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

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Abstract:

Growth hormone (GH) had direct effects on the glomerular podocyte. Increased levels of GH are implicated in the pathogenesis of renal abnormalities in acromegaly and in diabetic nephropathy in Type 1 diabetes mellitus. We investigated the role of Transforming growth factor-β1 (TGF-β1) in GH's effects on the glomerular podocyte. Exposure of podocytes to GH resulted in elevated expression of TGF-β1 and was concurrent with increased phosphorylation of SMAD2/3 and nuclear accumulation of SMAD4. Conditioned media from podocytes exposed to GH also triggered SMAD signaling. Podocytes treated with GH showed increased permeability to albumin. Mice injected with GH demonstrated increased kidney size, glomerular hypertrophy, and proteinuria. The renal manifestations in GH injected mice were associated with increased TGF-β1 expression and activation of TGF-β1 signaling pathways. Concurrent administration of TGF-β receptor antagonist (SB431542) with GH abrogated these renal effects of GH administration. These results reveal the role of TGF-β1 in GH's actions on the glomerular podocyte and provide a novel mechanistic basis for GH-induced glomerular dysfunction in clinical conditions such as diabetes and acromegaly.

Introduction:

Diabetic nephropathy (DN) is characterized by an array of structural and functional aberrations in the nephron. Structural changes include podocyte hypertrophy, glomerular sclerosis, and thickening of the glomerular basement membrane (GBM). The functional manifestations include a transient increase in glomerular filtration rate (GFR) and proteinuria ranging from microalbuminuria to overt proteinuria. The magnitude of proteinuria reflects the extent of damage to the glomerular filtration barrier (GFB), which consists of endothelial cells, basement membrane and podocytes [1]. Podocytes are the specialized cells that offer epithelial coverage to renal capillaries and serves as a final barrier to curb the loss of protein into the urine. Injury to the podocytes including hypertrophy, foot process effacement, and epithelial-mesenchymal-transition (EMT) that eventually culminates into reduced podocyte count [2]. However, the molecular signals that initiate these podocyte abnormalities remain incompletely understood.

Elevated growth hormone (GH) levels in poorly controlled type 1 diabetes mellitus (DM) is implicated as a causative factor in the development of renal aberrations and proteinuria in DM [2-4]. GH transgenic animals developed significant renal hypertrophy and glomerulosclerosis [5]. Although certain biological effects of GH are mediated by insulin-like growth factor 1 (IGF-1), the glomerular hypertrophy and significant renal manifestations are evidenced in GH transgenics with IGF-1 null background [6], suggesting that IGF-1 is not necessary for these effects of GH on the glomerulus. In GH transgenic animals, podocytes undergo hypertrophy while mesangial and endothelial cells undergo proliferation [6]. GH administration enhanced the effects of streptozotocin (STZ)-induced diabetes on renal hypertrophy in rats [5]. Conversely, mice with mutant GHR were protected from STZ-induced diabetic renal complications [7]. Similarly, in animal models, somatostatin analogs (lanreotide and octreotide) that decrease GH secretion offered substantial protection against DN [8]. Long-term treatment of acromegaly patients with

somatostatin analogues prevented GH-induced renal alterations [9]. This evidence argues for a strong association between elevated GH levels and renal aberrations. Our earlier studies identified that podocyte as a direct target for GH action and our subsequent studies demonstrated that GH regulates expression of Smad interacting protein (SIP1) and transforming growth factor β interacting protein (TGFBIP, also known as Bigh3) in podocytes [10-12].

Transforming growth factor-β (TGF-β) is a master regulator of ECM turnover that is secreted from most cell types in the body. Three TGF-β isoforms have been identified among which isoform 1 (TGF-β1) has been associated with the pathophysiology of DN. Earlier studies implicated TGF-β1 as a central molecule responsible for the excess deposition of ECM proteins in diabetes [13]. Both mRNA and protein levels of TGF-β1 are significantly elevated in DN patients [14, 15]. Studies from experimental diabetic animals further substantiated the role of TGF-β1 in the pathogenesis of DN [16]. Neutralizing TGF-β1 with anti-TGF-β1 antibodies reversed the established type II diabetic renal injury [17]. In the present study, we provide evidence to substantiate the hypothesis that GH's effect on the glomerular podocyte is transduced via the TGF-β/SMAD pathway. Our findings suggest a novel mechanism for GH's actions on the glomerular podocyte

Materials and methods:

Materials: TGF-β1 antibody (#MA5-16949) and Lipofectamine (#11668019) were purchased from Invitrogen. SMAD pathway antibodies were purchased from Novus Biologics (#NBP2-54771, NBP1-77836, AF3797, NBP2-67372). All secondary antibodies used for western blotting in this study were from Jackson Laboratories (#111-035-144, 115-035-062) and fluorescent-tagged secondary antibodies from Vector Labs (#DI-1094, CY-2500). Human GH was Genotropin (Pfizer) and recombinant TGF-β1 (#240-B-002) and ELISA kit for TGF-β1 (#DB100B) were procured from R&D systems. TGF-βR1 inhibitor (SB431542) was purchased

from Tocris Bioscience (#1614-10MG). Signal SMAD4 reporter plasmid encodes GFP gene under minimal CMV promoter and tandem repeats of SMAD4 transcriptional response elements (SA Biosciences).

Animal experiments and animal care: Swiss-albino mice were purchased from the National Center for Laboratory Animal Sciences (NCLAS, NIN, Hyderabad). All experimental group animals were maintained under 12hr day and night cycles throughout the experimental procedure. After acclimatization animals are grouped into three groups: control, GH, GH+SB. On day fifth of acclimatization, the GH group received intraperitoneal injections of GH (2mg/kg body weight/day) and the GH+SB group received intraperitoneal injections of SB431542 (1mg/kg body weight/day) 1h prior to the GH treatment. All animals were sacrificed on day 14th post GH treatment, perfused with PBS and internal body fixation achieved by 4% PFA (paraformaldehyde). The kidneys were collected and one kidney was used for histological analysis and the other kidney was used for molecular signaling studies. Institutional Animal Ethics Committee of University of Hyderabad approved the animal protocols.

Cell culture and treatments: Immortalized human podocytes were gifted by Prof. Moin Saleem (University of Bristol, UK) cultured in 10% serum containing media prepared in RPMI 1640 with 1% antibiotic-antimycotic at 33ºC under 5% CO_2. After cells reached 70% confluence, the cells were transferred to 37ºC and maintained for 6-8 days for differentiation. Differentiated podocytes were used for all treatments. Cells were serum starved in glucose-free RPMI media for 24hr before being treated by human GH. All treatments were performed in serum and glucose-free media. Before treating cells with GH or TGF-β1, cells were treated with SB431542 for 24h in serum and glucose-free medium.

Preparation of conditioned media: Differentiated podocytes were treated with or without GH (500ng/ml) for 30 min. Subsequently these podocytes are thoroughly washed with RPMI1640 media to remove trace of GH and then cultured in fresh serum free RPMI1640 media, which

was collected 30 min later (Conditioned media). The conditioned media from GH-treated (CM-GH) and untreated (Con-CM) podocytes were centrifuged 6000xg for 5 min to remove cell debris and resulting supernatant was further purified with 0.2 µm membrane filters. Fresh batch of differentiated podocytes were treated with Con-CM or GH-CM for 30 min to assess activation of SMAD signaling or treated for 12 h for assessing morphological parameters.

SDS-PAGE & Western Blotting: Unless otherwise mentioned, 10% SDS-PAGE gels were used in this study. Cell lysates were prepared in RIPA lysis buffer and protein estimation was done using Bradford standard method. Equal amounts of protein were loaded and gels were run at 100 volts for 2hrs or until the dye was released. Western blotting was performed using standard methods at 200mA for 1hr at $4^{o}C$ and PVDF membrane was used for all western transfers. Blots were incubated with the respective primary antibody for overnight at $4^{o}C$ before being incubated with respective secondary antibody for 1hr at room temperature. Blots were developed using the ECL substrate employing Bio-Rad Versa Doc 5000 MFP and results were analyzed in Image Lab and band intensities were quantified in Image J software developed by NIH, Bethesda.

Immunohistochemistry: Paraffin-embedded tissues were processed into 6µm sections using a Leica systems microtome. Sections were deparaffinized in xylene and rehydrated with ethanol from 100% to 50%. Peroxidase blocking and permeabilization were performed in 30% H_2O_2 for 25 min at room temperature. Antigen retrieval was done in Tris-EDTA-Tween buffer in the micro oven (three cycles of five minutes with intervals of two minutes). Blocking was performed in 5% BSA in PBS for 1hr at room temperature. Sections were incubated with primary antibody for two hours followed by two washes with PBS and incubated with secondary antibody either with fluorescent tagged or HRP (horseradish peroxidase) conjugate. Sections were developed using DAB stain and counterstained with hematoxylin. All washes are done with PBS. Sections were dehydrated and mounted with DPX mounting media.

Immunofluorescence: Immunofluorescence was performed according to a previous protocol [12]. Briefly, cells were fixed with 4% PFA for 10 min on a glass coverslip and permeabilized with 0.5% Triton X-100 for 15min at room temperature. Blocking was performed with 5% BSA in PBS for 1hr and primary antibody was used at a 1:100 dilution. Cells were mounted with prolong gold antifade with DAPI (Molecular Probes, #P36941). All washes are done with PBS. Imaging was done in a Leica Microsystems trinocular or Zeiss confocal microscopy.

SMAD4-GFP Reporter Assay: SMAD4-GFP reporter assay was performed in HEK293T cells according to the earlier published protocols with minor modifications [18]. In a 96 well plate, $6X10^3$ cells/well were seeded prior to the day of transfection. Cells were transfected either with SMAD4-GFP, positive control and negative control vectors in triplicates. After transfection cells were left untreated in complete medium for 16h, followed by serum starvation for 12h. The cells were then treated with TGFβ, GH CM or GH in serum-free medium. After 16h of treatment, images were taken in Olympus 5000 fluorescence microscope and the fluorescence emission spectrum from 510-520nm recorded by exciting at 488nm. Fluorescence values were plotted after normalizing with mock test control values.

Transient transfection: All transient transfections were performed according to the manufacturer protocol. 95% confluent HEK293T cells were transfected using lipofectamine 2000, Invitrogen. After 6hr of transection, cells were briefly washed with PBS and incubated in 10% serum containing media for 24hr followed by which all treatments were performed in serum-free medium.

ELISA: Cells were serum starved in glucose-free RMPI 1640 medium for 24h under standard culture conditions. Cells were treated with GH 250 and 500ng concentrations in the starvation medium (not supplement with glucose) for 30min. Briefly, dead cells were removed from the cell supernatants by centrifugation at 16000g for 10min at 4^oC and the supernatants were passed through 0.2µm membrane filters. The resultant supernatants were directly subjected to the TGF-

β1 activation procedures as the manufacturer's protocol. Activated samples were assayed by indirect sandwich Elisa kit (R&D systems # DB100B).

Paracellular albumin influx assay: Albumin influx assay was performed in podocytes as per published methods [11]. $1.2X10^5$ cells per well were seeded on to the top chamber of a 6-well insert. The wells of the six-well plate were filled with 6ml of RPMI complete medium and incubated under permissive conditions for 24h. Then cells were shifted to 37^oC and incubated for 6-8 days for differentiation into mature podocytes before being serum starved for 24h and the indicated treatments applied to the cells. After 48hr of treatment, cells were washed with PBS supplemented with 1mM MgCl2 and 1mM CaCl2 to preserve cadherin junctions. Then, six-well plates were filled with 6ml of 40mg/ml BSA containing serum-free RPMI medium and the top chamber filled only with 0.7ml of RPMI. After incubation, a small aliquot of a medium from the top chamber was collected to measure BSA influx from bottom to top chamber and concentration assessed by the BCA protein assay method.

Statistics Analysis: Data are presented as standard error mean (SEM) unless otherwise indicated. Graphpad Prism 8 software was used to analyze statistical differences between the distributions of two (Unpaired non-parametric-Mann Whitney test) or multiple independent samples (Tukey's Multiple comparison test), respectively. $P \leq 0.05$ was considered as significant.

Results:

GH induces TGF-β1 and the cognate TGF-β-SMAD pathway in human podocytes: Considering both the central role of podocytes in glomerular permselectivity and the established role of TGF-β1 in increasing podocyte permeability, we investigated the direct action of GH on the TGF-β/SMAD pathway in human podocytes. We observed that GH (100-500 ng/ml) induces TGF-β1 protein levels in human podocytes as analyzed by immunoblotting (Fig 1A&B). Since TGF-β1 is a secretory molecule, we next estimated TGF-β1 concentration in conditioned

medium from GH treated podocytes. GH increased TGF-β1 concentration in both a dose (250 and 500 ng/ml) and time (0-60 min) dependent manner (Fig 1C&D). Furthermore, GH also increased phosphorylation of SMAD2&3 in podocytes (Fig 1E&F). These results indicate that GH induces expression of TGF-β1 and activation of TGF-β1 down-stream signaling in podocytes.

GH induced TGF-β in podocytes acts in both autocrine and paracrine manner: TGF-β1 is known to act in a both autocrine and paracrine manner. To elucidate the paracrine action of GH induced TGF-β1, we treated podocytes with GH and collected conditioned media. Conditioned media from GH-treated podocytes induced phosphorylation of SMAD2&3 in podocytes naïve to GH (Fig 2A). This suggests the possibility of GH-dependent secretion of TGF-β1 into the conditioned media, which then elicited activation of SMAD2&3. TGF-β dependent regulation of its target genes requires the interaction of SMAD4 with SMAD2&3 and the localization of the SMAD2/3/4 complex to the nucleus. We observed increased accumulation of SMAD4 in the nucleus following treatment with GH or conditioned media from GH-treated podocytes (Fig 2B). We next performed TGF-β neutralization studies with podocytes transfected with a GFP expression plasmid under the control of SMAD binding element. Both, GH and conditioned media from GH treated podocytes induced GFP expression in this model (Fig 2C). However, conditioned media from GH treated podocytes that were pre-incubated with anti-TGF-β1 antibody failed to induce GFP expression (Fig 2C&D). Furthermore, to verify that observed effects of GH on activation of SMADs is mediated via GH-dependent secretion of TGF-β1 in podocytes; we employed the TGF-βR1 inhibitor, SB431542 (hereafter named as SB). SB is a small molecule that targets the TGF-β signaling pathway by competing for ATP binding domains in the ALK5 (TGF-βR1) receptor [19]. SB treatment significantly reduced the TGF-β1 levels in GH treated podocytes as measured by ELISA (Fig 3A) and immunoblotting (Fig 3B). In addition,

we observed reduced phosphorylation of SMAD2&3 (Fig 3C) and decreased nuclear levels of SMAD4 (Fig 3D) in podocytes treated with GH and SB. We next sought to study the functional consequence of GH-dependent expression of TGF-β1 and down-stream activation of SMAD signaling in podocytes using a paracellular albumin influx assay. Podocytes treated with GH showed increased permeability to albumin across a podocyte monolayer (Fig 3E). Interestingly, treatment with SB abrogated the observed effect of GH on podocyte permeability (Fig 3E). Together, these data from either direct treatment with GH or from conditioned media from GH-treated podocytes demonstrate that TGF-β1 induced by GH acts in a both autocrine and paracrine manner to activate SMAD signaling, which results in increased podocyte permeability.

GH alters podocyte morphology and regulates expression of ECM components in podocytes: GH treated podocytes show increased cell size compared with podocytes naïve to GH (Fig 4A). Similar to GH, TGF-β1 also induced an increase in podocyte cell size (Fig 4A). Changes in the structural organization of the actin cytoskeleton usually accompany changes in cell size. Profiling of F-actin polymerization in podocytes by Phalloidin staining revealed that both TGF-β1 and GH increased F-actin polymerization in podocytes (Fig 4B&C). TGF-βR1 inhibitor (SB) attenuated GH induced increase in podocyte cell size (Fig 4B&C). Cell morphology is also influenced by its interactions with ECM proteins and TGF-β1 is known to play a critical role in ECM turnover. Next, we found increased expression of extracellular matrix molecules in podocytes exposed to GH or TGF-β1 compared with cells naïve to these molecules (Fig 4D&E). Increased expression of ECM proteins in podocytes correlates with increased thickening of GBM observed in mice injected with GH (Fig 4D&E Vs. Fig 6A&B).

Activation of TGF-β/SMAD pathway in GH injected mice: To delineate the *in vivo* effect of GH on TGF-β in the podocyte we administered GH to mice for two weeks. Compared with control mice, GH injected mice had higher kidney (mg) to body weight (g) ratio (7.2 Vs. 8.46)

and increased glomerular size (Fig 5A&B). Histological analysis of kidney from GH injected mice revealed mild to moderate renal fibrosis, mesangial expansion, and synechiae formation between bowman's space and glomerular tufts (Fig 5C). There was also a significant decrease in GFR in GH injected animals (Fig 5D). Analysis of podocyte architecture by transmission electron microscopy revealed disruption of podocyte foot-processes and thickening of GBM (Fig 6A&B). Urine from GH administered mice has higher albumin to creatinine ratio (UCAR, Fig 6C) and more abundance of high molecular weight proteins (Fig 6D) compared with control mice. These results demonstrate that GH induces glomerular hypertrophy, alterations in podocyte morphology, and impairment of renal function as evidenced by increased UCAR and decreased GFR.

We conducted further analysis to understand the mechanism for the glomerular hypertrophy and proteinuria in mice administered with GH. TGF-β1 levels were elevated in platelet poor plasma preparations from GH injected mice (Fig 7A). Furthermore, glomerular lysates from GH injected mice revealed an increased abundance of TGF-β1 protein (Fig 7B&C). Immunohistochemistry analysis demonstrated increased expression of TGF-β1 in both glomerulus and renal tubule (Fig 7D). Increased phosphorylation of SMAD2&3 was also observed in glomerular lysates from mice administered with GH (Fig 7E). These results suggest that TGF-β1 mediates effects of GH on the glomerular podocyte.

TGF-βR1 inhibitor attenuates GH induced renal anomalies: In order to ascertain that the observed GH-dependent glomerular injury is specifically due to TGF-β1, we employed the TGF-βR1 inhibitor (SB) in our further studies. SB administration ameliorated alterations in kidney (mg) to body weight (g) ratio in GH injected mice (7.9 Vs. 8.4) Administration of SB to mice prior to the GH injection prevented the increase in TGF-β1 levels induced by GH injection (Fig 8A). SB treatment also alleviated the GH-induced glomerular hypertrophy and increases in

glomerular tuft area (Fig 8B&C) and resulted in decreased activation of SMAD2&3 (Fig 8D). Furthermore, SB treatment improved proteinuria in the GH-administered animals (Fig 8E). These results support the hypothesis that TGF-β1 mediates effects of GH on the podocyte and that inhibition of TGF-βR1 could prevent certain GH mediated adverse effects on the podocyte and the glomerulus and the podocyte.

Discussion:

The current investigation reveals a novel mechanism for GH action on glomerular podocytes and in the pathogenesis of diabetic nephropathy. The major findings of this study are that GH induces TGF-β1 and the canonical TGF-β1/SMAD signaling pathway in glomerular podocytes. GH impairs podocyte permeability as measured in paracellular albumin influx assay and the inhibitor of TGF-β1R ameliorated this effect. GH induces glomerular hypertrophy, accumulation of ECM proteins, and proteinuria in mice whereas inhibition of TGF-β1 receptor prevented GH-induced degenerative changes in the kidney and improved proteinuria.

Transient renal hypertrophy, podocyte hypertrophy, and glomerulosclerosis are hallmarks of diabetic kidney disease. Elevated levels of GH were implicated in the early renal hypertrophy and proteinuria in type I diabetes [20]. GH transgenic mice also showed significant podocyte hypertrophy [6]. A direct relationship was established between the activity of the GH/GHR axis and renal hypertrophy and glomerulosclerosis [6]. In our earlier study, we reported the presence of GHR and activation of canonical JAK/STAT signaling in podocytes [12]. However, it was not clear whether this causal role of the GH in the pathogenesis of renal hypertrophy and sclerosis in conditions such as acromegaly and DN is due to direct actions of GH on the podocytes or via GH's effector molecules. Among several host of mediators in the diabetic milieu, TGF-β1 has emerged to have a key role in the development of renal hypertrophy and accumulation of ECM

proteins via both stimulations of matrix synthesis and inhibition of matrix degradation. Several lines of evidence revealed TGF-β1's role in morphologic manifestations and clinical characteristic of DN [13, 20]. Despite knowing the fact that TGF-β/SMAD pathway activation is crucial in most of the kidney diseases, the stimuli that activate the TGF-β/SMAD pathway in the podocyte remain unclear. Previous studies proposed that high glucose and angiotensin II induces TGF-β1 expression in mesangial cells, however, in the podocytes, angiotensin II does alter TGF-β expression. Although, elevated GH levels and overactivity of GH/GHR axis is implicated in renal manifestations and CKD [2, 4] the temporal association between GH and TGF-β is 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 [5] and suppressing JAK2, an immediate downstream target of GH reduced TGF-β mRNA expression [21]. In the present study, our results establish that GH stimulates expression of TGF-β1 in podocytes and to best of our knowledge this is the first study to demonstrate GH induces TGF-β1 expression. TGF-β1 is secreted from cells as a biologically inactive large latent complex (LLC) [22]. LLC is composed of a family of latent TGF-β binding proteins (LTBP) that binds covalently to TGF-β and facilitate efficient secretion and targeting TGF-β to the ECM. In our earlier study, we reported that GH regulates expression of TGFBIp (TGF-beta interacting protein, also known as BigH3), which is a member of the LTBP complex [10]. Hence, GH increases TGF-β1 mediated renal effects by both increasing TGF-β1 and by regulating the bioavailability of TGF-β1.

TGF-β family proteins are secreted proteins and elicit pleiotropic actions on target cells. Our study suggests GH-dependent secretion of TGF-β1 elicits its action in a both autocrine and paracrine manner. TGF-β1 binds and activate heteromeric complex (type I and II) of transmembrane receptors. Upon binding with the ligand, type II receptor, which is a

constitutively active kinase phosphorylates the type I receptor. Activated type I receptor kinases phosphorylate two serine residues in the “SSXS motif” of receptor-regulated SMADs (R-SMADs; SMAD2&3). Phosphorylation enables the formation of heteromeric complexes between SMAD2&3 and one common-SMAD, SMAD4, and translocation of the entire complex to the nucleus [23]. We demonstrated GH-dependent activation of R-SMADs and localization of SMAD4 in the podocyte nucleus. Nuclear translocation of SMAD4 is dependent on phosphorylation of SMAD2&3 that form complex with SMAD4. Both direct exposure of podocytes to GH and GH-conditioned media induced GFP expression from a transfected plasmid containing GFP controlled by a putative SMAD4 binding element. The former actions support autocrine action and later indicate paracrine action of TGF-β1. Furthermore, pre-treatment of GH-conditioned media with TGF-β1 neutralizing antibodies abolished the potential of GH-conditioned media to activate SMAD4. It is noteworthy that podocytes exposed to GH-conditioned media that are prior exposed to TGF-βR1 inhibitor (SB) reduced the phosphorylation of SMAD2&3. SMAD4 nuclear translocation also was prevented by treatment with SB. These observations are congruent with the diminished levels of TGF-β1 in the podocytes exposed to GH and SB. Our study also demonstrates that perturbing the TGF-βR1/SMAD pathway by SB prevented GH-mediated renal manifestations in mice suggesting the autoregulation of TGF-β1. Our observation is supported by an earlier study where administration of neutralizing TGF-β antibodies decreased the total production of TGF-β [22]. GH induces the expression of ZEB2 (also known as Smad Interacting protein, SIP1) [11]. ZEB2 binds tightly to activated RSMADs (SMAD2&3) and regulate Smad-mediated transcription even when ZEB binding sites are absent from a target gene [24]. Together the data suggest GH regulates TGF-βR1/SMAD signaling at multiple levels.

Our study indicates that TGF-β1 elicits reorganization of the actin cytoskeleton in podocytes. Podocytes are terminally differentiated cells and offer epithelial coverage to the

glomerular basement membrane. Neighboring foot-processes of podocytes are tethered by a modified tight-junction known as slit-diaphragm (SD) that forms the major filtration-barrier at the blood-urine interface. Podocyte foot-processes composed of the actin cytoskeleton. Therefore actin cytoskeleton plays a vital role in normal biology of the podocyte and reorganization of the actin cytoskeleton is expected to cause podocyte dysfunction, which results in glomerulosclerosis and progressive kidney failure [25]. TGF-β1 induced reorganization of the actin cytoskeleton and abnormal functioning of SD suggests a mechanistic link between increased permeability to albumin across podocyte monolayer. Concomitantly, mice injected with GH showed elevated TGF-β1 levels, effacement of podocyte foot-processes and proteinuria. The validation of our hypothesis and the decisive role of TGF-β1 in transducing GH's action on podocyte dysfunction will be investigated in future studies in the presence and absence of TGF-β1 receptors.

The results of the current study demonstrate that GH's role in the pathogenesis of nephropathy is at least in part is mediated by TGF-β1. A proposed model for GH and TGF-β1 axis is glomerular podocytes is depicted in Fig 9. However, further studies are required to delineate the contribution of specific STATs in GH mediated TGF-β1 production. Based on our observations, we propose that GH induces TGF-β1 expression and it could be implicated as a causative factor in the development of renal hypertrophy, ECM accumulation, podocytopathy, and proteinuria during GH-induced kidney diseases. In summary, the present report establishes that GH induces TGF-β1 expression in podocytes and that some of the actions of GH on the podocytes are mediated through TGF-β1 in a both autocrine and paracrine manner. Our data provide a mechanistic link between GH and podocyte dysfunction in diseases like type I diabetes mellitus and acromegaly.

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Figure legends

Figure 1: GH treatment induces TGF-β/SMAD pathway in podocytes. (A) Human podocytes were treated with GH in a dose-dependent manner and cell lysates subjected to 10% SDS-PAGE and western blotting performed to assess TGF-β1 expression. (B) Densitometric analysis of TGF-β1 band normalized for actin expression. TGF-β1 levels were estimated in conditioned media from podocytes treated with GH in dose (C) and time (D) dependent manner. Error bars indicate mean ± SE; n=4; * $p<0.05$ and ** $p<0.003$. (E) Western blotting analysis for TGF-β1 and SMAD2/3 expression in podocytes exposed to GH (500ng/ml) for varying periods of time. (F) Densitometric analysis of TGF-β1 (normalized to actin) and pSMAD2 and 3 (normalized to total SMAD2/3). Error bars indicate mean ± SE; n=3-4.

Figure 2: Autocrine and paracrine activity of GH induced TGF-β1 in podocytes. (A) Conditioned media from GH (CM-GH) treated podocytes and podocyte naïve to GH (CM-Con) was collected as described in methods and added to the fresh batch of podocytes that are naïve to the GH treatment. Total cell extracts prepared from podocytes treated with CM for 30 min and assessed for activation of SMAD2&3. (B) SMAD4 nuclear localization in podocytes treated CM. (C) SMAD-GFP signal reporter assay was performed in HEK293T cells treated for 12h with GH, CM-GH, and rTGF-β1. (D) GFP fluorescence intensities were measured and plotted after normalizing with the mock test control. Error bars indicate mean ± SE; n=4; $p<0.005$ is applicable to all data set points.

Figure 3: SB treatment attenuated SMAD4 nuclear localization in GH treated podocytes. (A) Podocytes were treated with GH for 30min in the presence or absence of TGF-βR1 inhibitor, SB431542 (SB) and conditioned media were collected from all experimental setups and processed as indicated in methods and subjected to ELISA for measuring the levels of TGF-β1. Error bars indicate mean ± SE; n=4; *** indicates $p<0.001$, statistical differences were analyzed by Tukey's multiple comparison test. (B) Cell debris free-culture supernatants were collected after 30 min of GH treatment and concentrated using 20% TCA precipitation. A total of 20µg of concentrate was subjected to SDS-PAGE and immunoblotting and probed with the TGF-β1 antibody. Ponceau stained blotting membrane was provided to demonstrate the equal loading of protein. (C) Western blotting analysis for pSMAD2 and pSMAD3 in podocyte lysate treated with GH in the presence or absence of SB431542 (SB). (D) Immunofluorescence detection of SMAD4 in podocytes treated with or without GH in the presence of SB (E) Paracellular albumin

influx assay performed in podocytes treated with either GH or rTGF-β1 in the presence or absence of SB. BSA protein levels are estimated by BCA protein assay. Error bars indicate mean ± SE; n=4; *** indicates p<0.001, statistical differences were analyzed by Tukey's multiple comparison test.

Figure 4: GH elicits podocyte size and regulates expression of ECM components: Differentiated podocytes that were cultured on coverslips treated with or without GH/ TGF-β1 for 30 min and were fixed with 4% paraformaldehyde and images were taken under bright field (A) and alternatively stained with (B) TRITC labeled phalloidin and images were acquired using Trinocular microscopy at 40X magnification, scale bars indicate 20µm. (C) Mean cell area was quantified from each group using Image J (NIH) analysis. Error bars indicate mean ± SE; n=20; *** indicates p<0.001. (D) Western blotting analysis for the ECM molecules treated with CM-GH for indicated duration. (E) Densitometric analysis of each band was normalized to actin expression. Error bars indicate mean ± SE, n=3-4.

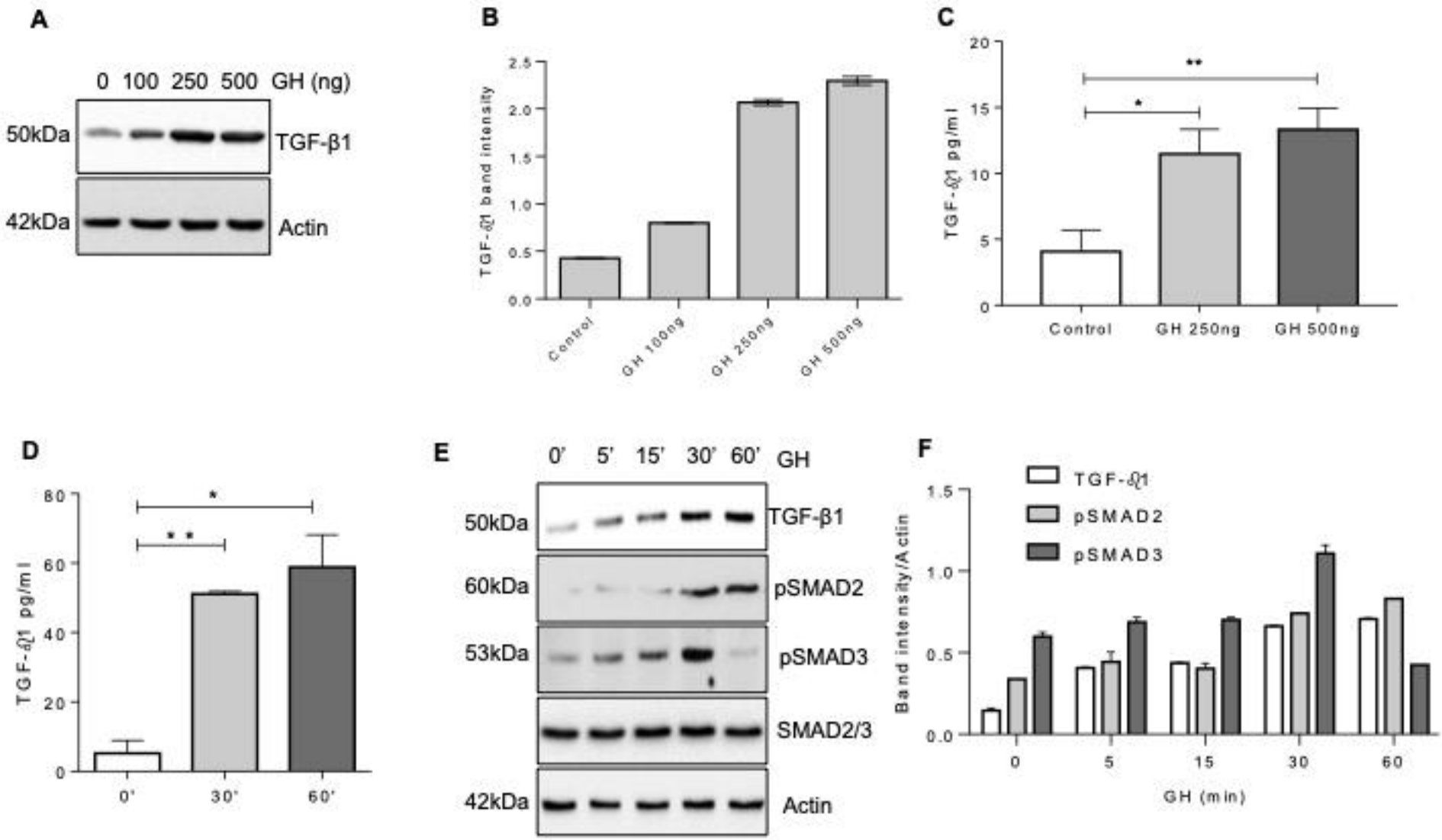
Figure 5: GH induces glomerular hypertrophy and impairs renal function: (A) Representative bright field images of isolated glomeruli from control and GH injected animals. (B) 20 images from control and GH injected mice were obtained and the area was quantified using Image J (NIH) analysis. Error bars indicate mean ± SE, and *** indicates p<0.001. (C) Representative image for renal cortical sections stained with PAS showing enlarged glomeruli with hypercellularity and mild fibrosis in GH treated group. (D) GFR was estimated in GH injected animals using Fit-GFR Inulin kit, BioPAL. Error bars indicates mean±SE; n=5-6; *** indicates p<0.001.

Figure 6: Histological analysis: (A) Kidneys were primarily fixed in 2.5% glutaraldehyde for overnight and the tissues were processed for TEM sectioning as detailed in methods. TEM imaging was carried out using a Tecnai G2 S Twin (200kV) system. (B) The thickness of GBM is quantified from TEM images using Image J (NIH) analysis and the values represent the area as the thickness of the GBM in arbitrary units. (C) Urinary albumin to creatinine ratio measured in control and GH injected animals. (D) Urine samples analyzed in SDS-PAGE gels using silver staining method and the unstained protein marker was loaded for reference (#Bio-Rad 11882144). Error bars indicate mean ± SE; n=4-6; *** p<0.005.

Figure 7: GH treatment induces the TGF-β/SMAD pathway *in vivo*: (A) Platelet-poor plasma preparations from GH injected and control animals were subjected to ELISA to measure TGF-β1 levels. (B) Western blotting analysis of TGF-β1 in the glomerular lysates from GH injected and control animals. (C) Densitometric analysis of TGF-β1 band normalized for respective actin expression. Error bars indicate mean ± SE,* $p<0.05$ and ***$p<0.001$, respectively compared with expression prior to exposure to GH. (D) Immunohistochemical analysis of TGF-β1 in paraffin-embedded sections obtained from GH and control animals and developed with DAB. (E) Western blotting analysis demonstrating SMADs activation in the glomerular lysates from control and GH injected animals.

Figure 8: SB431542 administration prevented renal injury: (A) Plasma preparations of GH injected and other experimental group animals were subjected to ELISA for measuring TGF-β1 levels. (B) Kidney cortical sections prepared from control, GH injected and GH+SB treated mice were subjected to PAS staining. (C) Mean glomerular area was calculated using Image J (NIH) analysis by measuring at least 10 glomeruli from each experimental group. (D) Western blotting analysis for activated SMADs in the glomerular preparations from mice treated with GH and other groups. (E) Urine samples collected from control and experimental groups subjected to direct urine analysis using silver staining method. 10µg of protein was loaded onto the SDS-PAGE. Statistical differences analyzed via non-parametric t-test, Error bars indicate mean ± SE,** $p<0.006$ (Fig A) and ***$p<0.001$ (Fig E).

Figure 9: Proposed model for GH induced TGF-β1 secretion: GH induces TGF-β1 secretion in podocytes and that TGF-β1 display both autocrine and paracrine effect on podocytes and elicits canonical TGF-β1/SMAD signaling pathway. SMAD signaling is implicated in the podocyte hypertrophy via increased actin polymerization. Alternatively, SMAD activation results in accumulation of ECM components. Together, these events manifest in renal hypertrophy and eventually culminate in decreased kidney function as evidenced by proteinuria.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0 100 250 500 GH (ng)
50kDa
TGF-β1
42kDa
Actin
B
TGF-β1 band intensity
2.5
2.0
1.5
1.0
0.5
0.0
Control
GH 100ng
GH 250ng
GH 500ng
C
TGF-β1 pg/ml
20
15
10
5
0
**
*
Control
GH 250ng
GH 500ng
D
TGF-β1 pg/ml
80
60
40
20
0
*
**
0'
30'
60'
E
0' 5' 15' 30' 60' GH
50kDa
TGF-β1
60kDa
pSMAD2
53kDa
pSMAD3
SMAD2/3
42kDa
Actin
F
TGF-β1
pSMAD2
pSMAD3
Band intensity/Actin
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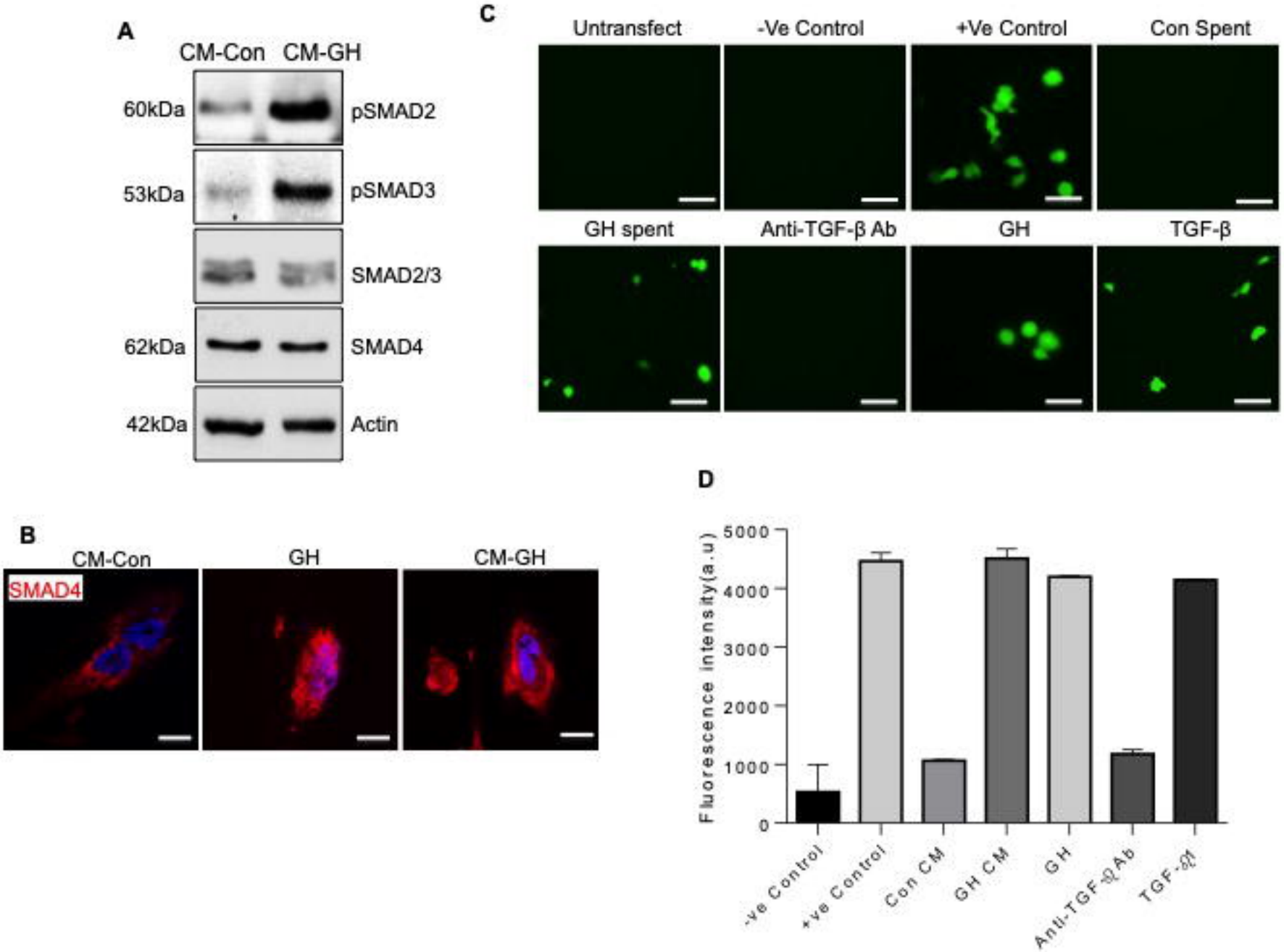
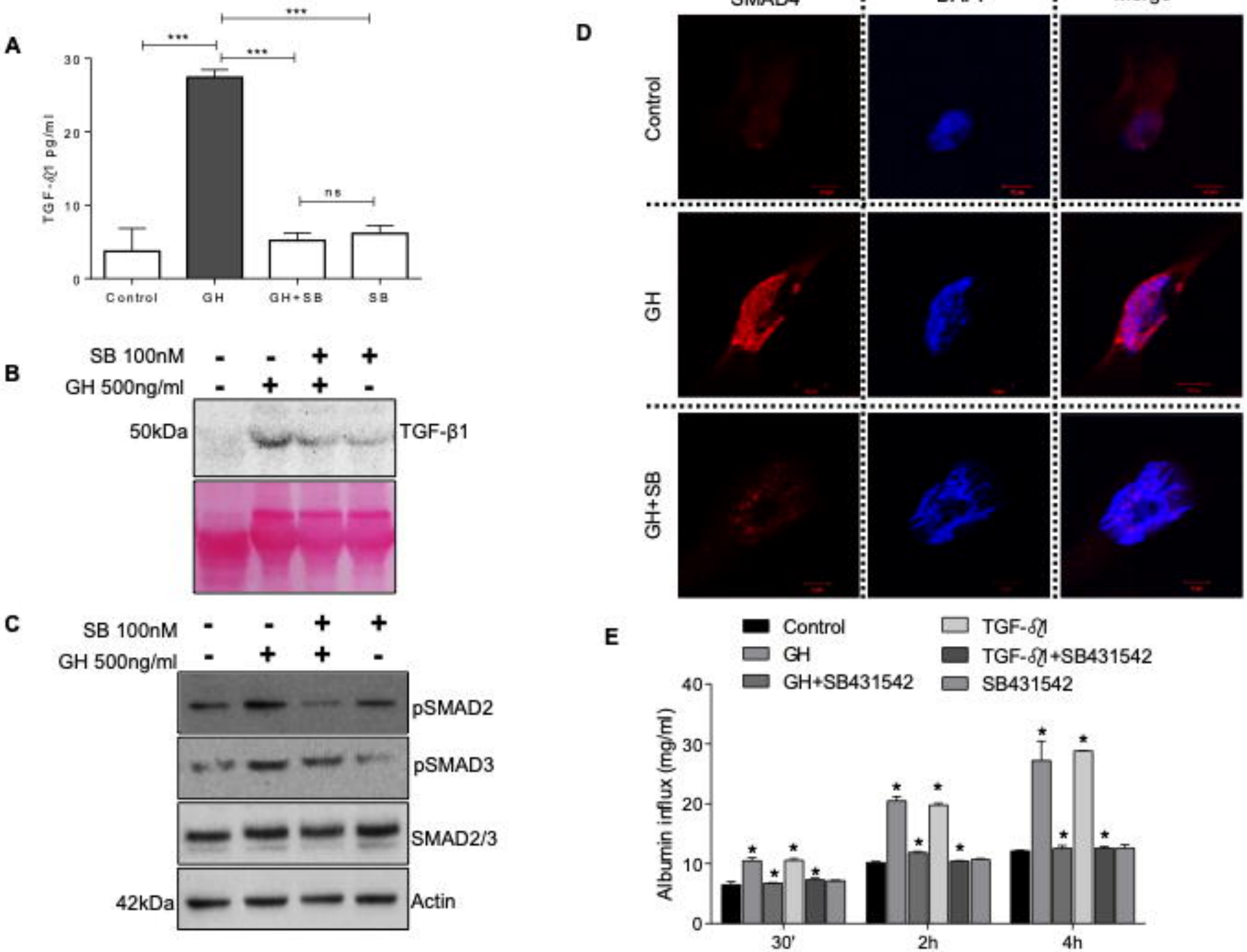
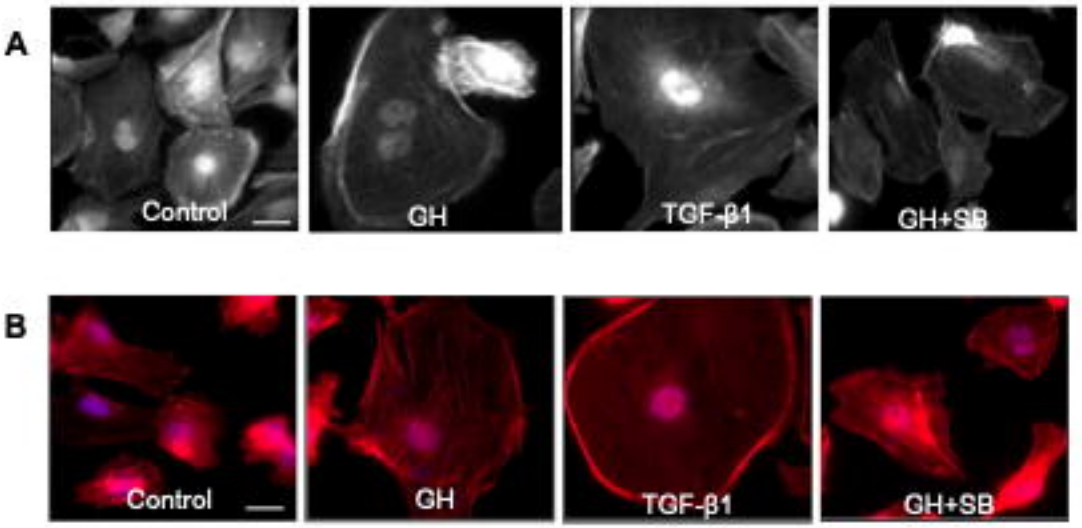
1.0
0.5
0.0
0
5
15
30
60
GH (min)


A
CM-Con
CM-GH
60kDa
pSMAD2
53kDa
pSMAD3
SMAD2/3
62kDa
SMAD4
42kDa
Actin
C
Untransfect
-Ve Control
+Ve Control
Con Spent
GH spent
Anti-TGF-β Ab
GH
TGF-β
B
CM-Con
GH
CM-GH
SMAD4
D
Fluorescence intensity(a.u)
5000
4000
3000
2000
1000
0
-ve Control
+ve Control
Con CM
GH CM
GH
Anti-TGF-β Ab
TGF-β1


A
TGF-β1 pg/ml
30
20
10
0
***
***
***
ns
Control
GH
GH+SB
SB
B
SB 100nM - - + +
GH 500ng/ml - + + -
50kDa
TGF-β1
C
SB 100nM - - + +
GH 500ng/ml - + + -
pSMAD2
pSMAD3
SMAD2/3
42kDa
Actin
D
SMAD4
DAPI
Merge
Control
GH
GH+SB
E
Control
GH
GH+SB431542
TGF-β1
TGF-β1+SB431542
SB431542
Albumin influx (mg/ml)
40
30
20
10
0
30'
2h
4h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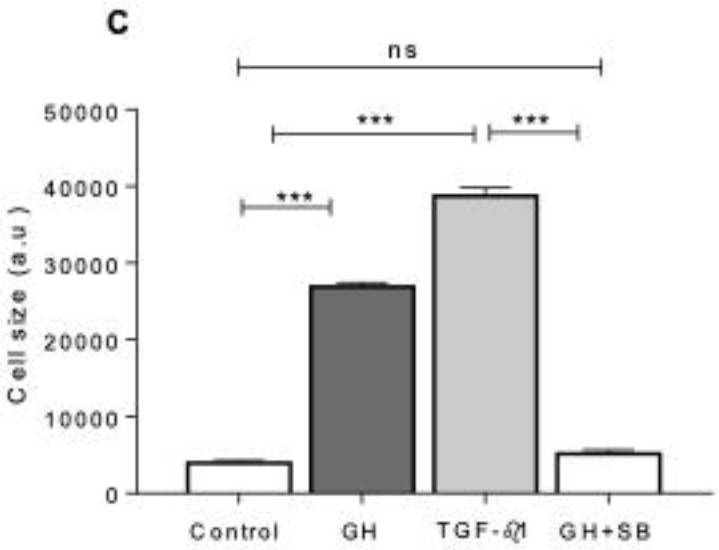
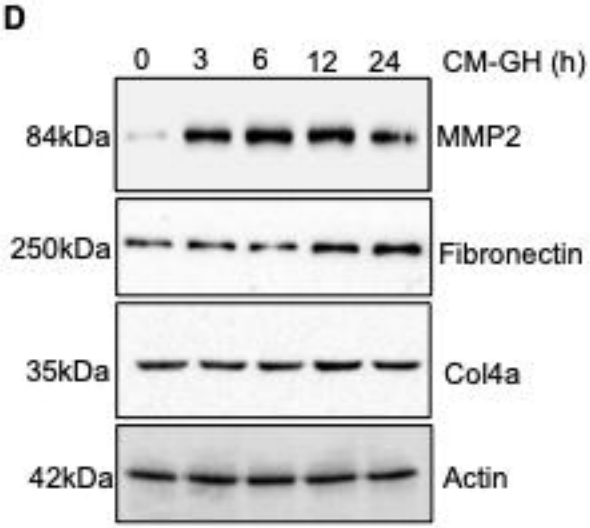
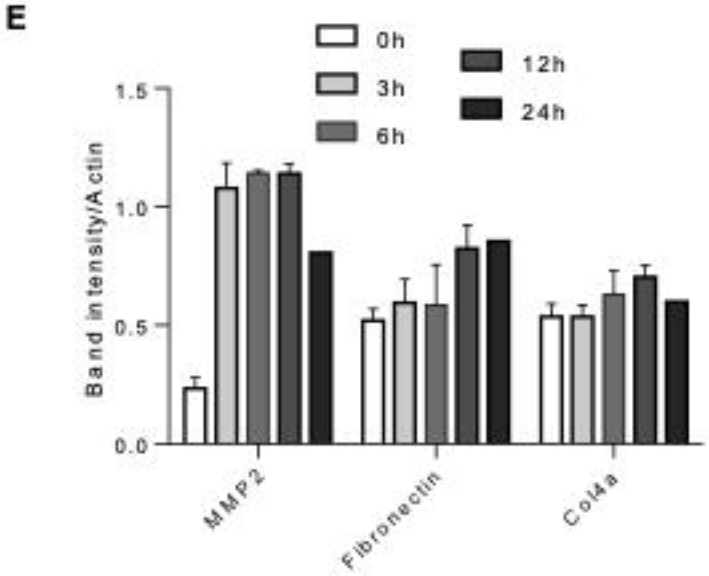
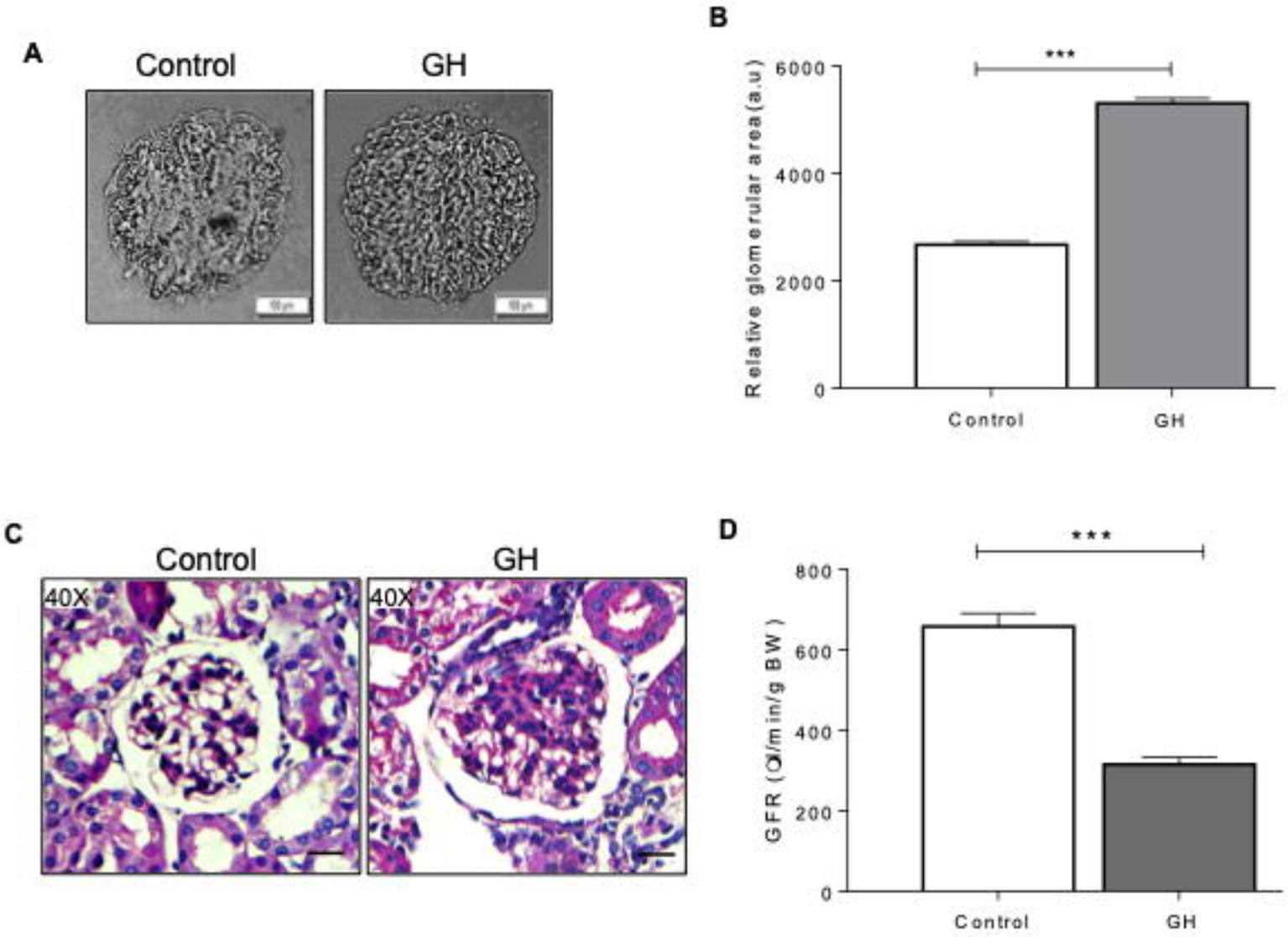
Control
GH
TGF-β1
GH+SB
B
Control
GH
TGF-β1
GH+SB

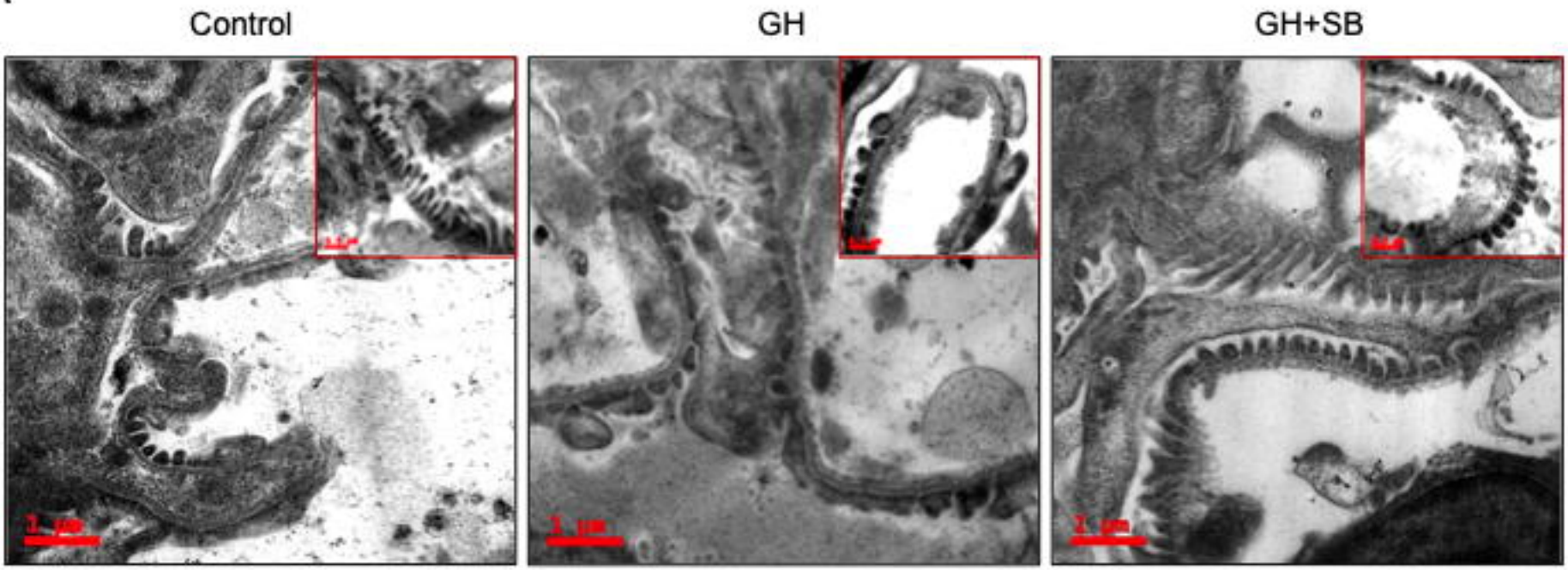

C
ns
***
***
***
Cell size (a.u)
50000
40000
30000
20000
10000
0
Control
GH
TGF-β1
GH+SB


D
0
3
6
12
24
CM-GH (h)
84kDa
MMP2
250kDa
Fibronectin
35kDa
Col4a
42kDa
Actin

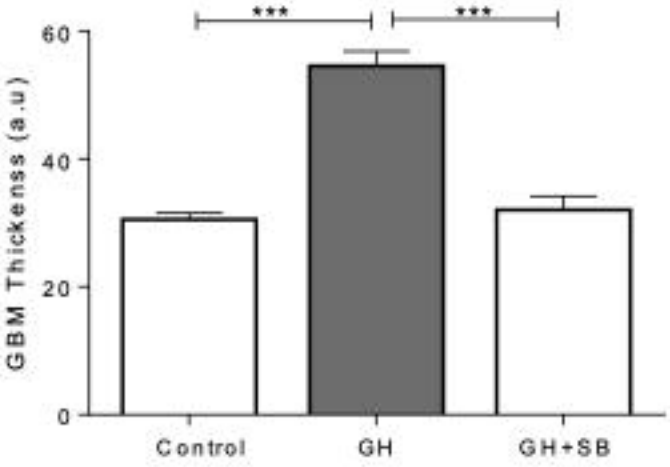

E
0h
3h
6h
12h
24h
Band intensity/Actin
1.5
1.0
0.5
0.0
MMP2
Fibronectin
Col4a


A
Control
GH
B
***
Relative glomerular area (a.u)
6000
4000
2000
0
Control
GH
C
Control
GH
40X
40X
D
***
GFR (Ø/min/g BW)
800
600
400
200
0
Control
GH

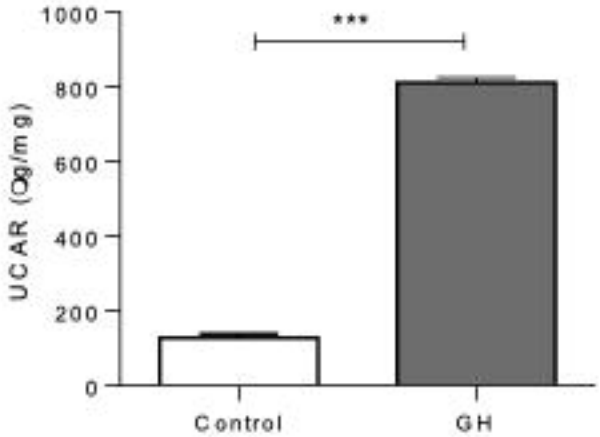
A


Control
GH
GH+SB

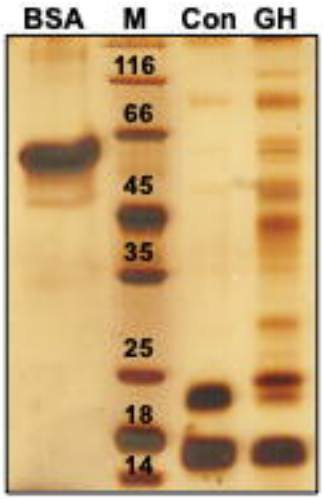
B

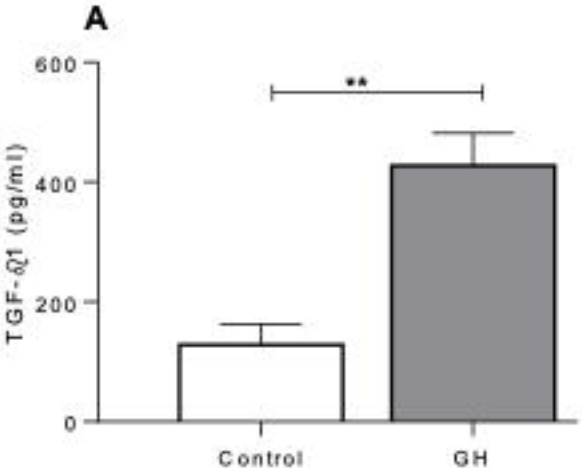

GBM Thickenss (a.u)
60
40
20
0
***
***
Control
GH
GH+SB

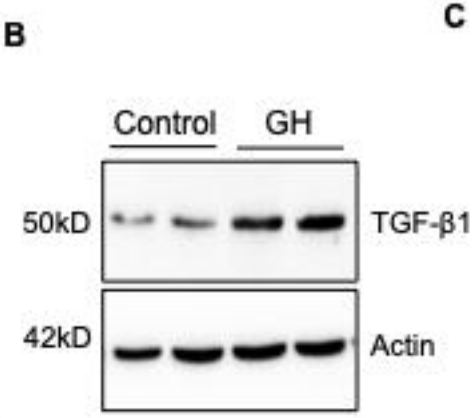
C

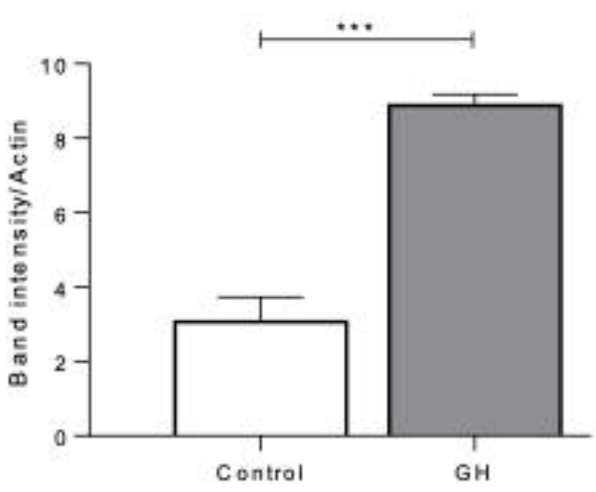

UCAR (Og/mg)
1000
800
600
400
200
0
***
Control
GH

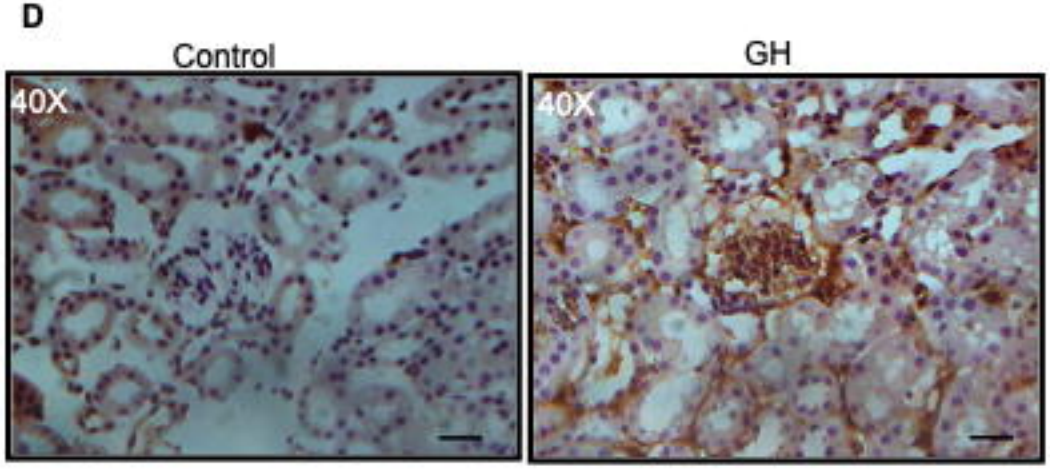
D

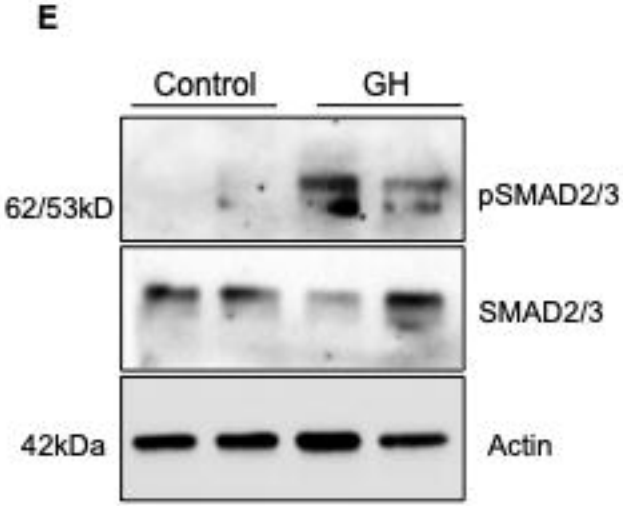

BSA
M
Con
GH
116
66
45
35
25
18
14


A
600
400
200
0
TGF-β1 (pg/ml)
**
Control
GH


B
Control
GH
50kD
TGF-β1
42kD
Actin


C
***
10
8
6
4
2
0
Band intensity/Actin
Control
GH


D
Control
GH
40X
40X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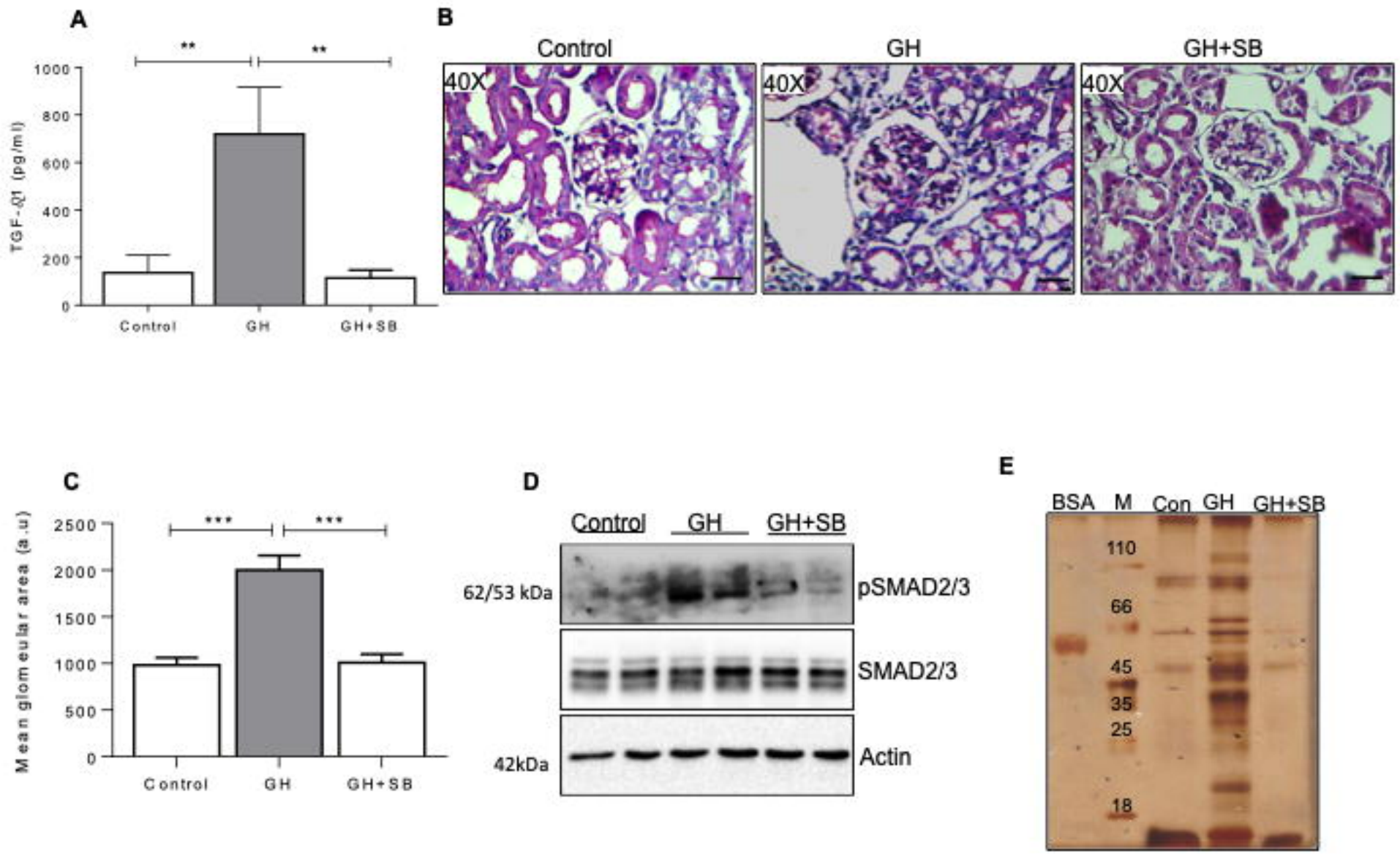
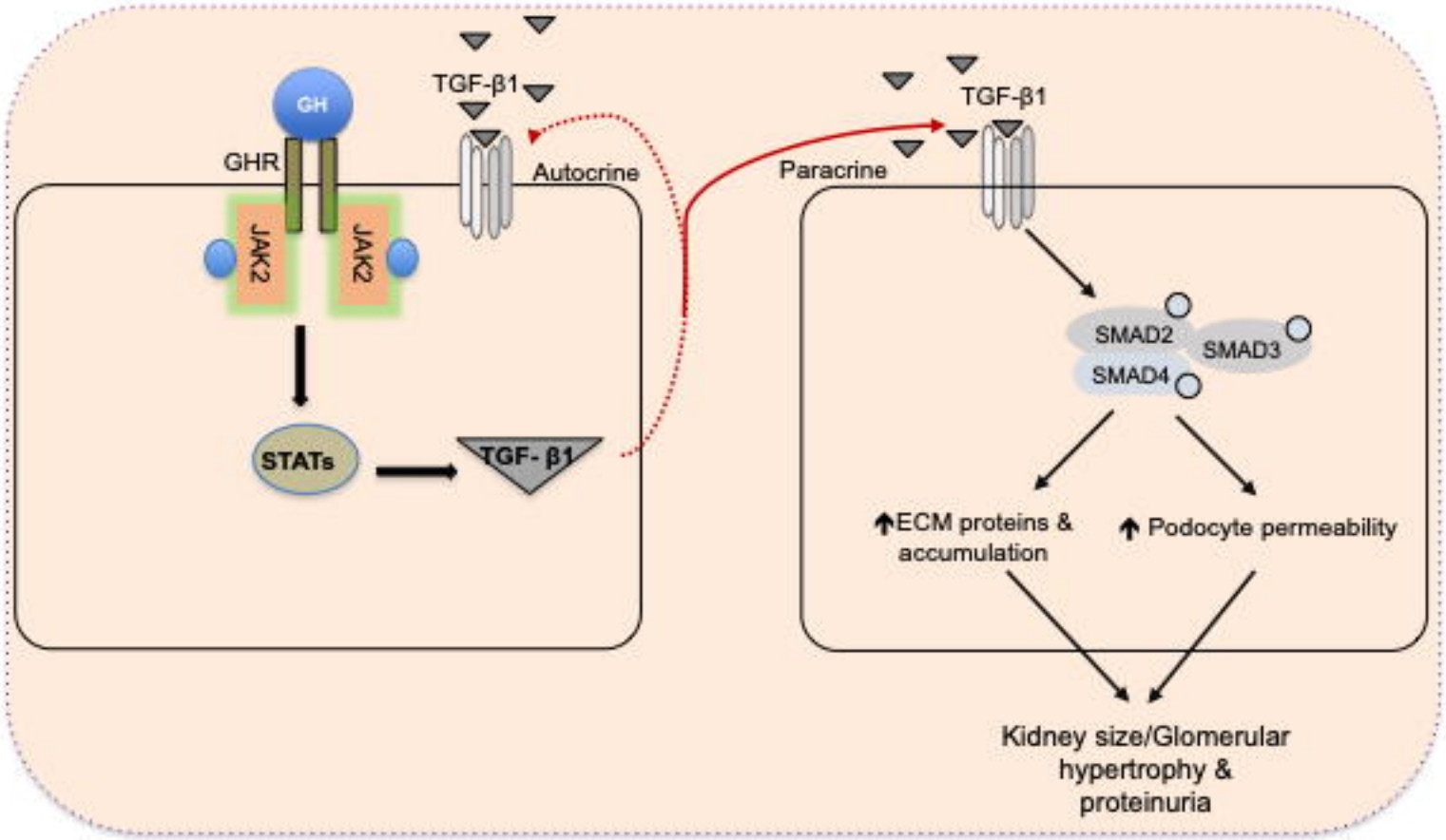
Control
GH
62/53kD
pSMAD2/3
SMAD2/3
42kDa
Actin


A
**
**
1000
800
600
400
200
0
TGF-β1 (pg/ml)
Control
GH
GH+SB
B
Control
GH
GH+SB
40X
40X
40X
C
***
***
2500
2000
1500
1000
500
0
Mean glomerular area (a.u)
Control
GH
GH+SB
D
Control
GH
GH+SB
62/53 kDa
pSMAD2/3
SMAD2/3
42kDa
Actin
E
BSA
M
Con
GH
GH+SB
110
66
45
35
25
18


GH
GHR
JAK2
JAK2
TGF-β1
Autocrine
STATs
TGF- β1
TGF-β1
Paracrine
SMAD2
SMAD3
SMAD4
↑ECM proteins & accumulation
↑ Podocyte permeability
Kidney size/Glomerular hypertrophy & proteinuria