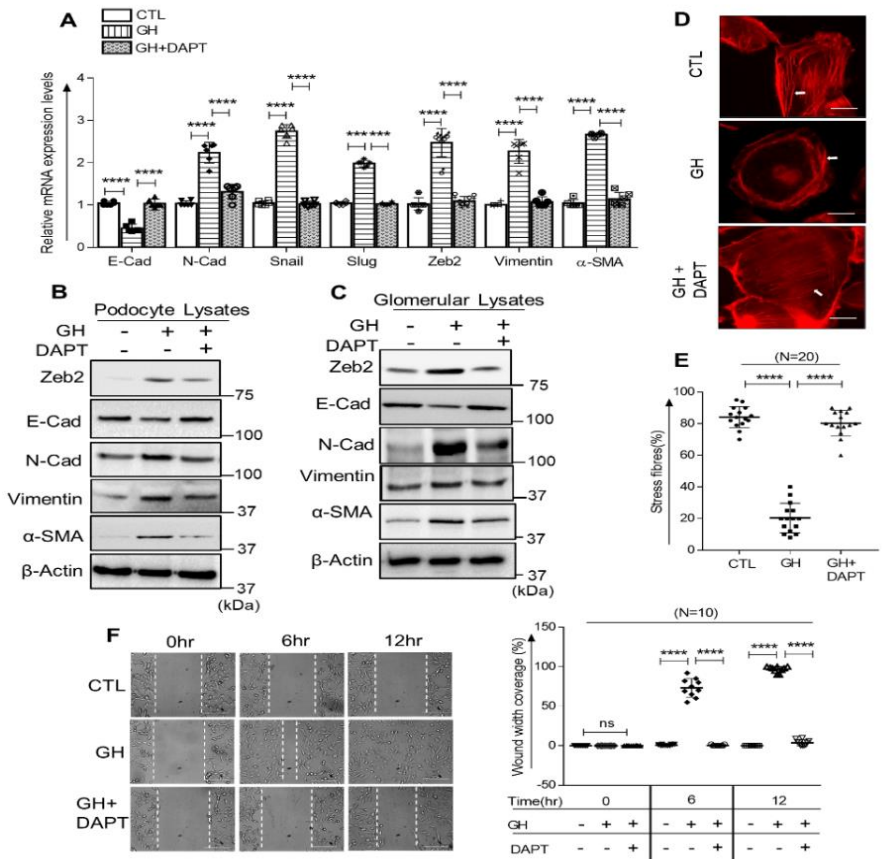

Figure 12: Notch activation elicits EMT in podocytes. ***A,B&C***, qRT-PCR and immunoblotting from HPC and glomerular lysates for EMT makres genes expression in with or without GH or GH+DAPT treatment. ***D,*** Phalloidin staining for cytoskeletal rearrangement in HPC from with or without GH and GH+DAPT treatement. ***E,*** Quantification of stress fibers formation from phallodin staining in with or without GH and GH+DAPT treatment. ***F***, Wound healing assay was performed in with or without GH and GH+DAPT for 0-12 hr. Quantification of area covered by cells were represepresnted with dot plot. *,p<0.05,**,p<0.01 and ***,p<0.001. Student t-test**.**. β-Actin as a internal control. Data are the mean ± SD .

(Fig. 12A) and protein levels (Fig. 12B&C). DAPT treatment abrogated this effect from GH on both humans (Fig. 12A, 4B) as well as in the glomerulus (Fig. 12C).

Remodeling of actin filaments is necessary for EMT [120]. To confirm the physiological function of EMT induced by GH, we next performed the phalloidin staining of the podocytes treated with GH, with or without DAPT treatment. The results presented in Fig. 12D show that there is complete disorganization of the actin filaments in the podocytes treated with GH, and can be rescued by co-treatment with the γ-secretase inhibitor, DAPT in these cells (Fig. 12D). Further, to confirm the role of Notch1 signaling in GH-induced EMT in the podocytes, we also performed the standard migration/ wound healing assay. While the GH treatment alone to the HPC completely covered the wound in 12 h., DAPT treatment completely abrogated this GH-induced phenotype (Fig. 12E). These results confirm that excess GH induces EMT in the podocytes, and activated Notch signaling is essential for GH-induced EMT in podocytes.

2.2.4 Activated Notch signaling is required for GH-induced interstitial infiltration of plasma-lymphocytic cells and fibrosis in kidneys

Diabetes is characterized by mild but significant glomerulosclerosis and tubulointerstitial fibrosis (TIF), and GH has been shown to increase both glomerulosclerosis and TIF [109]. Next, we treated the mice with GH and prepared the paraffin-embedded kidney sections to look for interstitial infiltration of plasma-lymphocytic cells. As previously noted, our Hematoxylin and eosin (H&E) staining of the kidney sections showed interstitial infiltration of plasma-lymphocytic cells in the mice treated with the GH (Fig. 13A). We also observed enhanced fibrosis in the mice kidneys treated with the GH, as analyzed by both Periodic acid-Schiff (PAS) (Fig. 13B) and Masson-Trichrome (MT) (Fig. 13C). Further, this data also correlated with increased fibrotic and decreased podocyte-specific markers expression, as measured at both mRNA (Fig.13D) and protein (Fig. 13E). This data also correlated with enhanced cytokine markers expression at mRNA levels (Fig. 13F).

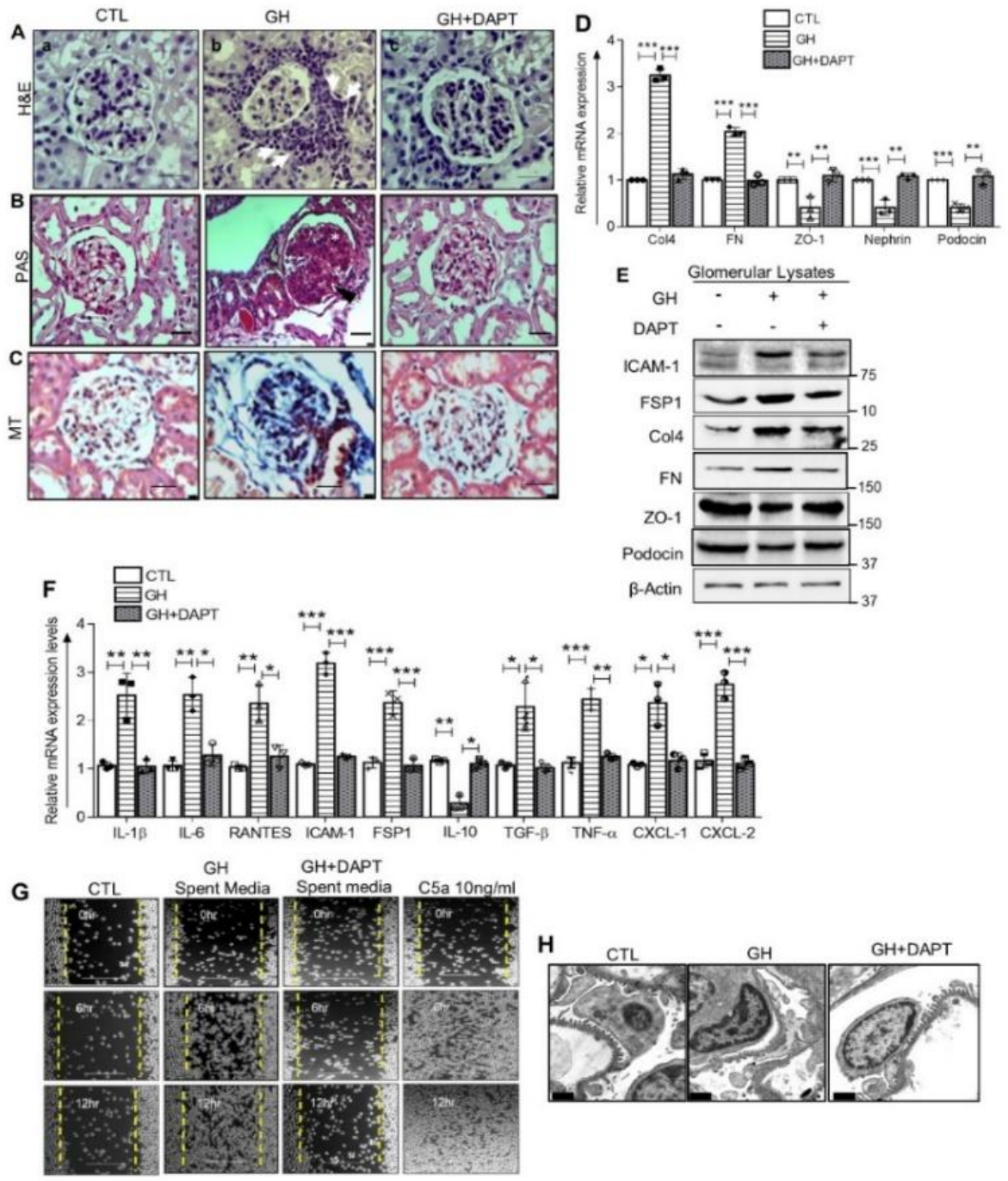

Figure 13: GH-induced Notch activation elicits immune cell infiltration and glomerulosclerosis. ***A,*** H&E staining for kidneys from with or without and GH+DAPT group showed interstitial infiltration of immune cells in GH treated group (indicated by white arrows). ***B&C,*** PAS and MT stained images of glomeruli from with or without and GH+DAPT indicating global change in GH treated mice. ***D,*** qRT-PCR expression of IL-1b, IL-6, RANTES, ICAM-1, FSP1, IL-10, TGF-β, TNFα, CXCL2 and CXCL1 in CTL, GH and GH+DAPT treated podocytes. ***E&F***, Immunobloting and mRNA expression for fibrotic and podocyte specific genes in with or without GH and GH+DAPT treatement. ***G.*** Macrophage migration assay with spent media from with or without GH and GH+DAPT treatment. ***H.*** TEM was used to study the changes in the glomerular basement membrane (GBM) and podocyte foot processes in with or without GH and GH+DAPT mice. n= 6. *,p<0.05,**,p<0.01 and ***,p<0.001. Student t-test. β-Actin as a internal control.

Indeed, the treatment of mice with DAPT had reversed the GH-induced non-resident cell infiltration into the kidneys (Fig. 13A) and was able to reverse the fibrosis, as evidenced by PAS (Fig. 13B) and MT (Fig. 13C) staining. DAPT treatment of the HPC also blocked the GH-induced cytokine, fibrotic gene induction, and reversal of podocyte-specific marker genes expression (Fig. 13B-E). To further evaluate whether the cytokines released from these podocytes are indeed functional, and DAPT blocked the function of these cytokines, we next performed a classical chemotaxis assay using J774A.1 macrophage. As expected, the macrophages could able to fill the gap when treated with conditioned media collected from podocytes treated with the GH. In contrast, this function was completely blocked when similar conditioned media from podocytes treated with GH+DAPT was used (Fig. 13G). The conditioned media from the untreated podocytes was used as a control Since kidney fibrosis leads to thickening of the glomerular basement membrane (GBM), it results in podocyte foot process effacement [121]. To confirm, whether a similar phenomenon also happens in our GH-induced proteinuria mice model, we next performed Transmission electron microscopy (TEM) thickening and podocyte foot process effacement, similar treatment of GH, along with DAPT, completely abrogated this effect (Fig. 13H). All these results confirm that excess GH activates Notch1 signaling, which in turn leads to infiltration of circulating cells in the kidneys and leading to its fibrosis.

2.2.5 Inhibition of activated Notch1 signaling abrogates GH-induced proteinuria in mice

Studies from our lab and others have shown that GH treatment to rodents induces loss of podocyte number [106]. GH treated mice kidney sections were used for the Diamino Benzidine (DAB) staining, for WT1 specific to podocyte nuclei and counted manually 20

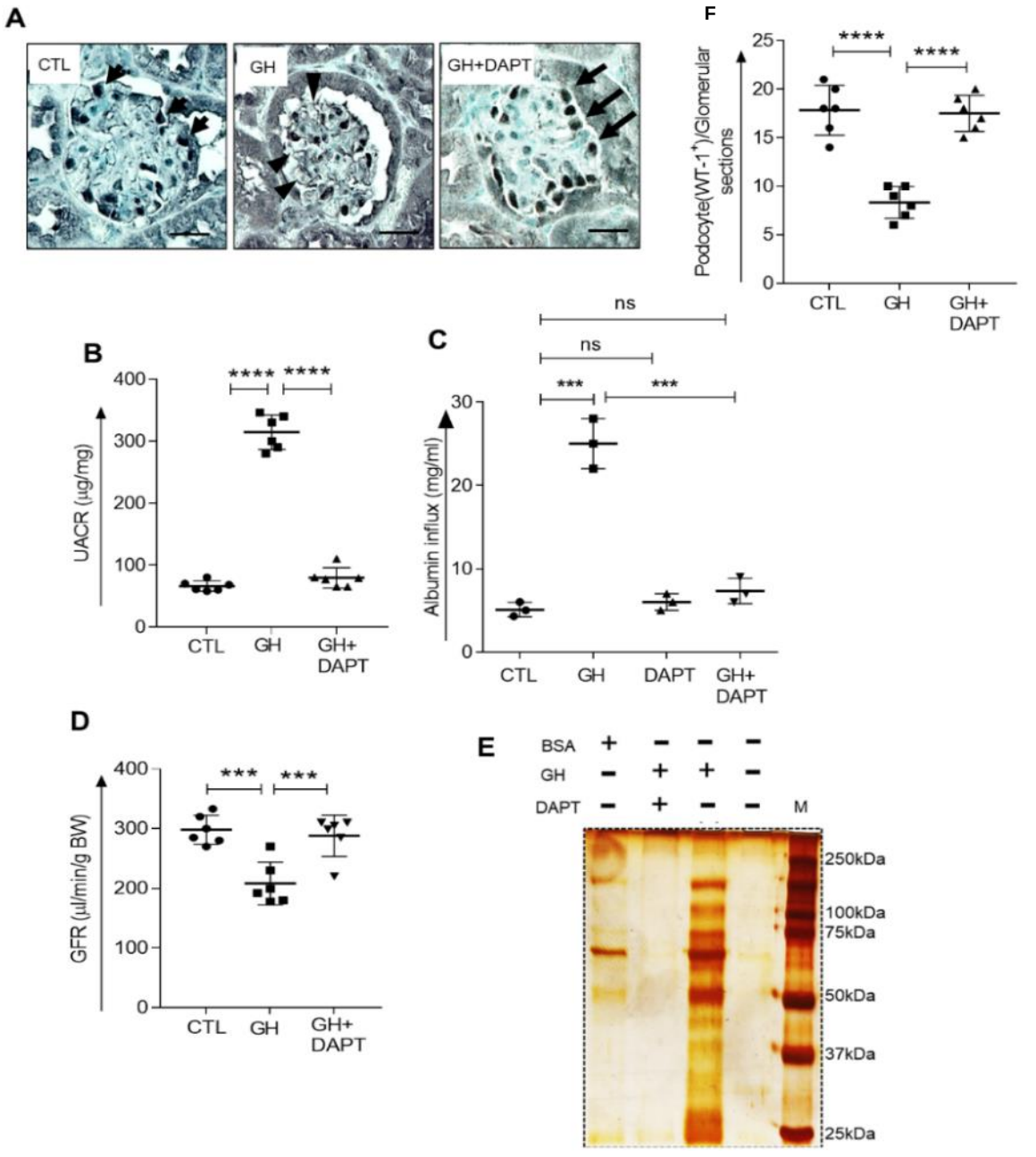

Figure 14: Abrogation of Notch signaling protect mice from GH- induced proteinuria. ***A,*** Representative image of $WT1^+$ (black dots) cells (podocytes) staining in glomerulus from with or without GH and GH+DAPT treated mice. ***B***, UCAR levels are estimated in with or without GH and GH+DAPT treated mice group. ***C***, Quantification of albumin influx across podocyte monolayer. ***D***, Representative result for GFR in with or without GH and GH+DAPT treated mice. *E*, Urinary protein levels were estimated in with or without GH and GH+DAPT group by silver staining. Qunatification of $WT1^+$ in with or without GH and GH+DAPT group.

glomeruli per section, we reconfirmed these results (Fig. 14A). We also confirmed that GH treatment reduced the physiological functional ability of the kidneys in the mice as analyzed

by increase in urinary albumin-creatinine ratio (Fig. 14B), increase in the albumin flux (Fig. 14C) and decrease in glomerular filtration rate (GFR) (Fig. 14D). We also measured the amount of protein secreted in the urine (proteinuria) of mice treated with GH by silver staining of mice urine samples on an SDS-PAGE gel. GH treatment had increased the amount of protein in urine, while simultaneous treatment with DAPT abrogated this effect (Fig. 14E). Treatment of mice with DAPT has rescued the decrease in podocyte number induced by the GH (Fig. 14A). We also observed that in the presence of DAPT, GH was not able to increase the albumin-creatinine ratio (Fig.14B) was not able to increase the albumin influx (Fig. 6C), and was not able to decrease the glomerular filtration rate (Fig. 14D). Finally, DAPT also blocked the proteinuria induced by the GH in these mice (Fig. 14E). All these data indicate that excess GH locks the physiological functionality of kidneys and induces proteinuria in mice through activated Notch1 signaling.

2.2.6 Notch signaling is hyperactivated in patients with diabetic nephropathy

Finally, to confirm the results obtained so far that excess GH induces activated Notch1 signaling, we evaluated the extent of NICD1 expression in people with DN. As expected, these patients showed higher albumin (Fig. 15A) and serum creatinine (Fig. 15B) levels and decreased glomerular filtration rate (Fig. 15C). We observed that there is more urinary protein in t hese patients, as observed by Coomassie brilliant blue staining of the urine samples (Fig. 15D). Immunohistochemical analysis of the kidney sections of the same patients showed that there is a more NICD1 expression in people with DN compared to the healthy controls (Fig. 15E) as well as JAG1 (Fig. 15D) and HES1 (Fig. 15D). Further study with these tissue samples for EMT markers showed elevated expression of α-SMA, N-CADH, and Vimentin (Fig. 15E).

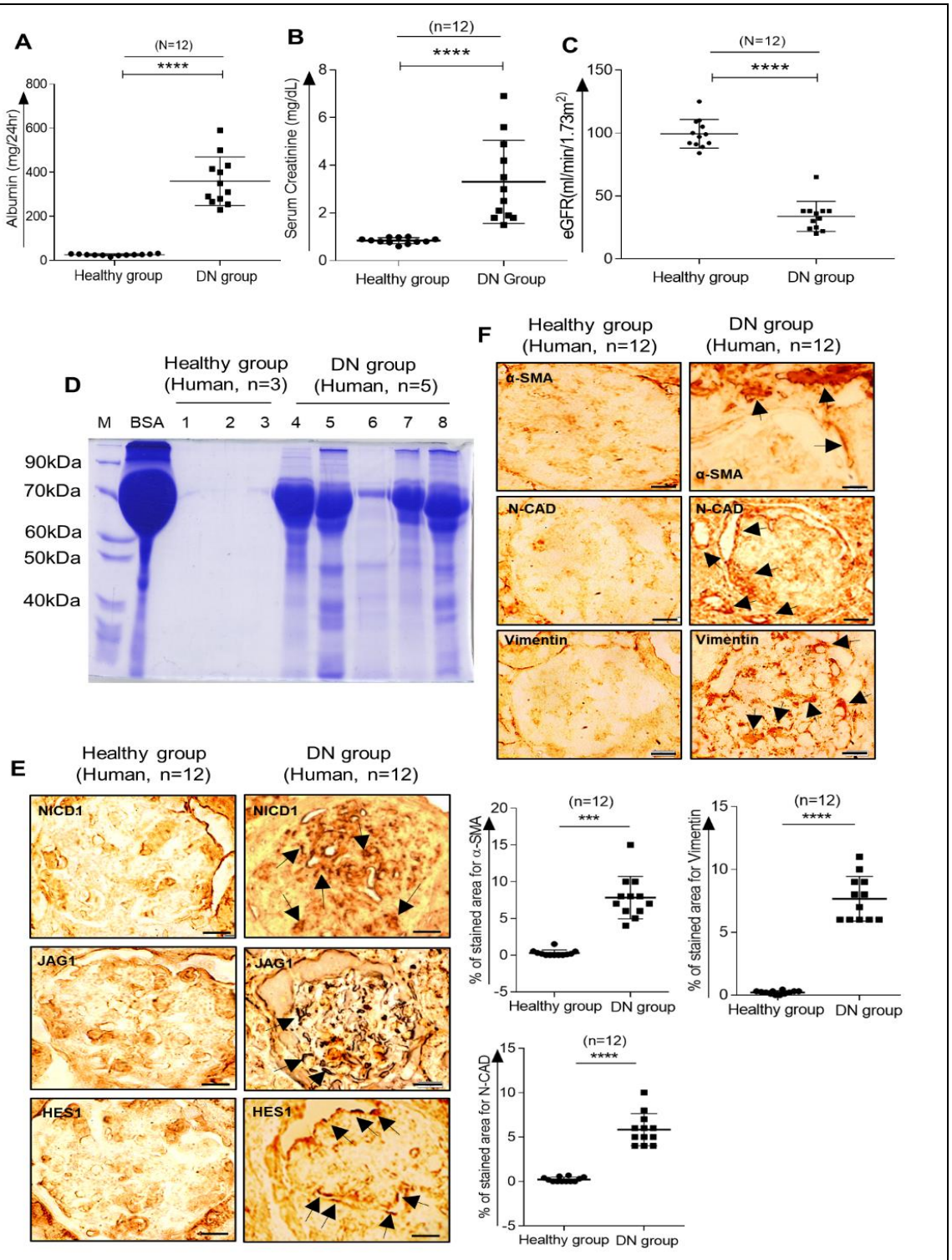

Figure 15: Activation of Notch and EMT is paralleled with proteinuria in humans with DN. ***A,B &C***, Estimation of serum albumin, serum creatinine and estimated GFR (eGFR) in healthy group vs DN group. ***D***, Coomassie blue stain of spot urine from people with DN (n= 5) and healthy group (n=3) was resolved by SDS-PAGE gel, which illustrates proteinuria in the DN group. ***E***, DAB staining for active NICD1, JAG1and HES1 were found reactivated in the DN group compared to the healthy group. ***F,*** DAB staining for α-SMA, N-CADH and Vimentin performed in DN biopsy sample in healthy (n=12) and DN group (n=12). Quantification of staining area by SMA, Vimentin and N-CAD were represented as a dot plot. M (Marker). 1, 2, 3, 4, 5, 6, 7 and 8 denote the number of wells where urine samples were loaded on SDS-PAGE gel.

2.3 Discussion:

In this study, we show that GH activates Notch1 signaling in podocytes and pharmacological inhibition of γ-secretase prevent the GH-induced EMT, infiltration of non-resident cells, fibrosis, GBM thickening and podocyte foot-process effacement (FPEs) of the kidneys. Glomerular sclerosis and albuminuria are major outcome to represent the failure of the normal kidney functions. GH had induced the albumin-creatinine ratio and the albumin influx, while the pharmalogical inhibitor for γ-secretase under similar conditions kept a constant check on these parameters and thus protected the mice kidneys from fibrosis. More importantly, GH treatment in the mice leads to proteinuria, a common symptom associated with diabetic nephropathy, which was successfully abrogated with the γ-secretase inhibitor treatment. Notch signaling pathway genes have been shown to be essential during kidney development i.e., during the embryonic development of proximal tubules and glomerular podocytes [122]. On the contrary, Notch signaling is not essential for podocyte development, beyond the stage of the S-shaped body [122, 123]. While, Niranjan et al., showed that activated ICN1 elevates podocyte apoptosis in rats [97], whereas in the experiments performed by Waters et al., showed that the podocyte-specific expression of the ICN1 promoted severe proteinuria and showed evidence of FPEs and progressive glomerulosclerosis [124]. The differences in these two results can be possible because of different mice strains used in their studies. Lasagni et al. observed that the administration of pharmacological inhibitor γ-secretase protected from worsened proteinuria and glomerulosclerosis in Adriamycin (ADR)-induced FSGS model mice [96]. Studies using knock-in mice models have given a clear indication of Notch pathway genes in podocytes diseased conditions. Podocyte-specific deletion of Notch1 in mice were shown to be protected from DN [125]. Similarly, podocyte-specific deletion of RBPjk, which is an important downstream component of canonical Notch signaling, has lowered the severity of proteinuria and reduced the podocyte injury in DN mice model [97].

However, the mechanism by which ICN1 or NICD1 is induced during the DN conditions is not yet known. The data presented in this study confirms that elevated levels of GH commonly observed during the DN [126], activates the Notch signaling in the podocytes and inhibition of this signaling by γ-secretase inhibitor blocks GH-induced proteinuria and FPEs in mice.

EMT and renal fibrosis, are the shared pathological hallmarks of progressive chronic kidney disease (CKD), which develops with diverse aetiologies. In mouse models with podocyte, EMT leads to proteinuria and FSGS, ultimately leading to kidney failure and death [97, 124]. Diabetes, especially hyperglycemia, was shown to stimulate podocyte EMT via several molecular mechanisms, including Notch signaling activation [127]. Podocyte-specific Notch activation leads to dedifferentiation and its decreased number in the glomerulus [97, 124]. In different models, either by using γ-secretase antagonist or weakening Notch transcriptional binding protein levels showed glomerular injury and fibrosis, demonstrating the role of Notch signaling in podocyte EMT [98, 99]. Several upstream master regulators like TGF-β and Wnt, etc., are shown to increase the Notch signaling [128-130].

Our data indicate that GH induces activated Notch1 signaling in podocytes, and since during DN, GH is upregulated [131]; and we observed increased NICD1 and its target gene expression in people with DN, we presume that during DN, GH activates Notch signaling in podocytes. Further, our data indicate that the activated Notch1 regulates the GH-induced EMT of the podocytes. Recent researches have also highlighted the critical role of hypoxia during the development of renal fibrosis as a final common pathway in end-stage kidney disease (ESKD) [132]. We had shown previously that GH induces the expression of EMT transcription factor, ZEB2, which transcriptionally downregulates P- and E-cadherin expressions in the podocytes [107, 133]. In the present manuscript, we show that GH induces NICD1, and treatment of podocytes with γ-secretase inhibitor blocks GH-induced EMT in podocytes.

Further studies are needed to understand the cross-talk between NICD1 signaling and ZEB2-dependent transcription in fine-tuning the EMT of the podocytes.

Transforming Growth Factor-beta (TGF-β) is a pro-sclerotic cytokine, and it is accepted that TGF-β and its downstream SMAD signaling is involved in the development and progression of renal fibrosis in people with DN [134, 135]. Studies from our laboratory report that GH regulates the bioavailability of TGF-β1 via regulating the expression of Transforming growth factor-beta-induced protein (TGFBIp) [106] and induces the expression of Smad-interacting protein, SIP1 [107]. Recently, we identified that GH directly stimulates TGF-β expression in podocytes (Mukhi et al., Manuscript communicated). Whereas DAPT treatment successfully abrogated GH-induced Notch signaling, our data shows that DAPT was not able to reverse GH-induced JAG1 expression. TGF-β has been shown to independently regulate JAG1 and HEY1 expression in renal epithelial cells [136, 137]. Further, TGF-β-induced EMT can be inhibited either by either HEY1 or JAG1 silencing as well as by chemical inactivation of Notch signaling [137]. All these data suggest that there is a significant cross-talk between Notch and TGF-β signaling in fine-tuning the EMT process. How this cross talk is specifically modulated during GH-dependant podocyte EMT, and renal fibrosis needs further studies.

Preclinical studies have shown that DAPT could suppress the Notch signaling (γ-secretase inhibitor) inhibitor, and also selective antibodies to preferentially target notch receptors and ligands have proven successfully [137]. Based on our data that people with DN show stronger NICD1 staining, as well as blocking NICD1 functions through γ-secretase inhibition prevent mice from proteinuria, we believe that DN can be controlled by using γ-secretase antagonist. However, for the commercial uses of γ-secretase inhibitors, there are clinical concerns remain over normal organ homeostasis and significant pathology in multiple organs. It is worth to note that Notch is not important for the podocytes after embryonic

development. Thus, based on our results, we believe that it is worth considering the possibility of localized tissue targeted delivery of such inhibitors for DN therapy.

2.4 Material and methods:

2.4.1 Reagents:

The primary antibodies are as following: anti-activated Notch1 (ab8925), anti-WT-1(ab212951), anti-pSTAT3a (ab76315), anti-t-STAT3a (ab5073), anti-Cleaved Notch1 (ab8925) and anti-FSP1 (ab41532), anti-HEY1(ab154077) were purchased from Abcam (Cambridge MA, USA). Notch1 Full-length (FL) (#3608), Anti-E-Cadherin (#3195), anti-N-Cadherin (#13116), anti-αSMA (#19245), anti-Cleaved Notch1 (#4147S), anti-ICAM-1 (#4915), anti-Actin (#4970) were purchased from Cell Signaling Technology (Danvers, MA, USA). The anti-HES1 (sc-166410) and anti-ZO-1 (sc-33725) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-Vimentin (PAB040Hu01), anti-Col4 (PAA180Hu01), anti-Fibronectin (PAA037Hu01), and anti-JAG1 (PAB807Hu01) were purchased from Cloud-clone (Houston, USA). anti-ZEB2 (PA5–20980) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). DAPT (D5942), SIGMAFAST DAB with Metal Enhancer Tablet Set (D8552), Phalloidin Fluorescein Isothiocyanate Labelled (P5282), Glutaraldehyde solution (G5882) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pink pre-stained marker protein (ABIN5662611, Nippon Genetics Europe), Protein marker (Precision Plus Protein Dual Color Standards, California) ProLong™ Diamond Antifade Mountant (P36961) purchased from molecular probes life technologies and DyLight 488 and DyLight 564, and Cy5-conjugated secondary antibody were obtained from Vector Laboratories (Burlingame, CA, USA). Primers are used in this study procured from Integrated DNA Technologies (Coralville, IA, USA). cDNA reverse transcription kit and SYBR Green Master Mix reagents were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All the chemicals used in this study were from analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.4.2 Animals and tissues:

All the experimental procedures for the animals were pre-approved by the Institutional Animal Ethics Committee of the University of Hyderabad, India. 8-week old swiss webster male mice weighing nearly 30±5g were used in this study. The mice were randomly assigned to three groups (6 mice per group): 1) control group (CTL), 2) GH treated group, and 3) GH+DAPT treated group. Experimental mice received a single i.p dose of hGH (1.5 mg/kg/day) whereas, control mice have received an equal volume of saline for four weeks. The inhibitor groups were received 10mg/kg body weight DAPT per day through i.p before the GH treatment. After four weeks of the experimental period, the mice were placed in individual metabolic cages for collecting 24 h urine to estimate albumin and creatinine. An aliquot of urine from mice was subjected to SDS-PAGE gel and performed silver staining to visualize proteins in the urine [133]. Briefly, the urinary protein was estimated using a 10% SDS-PAGE gel. 1µl of BSA standard of 1mg/ml and 5 µl of a urine sample from CTL, GH, and GH+DAPT treated mice (n= six each group) were used to estimate the quantity of protein in the urine. We have also estimated the glomerular filtration rate (GFR) in these mice, as described previously [133]. Briefly, mice were sacrificed, and kidneys were collected, and glomeruli were isolated. Alternatively, some kidneys were processed for histological analysis, protein, and RNA isolation. For histological analysis, kidney cortex from mice was fixed with 4% paraformaldehyde prior to embedding in paraffin. Paraffin-embedded tissues were sliced longitudinally into 3 µm thick sections, subjected to staining with Haematoxylin and Eosin, periodic acid-Schiff (PAS) and Masson's trichrome (MT) staining's. We also performed immunohistochemistry for analyzing the expression of specific markers. Transmission Electron Microscopic (TEM) images were obtained for glomerular sections from experimental mice groups, as described earlier [138].

2.4.3 Human kidney:

Kidney samples were collected without patient identifiers from archived kidney biopsies at Guntur Medical College and Government General Hospital, Andhra Pradesh, India. We selected cases with biopsy-proven diabetic nephropathy and with significant proteinuria. This study was approved by the Institutional Review Board of Guntur Medical College and Government General Hospital, Guntur, Andhra Pradesh, India (Application number GMC/IEC/120/2018).

2.4.4 Morphological studies:

All histological quantifications were evaluated in a blinded manner by two independent investigators. Using kidney sections from these mice (n=6 each group), we were examined 20 consecutive glomeruli per mouse for evaluation of glomerular mesangial expansion and the average value of 120 glomeruli from each group presented as a dot plot. The index of the mesangial expansion was defined as the ratio of mesangial area/glomerular tuft area. The mesangial area was determined by assessment of the PAS-positive and nucleus-free area in the mesangium using Image J (NIH). For measurement of GBM widths in TEM picture, we have randomly taken nearly 3-4 glomeruli per animal (n=6 each group), 5 pictures per glomerulus (20 shots per animal) at 5 points, evenly distributed per loop, and average value from each group were represented as a dot plot. All the TEM Images were taken at X14000 magnification using JEM-1400TEM (Jeol, Peabody, MA) using a Gatan ultrascan CCD camera (Gatan Inc, Pleasanton, CA).

2.4.5 Estimation of Glomerular Filtration Rate:

Glomerular filtration rate (GFR) in mice was performed at 7 weeks of age using a FIT GFR Test Kit for Inulin according to the manufacturer's instructions (BioPal, Worcester, MA, USA). Briefly, 5 mg/kg inulin was injected intraperitoneal, followed by serial saphenous bleeds at 30, 60, and 90 minutes. Next, serum isolation was done and quantified on an inulin

ELISA kit. Serum inulin clearance estimation was performed by the nonlinear regression method using a one-phase exponential decay formula ($y = Be{-}bx$). GFR was calculated (GFR = ((I)/(B/b))/KW, where I is the amount of inulin delivered by the bolus injection, B is the y-intercept, b is the decay constant, x is time, and KW is kilo weight of the animal). Alternatively, spot urine collection was performed for CTL, GH and GH+DAPT groups and urinary albumin (#COD11573) and creatinine (#COD11502) levels estimated using available assay kits (Biosystems, Barcelona, Spain).

2.4.6 Podocyte culture and experimentation:

Conditionally immortalized HPC were cultured as described earlier [107]. Differentiated podocytes were maintained for 12-16 h in serum-free medium before treating with hGH (Pfizer, NY, USA) or pegvisomant (Pfizer, NY, USA) and DAPT (Sigma-Aldrich, St. Louis, MO, USA). Unless otherwise mentioned, all the experiment conditions for podocyte cells were given for 12 h. The cell lysate was prepared for RNA isolation or immunoblotting. For immunofluorescence, cells were cultured on coverslips, followed by treatment as mentioned above, subsequent fixation with paraformaldehyde (4%), and blocking with PBS containing normal BSA (5%) prior to incubation with primary antibodies. The next day, the samples were incubated with Alexa Fluor-conjugated secondary antibodies, DAPI for 1 h. Images were acquired using a confocal microscope (Zeiss, NY, USA). Albumin influx assay across podocyte monolayer was performed as described earlier [106].

2.4.7 Immunoblotting:

Glomerular protein lysate from kidney tissues or human podocytes cells were extracted with lysis buffer containing protease inhibitor (150mM NaCl, 1% NP-40, 0.1% SDS, 2 μg/mL aprotinin, 1mM PMSF) for 30 min at 4^{o}C. The supernatants for 25 min at 4^{o}C were centrifuged at 12000g and total protein harvested. Protein estimations were performed by BCA assay (#23225, Thermo Fisher Scientific, Waltham, MA, USA). An equal amount of protein (50 μg)

from samples was electrophoresed through 10 to 15% SDS-PAGE gel and transferred to nitrocellulose membranes at 50 v for two h by electroblotting. The membranes were soaked in blocking buffer 5% non-fat milk for one h at room temperature. Subsequently, protein detection was performed using different primary antibodies overnight at 4^{o}C, they visualized using anti-rabbit, anti-mouse IgG conjugated with horseradish peroxidase (1:10000, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. Blots were developed using the ECL western blotting substrate (#1705060, Bio-rad, Hercules, CA, USA) and chemiluminescence device by Bio-Rad Versa Doc 5000 MFP.

2.4.8 RNA extraction and quantitative RT-PCR:

The total transcripts were extracted by using Trizol reagent (Invitrogen, Waltham, MA, USA) and isolated using an RNA isolation kit (Qiagen, Hilden, Germany). We examined the RNA quality on 2% agarose gels and quantification performed by NanoDrop (#ND2000LAPTOP, USA). Next, 1 μg of total RNA was reverse transcribed using the cDNA synthesis kit (Thermo Fisher Scientific). qPCR analysis was performed by QuantStudio 3 system (Applied Biosystem) with the SYBR Green (KAPPABIOSYSTEM, USA) Master Mix using three-step standard cycling conditions with sequence-specific primers. To ensure a single PCR product was amplified, we examined the melting curve for each primer. mRNA expression of each gene was normalized using the expression of β-actin as a housekeeping gene.

2.4.9 Enzyme-Linked Immunosorbent Assay:

γ-Secretase activity was quantified according to the manufacturer's instructions (ImmunoTag, G-Biosciences, St. Louis, MO, USA). Briefly, the γ-secretase activity was determined by quantification of human APH1A (γ-Secretase subunit APH-1A) with biotin-conjugated anti-APH1A antibody as a detection antibody. The cleavage-dependent release was measured at 450 nm by using a fluorescent plate reader (Thermo Scientific).

2.4.10 Chemotaxis assay:

J774A.1 macrophage were cultured as a monolayer and were scratched using the sterile 10 µl tip and washed with PBS to remove cell debris. Conditioned media from podocytes treated with GH, GH+DAPT or naïve to any treatment was added to J774A.1 macrophage. C5a was used as a positive control with a concentration of 10ng/ml [139]. After the scratch, the images of the wounded area were captured on 0 h, 6 h and 12 h intervals to monitor J774A. 1 macrophage migration into the wounded area. The migratory abilities were quantified by measuring the distance between cells in the scratch zone.

2.4.11 F-actin staining:

Phalloidin staining for F-actin was performed to visualize the distribution of stress fibers in differentiated podocytes as describe previously [140]. Briefly, the cells were fixed in 4% paraformaldehyde at room temperature for 15 min. After washing, and then permeabilized for 15 min. with 0.3% Triton X-100 in PBS followed by 5% BSA blocking. Cells were incubated with rhodamine-phalloidin (Invitrogen Corp., Carlsbad, CA, USA) for 15 min. at room temperature to stain F-actin. The slides were examined using confocal laser scanning microscopy. Fifty cells per group were counted to calculate the ratio of cells retaining distinct F-actin fibres actin. The slides were examined by Leica Triocular Microsystems.

2.4.12 Wound healing assay:

The phenomenon of EMT in podocyte cells was assessed using a “wound-healing” migration assay. A confluent monolayer of podocytes in 6-well plates was wounded with 10 µl pipette tip following two perpendicular diameters, giving rise to two acellular clear areas per well. After washing with PBS, podocyte cells were treated with GH and GH+DAPT and incubated for 0,6 and 12 h. Photographs were taken at different times of incubation for GH and GH+DAPT along with CTL (after 0,6 and 12 h). The extent of migration at these time points was determined by image analysis (ImageJ software) in both control and treated wells.

2.4.13 Primer table:

TGFBR1(Human)	FP= TCAATTGTAAGCACATTGAAAGGG
	RP=TTCGCCCGGCAGATCTAAAC
TGFBR1(Mouse)	FP=AAGACAACTGCCAGCCCTTAG
	RP=TCATTTAGTGCCACACCCCA
TGF-β1(Human)	FP=GTTCAGGTACCGCTTCTCGG
	RP=CCTGATCGCCTCCCTTCATTT
TGF-β1(Mouse)	FP=AAATCAACGGGATCAGCCCC
	RP=CGCACACAGCAGTTCTTCTC
Notch1(Human)	FP=TGAATGGCGGGAAGTGTGAA
	RP=CACAGCTGCAGGCATAGTCT
Notch1(Mouse)	FP=AGACATGTAGGGCAGTCAGC
	RP=CCAGAGCTTACGTCATCCCA
HES1(Human)	FP=ATGACAGTGAAGCACCTCCG
	RP=GAGTGCGCACCTCGGTATTA
HES1(Mouse)	FP=TCCCACGGTCTGGGTCTTAT
	RP= GTGCTAAACCACTGACCCCT
JAG1(Human)	FP=CCTGTCCATGCAGAACGTGA
	RP=CGCGGGACTGATACTCCTT
JAG1(Mouse)	FP=GTTTCGCAGGAGGCCTGTTT
	RP=CTGGGTCAGCACCGAGAATG
BAX(Human)	FP=CTGACGGCAACTTCAACTGG
	RP=GCAGGGGGTTGATACCACG
Bcl2(Human)	FP=CGGGTTGTCGCCCTTTTCTA
	RP=TCACAGATCTGAGGGGGAGC
CTGF(Mouse)	FP=GCATCTCCACCCGAGTTACC
	RP=TAGGGGCAGAGGATGTACCTT
BMP7(Mouse)	FP=GTCTGCCAGGAAAGTGTCCA
	RP=CGAGGCTTGCGATTACTCCT

2.4.14 Statistical Analysis:

The results are presented as mean ± DS of at least three independent experiments unless otherwise mentioned. Prism software (GraphPad Software Inc.) was used to analyze the data. Statistical differences between the groups made using Student's *t*-test. Statistical significance was determined as $P<0.05$.

Chapter 3: Growth hormone induces podocyte mitotic catastrophe

3.1 Introduction:

Albuminuria is a marker for renal dysfunction in the general population and is an early marker for overt nephropathy in diabetic subjects. Elevated circulating growth hormone (GH) levels and increased renal expression of the GH receptor (GHR) are associated with nephropathy in poorly controlled type1 diabetes [101, 102]. Excess GH conditions are characterized by glomerular hypertrophy, sclerosis, and albuminuria, whereas blunting GH action is protected from glomerulopathy [104]. Gaddamedi et al. showed that podocytes express GHR, and canonical JAK-STAT signaling is activated when podocytes are exposed to GH [105]. Our previous work showed that GH activates Notch signaling [108] and promotes epithelial to mesenchymal transition (EMT) of podocytes by inducing ZEB2 (Zinc Finger E-Box Binding Homeobox 2) [106, 107].

GH-induced glomerulosclerosis and interstitial fibrosis in diabetic rats is associated with increased TGF-β1 levels [109], whereas inhibition of JAK2, an immediate downstream target of GH, reduced TGF-β1 expression [110]. Although multiple studies revealed TGF-β1's role in morphologic manifestations and clinical characteristics of DN, the stimuli that activate the TGF-β/SMAD pathway in the podocytes remain unclear [110, 111]. In the present study, we demonstrate that GH induces TGF-β1 expression, which in-turn transduces Notch activation. GH and TGF-β1 dependent Notch activation stimulated podocyte re-entry into the cell cycle. Nevertheless, persistent activation of Notch signaling resulted in cytokinesis failure and podocyte apoptosis.

3.2 Results:

3.2.1 GH induces TGF-β1 and cognate TGF-β-SMAD pathway in podocytes:

Considering the established role of GH and TGF-β1 in eliciting podocyte injury, we investigated the direct action of GH on the TGF-β/SMAD pathway. TGF-β1 mRNA (Fig.1A&B) and protein (Fig.1C&D) levels were up-regulated in both dose (0-500 ng/ml) and

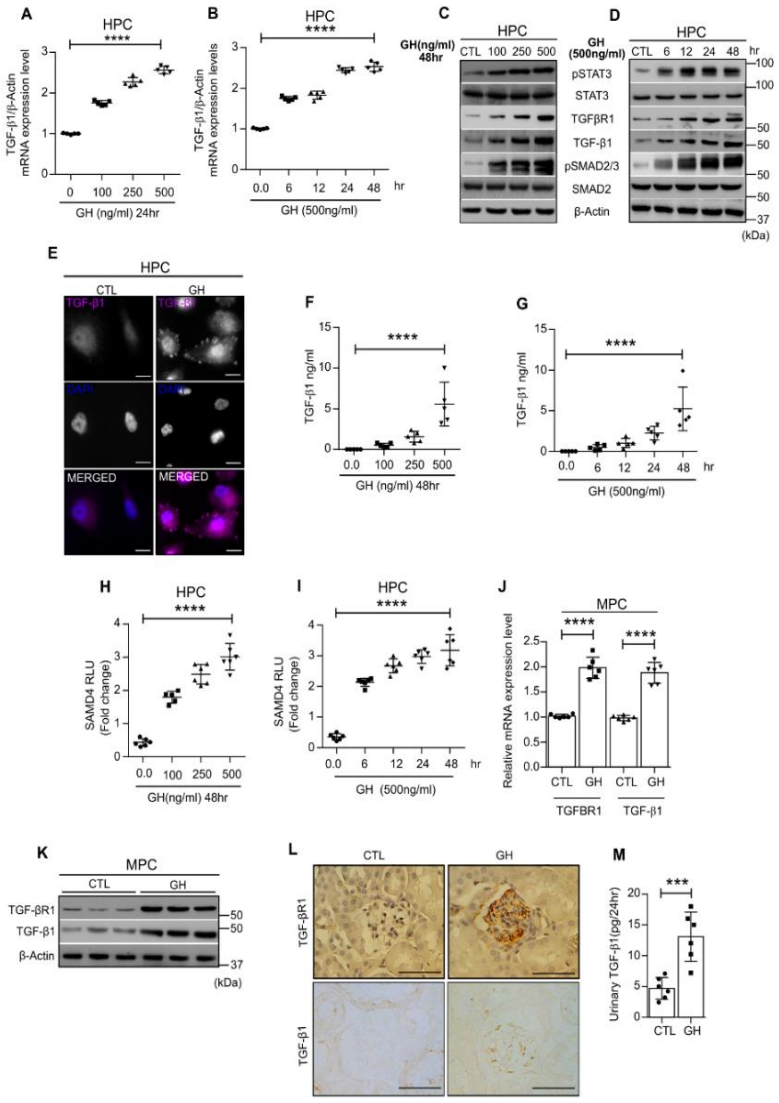

Figure 16: GH induces TGF-β/SMAD pathway in podocytes: ***(A&B)*** qRT-PCR for TGF-β1 in HPC treated with or without GH in concentration (100 to 500 ng/ml) and time (up to 48 hr) dependent manner. ***(C&D)*** Immunoblotting for HPC treated with or without GH in concentration (100 to 500 ng/ml) and time (up to 48hr) dependent manner. ***(E)*** Immunofluorescence for TGF-β1 in HPC treated with or without GH (500 ng/ml, 48hr). ***(F&G)*** Estimation of TGF-β1 in conditioned media (CM) from HPC treated with GH in concentration (100 to 500 ng/ml) and time (up to 48hr) dependent manner. ***(H&I)*** SMAD4 luciferase activity in HPC was treated with GH in concentration (100 to 500 ng/ml) and time (up to 48 hr) dependent manner. ***(J&K)*** qRT-PCR and immunoblotting for expression of TGFBR1 and TGF-β1 in CTL or GH-treated (1.5mg/Kg bw) mice podocytes (MPC). ***(L)*** TGFBR1 and TGF-β1 expression by DAB staining in mice glomerular sections from CTL vs GH group. (M) Quantification of TGF-β1 in urine from CTL or GH treated mice. Mean±SD. Scale bar:100μm.. (n=3) ****p<0.0001 by Student t-test. β-Actin served as internal control.

time-dependent (0-48 h) manner in GH treated human podocytes (HPC). Immunofluorescence analysis also revealed GH induced TGFβ1 expression in podocytes (Fig.1E). We also observed GH dependent expression of pSTAT3 (Tyr705) and components of TGF-β1 signaling such as

TGFBR1 (TGF-β Receptor 1) and pSMAD2/3 in podocytes (Fig.1C&D). As TGF-β1 is a secretory molecule, we estimated it levels in conditioned medium from GH (GH- CM) treated podocytes and observed that TGF-β1 is induced by GH in both dose and time-dependent manner (Fig.1F&G). We verified the TGF-β1 activation in GH treated podocytes by performing SMAD4-Luciferase activity assay (Fig.1H&I). Furthermore, podocytes isolated from mice (MPC) administered with GH showed elevated expression of TGFBR1 and its ligand TGF-β1 on mRNA and protein levels (Fig.1J&K) and their enhanced staining in the glomerulus (Fig.1L). TGF-β1 was also detected in urine from mice administered with GH (Fig.1M). Together, these data support the activation of cognate TGF-β1/SMAD signaling in podocytes by GH.

3.2.2 TGF-β1 signaling is required for GH induced Notch reactivation in podocytes:

Notch activation was virtually undetectable in glomeruli from heathy adult kidney, unlike their progenitors in the fetal kidney which show enhanced Notch activity [96]. Previously, we showed that GH activates Notch signaling in adult podocytes [108]. Earlier to us, Niranjan et al. 2008 reported that TGF-β1 induces Notch signaling in podocytes from diabetic mice [97]. Since circulating levels of GH elevate in type1 diabetes milieu, and both GH and TGF-β1 were shown to induce Notch signaling we sought to investigate whether GH activates Notch signaling via TGF-β1. When podocytes naive to GH were exposed to GH-CM, activation of Notch signaling was observed similar to that of podocytes treated with GH (Fig.2A&B). It is noteworthy that a TGFBR1 inhibitor (SB431542) ameliorated the effect of GH-CM to induce Notch activation (Fig.2C). Interestingly, expression of Notch1 and its downstream targets were ameliorated when podocytes were treated with GH in the presence of inhibitors for either GHR (AG490) or TGFBR1 (Fig.2D).

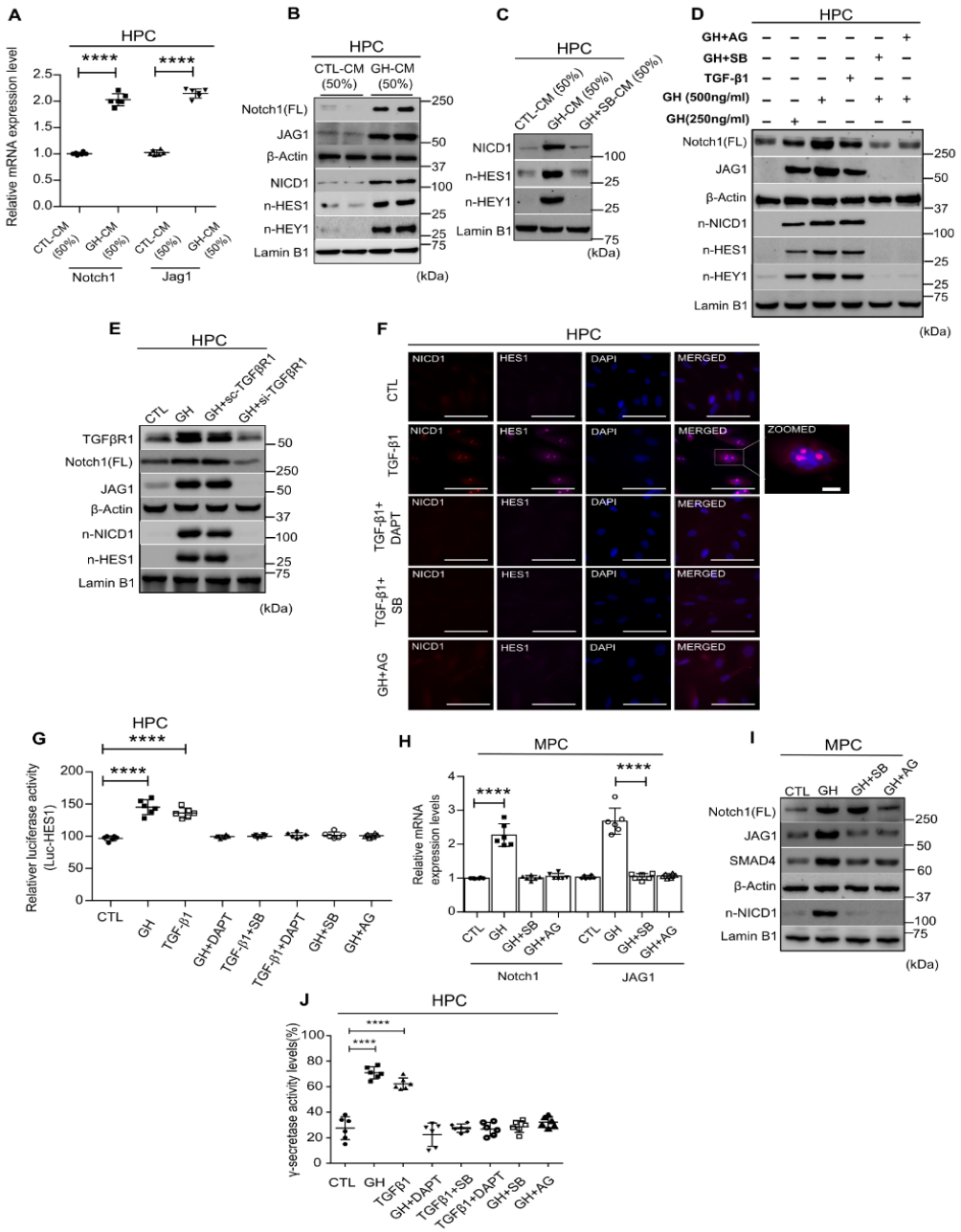

Figure 17: GH induces TGF-β/SMAD pathway mediated Notch signaling in podocytes: ***(A)*** qRT-PCR for Notch1 and Jag1 in HPC treated with or without conditioned medium (CM) of 50% from CTL and 50% GH treated podocytes for 48h. ***(B)*** Immunoblotting for HPC treated with or without CM of 50% from CTL and 50% GH treated podocytes for 48hr. ***(C)*** Immunoblotting for HPC treated with or without GH (250 and 500ng/ml), TGFβ-1 (5ng/ml), GH (500ng/ml) + SB431542 (100nM/ml) and GH (500ng/ml) + AG490 (10μM/ml) for 48hr. ***(D)*** HPC cells transfected with specific siRNA targeting TGFBR1 or scramble RNA (Scr-RNA) were subjected to immunoblotting. ***(E)*** Immunofluorescence for nuclear co-localization of NICD1 (red color), HES1 (purple color) and DAPI (blue color) in HPC from CTL vs treatments for 48hr. ***(F)*** HES1 reporter activity was measured in HPC from CTL vs treatment for 48h. ***(G)*** qRT-PCR analysis of Notch1 and JAG1 expression in MPC from CTL or GH (1.5mg/kg bw), GH+ SB431542 (1mg/kg bw) and GH+AG490 (1mg/kg bw) administered mice (each group, n=6). ***(H)*** Immunoblotting analysis for MPC from CTL vs treatment group of mice. (n=3). β-Actin and Lamin-B1 served as an internal control.

Also, GH fails to induce Notch signaling components in engineered podocytes with a specific knockdown of TGFBR1(Fig.2E). Further, the transcriptional activity of NICD1 (Notch intracellular domain) as determined by both nuclear localization of NICD1 and HES1-Luciferase reporter assay is elevated upon GH or TGF-β1 treatment (Fig.2F&G). Nuclear localization of NICD in podocytes primed with GH or TGF-β1 was reduced in the presence of AG490 or SB431542 (Fig.2H). Similarly, the observed increase (~45%) in the transcriptional activity of HES1 by GH or TGF-β1 was abrogated by AG490 or SB431542 (Fig.2I). γ-Secretase is an intracellular protease that cleaves NICD from the Notch receptor and triggers the Notch cascade. Increased γ-secretase activity by GH and TGF-β1 was abolished when podocytes were treated with AG490 or SB431542 (Fig.2J). Indeed, inhibition of γ-secretase activity by DAPT abolished HES1 promoter activity in podocytes exposed to GH or TGF-β1 (Fig.2G). Notch activation in podocytes isolated from GH administered mice was also ameliorated in the presence of SB431542 or AG490 (Fig.2H&I). Together, these data suggest that GH activates Notch signaling in podocytes, and it is mediated via TGF-β1.

3.2.3 Both GH and TGF-β1 induce cell cycle re-entry of quiescent podocytes in a Notch1 dependent manner:

In healthy mice and human; mature podocytes are in the quiescent stage (G0 phase), a prerequisite for their highly specialized functions [113]. Mature podocytes are terminally differentiated and express high levels of cyclin-dependent kinase (CDK) inhibitors suggesting that these cells lack the ability to renew during adult life [114]. Since Notch signaling was shown to induce proliferation of embryonic stem cells and cell cycle re-entry of terminally differentiated cells [112, 115], we assessed whether activated Notch signaling induces cell cycle re-entry of podocytes in our experimental setting. When podocytes were stained for α-tubulin, we found that 27±10% of GH or TGF-β1 treated cells were in anaphase as suggested

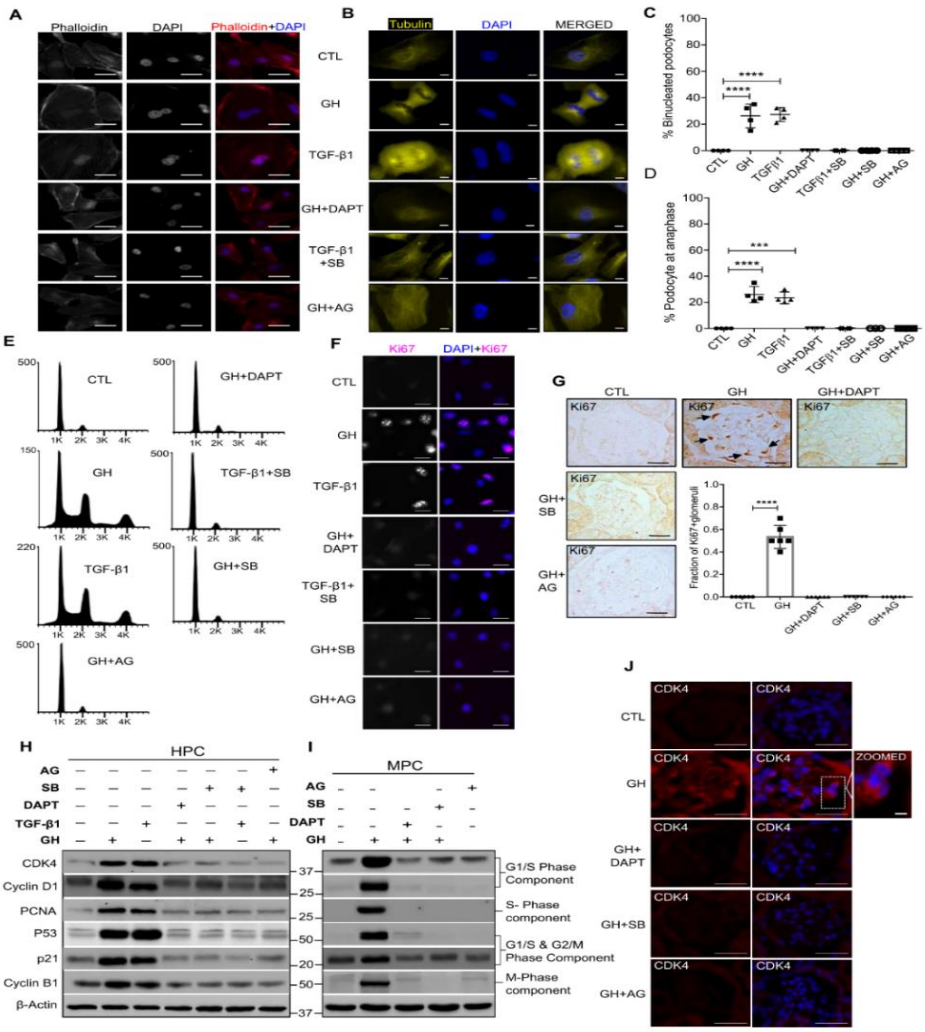

Figure 18: GH stimulates cell cycle re-entry and binucleation in differentiated podocytes: ***(A&B)*** F-actin staining, α-Tubulin, and counterstained with DAPI in HPC from CTL vs treatment for 48hr. ***(C)*** The representative graph showed the percentage of binucleated HPC from CTL vs treatment for 48hr. ***(D)*** Percentage of HPC at anaphase from indicated CTL vs treatment for 48hr. ***(E)*** Cell cycle phases of HPC from CTL vs treatment for 48hr. ***(F)*** Immunofluorescence for the Ki67 and counterstained with DAPI in HPC from CTL vs treatment for 48hr. ***(G)*** Representative images for anti-Ki67 expression by DAB staining in mice glomerular sections from CTL vs treatment group and graph represent the quantification of $Ki67^{+}$ glomeruli. Black arrow indicates specific expression of Ki67 in podocytes. ***(H&I)*** Immunoblotting analysis from HPC (48hr treatment) and MPC from CTL vs treatment group. (n=3). ***(J)*** Representative images for CDK4 and counterstained with DAPI in glomeruli from CTL vs treatment group. Magnification x630 and x400. Scale bar:20 μm. (n=3). White arrowhead indicates specific expression of CDK4 in podocyte. β-Actin served as internal control.

by microtubule formation (Fig.3A&C), whereas DAPT prevented GH-induced cell cycle progression (Fig.3A&C). The morphological screening of podocytes by phalloidin staining revealed 24±7% of GH or TGF-β1 treated cells were bi-nucleated and hypertrophic (Fig.3A&C). This aberrant phenotype was not observed in podocytes treated with AG490 and

SB431542 (Fig.3A&C). Interestingly, inhibition of Notch by DAPT mitigated GH induced podocyte binucleation vis-a-vis hypertrophy (Fig.3A&C). To further ascertain the activation of cell cycle events with GH or TGF-β1, we performed flow cytometric analysis (Fig.3E). Flow cytometry data revealed that ~40% & 31% of GH treated podocytes were in S and G2/M phase, respectively. Similarly, ~35%&28% of TGF-β1 treated podocytes were also accumulated in the S and G2/M phase, respectively (Fig.3E). As anticipated, AG490 and SB431542 abrogated cell cycle progression (Fig.3E). We observed elevated Ki67 expression, which is strongly associated with cell proliferation in GH-treated podocytes, both *in vitro* and *in vivo* (Fig.3F&G). Despite podocytes displaying proliferative phenotype when exposed to GH and TGF-β1, they also showed bi-nucleation, suggesting only successful karyokinesis, but not cytokinesis. Therefore, in addition to proliferating markers (PCNA), we analyzed the expression of cell cycle regulators and checkpoints. Interestingly, in addition to cell cycle activators (CDK4 & CyclinD1), we also observed elevated expression of both G1/S and G2/M checkpoints, implying a complex two-tier regulation of cell-cycle events in podocytes exposed to GH or TGF-β1 (Fig.3H-J). Inhibition of GHR or TGF-βR1 mitigated Ki67 expression and also attenuated cell-cycle regulators in both human and mouse podocytes (Fig.3H-J). Strikingly, inhibition of Notch activation by DAPT abrogated GH-induced proliferating markers and, in turn activation of cell cycle events (Fig.3F-J). Together, these data reveal that podocytes overcome quiescent stage and re-enter the cell cycle during stimuli such as exposure to GH or TGF-β1 in a Notch1 dependent manner.

3.2.4 Cytokinesis failure induces apoptosis in GH, or TGF-β1 treated podocytes:

Incomplete cytokinesis could be one of the predominant possibilities for binucleation that might arise as a result of aberration in contractile ring assembly or ingression phase of cytokinesis. RhoA, a member of the Rho GTPase family, is essential for cytokinesis via acting at the mid-body during cleavage furrow ingression and thriving generation of two daughter

cells [116, 118]. Elevated expression of RhoA was observed in podocytes either treated with GH or TGF-β1 and also during ectopic expression of NICD1 (Fig.4A-C). On the other hand, inhibition of Notch reduced GH or TGF-β1 induced RhoA expression (Fig.4A-B). Interestingly, abnormal localization of RhoA expression (away from the contractile ring) was observed in podocytes treated with GH (Fig.4D). Although GH elicited cell-cycle entry of quiescent podocytes, these cells fail to accomplish successful cell division.

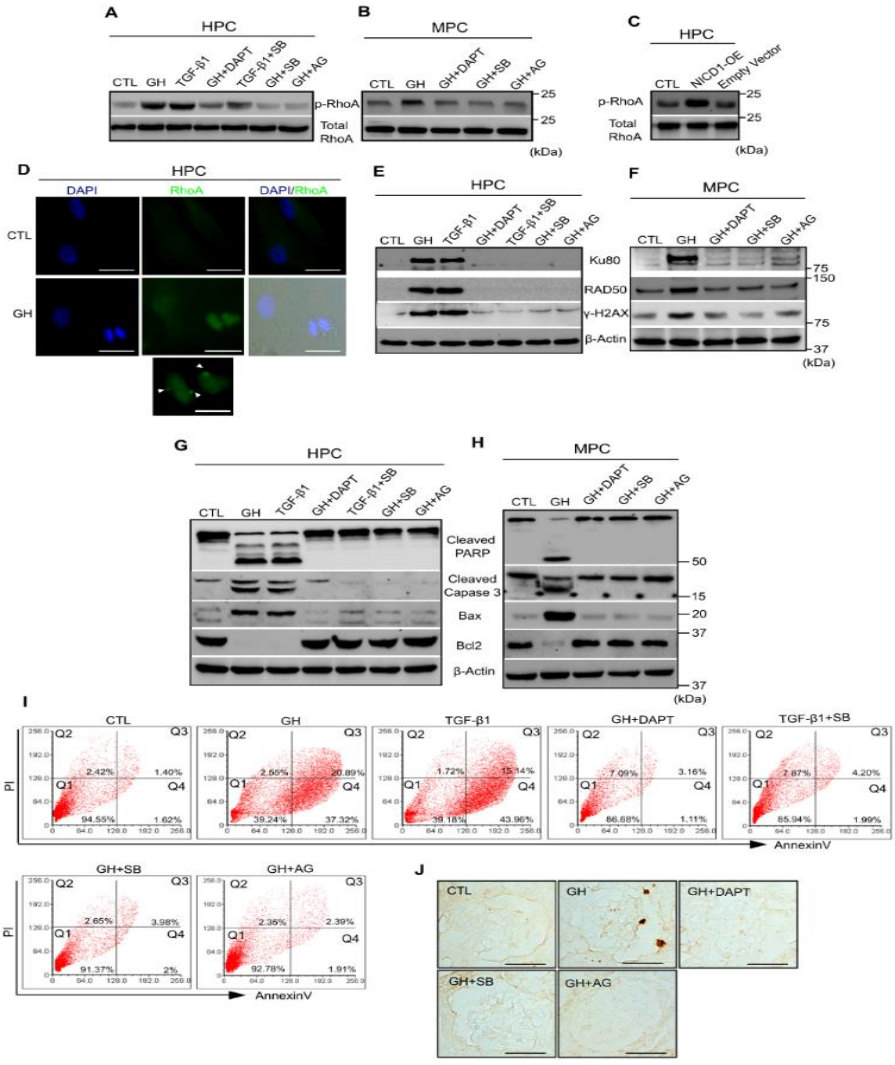

Figure 19: GH induced TGF-β leads to podocyte DNA damage and apoptosis: ***(A&B)*** Immunoblotting for HPC (48hr treatment) and MPC from CTL vs treatment group. ***(C)*** Immunoblotting for HPC under ectopic expression of NICD1 (NICD1-OE). ***(D)*** Immunofluorescence for the RhoA (green color) in HPC from CTL vs treatment for 48hr. ***(E-H)*** Immunoblotting from HPC and MPC from CTL vs treatment group. ***(I)*** HPC from CTL vs treatment for 48hr, stained with FITC AnnexinV and PI, and analyzed by flow cytometry. The lower left quadrant (Live cells), lower right quadrant (early apoptosis), upper right quadrant (late apoptosis) and upper left quadrant (necrotic cells). ***(J)*** Representative TUNEL staining by DAB in glomerular sections from CTL vs treatment group. Magnification x630, Scale bar=20μm.(n=3). β-Actin served as internal control.

Often, the failure of cell cycle progression is accompanied by the induction of cell death. Therefore, we next investigated markers of DNA damage and apoptosis in podocytes exposed to GH or TGF-β1. Elevated expression of γ-H2X (a marker for DNA double-strand break), Ku80, and Rad50 (markers for double-strand repair) was observed in GH and TGF-β1 treated human podocytes and also in podocytes isolated from mice administered with GH (Fig.4E&F). Activated PARP & Caspase-3, Bax (pro-apoptotic markers) enhanced and suppression of Bcl2 expression were observed in podocytes exposed to GH or TGF-β1 (Fig.4G&H). Interestingly, the expression of GH or TGF-β1 induced DNA damage (Fig. 4E&F), and apoptotic (Fig.4G&H) markers were ameliorated by DAPT and AG490 or SB431542.

The majority of GH or TGF-β1 treated podocytes are early apoptotic (40±5% vs. late 15±7%) (Fig.4I). However, DAPT, SB431542, and AG490 prevented podocyte apoptosis (Fig.4I). Similarly, TUNEL staining also showed increased podocyte apoptosis in GH treated mice, whereas DAPT, SB431542, and AG490 treatment ameliorated GH-induced apoptosis (Fig.4J). Our data suggest that GH or TGF-β1 treatment, despite inducing the activation of mitosis, evoked cell death, a phenomenon is known as a mitotic catastrophe [117].

3.2.5 Blocking of TGFB R1 or Notch1 signaling abrogates GH-induced podocytopathy and proteinuria:

GH administered mice showed increased urinary albumin creatinine ratio (UACR) and proteinuria, and a decline in glomerular filtration rate (GFR) (Fig.5A-D). Elevated TGBR1 and CTGF and decreased BMP-7 expression was noticed in podocytes isolated from GH-treated mice (Fig.5E&F). CTGF is a TGF-β1 target, whereas BMP-7 is an antagonist of TGF-β1. Furthermore, we also noticed the activation of canonical SMAD signaling in podocytes isolated from GH-treated mice (Fig.5F). As expected, blocking GHR (by AG490) or TGBR1 (by

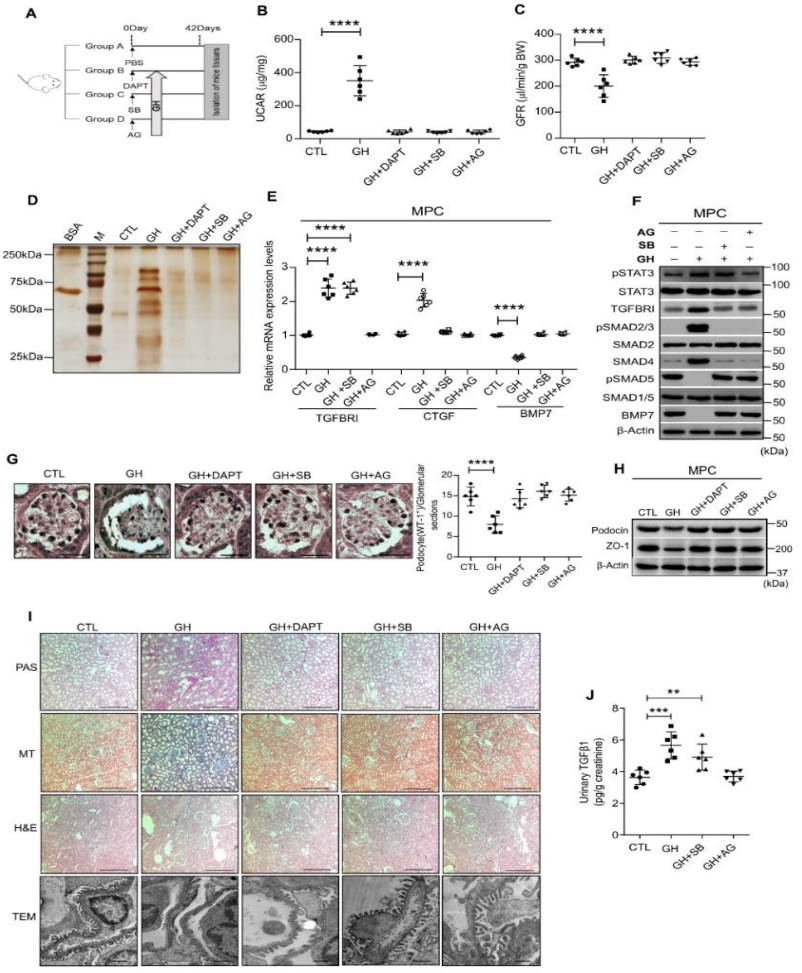

Figure 20: Blockade of GHR and TGFβR1 protects mice from GH-induced proteinuria: ***(A)*** Schematic presentation of mouse experimentation. ***(B)*** UACR and ***(C)*** GFR were estimated in CTL vs treatment group of mice. ***(D)*** Silver staining was performed to the urine samples from CTL vs treatment group of mice. BSA; Bovine Serum Albumin, M; protein standard marker. ***(E)*** qRT-PCR in MPC from CTL vs treatment group of mice. ***(F)*** Immunoblotting for MPC from CTL vs treatment group of mice. ***(G)*** *Left panel;* Representative images of immunohistochemical staining for anti-WT1 (podocytes) by DAB in the glomerulus sections from CTL vs treatment group of mice. *Right panel;* Average number of WT1+ cells in the glomerulus was quantified in mice from CTL vs treatment group with the help of ImageJ (NIH). ***(H)*** PAS, MT, H&E staining in kidney tissue, and TEM analysis in podocytes from CTL vs treatment group of mice. Magnification x100. Scale bar= 100µm, TEM scale bar 1µm. β-Actin served as internal control.

SB431542) prevented activation of SMAD signaling in GH administered mice (Fig.5E&F). Since GH treated podocytes showed bi-nucleation and consequent apoptosis *in vitro*, to ascertain *in vivo* confirmation, we counted the average number of podocytes per glomerulus in mice administered with GH.

The number of podocytes (WT1 positive) in GH treated mice decreased significantly ($P<0.005$) compared with mice naive to GH treatment (Fig.5G, left & right panel). Blunting

the Notch activation or inhibition of GHR/TGFBR1 mitigated GH-induced podocyte loss. Expression of slit-diaphragm proteins (podocin and ZO-1) were decreased in podocytes from GH treated mice (Fig.5H). Since podocyte loss and damage to the slit-diaphragm eventually manifest in glomerulosclerosis in addition to proteinuria [141, 142], we measured the histopathological changes by PAS, MT, H&E staining, and TEM imaging. Severe glomerulosclerosis (PAS and MT staining) and altered morphology (H&E staining) of the nephron was observed in GH treated mice, whereas TEM images revealed a podocyte foot process effacement and thickening of the glomerular basement membrane (Fig.5I). Blunting of GH or TGF-β1 action or blocking Notch activation prevented GH-induced renal manifestations (Fig.5I). Suppression of GH or TGF-β1 action or preventing Notch signaling preserved the expression of slit-diaphragm proteins (podocin & ZO1) and prevented proteinuria. Blunting GH or TGF-β1 action also ameliorated TGF-β1 loss into the urine (Fig.5J). Together, our data demonstrate that GH's role in the pathogenesis of nephropathy is mediated by TGF-β1.

3.2.6 Hyperactivated Notch signaling and binucleated podocytes in patients with diabetic nephropathy:

We evaluated the extent of NICD1 expression and binucleation of podocytes in subjects with diabetic nephropathy (DN). Kidney biopsy sections from diabetics showed increased TGF-β1 and active Notch (NICD1) expression (Fig.6A). Furthermore, we observed both binucleated podocytes and also detached podocytes localized to urinary space in renal sections from people with DN (Fig.6B&C). As anticipated, glomerulosclerosis was observed in kidney sections from people with DN (Fig.6D). We have also observed elevated urinary TGF-β1 levels from these subjects with DN (Fig.6E&F). As expected, these people with diabetes showed severe proteinuria (Fig.6G). Interestingly, Nephroseq (https://www.nephroseq.org) analysis revealed co-expression of TGFBR1, Notch signaling components (HES1), cell proliferating markers (Ki67 & PCNA), cell cycle regulator (TP53), and regulator of cytokinesis (RhoA) in

human diabetes glomerulus dataset (Woroniecka) (Fig.6H).These data confirm that people with DN have elevated functional Notch signaling in their glomeruli, podocytes with aberrant cell-cycle entry, and enhanced podocyte injury markers.

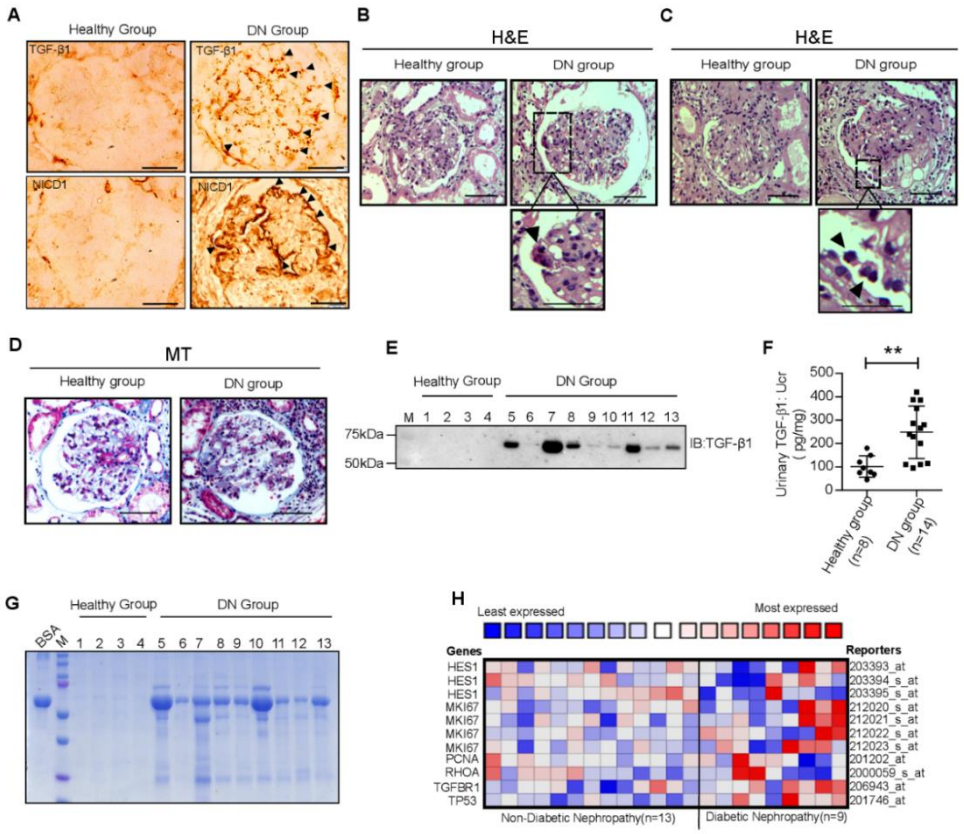

Figure 21: Elevated TGF-β1 signaling and proteinuria correlated in people with DN: ***(A)*** Immunohistochemical staining for TGFβ-1 and NICD1 by DAB in the glomerulus sections from healthy (n=8) and DN group (n=14). ***(B&C)*** H&E staining in glomerular sections from healthy and DN group. Zoomed picture emphasizes a bi-nucleated and detached podocyte. ***(D)*** Representative image of MT stain in glomerular sections from healthy and DN group. ***(E)*** Immunoblotting for TGF-β1 in the urine samples from healthy and DN group. ***(F)*** Quantification of TGF-β1 in the urine samples from healthy and DN. (G) Urine samples from healthy and DN were resolved on SDS-PAGE and stained with Coomassie Blue. BSA= Bovine serum albumin. M=protein marker. ***(H)*** Nephroseq comparing HES1, MIKI67, PCNA, RHOA, TGFBR1, and TP53 expression levels in non-diabetic versus DN.. Magnification x630. Scale bar=20μm.

3.3 Discussion:

The current investigation reveals a novel mechanism for GH action on glomerular podocytes and in the pathogenesis of DN. The major findings of this study are that GH induces TGF-β1 and the canonical TGF-β1/SMAD signaling in podocytes. GH and TGF-β1 activate Notch signaling, which is implicated in the cell cycle re-entry of podocytes. However, these activated podocytes fail to complete the mitotic cycle and as a consequence, binucleated

podocytes are accumulated. Indeed, renal biopsies from patients with DN also revealed binucleated podocytes. Podocytes exposed to GH or TGF-β1 fail to accomplish mitosis due to cytokinesis failure and susceptible to cell death. Importantly, inhibition of GHR and TGFBR1 successfully protected mouse podocytes from GH and TGF-β1 induced Notch activation, cell cycle re-entry, and apoptosis. Furthermore, we observed podocyte loss, glomerulosclerosis, and proteinuria in GH treated mice, a common pathological feature associated with DN. The current study demonstrates that GH's role in the pathogenesis of nephropathy is at least partly mediated by the TGF-β1/SMAD signaling. Interestingly, inhibition of Notch with DAPT significantly ameliorated both GH and TGF-β1 induced podocyte injury and apoptosis.

The first important observation from our study was that GH induces TGF-β1 expression in podocytes. Elevated GH levels are implicated in the early renal hypertrophy, depletion of podocytes, and proteinuria [143, 144]. However, it was not clear whether this causal role of the GH in the pathogenesis of nephropathy is due to direct actions of GH on the podocytes or via GH's effector molecules. Among several hosts of mediators in the diabetic milieu, TGF-β1 has emerged to have a key role in the development of morphologic manifestations and clinical characteristics of diabetic nephropathy [118, 145-147]. Inhibition of TGF-β1 or ablation of SMAD3 ($SMAD^{-/-}$) showed promising protection from glomerulosclerosis and renal dysfunction [42, 110]. Despite knowing the fact that activation of TGF-β/SMAD signaling is crucial in most kidney diseases, the stimuli that activate this pathway in the podocyte remain unclear. Previous studies proposed that high glucose and angiotensin II induces TGF-β1 expression in glomerular cells [147, 148]. Although, elevated GH levels and overactivity of the GH/GHR axis are implicated in renal manifestations, and CKD [149], the temporal association between GH and TGF-β was unclear. It is noteworthy that GH-induced mild glomerulosclerosis and interstitial fibrosis in diabetic Sprague-Dawley rats is associated with an elevation in TGF-β1 levels [109] and suppressing JAK2, an immediate downstream target

of GH reduced TGF-β mRNA expression [110]. In the present study, we establish that GH stimulates TGF-β1 in podocytes, and to the best of our knowledge, this is the first study to demonstrate that GH induces TGF-β1 expression.

Another key finding that emerged from our study is that both GH and TGF-β1 trigger cell cycle re-entry of podocytes by Notch activation. Notch is a highly conserved juxtracrine signaling cascade, which transduces short-range signals between neighboring cells. This pathway comprises four transmembrane receptors (Notch 1-4) and five ligands (Jagged 1&2; Delta-like 1, 3, & 4). Binding of ligand to Notch receptor results in shedding of both the Notch extracellular domain by ADAM protease and cleavage of the Notch intracellular domain (NICD) by the γ-secretase complex. Subsequent nuclear translocation of NICD activates expression of target genes such as Hes1 and Hey1. We had previously demonstrated that GH induces Notch activation [108]. Since TGF-β1 is a powerful Notch activator, we investigated whether the observed Notch activation in GH treated conditions could be due to TGF-β1 secreted under GH stimuli. Although active Notch signaling is required till the stage of S-shaped body formation during glomerulogenesis, it is almost undetectable in healthy adult glomeruli. Indeed, the down-regulation of the Notch pathway is required for renal progenitors to differentiate towards podocyte lineage [96]. Mature podocytes exit from the cell cycle which is evidenced by reduced expression of proliferating markers (eg, Ki67, PCNA, and CyclinB1) and increased expression of cell-cycle inhibitors p57and p27 [150]. The persistent expression of p57 and p27 enable the mature podocytes remain quiescent [151, 152]. Our data reveal that GH or TGF-β1 stimulated quiescent podocytes re-enter the cell-cycle and progressed to the S- and G2/M phase, and the progression of cell cycle events is concomitant with activation of Notch signaling. It was reported that Notch activation stimulates cell-division in renal progenitors, whereas in differentiated podocytes, it helps cells overcome the G2/M checkpoint [96]. Increased Notch activity was observed in podocytes of patients with glomerular disorders,

and blunting Notch activity ameliorated glomerulosclerosis, prevented podocyte death during the initial phases of glomerular injury, and proteinuria [97].

A significant observation from this study is that podocyte exposed to GH becomes binucleated and hypertrophic. Normally, post-mitotic cells do not re-enter the cell cycle when exposed to growth signals. In the case of podocytes, we observed an increase in cell size with GH treatment, and it caused increased kidney mass in these animals. Stressed podocytes were considered to re-enter the cell cycle and are arrested at G2/M restriction point by CDK inhibitors, and become hypertrophic [153]. Multi-nucleation of podocytes contributes to the increased cell size. The hypertrophic phenotype of podocytes appears to be transient as podocytes with cytokinesis failure and aneuploidy are susceptible to cell death [117]. Podocyte depletion has been considered as a hallmark of both primary and secondary forms of glomerulosclerosis [75, 154, 155]. Decreased podocyte density strongly correlates with the severity of proteinuria and DN progression [156-158]. A large body of evidence suggests that podocytes undergo apoptosis, which is considered as a major form of podocyte loss that culminates in glomerular injury [159, 160]. EMT of podocytes is considered an alternative cause for podocyte loss [161]. We have noticed both early and late phase apoptosis in podocytes exposed to GH or TGF-β1. Cell cycle transition from G1 to S phase leads to extensive DNA damage that culminates in the early apoptosis of podocytes. Whereas late apoptosis suggests that the podocytes with DNA damage that overcome the G2/M checkpoint eventually failed to complete cytokinesis. These cells accumulated at the G2/M phase of the cell cycle with increased DNA content per cell and eventually undergo late apoptosis. Most of the podocytes in our experiments underwent early apoptosis in response to GH treatment, suggesting that these terminally differentiated podocytes are not sufficiently competitive to carry cell cycle events successfully despite mitogenic stimuli by GH or TGF-β1. Wu et al. reported that TGF-β at a lower concentration promotes podocyte differentiation, whereas TGF-β levels beyond a

critical threshold induce G2/M block and apoptosis [162]. Whereas GH plays a critical role in normal kidney function, and hyperactive GH signaling has been implicated in proteinuria in diabetes. Together these studies suggest in a dose-dependent manner, GH and TGF-β specify podocyte fate.

Differentiated podocytes possess high cytoplasm to nucleus ratio and express highly organized myofibrils that prevent from cell division. Disorganization of cytoskeletal filaments during mitogenic stimuli would adversely affect their function. Another peculiar property of differentiated podocytes is that they express a wide range of cell cycle proteins, which could be a prerequisite for executing mitotic catastrophe in response to a noxious stimuli such as GH and TGF-β1. The mechanisms by which cell cycle re-entry causes cell death are not completely explained, but cytokinesis failure and abnormal Rho distribution, as observed in our study, could be one of the key reasons. Further studies are needed to delineate the contribution of specific STATs in GH mediated activation of TGF-β1 signaling and whether the source of GH *in vivo* is more local than endocrine or opposite. Based on our observations, we propose that GH induces TGF-β1 expression and is a causative factor in the progression of podocyte podocyte injury, hypertrophy, and consequent proteinuria during GH-induced kidney diseases. In summary, the present study establishes that GH elevates TGF-β1 expression in podocytes, and further, the downstream signaling of TGF-β1 get enhanced by in both autocrine and paracrine manner. Our data provide a mechanistic link between GH and podocyte pathology in diseases like type 1 diabetes and acromegaly.

3.4 Materials and Methods:

3.4.1 Antibodies and Reagents:

The primary antibodies are as follows: anti-activated Notch1(ab8925), anti-pSTAT3 (ab76315), anti-STAT3(ab5073), TGF-βR1(ab31013), anti-HEY1(ab154077), p53 (ab26), RhoA(ab54835), Cyclin B1(ab72), p21(ab109520), α-Tubulin(ab7291), CDK4 (ab137675),

Ki67(ab16667), CyclinD1(ab16663) and Laminin-B1(ab16048) were purchased from Abcam (Cambridge, MA). Anti-Notch1-Full-length(#3608), anti-cleaved-Notch1(#4147S), pSMAD2/3(#8828), anti-SMAD2(#5339), anti-pSMAD1/5(#9516), anti-SMAD5(#12534), anti-Cleaved-PARP(#5625), anti-Cleaved Caspase3(#9664), anti-BMP7(#4693), anti-BAX(#89477), anti-Bcl2(#15071) and anti-β-Actin(#4970) were purchased from Cell Signaling Technology (Danvers, MA). Anti-Ku80(NBP156408), anti-RAD50(NB100-147), anti-γ-H2AX(NB100-384), anti-Nephrin (NBP1-77303) and anti-PCNA(NBP500-106). The anti-TGF-β1(MAB240) from R&D Systems (Minneapolis, Minnesota). Anti-HES1(sc-166410), anti-WT-1(sc-393498), and anti-ZO-1(sc-33725) were obtained from Santa Cruz Biotechnology (Dallas, TX). Anti-JAG1(PAB807Hu01) was purchased from Cloud-clone (Houston, TX). Anti-Caspase3(9H19L2) and TGF-β1(BMS249-4) were purchased from Thermo Fischer Scientific (Waltham, Massachusetts). Mouse/Rabbit PolyDetector DAB HRP Brown- Bio SB (BSB020, Santa Barbara, CA). Phalloidin fluorescein isothiocyanate labelled (P5282) and glutaraldehyde solution (G5882) were obtained from Sigma chemicals (St. Louis, MO, USA). Precision Plus Protein Dual Color Standards (Bio-Rad, Hercules, CA), and ProLongTM Diamond Antifade Mountant (P36961) were purchased from Molecular Probes Life Technologies. DyLight 488 and DyLight 564, and Cy5-conjugated secondary antibody were purchased from Vector Laboratories (Burlingame, CA). Primers used in this study procured from Integrated DNA Technologies (Coralville, IA). All other reagents used were of analytical grade and obtained from Sigma chemicals (St. Louis, MO, USA).

3.4.2 Experimental drugs:

DAPT(D5942) was purchased from Sigma Chemicals (St. Louis, MO, USA), TGF-βR1 inhibitor (SB431542), and JAK2 inhibitor (AG490) were purchased from Tocris Bioscience (1614-10MG). Recombinant TGF-β1 (#240-B-002) and human growth hormone Genotropin (Pfizer) procured from R&D Systems.

3.4.3 Podocyte culture and experimentation:

In this study, conditionally immortalized human podocytes (A gift from Prof. Moin Saleem, University of Bristol) cells were cultured essentially as described earlier [108]. Briefly, after 14 days of differentiation at 37°C, podocytes were treated with or without GH, GH+DAPT, TGF-β1, TGF-β1+SB431542, GH+SB431542, and GH+AG490. Unless otherwise mentioned, all the experimental conditions for podocyte cells were given for 48 hr. The cell lysate was prepared for RNA isolation, immunoblotting, and Enzyme Immunosorbent Assay (ELISA). For immunofluorescence, cells were cultured on coverslips, followed by treatment as mentioned above, subsequent fixation with paraformaldehyde (4%), and blocking with PBS containing normal BSA (5%) before incubation with primary antibodies. The next day, the samples were incubated with Alexa Fluor-conjugated secondary antibodies, and DAPI for nuclear stain, for 1hr at room temperature. Images were acquired using a laser scanning microscope (ZEISS, Germany). For F-actin staining in podocytes cells essentially as described earlier [108]. Briefly, HPC cells were incubated with Fluorescent phalloidin-TRITC conjugate (P1951) for 40 min at room temperature. Next counterstaining by DAPI (P36971), mounting and images were acquired using a Leica trinocular microscope or Apotome Axio Imager Z2 (Zeiss). Images were analyzed with LASX Industry Software and ImageJ. For Live-cell imaging, cells were grown on µ-Dish 35 mm (#81156) at 60% confluency treatment performed for 48 hr and then imaged for 2 hr on a Leica SP5 confocal laser scanning microscope with a HCX PL APO CS 63×, 1.40-NA oil-immersion lens.

3.4.4 Animal and Tissues:

All the experimental procedures for the animals were approved by the Institutional Animal Ethics Committee of the University of Hyderabad, India. 8-Week-old Swiss Webster male mice weighing nearly 30±5 g were used in this study. The mice were randomly assigned to five groups (6 mice per group): 1) control group (CTL), 2) GH-treated group, 3) GH+DAPT-

treated group, 4) GH+SB431542, and 5) GH+AG490. Experimental mice received a single i.p. hGH (1.5 mg/kg/day), whereas control mice have received an equal volume of saline for six weeks. The inhibitor groups were received DAPT (10 mg/kg of body weight) per day before the GH treatment. After six weeks of the experimental period, the mice were placed in individual metabolic cages for collecting 24 hr urine to estimate albumin (#COD11573) and creatinine (#COD11502) levels as recommended by the manufacturer's protocol (Biosystems, Barcelona, Spain). An aliquot of urine from mice was subjected to SDS-PAGE gel, and silver staining was performed to compare the urinary protein profile for all the five groups.

Further, we have also measured the GFR in these mice, as described previously [108]. Mouse podocytes were isolated from the kidney of mice, as described in earlier protocol [163]. Briefly, glomeruli were prepared by filtration of the cortex of the kidney with mesh sieves, whose holes were 100, 76, and 54 μm in diameter. The tissues left on the mesh sieve with 54 μm holes were collected and prepared for the RT-PCR and immunoblotting. For histological analysis, the kidney cortex was fixed with 4% paraformaldehyde before embedding in paraffin. Paraffin-embedded tissues were sliced longitudinally into 3-4 μm thick sections, subjected to staining with Periodic-acid Schiff Base (PAS), Masson’s trichrome (MT), and Haematoxylin and Eosin (H&E) staining. Transmission electron microscopic (TEM) images were obtained for glomerular sections from all the experimental mice groups, as described earlier [108].

3.4.5 RNA extraction and Quantitative RT-PCR assay:

The total RNA was prepared from HPC and MPC by using a TRIzol RNA isolation reagent (Thermo Scientific, Waltham, MA). Next, 1 μg of total RNA was reverse transcribed using the cDNA synthesis kit (PrimeScript 1st strand cDNA Synthesis). the qRT-PCR analysis was performed by the QuantStudio 3 system (Applied Biosystems) with SYBR Green (Kappa Biosystem) Master Mix as mentioned in the following protocol: initial denaturation at 95°C for

3 min, followed by 35 cycles of three steps each at 95°C for 30s, 60°C for 30s, and 72°C for 30s. mRNA expression of each gene was normalized using the expression of β-actin.

3.4.6 Western Blotting:

Cytoplasmic extract for immunoblotting was prepared as described previously [108]. Briefly, Human podocytes and isolated mouse primary podocytes were lysed by RIPA buffer (Cell Signaling) containing protease inhibitor mixture (Sigma–Aldrich) and phosphatase inhibitor tablets (Roche), centrifuged, and collection of supernatants. However, for nuclear extract protein sample preparation, pellet was resuspended with 20 mM HEPES (pH 7.9), 25 % Glycerol, 0.42 M NaCl, 0.2 mM EDTA, 1.5 mM MgCl2, 1 mM DTT, 0.2 mM PMSF) and vortex for 20 sec. Incubate the cell lysate for 25 min on ice and vortex every 10 min for 10 sec. Next, cell lysate was centrifuged for 12 min at 13,500 rpm at 4°C and collect the supernatant (Nuclear). The protein concentrations of cell and mouse podocyte lysates were determined using a bicinchoninic acid reagent (Thermo Scientific) using bovine serum albumin as a standard. A total of 20 to 25 μg of protein samples were resolved by sodium dodecyl sulfate polyacrylamide electrophoresis followed by Western blot.

3.4.7 Enzyme-Linked Immunosorbent Assay:

γ-Secretase activity was quantified as describe earlier [108]. Briefly, HPC cells were treated with or without GH, TGF-β1, GH+DAPT, TGF-β1+SB431542, GH+SB431542, and GH+AG490 for 48 hr and cleavage-dependent release of APH-1A measured at 450 nm by using a fluorescent microplate reader (Multiskan O Microplate Spectrophotometer, ThermoFisher Scientific). For TGF-β1 detection in condition, media from HPC treated with different experimental conditions and in urine samples from mice and humans were analyzed by TGF-β1 ELISA commercial kit (R&D Systems) according to the manufacturer's protocol.

3.4.8 Cellular DNA Flow Cytometric Analysis:

The single-cell suspension of HPC (5×10^5 to 1×10^6 cells) from with or without GH, TGF-β1, GH+DAPT, TGF-β1+SB431542, GH+SB431542, and GH+AG490 for 48hrs were prepared in 300 μL PBS, fixed by cold 70% ethanol for 30 min at 4°C and then washed and resuspended in 300 μL PBS, followed by treatment with three μL RNase at 37°C for 30 min, chilled on ice, and 30 μL PI (propidium iodide; Roche) treatment in the dark at room temperature for 1hr. DNA contents were acquired by an S3e Cell Sorter flow cytometer (Bio-Rad) using FCS Express 7 program.

3.4.9 Apoptosis analysis in podocytes:

Apoptotic cell death was measured by an Alexa Fluor® 488 annexin V and propidium iodide (PI) apoptosis detection kit (ThermoFisher Scientific) according to the manufacturer's protocol. Briefly, HPC cells were plated on 6 cm dishes at 1×10^5 cells per dish with or without GH, TGF-β1, GH+DAPT, TGF-β1+ SB431542, GH+SB431542, and GH+AG490 for 48 hr. Next, podocytes were harvested with the help of trypsin, washed with cold PBS twice, resuspended in binding buffer, and stained with FITC-Annexin V and PI in the dark at room temperature for 15 min. After incubation, the binding buffer was added, and the podocytes were analyzed by an S3e Cell Sorter flow cytometer (Bio-Rad). Unstained cells and cells stained with FITC-Annexin V or PI alone were used as controls to set up compensation and quadrants in flow cytometry. The results were analyzed by the FCS Express 7 program.

3.4.10 Reporter assay:

HES1 promoter activity luciferase assay was performed as described earlier [133]. Briefly, HPC cells were transfected with a pHES1(467)-Luc (procured from Addgene) and internal control expressing the *Renilla luciferase*, pRL-TK (Promega). HPC cells were transfected using Xfect polymer (DSS Takara Bio, New Delhi, India) as per the manufacturer's instructions. After 48 hr of transfection, cells were treated with or without GH, TGF-β1,

GH+DAPT, TGF-β1+ SB431542, GH+SB431542, and GH+AG490 for 48 hr, PBS wash and harvested . After cell lysis, the debris was removed by centrifugation at 12,000g (4°C) for 5 min, and 20 µl of supernatant was used for luciferase assay. The co-transfected luciferase reporter plasmid activity was used to normalize transfection efficiency.

SMAD4 signal-GFP reporter assay was performed in podocyte cells according to the earlier published protocols with minor modifications [164]. Briefly, podocyte cells in a 96 well plate, 1000 cells/well were seeded before the day of transfection. Cells were transfected either with SMAD4-GFP, positive control, and negative control vectors in triplicates. After transfection, cells were left untreated in a complete medium for 16hr. Then cells were treated with GH-CM, Anti-TGFβ1 antibody (#AB-100-NA), GH, and TGFβ1. After 48hr of treatment, images were taken by using a Leica trinocular microscope. The fluorescence emission spectrum for these samples was acquired at 510-520nm by using a fluorescent microplate reader (Multiskan O Microplate Spectrophotometer, ThermoFisher Scientific).

For SMAD4 luciferase assay, Podocyte cells were seeded into a 6-well plate (2×10^5 cells per well). The cells were then co-transfected with either 0.3 µg/well of Smad4 firefly luciferase reporter plasmid constructs (pLuc366 or pLuc207) or the control pGL3-Basic vector (Promega, San Luis Obispo, USA). The renilla luciferase plasmid was also co-transfected to correct variations in transfection efficiency (45 ng/well). After incubating for 24hr, treatment was given for 48 hr. Next, cells were harvested from each experimental condition, and the luciferase activity was measured using a fluorescent microplate reader. The final activity was calculated as the ratio of firefly luciferase activity versus renilla luciferase activity units.

3.4.11 Transfection of podocytes for knockdown and overexpression:

HPC cells were transfected with siRNA as described earlier [133]. Briefly, transfection was done using jetPEI reagent (Polyplus, Illkirch, France). HPC cells were seeded at 70-90%

confluency in 6-well cell culture plates and transiently transfected with siRNA specific to TGFBR1 (#EHU051131) and its parental negative control siRNA by mixing with NaCl-jetPEI complexes. After 72 hr of transfection, cells were treated with or without GH, TGF-β1, GH+DAPT, TGF-β1+SB431542, GH+SB431542, and GH+AG490 for 48 hr. Next, cells were washed twice with PBS and lysed with RIPA buffer; the expression levels measured by western blotting, as described above. The transient transfection of pT3-EF1aH NICD1; an overexpressing vector from Addgene (#86500) and its empty parental vector, pT3-EF1aH the podocytes using Xfect polymer (DSS Takara Bio, New Delhi, India) as per manufacturer's instructions. After 24 hr of transfection incubation, cells were harvested, and immunoblotting was performed.

3.4.12 Ethics approval:

The study was approved by the Institutional Review Board of Guntur Medical College and Government General Hospital, Guntur, India (#GMC/ IEC/120/2018), and adhered to the principles and the guidelines of the Helsinki Declaration. The animal experimental procedures were performed in adherence to the Institutional Animal Ethics Committee of the University of Hyderabad.

Chapter 4: Summary

The kidneys are multifunctional and very crucial organs, and understanding its pathophysiology and exploring the modes of treatments are challenging due to the inherent complexity of the organ. Podocytes are the critical cell types that primarily involve in kidney permselectivity besides offering structural support to the glomerular filtration apparatus. Being a vital cell type and central to the diverse functions of the glomerulus, podocytes seek great interest as a critical regulator of the glomerular biology. It is evident that podocytes vis-à-vis glomeruli become injured during diabetic kidney diseases. Hallmarks of diabetic kidney diseases are podocyte loss, glomerulosclerosis, and proteinuria. Diabetes mellitus, mainly T1DM, is presented with elevated GH levels [5]. Several components of the GH system, including GH and GHR, are elevated in the diabetic kidney [102, 131]. Studies from our laboratory reported a direct action of GH on podocytes and activation of canonical JAK-STAT signaling [105] . Administration of GH to mice elicited podocyte loss with a significant increase in the thickness of the glomerular basement membrane (GBM) [108].

Increasing evidence suggests that podocyte loss in diabetic subjects is associated with podocyte detachment [131]. Since podocytes adhere to the GBM, and altered composition of this basement membrane during diabetic milieu negates podocyte adherence, which may lead to impaired podocyte function and consequently results in proteinuria. Interestingly, the composition of GBM is determined by podocytes and endothelial cells, which together contribute to >50% of the core GBM. Podocyte loss and enhanced secretion of ECM proteins, in turn, thickening GBM could be one of the possibilities for compromised kidney function.

Our laboratory longed an interest in understanding the molecular and cellular basis for podocyte injury, migration, and cell death during diabetic kidney diseases. Since elevated GH levels are implicated in the progression of DN, and GH elicits its action directly on podocytes

via GHR, it is obvious to suspect the contribution of GH towards podocyte loss and glomerulosclerosis.

The essence of my doctoral study is to demonstrate that GH induced TGF-β1 mediated reactivation of Notch signaling in podocytes and its consequences. The salient findings of my study are:

- ✓ GH induces cognate TGF-β1/SMAD signaling in glomerular podocytes *in vitro* and *in vivo*.
- ✓ GH reactivates Notch signaling in adult podocyte *in vitro* and *in vivo*.
- ✓ GH induced reactivation of Notch signaling in podocytes could be mediated through the TGF-β1.
- ✓ Notch reactivation in podocytes leads to de-differentiation (i.e. recapitulate embryological features) or transdifferentiation (i.e Epithelial-Mesenchymal Transition).
- ✓ GH or TGF-β1 stimulated quiescent (G0-phase) podocyte cell cycle re-entry and progressed to the S- and G2/M phase but fails to complete the cytokinesis as a result podocyte develop aneuploidy.
- ✓ The antagonist for GH induced the TGF-β1/Notch axis in podocytes protected from the formation of aneuploidy and also from mitotic catastrophe.
- ✓ GH induces decreased podocyte number, glomerular filtration rate and, increased UACR and proteinuria, whereas administration of GHR, TGFBR1, or Notch inhibitors protected mice from GH-induced kidney dysfunction.
- ✓ In summary, our study proposes that podocyte undergoes both mitotic failure and EMT ; the phenomenon is also known as a mitotic catastrophe.

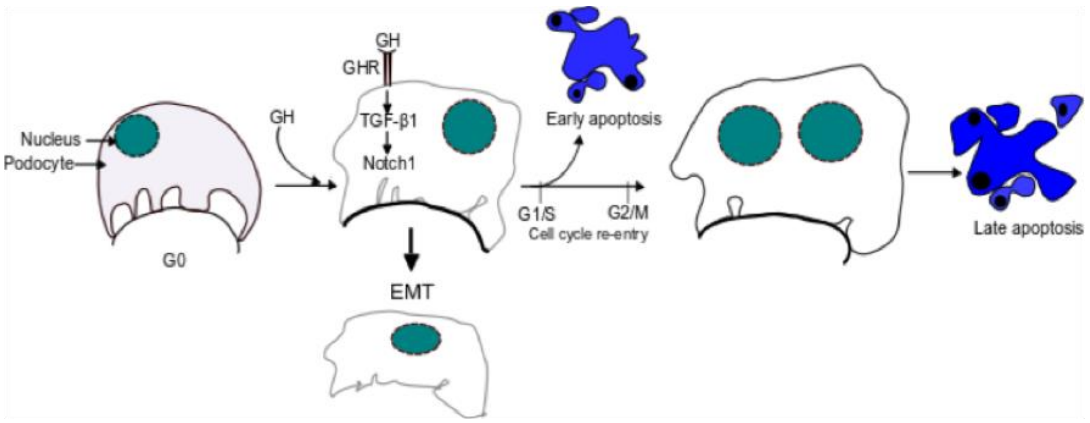

Figure 22: Schematic illustration of GH action on podocyte cell cycle entry, EMT, podocyte binucleation and apoptosis via TGF-β1 mediated Notch1 activation.

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2. **Rajkishor Nishad** et al., Growth hormone induces Notch signaling and mitotic catastrophe in podocytes via TGF-β1 signaling. PNAS, 2020 Sep (Manuscript submitted. ID.: 202010065).

Chapter 5: References

1. Kumar, P.A., et al., *Carboxymethyl lysine induces EMT in podocytes through transcription factor ZEB2: Implications for podocyte depletion and proteinuria in diabetes mellitus.* Arch Biochem Biophys, 2016. **590**: p. 10-19.
2. Ogobuiro, I. and F. Tuma, *Physiology, Renal*, in *StatPearls*. 2020: Treasure Island (FL).
3. American Diabetes, A., *Standards of medical care in diabetes.* Diabetes Care, 2005. **28 Suppl 1**: p. S4-S36.
4. Kitching, A.R. and H.L. Hutton, *The Players: Cells Involved in Glomerular Disease.* Clin J Am Soc Nephrol, 2016. **11**(9): p. 1664-74.
5. Mukhi, D., et al., *Novel Actions of Growth Hormone in Podocytes: Implications for Diabetic Nephropathy.* Front Med (Lausanne), 2017. **4**: p. 102.
6. Larson, T.S., *Evaluation of proteinuria.* Mayo Clin Proc, 1994. **69**(12): p. 1154-8.
7. Reiser, J. and M.M. Altintas, *Podocytes.* F1000Res, 2016. **5**.
8. Pavenstadt, H., W. Kriz, and M. Kretzler, *Cell biology of the glomerular podocyte.* Physiol Rev, 2003. **83**(1): p. 253-307.
9. Mundel, P., et al., *Rearrangements of the cytoskeleton and cell contacts induce process formation during differentiation of conditionally immortalized mouse podocyte cell lines.* Exp Cell Res, 1997. **236**(1): p. 248-58.
10. Gelberg, H., et al., *In vivo enzymatic removal of alpha 2-->6-linked sialic acid from the glomerular filtration barrier results in podocyte charge alteration and glomerular injury.* Lab Invest, 1996. **74**(5): p. 907-20.
11. Kerjaschki, D., D.J. Sharkey, and M.G. Farquhar, *Identification and characterization of podocalyxin--the major sialoprotein of the renal glomerular epithelial cell.* J Cell Biol, 1984. **98**(4): p. 1591-6.
12. Mundel, P. and S.J. Shankland, *Podocyte biology and response to injury.* J Am Soc Nephrol, 2002. **13**(12): p. 3005-15.
13. Kriz, W., *Glomerular diseases: podocyte hypertrophy mismatch and glomerular disease.* Nat Rev Nephrol, 2012. **8**(11): p. 618-9.
14. Kerjaschki, D., *Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis.* J Clin Invest, 2001. **108**(11): p. 1583-7.
15. Mundel, P. and W. Kriz, *Structure and function of podocytes: an update.* Anat Embryol (Berl), 1995. **192**(5): p. 385-97.
16. Greka, A. and P. Mundel, *Cell biology and pathology of podocytes.* Annu Rev Physiol, 2012. **74**: p. 299-323.
17. Durvasula, R.V. and S.J. Shankland, *Podocyte injury and targeting therapy: an update.* Curr Opin Nephrol Hypertens, 2006. **15**(1): p. 1-7.
18. Blutke, A., et al., *Growth hormone (GH)-transgenic insulin-like growth factor 1 (IGF1)-deficient mice allow dissociation of excess GH and IGF1 effects on glomerular and tubular growth.* Physiological reports, 2016. **4**(5): p. e12709.
19. Sharma, K. and F.N. Ziyadeh, *Renal hypertrophy is associated with upregulation of TGF-beta 1 gene expression in diabetic BB rat and NOD mouse.* American Journal of Physiology-Renal Physiology, 1994. **267**(6): p. F1094-F1001.
20. Chen, S., et al., *THE KEY ROLE OF THE TRANSFORMING GROWTH FACTOR-β SYSTEM IN THE PATHOGENESIS OF DIABETIC NEPHROPATHY.* Renal Failure, 2001. **23**(3-4): p. 471-481.
21. Chen, S., B. Jim, and F.N. Ziyadeh, *Diabetic nephropathy and transforming growth factor-β: transforming our view of glomerulosclerosis and fibrosis build-up.* Seminars in Nephrology, 2003. **23**(6): p. 532-543.
22. Wang, S.-N. and R. Hirschberg, *Growth factor ultrafiltration in experimental diabetic nephropathy contributes to interstitial fibrosis.* American Journal of Physiology-Renal Physiology, 2000. **278**(4): p. F554-F560.

23. Ding, G., et al., *Angiotensin II induces apoptosis in rat glomerular epithelial cells.* American Journal of Physiology-Renal Physiology, 2002. **283**(1): p. F173-F180.
24. Wolf, G., S. Chen, and F.N. Ziyadeh, *From the Periphery of the Glomerular Capillary Wall Toward the Center of Disease.* Podocyte Injury Comes of Age in Diabetic Nephropathy, 2005. **54**(6): p. 1626-1634.
25. Ziyadeh, F.N., et al., *Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-β antibody in db/db diabetic mice.* Proceedings of the National Academy of Sciences, 2000. **97**(14): p. 8015-8020.
26. Ling, H., et al., *Therapeutic Role of TGF-β–Neutralizing Antibody in Mouse Cyclosporin A Nephropathy: Morphologic Improvement Associated with Functional Preservation.* Journal of the American Society of Nephrology, 2003. **14**(2): p. 377-388.
27. Liang, X., et al., *Anti-TGF-β Antibody, 1D11, Ameliorates Glomerular Fibrosis in Mouse Models after the Onset of Proteinuria.* PloS one, 2016. **11**(5): p. e0155534-e0155534.
28. Wang, A., et al., *Interference with TGF-β signaling by Smad3-knockout in mice limits diabetic glomerulosclerosis without affecting albuminuria.* American Journal of Physiology-Renal Physiology, 2007. **293**(5): p. F1657-F1665.
29. Voelker, J., et al., *Anti–TGF-β1 Antibody Therapy in Patients with Diabetic Nephropathy.* Journal of the American Society of Nephrology, 2017. **28**(3): p. 953-962.
30. Donate-Correa, J., et al., *Inflammatory cytokines in diabetic nephropathy.* Journal of diabetes research, 2015. **2015**: p. 948417-948417.
31. Reiser, J., et al., *Induction of B7-1 in podocytes is associated with nephrotic syndrome.* The Journal of clinical investigation, 2004. **113**(10): p. 1390-1397.
32. Mauer, S.M., et al., *Structural-functional relationships in diabetic nephropathy.* J Clin Invest, 1984. **74**(4): p. 1143-55.
33. Osterby, R., et al., *Glomerular structure and function in proteinuric type 2 (non-insulin-dependent) diabetic patients.* Diabetologia, 1993. **36**(10): p. 1064-70.
34. White, K.E. and R.W. Bilous, *Type 2 diabetic patients with nephropathy show structural-functional relationships that are similar to type 1 disease.* J Am Soc Nephrol, 2000. **11**(9): p. 1667-73.
35. Wolf, G. and F.N. Ziyadeh, *Molecular mechanisms of diabetic renal hypertrophy.* Kidney Int, 1999. **56**(2): p. 393-405.
36. Deckert, T., et al., *Albuminuria reflects widespread vascular damage. The Steno hypothesis.* Diabetologia, 1989. **32**(4): p. 219-26.
37. Vernier, R.L., et al., *Heparan sulfate proteoglycan in the glomerular basement membrane in type 1 diabetes mellitus.* Kidney Int, 1992. **41**(4): p. 1070-80.
38. Wolf, G., S. Chen, and F.N. Ziyadeh, *From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy.* Diabetes, 2005. **54**(6): p. 1626-34.
39. Ziyadeh, F.N., et al., *Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in db/db diabetic mice.* Proc Natl Acad Sci U S A, 2000. **97**(14): p. 8015-20.
40. Ling, H., et al., *Therapeutic role of TGF-beta-neutralizing antibody in mouse cyclosporin A nephropathy: morphologic improvement associated with functional preservation.* J Am Soc Nephrol, 2003. **14**(2): p. 377-88.
41. Liang, X., et al., *Anti-TGF-beta Antibody, 1D11, Ameliorates Glomerular Fibrosis in Mouse Models after the Onset of Proteinuria.* PLoS One, 2016. **11**(5): p. e0155534.
42. Wang, A., et al., *Interference with TGF-beta signaling by Smad3-knockout in mice limits diabetic glomerulosclerosis without affecting albuminuria.* Am J Physiol Renal Physiol, 2007. **293**(5): p. F1657-65.
43. Voelker, J., et al., *Anti-TGF-beta1 Antibody Therapy in Patients with Diabetic Nephropathy.* J Am Soc Nephrol, 2017. **28**(3): p. 953-962.

44. Dehkhoda, F., et al., *The Growth Hormone Receptor: Mechanism of Receptor Activation, Cell Signaling, and Physiological Aspects.* Frontiers in Endocrinology, 2018. **9**(35).
45. Brooks, A.J., et al., *Mechanism of activation of protein kinase JAK2 by the growth hormone receptor.* Science, 2014. **344**(6185): p. 1249783.
46. Frank, S.J., et al., *Regions of the JAK2 tyrosine kinase required for coupling to the growth hormone receptor.* J Biol Chem, 1995. **270**(24): p. 14776-85.
47. Herrington, J., et al., *The role of STAT proteins in growth hormone signaling.* Oncogene, 2000. **19**(21): p. 2585-97.
48. Hansen, L.H., et al., *Identification of tyrosine residues in the intracellular domain of the growth hormone receptor required for transcriptional signaling and Stat5 activation.* J Biol Chem, 1996. **271**(21): p. 12669-73.
49. Landau, D., et al., *Differential expression of renal growth hormone receptor and its binding protein in experimental diabetes mellitus.* Growth Horm IGF Res, 1998. **8**(1): p. 39-45.
50. Yakar, S., et al., *Inhibition of growth hormone action improves insulin sensitivity in liver IGF-1-deficient mice.* The Journal of clinical investigation, 2004. **113**(1): p. 96-105.
51. Kumar, P.A., F.C. Brosius, 3rd, and R.K. Menon, *The glomerular podocyte as a target of growth hormone action: implications for the pathogenesis of diabetic nephropathy.* Current diabetes reviews, 2011. **7**(1): p. 50-55.
52. Hirschberg, R. and J.D. Kopple, *Effects of growth hormone and IGF-I on renal function.* Kidney Int Suppl, 1989. **27**: p. S20-6.
53. Sonksen, P.H., D. Russell-Jones, and R.H. Jones, *Growth hormone and diabetes mellitus. A review of sixty-three years of medical research and a glimpse into the future?* Horm Res, 1993. **40**(1-3): p. 68-79.
54. Ogle, G.D., A.R. Rosenberg, and G. Kainer, *Renal effects of growth hormone. I. Renal function and kidney growth.* Pediatr Nephrol, 1992. **6**(4): p. 394-8.
55. Bak, M., K. Thomsen, and A. Flyvbjerg, *Effects of the somatostatin analogue octreotide on renal function in conscious diabetic rats.* Nephrol Dial Transplant, 2001. **16**(10): p. 2002-7.
56. Jacobs, M.L., et al., *Effect of long-acting somatostatin analog (Somatulin) on renal hyperfiltration in patients with IDDM.* Diabetes Care, 1997. **20**(4): p. 632-6.
57. Auriemma, R.S., et al., *The kidney in acromegaly: renal structure and function in patients with acromegaly during active disease and 1 year after disease remission.* Eur J Endocrinol, 2010. **162**(6): p. 1035-42.
58. Doi, T., et al., *Glomerular lesions in mice transgenic for growth hormone and insulinlike growth factor-I. I. Relationship between increased glomerular size and mesangial sclerosis.* The American journal of pathology, 1990. **137**(3): p. 541-552.
59. Doi, T., et al., *Progressive glomerulosclerosis develops in transgenic mice chronically expressing growth hormone and growth hormone releasing factor but not in those expressing insulinlike growth factor-1.* The American journal of pathology, 1988. **131**(3): p. 398-403.
60. Bellush, L.L., et al., *Protection against Diabetes-Induced Nephropathy in Growth Hormone Receptor/Binding Protein Gene-Disrupted Mice1.* Endocrinology, 2000. **141**(1): p. 163-168.
61. Reddy, G.R., et al., *Identification of the Glomerular Podocyte as a Target for Growth Hormone Action.* Endocrinology, 2007. **148**(5): p. 2045-2055.
62. Yang, C.W., et al., *Glomerulosclerosis in mice transgenic for native or mutated bovine growth hormone gene.* Kidney Int Suppl, 1993. **39**: p. S90-4.
63. BAK, M., et al., *Renal Enlargement Precedes Renal Hyperfiltration in Early Experimental Diabetes in Rats.* Journal of the American Society of Nephrology, 2000. **11**(7): p. 1287-1292.
64. Thirone, A.C.P., et al., *Modulation of Growth Hormone Signal Transduction in Kidneys of Streptozotocin-Induced Diabetic Animals.* Effect of a Growth Hormone Receptor Antagonist, 2002. **51**(7): p. 2270-2281.

65. Pantaleon, M., et al., *Functional growth hormone (GH) receptors and GH are expressed by preimplantation mouse embryos: a role for GH in early embryogenesis?* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(10): p. 5125-5130.
66. Mundel, P. and S.J. Shankland, *Podocyte Biology and Response to Injury.* Journal of the American Society of Nephrology, 2002. **13**(12): p. 3005-3015.
67. KIM, N.H., *Podocyte hypertrophy in diabetic nephropathy.* Nephrology, 2005. **10**(s2): p. S14-S16.
68. Schwieger, J. and L.G. Fine, *Renal hypertrophy, growth factors, and nephropathy in diabetes mellitus.* Semin Nephrol, 1990. **10**(3): p. 242-53.
69. May, C.J., M. Saleem, and G.I. Welsh, *Podocyte dedifferentiation: a specialized process for a specialized cell.* Front Endocrinol (Lausanne), 2014. **5**: p. 148.
70. Lv, Z., et al., *Rac1/PAK1 signaling promotes epithelial-mesenchymal transition of podocytes in vitro via triggering beta-catenin transcriptional activity under high glucose conditions.* Int J Biochem Cell Biol, 2013. **45**(2): p. 255-64.
71. Li, Y., et al., *Epithelial-to-mesenchymal transition is a potential pathway leading to podocyte dysfunction and proteinuria.* Am J Pathol, 2008. **172**(2): p. 299-308.
72. Shankland, S.J., *The podocyte's response to injury: role in proteinuria and glomerulosclerosis.* Kidney Int, 2006. **69**(12): p. 2131-47.
73. Bridgewater, D.J., et al., *Insulin-like growth factors inhibit podocyte apoptosis through the PI3 kinase pathway.* Kidney International, 2005. **67**(4): p. 1308-1314.
74. Welsh, G.I., et al., *Insulin signaling to the glomerular podocyte is critical for normal kidney function.* Cell metabolism, 2010. **12**(4): p. 329-340.
75. Lee, S.H., et al., *Podocyte hypertrophy precedes apoptosis under experimental diabetic conditions.* Apoptosis, 2015. **20**(8): p. 1056-71.
76. Swathi Chitra, P., et al., *Growth Hormone Induces Transforming Growth Factor-Beta-Induced Protein in Podocytes: Implications for Podocyte Depletion and Proteinuria.* Journal of Cellular Biochemistry, 2015. **116**(9): p. 1947-1956.
77. Schnaper, H.W., et al., *TGF-β signal transduction and mesangial cell fibrogenesis.* American Journal of Physiology-Renal Physiology, 2003. **284**(2): p. F243-F252.
78. Mahimainathan, L., et al., *Mesangial cell hypertrophy by high glucose is mediated by downregulation of the tumor suppressor PTEN.* Diabetes, 2006. **55**(7): p. 2115-25.
79. Choi, M.E., et al., *Rat mesangial cell hypertrophy in response to transforming growth factor-β1.* Kidney International, 1993. **44**(5): p. 948-958.
80. Sharma, K., et al., *Neutralization of TGF-beta by anti-TGF-beta antibody attenuates kidney hypertrophy and the enhanced extracellular matrix gene expression in STZ-induced diabetic mice.* Diabetes, 1996. **45**(4): p. 522-30.
81. Ziyadeh, F.N., *Mediators of Diabetic Renal Disease: The Case for TGF-β as the Major Mediator.* Journal of the American Society of Nephrology, 2004. **15**(1 suppl): p. S55-S57.
82. Russo, L.M., et al., *Evidence for a role of transforming growth factor (TGF)-beta1 in the induction of postglomerular albuminuria in diabetic nephropathy: amelioration by soluble TGF-beta type II receptor.* Diabetes, 2007. **56**(2): p. 380-8.
83. Li, Y., et al., *Epithelial-to-mesenchymal transition is a potential pathway leading to podocyte dysfunction and proteinuria.* The American journal of pathology, 2008. **172**(2): p. 299-308.
84. Wang, S., et al., *Renal bone morphogenetic protein-7 protects against diabetic nephropathy.* J Am Soc Nephrol, 2006. **17**(9): p. 2504-12.
85. Weigert, C., et al., *Angiotensin II induces human TGF-β1 promoter activation: similarity to hyperglycaemia.* Diabetologia, 2002. **45**(6): p. 890-898.
86. Mingyuan, X., et al., *Hypoxia-inducible factor-1α activates transforming growth factor-β1/Smad signaling and increases collagen deposition in dermal fibroblasts.* Oncotarget, 2017. **9**(3): p. 3188-3197.
87. Croneigh, C., *New directions in home care quality assurance.* Chart, 1987. **84**(7): p. 6.

88. Andersson, E.R., R. Sandberg, and U. Lendahl, *Notch signaling: simplicity in design, versatility in function.* Development, 2011. **138**(17): p. 3593-612.
89. Lewis, J., *Notch signalling and the control of cell fate choices in vertebrates.* Semin Cell Dev Biol, 1998. **9**(6): p. 583-9.
90. Collier, J.R., et al., *Pattern formation by lateral inhibition with feedback: a mathematical model of delta-notch intercellular signalling.* J Theor Biol, 1996. **183**(4): p. 429-46.
91. de Celis, J.F., S. Bray, and A. Garcia-Bellido, *Notch signalling regulates veinlet expression and establishes boundaries between veins and interveins in the Drosophila wing.* Development, 1997. **124**(10): p. 1919-28.
92. de Celis, J.F. and S.J. Bray, *The Abruptex domain of Notch regulates negative interactions between Notch, its ligands and Fringe.* Development, 2000. **127**(6): p. 1291-302.
93. Goodyear, R. and G. Richardson, *Pattern formation in the basilar papilla: evidence for cell rearrangement.* J Neurosci, 1997. **17**(16): p. 6289-301.
94. Sirin, Y. and K. Susztak, *Notch in the kidney: development and disease.* J Pathol, 2012. **226**(2): p. 394-403.
95. Walsh, D.W., et al., *Co-regulation of Gremlin and Notch signalling in diabetic nephropathy.* Biochim Biophys Acta, 2008. **1782**(1): p. 10-21.
96. Lasagni, L., et al., *Notch activation differentially regulates renal progenitors proliferation and differentiation toward the podocyte lineage in glomerular disorders.* Stem Cells, 2010. **28**(9): p. 1674-85.
97. Niranjan, T., et al., *The Notch pathway in podocytes plays a role in the development of glomerular disease.* Nat Med, 2008. **14**(3): p. 290-8.
98. Lin, C.L., et al., *Modulation of notch-1 signaling alleviates vascular endothelial growth factor-mediated diabetic nephropathy.* Diabetes, 2010. **59**(8): p. 1915-25.
99. Ahn, S.H. and K. Susztak, *Getting a notch closer to understanding diabetic kidney disease.* Diabetes, 2010. **59**(8): p. 1865-7.
100. Teachey, D.T., et al., *Targeting Notch signaling in autoimmune and lymphoproliferative disease.* Blood, 2008. **111**(2): p. 705-14.
101. Herlihy, O.M. and P. Perros, *Elevated serum growth hormone in a patient with Type 1 diabetes: a diagnostic dilemma.* Diabetes Metab Res Rev, 2000. **16**(3): p. 211-6.
102. Kumar, P.A., F.C. Brosius, 3rd, and R.K. Menon, *The glomerular podocyte as a target of growth hormone action: implications for the pathogenesis of diabetic nephropathy.* Curr Diabetes Rev, 2011. **7**(1): p. 50-5.
103. Marshall, C.B. and S.J. Shankland, *Cell cycle and glomerular disease: a minireview.* Nephron Exp Nephrol, 2006. **102**(2): p. e39-48.
104. Wanke, R., et al., *[Role of podocyte damage in the pathogenesis of glomerulosclerosis and tubulointerstitial lesions: findings in the growth hormone transgenic mouse model of progressive nephropathy].* Verh Dtsch Ges Pathol, 2001. **85**: p. 250-6.
105. Reddy, G.R., et al., *Identification of the glomerular podocyte as a target for growth hormone action.* Endocrinology, 2007. **148**(5): p. 2045-55.
106. Chitra, P.S., et al., *Growth Hormone Induces Transforming Growth Factor-Beta-Induced Protein in Podocytes: Implications for Podocyte Depletion and Proteinuria.* J Cell Biochem, 2015. **116**(9): p. 1947-56.
107. Kumar, P.A., et al., *Growth hormone (GH)-dependent expression of a natural antisense transcript induces zinc finger E-box-binding homeobox 2 (ZEB2) in the glomerular podocyte: a novel action of gh with implications for the pathogenesis of diabetic nephropathy.* J Biol Chem, 2010. **285**(41): p. 31148-56.
108. Nishad, R., et al., *Growth hormone induces Notch1 signaling in podocytes and contributes to proteinuria in diabetic nephropathy.* J Biol Chem, 2019. **294**(44): p. 16109-16122.
109. Whitney, J.L., et al., *Growth hormone exacerbates diabetic renal damage in male but not female rats.* Biol Sex Differ, 2013. **4**: p. 12.

110. Wang, X., et al., *Inhibition of the Jak/STAT signaling pathway prevents the high glucose-induced increase in tgf-beta and fibronectin synthesis in mesangial cells.* Diabetes, 2002. **51**(12): p. 3505-9.
111. Callahan, J.F., et al., *Identification of novel inhibitors of the transforming growth factor beta1 (TGF-beta1) type 1 receptor (ALK5).* J Med Chem, 2002. **45**(5): p. 999-1001.
112. Campa, V.M., et al., *Notch activates cell cycle reentry and progression in quiescent cardiomyocytes.* J Cell Biol, 2008. **183**(1): p. 129-41.
113. Griffin, S.V., et al., *Podocyte proliferation and differentiation in glomerular disease: role of cell-cycle regulatory proteins.* Nephrol Dial Transplant, 2003. **18 Suppl 6**: p. vi8-13.
114. Thomasova, D. and H.J. Anders, *Cell cycle control in the kidney.* Nephrol Dial Transplant, 2015. **30**(10): p. 1622-30.
115. Das, D., et al., *Notch induces cyclin-D1-dependent proliferation during a specific temporal window of neural differentiation in ES cells.* Dev Biol, 2010. **348**(2): p. 153-66.
116. Hu, C.K., M. Coughlin, and T.J. Mitchison, *Midbody assembly and its regulation during cytokinesis.* Mol Biol Cell, 2012. **23**(6): p. 1024-34.
117. Liapis, H., P. Romagnani, and H.J. Anders, *New insights into the pathology of podocyte loss: mitotic catastrophe.* Am J Pathol, 2013. **183**(5): p. 1364-1374.
118. Yoshida, S., S. Bartolini, and D. Pellman, *Mechanisms for concentrating Rho1 during cytokinesis.* Genes Dev, 2009. **23**(7): p. 810-23.
119. Beel, A.J. and C.R. Sanders, *Substrate specificity of gamma-secretase and other intramembrane proteases.* Cell Mol Life Sci, 2008. **65**(9): p. 1311-34.
120. Haynes, J., et al., *Dynamic actin remodeling during epithelial-mesenchymal transition depends on increased moesin expression.* Mol Biol Cell, 2011. **22**(24): p. 4750-64.
121. Song, K., et al., *Loss of mucin-type O-glycans impairs the integrity of the glomerular filtration barrier in the mouse kidney.* J Biol Chem, 2017. **292**(40): p. 16491-16497.
122. Cheng, H.T. and R. Kopan, *The role of Notch signaling in specification of podocyte and proximal tubules within the developing mouse kidney.* Kidney Int, 2005. **68**(5): p. 1951-2.
123. Asanuma, K., J.A. Oliva Trejo, and E. Tanaka, *The role of Notch signaling in kidney podocytes.* Clin Exp Nephrol, 2017. **21**(1): p. 1-6.
124. Waters, A.M., et al., *Ectopic notch activation in developing podocytes causes glomerulosclerosis.* J Am Soc Nephrol, 2008. **19**(6): p. 1139-57.
125. Sweetwyne, M.T., et al., *Notch1 and Notch2 in Podocytes Play Differential Roles During Diabetic Nephropathy Development.* Diabetes, 2015. **64**(12): p. 4099-111.
126. Press, M., W.V. Tamborlane, and R.S. Sherwin, *Importance of raised growth hormone levels in mediating the metabolic derangements of diabetes.* N Engl J Med, 1984. **310**(13): p. 810-5.
127. Ying, Q. and G. Wu, *Molecular mechanisms involved in podocyte EMT and concomitant diabetic kidney diseases: an update.* Ren Fail, 2017. **39**(1): p. 474-483.
128. Wang, Y. and B.P. Zhou, *Epithelial-mesenchymal transition in breast cancer progression and metastasis.* Chin J Cancer, 2011. **30**(9): p. 603-11.
129. Collu, G.M. and K. Brennan, *Cooperation between Wnt and Notch signalling in human breast cancer.* Breast Cancer Res, 2007. **9**(3): p. 105.
130. Leong, K.G., et al., *Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin.* J Exp Med, 2007. **204**(12): p. 2935-48.
131. Pasupulati, A.K. and R.K. Menon, *Growth hormone and chronic kidney disease.* Curr Opin Nephrol Hypertens, 2019. **28**(1): p. 10-15.
132. Liu, M., et al., *Signalling pathways involved in hypoxia-induced renal fibrosis.* J Cell Mol Med, 2017. **21**(7): p. 1248-1259.
133. Nakuluri, K., et al., *Hypoxia induces ZEB2 in podocytes: Implications in the pathogenesis of proteinuria.* J Cell Physiol, 2019. **234**(5): p. 6503-6518.
134. Hills, C.E. and P.E. Squires, *The role of TGF-beta and epithelial-to mesenchymal transition in diabetic nephropathy.* Cytokine Growth Factor Rev, 2011. **22**(3): p. 131-9.

135. Sutariya, B., D. Jhonsa, and M.N. Saraf, *TGF-beta: the connecting link between nephropathy and fibrosis.* Immunopharmacol Immunotoxicol, 2016. **38**(1): p. 39-49.
136. Zavadil, J., et al., *Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition.* Embo j, 2004. **23**(5): p. 1155-65.
137. Geling, A., et al., *A gamma-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish.* EMBO Rep, 2002. **3**(7): p. 688-94.
138. Nakuluri, K., et al., *Stabilization of hypoxia-inducible factor 1alpha by cobalt chloride impairs podocyte morphology and slit-diaphragm function.* J Cell Biochem, 2018.
139. Das, D., M.A. Barnes, and L.E. Nagy, *Anaphylatoxin C5a modulates hepatic stellate cell migration.* Fibrogenesis Tissue Repair, 2014. **7**: p. 9.
140. Zhou, H., et al., *Loss of the podocyte glucocorticoid receptor exacerbates proteinuria after injury.* Sci Rep, 2017. **7**(1): p. 9833.
141. Kriz, W., N. Gretz, and K.V. Lemley, *Progression of glomerular diseases: is the podocyte the culprit?* Kidney Int, 1998. **54**(3): p. 687-97.
142. Lopes, T.G., et al., *Markers of renal fibrosis: How do they correlate with podocyte damage in glomerular diseases?* PLoS One, 2019. **14**(6): p. e0217585.
143. Doi, T., et al., *Glomerular lesions in mice transgenic for growth hormone and insulinlike growth factor-I. I. Relationship between increased glomerular size and mesangial sclerosis.* Am J Pathol, 1990. **137**(3): p. 541-52.
144. Doi, T., et al., *Progressive glomerulosclerosis develops in transgenic mice chronically expressing growth hormone and growth hormone releasing factor but not in those expressing insulinlike growth factor-1.* Am J Pathol, 1988. **131**(3): p. 398-403.
145. Hellmich, B., et al., *Activation of transforming growth factor-beta1 in diabetic kidney disease.* Metabolism, 2000. **49**(3): p. 353-9.
146. Sharma, K., et al., *Increased renal production of transforming growth factor-beta1 in patients with type II diabetes.* Diabetes, 1997. **46**(5): p. 854-9.
147. Zhu, Y., H.K. Usui, and K. Sharma, *Regulation of transforming growth factor beta in diabetic nephropathy: implications for treatment.* Semin Nephrol, 2007. **27**(2): p. 153-60.
148. Iglesias-de la Cruz, M.C., et al., *Effects of high glucose and TGF-beta1 on the expression of collagen IV and vascular endothelial growth factor in mouse podocytes.* Kidney Int, 2002. **62**(3): p. 901-13.
149. Mahesh, S. and F. Kaskel, *Growth hormone axis in chronic kidney disease.* Pediatr Nephrol, 2008. **23**(1): p. 41-8.
150. Nagata, M., et al., *Cell cycle regulation and differentiation in the human podocyte lineage.* Am J Pathol, 1998. **153**(5): p. 1511-20.
151. Grahammer, F. and T.B. Huber, *Aberrant podocyte cell cycle in glomerular disease.* Cell Cycle, 2016. **15**(17): p. 2237-8.
152. Liu, Y., et al., *YAP modulates TGF-beta1-induced simultaneous apoptosis and EMT through upregulation of the EGF receptor.* Sci Rep, 2017. **7**: p. 45523.
153. Olivetti, G., et al., *Morphometry of the renal corpuscle during postnatal growth and compensatory hypertrophy.* Kidney Int, 1980. **17**(4): p. 438-54.
154. Fries, J.W., et al., *Glomerular hypertrophy and epithelial cell injury modulate progressive glomerulosclerosis in the rat.* Lab Invest, 1989. **60**(2): p. 205-18.
155. Ichikawa, I. and A. Fogo, *Focal segmental glomerulosclerosis.* Pediatr Nephrol, 1996. **10**(3): p. 374-91.
156. Coimbra, T.M., et al., *Early events leading to renal injury in obese Zucker (fatty) rats with type II diabetes.* Kidney Int, 2000. **57**(1): p. 167-82.
157. Pagtalunan, M.E., et al., *Podocyte loss and progressive glomerular injury in type II diabetes.* J Clin Invest, 1997. **99**(2): p. 342-8.
158. Steffes, M.W., et al., *Glomerular cell number in normal subjects and in type 1 diabetic patients.* Kidney Int, 2001. **59**(6): p. 2104-13.
159. Ding, G., et al., *Angiotensin II induces apoptosis in rat glomerular epithelial cells.* Am J Physiol Renal Physiol, 2002. **283**(1): p. F173-80.

160. Schiffer, M., et al., *Apoptosis in podocytes induced by TGF-beta and Smad7.* J Clin Invest, 2001. **108**(6): p. 807-16.
161. Kretzler, M., *Regulation of adhesive interaction between podocytes and glomerular basement membrane.* Microsc Res Tech, 2002. **57**(4): p. 247-53.
162. Wu, D.T., et al., *TGF-beta concentration specifies differential signaling profiles of growth arrest/differentiation and apoptosis in podocytes.* J Am Soc Nephrol, 2005. **16**(11): p. 3211-21.
163. Murakami, A., et al., *A novel method for isolating podocytes using magnetic activated cell sorting.* Nephrol Dial Transplant, 2010. **25**(12): p. 3884-90.
164. Maruthi, M., et al., *Modulation of host cell SUMOylation facilitates efficient development of Plasmodium berghei and Toxoplasma gondii.* Cell Microbiol, 2017. **19**(7).

JBC ARTICLE

Growth hormone induces Notch1 signaling in podocytes and contributes to proteinuria in diabetic nephropathy

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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 article, using immortalized human podocytes *in vitro* and a mouse model *in vivo*, we show that excess GH activates Notch1 signaling in a γ-secretase–dependent manner. Pharmacological inhibition of Notch1 by 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. Furthermore, 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 up-regulated 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, epithelial cell dysfunction, leukocyte migration, excessive production, and deposition of extracellular matrix (2). In response to kidney damage, there will be infiltration of mature myofibroblasts from various sources including interstitial fibroblasts, pericytes, endothelial cells, and circulating fibrocytes (2). Previous studies have shown that multiple pathways such as the transforming growth factor-β (TGF-β)[2]/Smad2/3 and Notch signaling are involved in epithelial cell dysfunction and fibroblast activation, which leads to the progression of kidney fibrosis (2).

In the early glomerular development, particularly at the S-shaped body formation, podocyte fate determination is regulated by the highly conserved Notch signaling, which transduces short-range signals between neighboring cells (3–6). The Notch pathway comprises 4 transmembrane Notch receptors (Notch 1–4) and 5 Notch ligands (Delta-like 1, 3, and 4, and Jagged 1 and 2). After ligand binding, Notch receptors undergo a series of cleavages catalyzed by the ADAM proteases and γ-secretase complex, which results in the release of the Notch intracellular domain (NICD, Fig. S1); this process can be inhibited by the γ-secretase inhibitor and dibenzoazepine (7). The resulting NICD translocates into the nucleus (8), wherein it forms a ternary complex by associating with the DNA-binding protein, retinol-binding protein-jk and the coactivator, Mastermind-like protein 1 and activates expression of target genes (9–12).

Vooijs *et al.* (13) have reported that Notch1 is highly active in the developing kidney; however, in the mature kidney detection of active Notch1 is very little. Inhibition of Notch signaling during early development of the mouse kidney results in a severe deficiency of glomerular podocytes, indicating the importance of Notch signaling during kidney development (3). On the other hand, persistent activation of Notch signaling in the mature kidney leads to podocyte damage and subsequent kidney failure (14). Further studies had also shown that ectopic Notch activation in terminally differentiated podocytes is correlated with both diffuse mesangial sclerosis and focal segmental glomerulosclerosis, which are associated with *de novo* Pax2 expression and p53-induced podocyte apoptosis, respectively (14, 15). It was also observed that the genetic deletion of the

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This article contains Figs. S1–S5.

The microarray data reported in this paper have been submitted to the Gene Expression Omnibus (GEO) database under GEO accession no. GSE21327.

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[2] The abbreviations used are: TGF-β, transforming growth factor β; NICD, Notch intracellular domain; GH, growth hormone; GBM, glomerular basement; EMT, epithelial to mesenchymal transition; qRT, quantitative RT; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; UACR, urinary albumin-creatinine ratio; GFR, glomerular filtration rate; DN, diabetic nephropathy; PAS, periodic acid-Schiff; TEM, transmission electron microscope; DAPI, 4′,6-diamidino-2-phenylindole; α-SMA, α-smooth muscle actin.

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Growth hormone induces mitotic catastrophe of podocytes and contributes to proteinuria

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Running title: Growth hormone induces podocyte apoptosis

Keywords: Podocytes, Growth hormone, TGF-β1, Notch, Apoptosis, Proteinuria.

Authors contributions: RN, DM and AKP planned and designed the study. RN, DM and AKS performed the experiments. RN, AKP and PT evaluated the data. RN and KC performed the FACS experimentation and analysis. RN, DM and AKP wrote the manuscript. RN and AKP are the guarantor of this work and as such had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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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

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Activation of Notch1 signaling in podocytes by glucose-derived AGEs contributes to proteinuria

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ABSTRACT

Introduction Advanced glycation end-products (AGEs) are implicated in the pathogenesis of diabetic nephropathy (DN). Previous studies have shown that AGEs contribute to glomerulosclerosis and proteinuria. Podocytes, terminally differentiated epithelial cells of the glomerulus and the critical component of the glomerular filtration barrier, express the receptor for AGEs (RAGE). Podocytes are susceptible to severe injury during DN. In this study, we investigated the mechanism by which AGEs contribute to podocyte injury.

Research design and methods Glucose-derived AGEs were prepared in vitro. Reactivation of Notch signaling was examined in AGE-treated human podocytes (in vitro) and glomeruli from AGE-injected mice (in vivo) by quantitative reverse transcription-PCR, western blot analysis, ELISA and immunohistochemical staining. Further, the effects of AGEs on epithelial to mesenchymal transition (EMT) of podocytes and expression of fibrotic markers were evaluated.

Results Using human podocytes and a mouse model, we demonstrated that AGEs activate Notch1 signaling in podocytes and provoke EMT. Inhibition of RAGE and Notch1 by FPS-ZM1 (N-Benzyl-4-chloro-N-cyclohexylbenzamide) and DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenyl glycine t-butylester), respectively, abrogates AGE-induced Notch activation and EMT. Inhibition of RAGE and Notch1 prevents AGE-induced glomerular fibrosis, thickening of the glomerular basement membrane, foot process effacement, and proteinuria. Furthermore, kidney biopsy sections from people with DN revealed the accumulation of AGEs in the glomerulus with elevated RAGE expression and activated Notch signaling.

Conclusion The data suggest that AGEs activate Notch signaling in the glomerular podocytes. Pharmacological inhibition of Notch signaling by DAPT ameliorates AGE-induced podocytopathy and fibrosis. Our observations suggest that AGE-induced Notch reactivation in mature podocytes could be a novel mechanism in glomerular disease and thus could represent a novel therapeutic target.

Significance of this study

What is already known about this subject?

- Diabetes is presented with elevated advanced glycation end-products (AGEs) in serum and tissues including the kidney.

What are the new findings?

- AGEs induce Notch activation in glomerular podocytes.
- Notch activation resulted in epithelial to mesenchymal transition of podocytes.
- Administration of AGEs resulted in glomerulosclerosis and proteinuria.
- Inhibition of receptor for AGEs or Notch activation abrogates AGE-induced proteinuria.

How might these results change the focus of research or clinical practice?

- Inhibitors of γ-secretase, a key enzyme that triggers Notch activation, could ameliorate AGE-induced Notch activation to prevent proteinuria in diabetic conditions.

INTRODUCTION

Glomerular podocytes are terminally differentiated visceral cells and provide epithelial coverage to the glomerular capillaries. Podocytes, owing to their unique structure and localization, regulate glomerular permselectivity, contribute to the glomerular basement membrane (GBM), and counteract intracapillary hydrostatic pressure. Therefore, podocytes are considered instrumental in regulating the normal function of the glomerulus and are indispensable for the ultrafiltration of blood and the formation of primary urine. Platelet-derived growth factor and vascular endothelial growth factor derived from podocytes are required for the maintenance of parietal epithelial cells and endothelial cells, respectively.[1 2] Podocyte injury and loss are the early cellular changes in glomerular diseases that are clinically evidenced by proteinuria and renal failure due to glomerulosclerosis.[3] The number of podocytes was found to decline in diabetic nephropathy (DN).[4] Since intact podocytes were identified from the urine of patients with proteinuria, it was proposed that podocytes could detach from underlying GBM.[5] The transition of podocytes from epithelial to highly motile

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REVIEW ARTICLE

Epithelial–mesenchymal Transition of Glomerular Podocytes: Implications in Proteinuria

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ABSTRACT

The kidneys play an essential role in filtration of blood plasma, regulation of water, electrolyte, and acid/base balance of the body, and thus maintain overall homeostasis. The glomerular filtration barrier serves as a size, shape, and charge barrier to ensue glomerular permselectivity, so that kidneys excrete almost protein-free urine. Podocytes are glomerular visceral epithelial cells and significantly contribute to the glomerular permeability owing to their unique structure and specialized function. Nevertheless, podocytes are susceptible to various insults, including altered metabolites, aberrant signaling molecules, and mutations to critical proteins that otherwise ensue normal function. Podocyte injury is a predominant indicator of several glomerular diseases that are manifested by proteinuria. Epithelial–mesenchymal transition (EMT) is considered as one of the responses of podocytes to the noxious stimuli, which consequently results in podocyte depletion and proteinuria. This review discusses the importance of podocytes in normal renal filtration and details the molecular and cellular events that lead to EMT of podocytes vis-à-vis impaired glomerular filtration.

Keywords: Epithelial–mesenchymal transition, Glomerulus, Kidney, Nephron, Podocytes, Proteinuria.

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KIDNEYS ENSUE EXCRETION OF PROTEIN-FREE URINE

Over a million nephrons in each kidney work in concert to regulate water and acid/base balance of the body and to ensue excretion of protein-free urine. Thus, kidneys become vital organs to ensure homeostasis of the body. The two essential segments of a nephron are glomerulus and renal tubule. The glomerulus is essential for filtering water and small molecules from plasma. The tubular system ensues both selective reabsorption of glomerular filtrate and selective secretion of ions into glomerular filtrate. Therefore, both glomerulus and renal tubule work in concert and dictate the final composition of urine. The potential of kidney to excrete almost protein-free ultrafiltrated urine gets compromised during disease conditions, and as a result varying amounts of plasma proteins get excreted in urine. Albuminuria is an index of adverse renal outcome, which can be assessed by measuring albumin levels in urine, collected for 24 hours. According to the American Diabetic Association, microalbuminuria describes levels of urine albumin ranging from 30 to 300 mg/24 hours; and macroalbuminuria describes a urinary albumin excretion of ≥300 mg/24 hours. The condition of macroalbuminuria often progresses to overt proteinuria and even further to end-stage renal disease (ESRD), warranting renal transplant therapy.

Appearance of protein in the urine indicates a structural and/or functional artifact, particularly in the glomerular region.[1] The glomerular filtration barrier (GFB) of the kidney serves as a size, shape, and charge selective molecular sieve. The three critical components that constitute GFB are: (a) Fenestrated endothelium of glomerular blood vessels; (b) basement membrane that covers the blood vessels; and (c) the podocytes that provide epithelial coverage to basement membrane (Figs 1A and B). Though, all the three components contribute to the integrity of GFB, there is much debate on the critical role of each component toward size, shape, and charge-dependent permselectivity of GFB. It was proposed that endothelial dysfunction is a causal factor in the pathogenesis of proteinuria.[2] Thickening of glomerular basement membrane (GBM) by excess deposition of collagen and altered charge selectivity implicates the pathogenesis of proteinuria.[3,4] The third and final barrier that restricts entry of proteins from circulation into the urine is the podocytes, also known as visceral epithelial cells. There is increasing evidence for the crucial role of podocytes in this glomerular filtration process.[5]

PODOCYTES ARE UNIQUE CELLS WITH SPECIALIZED PROPERTIES

Podocytes are the major cell type of glomerulus and account for about 30% of all glomerular cells. Podocytes are highly branched epithelial cells and cover the urinary

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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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Cerebral ischemia induces TRPC6 via HIF1α/ZEB2 axis in the glomerular podocytes and contributes to proteinuria

Krishnamurthy Nakuluri[1,5], Rajkishor Nishad[1,5], Dhanunjay Mukhi[1], Sireesh Kumar[2], Venkata P. Nakka[3], Lakshmi P. Kolligundla[1], Parimala Narne[2], Sai Sampath K. Natuva[4], Prakash Babu Phanithi[2*] & Anil K. Pasupulati[1*]

Podocytes are specialized cells of the glomerulus and key component of the glomerular filtration apparatus (GFA). GFA regulates the permselectivity and ultrafiltration of blood. The mechanism by which the integrity of the GFA is compromised and manifest in proteinuria during ischemic stroke remains enigmatic. We investigated the mechanism of ischemic hypoxia-induced proteinuria in a middle cerebral artery occlusion (MCAO) model. Ischemic hypoxia resulted in the accumulation of HIF1α in the podocytes that resulted in the increased expression of ZEB2 (Zinc finger E-box-binding homeobox 2). ZEB2, in turn, induced TRPC6 (transient receptor potential cation channel, subfamily C, member 6), which has increased selectivity for calcium. Elevated expression of TRPC6 elicited increased calcium influx and aberrant activation of focal adhesion kinase (FAK) in podocytes. FAK activation resulted in the stress fibers reorganization and podocyte foot process effacement. Our study suggests overactive HIF1α/ZEB2 axis during ischemic-hypoxia raises intracellular calcium levels via TRPC6 and consequently altered podocyte structure and function thus contributes to proteinuria.

Extreme physiological and pathological conditions impose challenges on human physiology. The normal functioning of the human body demands both continuous and adequate supply of oxygen whereas relative (hypoxia) and the absolute deficiency (anoxia) of oxygen are a risk to human health. Human organs vary in their oxygen dependency and susceptibility to oxygen deficiency. Brain and kidney are most hypoxia-sensitive organs. Oxygen is involved in the formation of ATP from ADP and ATP-dependent active salt reabsorption in kidney demands high oxygen supply[1]. Kidney carries out its functions within a narrow range of partial pressure of oxygen, which is very low in the inner medulla (5 mmHg) compared with the outer cortex (50 mmHg)[2]. Furthermore, renal vasculature despite its low-resistance subjected to continuous perfusion[3,4]. Vascular architecture of the kidney and surplus demand for oxygen together let the kidneys highly sensitive to oxygen-deprived conditions[1,5,6]. Limitations in oxygen supply impose kidneys to undergo hypoxia-induced maladaptation, which likely reflects in the pathophysiology of acute kidney injury and proteinuria[6–12].

The vertebrate kidneys regulate homeostasis predominantly by controlling acid-base, electrolyte, and water balance. Kidneys are also instrumental in ultrafiltration of plasma components and regulating the composition of urine. Proteinuric condition suggests abnormalities in the glomerular filtration apparatus (GFA)[13]. Three layers of GFA are podocytes, glomerular basement membrane (GBM), and perforated endothelium[13]. Clinical conditions such as stroke and sleep apnea are associated with proteinuria and are presented with reduced renal perfusion and moderate to severe hypoxia[12,14]. Accumulated evidence suggests that hypoxia contributes to the proteinuria and pathogenesis of chronic kidney disease (CKD)[6,7,10,15–17]. The prevalence of CKD is more than 30% among stroke subjects[18]. Renal dysfunction is a worse clinical outcome in patients with ischemic stroke[19,20] and it is an independent predictor of stroke mortality[18].

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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.

Research Communication

In Silico Structural Characterization of Podocin and Assessment of Nephrotic Syndrome-Associated Podocin Mutants

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Abstract

Nephrotic syndrome (NS) is manifested by hyperproteinuria, hypoalbuminemia, and edema. *NPHS2* that encodes podocin was found to have most mutations among the genes that are involved in the pathophysiology of NS. Podocin, an integral membrane protein belonging to stomatin family, is expressed exclusively in podocytes and is localized to slit-diaphragm (SD). Mutations in podocin are known to be associated with steroid-resistant NS and rapid progression to end-stage renal disease, thus signifying its role in maintaining SD integrity and podocyte function. The structural insights of podocin are not known, and the precise mechanism by which podocin contributes to the architecture of SD is yet to be elucidated. In this study, we deduced a model for human podocin, discussed the details of transmembrane localization and intrinsically unstructured regions, and provide an understanding of how podocin interacts with other SD components. Intraprotein interactions were assessed in wild-type podocin and in some of its mutants that are associated with idiopathic NS. Mutations in podocin alter the innate intraprotein interactions affecting the native structure of podocin and its ability to form critical complex with subpodocyte proteins. © 2016 IUBMB Life, 68(7):578–588, 2016

Keywords: *nephrotic syndrome; proteinuria; podocytes; podocin; slit diaphragm; molecular modeling*

Introduction

The kidneys are vital organs that help to maintain body homeostasis by regulating blood pressure, acid–base, electrolyte, and water balance. Human kidney constitutes a million nephrons that collectively perform three key events including (*a*) glomerular filtration of water and small molecules from renal plasma; (*b*) tubular reabsorption of glomerular filtrate; and (*c*) tubular secretion of metabolic waste products into the filtrate. Thus, glomerulus in concert with tubular region of the nephron tightly regulates the composition of glomerular filtrate and ensures almost protein-free ultrafiltrated urine. Glomerulus, where initiation of filtration occurs, contains a tuft of capillaries and several resident cell types that include mesangial cells, endothelial cells, and glomerular visceral epithelial cells, also known as podocytes.

Proteinuria is a hallmark of renal damage in several glomerular diseases due to the alterations in glomerular filtration barrier (GFB; refs. 1 and 2). The three components that constitute GFB include fenestrated glomerular endothelial cells, glomerular basement membrane (GBM), and glomerular visceral epithelial cells, known as podocytes. A wealth of literature highlighted that podocytes are critical for glomerular filtration (3,4). Podocytes are terminally differentiated epithelial cells

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Growth hormone induces Notch reactiovation in podocytes.

by Rajkishor Nishad

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