ACTIVIN B IN DIABETIC KIDNEY DISEASE.

THE CONTRIBUTIONS OF ACTIVIN B SIGNALING TO DIABETIC KIDNEY DISEASE.

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Descriptive Note

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Lay Abstract

As the leading cause of end stage renal disease, diabetic kidney disease (DKD) is described as the reduction in renal function due to chronic exposure to diabetes. This thesis is aimed to understand the pathways and mechanisms that contribute to the development and progression of DKD to help identify novel therapeutic options. This project identified activin B (ActB) as a contributor to the disease and gives evidence that blocking the actions of ActB can prevent profibrotic effects in cells, similar to the profibrotic effects seen in DKD. Furthermore, this thesis demonstrates preliminary evidence for the beneficial effects of anti-ActB therapy, providing a potential alternative therapeutic option for DKD patients.

Abstract

DKD is the leading cause of kidney failure in Canada and its patients suffer the highest morbidity and mortality rates of any kidney failure patient group. Current interventions including strict glycemic control only delay DKD. Thus, there is a major need to identify new therapeutic targets. High glucose (HG) is identified as a major pathogenic factor, inducing the release of growth factors leading to kidney fibrosis. Although treatments have been developed to target these factors, their effectiveness is accompanied by adverse effects due to the lack of specificity. Recently, activins have been suggested to have a prominent role in promoting renal fibrosis and developing a specific anti-activin therapy can avoid potential side effects. Although there is evidence supporting an important role for activin A (ActA) in the induction of fibrosis in DKD, whether ActB also contributes is unknown.

In this study, we aim to determine the potential contribution of ActB to promoting fibrosis. Our results show that ActA and ActB are upregulated in rodent and human DKD. We show that hyperglycemia leads to the secretion of ActA and ActB by mesangial cells (MC), whereas only ActB is secreted by renal fibroblasts (RF). Similar to HG, treatment with ActA or ActB leads to Smad2/3 activation and upregulation of extracellular matrix proteins, whereas specific inhibition of either ActA or ActB attenuates these effects. We show that ActA and ActB regulate HG-induced activation of MRTF-A/SRF in MC, leading to an activated phenotype characterized by increased α-SMA expression and ECM production. Lastly, we confirm the specificity and functionality of the activin propeptides

in vitro, providing evidence for their effectiveness in vivo. This study will help further our knowledge of the role activins in DKD, potentially providing an alternative therapy.

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Table of Contents

List of Figures and Tables

List of Abbreviations

DKD	Diabetic kidney disease
ActB	Activin B
HG	High glucose
ActA	Activin A
MC	Mesangial cells
RF	Renal fibroblasts
RAAS	Renin-angiotensin-aldosterone system
SGLT-2	Sodium glucose linked transporter 2
GFR	Glomerular filtration rate
ESRD	End stage renal diseases
ECM	Extracellular matrix
GBM	Glomerular basement membrane
TGF	Transforming growth factor
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
AGE	Advanced glycation end-product
Ang II	Angiotensin II
CTGF	Connective tissue growth factor
VEGF	Vascular endothelial growth factor

BMP	Bone morphogenic protein
SLC	Small latent complex
LTBP	Latent TGF-β binding proteins
Smad	Small mothers against decapentaplegic
ERK	Extracellular signal-regulated kinase
MAPK	Mitogen-activated protein kinase
EMT	Epithelial-mesenchymal transition
sTβRII.Fc	Soluble human TβRII
ActAB	Activin AB
ActR-I	Activin type I receptor
ActR-II	Activin type II receptor
ALK	Activin receptor-like kinases
CKD	Chronic kidney disease
INHBB	Inhibin-βB
INHBA	Inhibin-βA
IRI	Ischemia-reperfusion injury
FST	Follistatin
GDF	Growth differentiation factor
IgG	Immunoglobin
ActC	Activin C
ActD	Activin D

ActE	Activin E
EGFR	Epidermal growth factor receptor
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
STZ	Streptozotocin
IP	Intraperitoneal
PMSF	Phenylmethylsulfonyl fluoride
PSB	Protein solubility buffer
ProA	Activin A propeptide
ProB	Activin B propeptide
α-SMA	α-smooth muscle actin
MRTF-A	Myocardin Related Transcription Factor-A
SRF	Serum response factor
FN	Fibronectin
Col-1	Collagen-1α1
CM	Conditioned media

Declaration of Academic Achievement

I, Mohammad Khajehei, declare all the work within this thesis to be my own. This work has not been submitted or published by any other institution. My supervisor, Dr. Joan Krepinsky as well as my supervisory committee, Dr. Kjetil Ask and Dr. Judith West-Mays supported and offered guidance to me for the duration of this thesis.

There are no conflicts of interest to declare.

Introduction

Diabetes

Characterized by the body's inability to produce or use insulin, diabetes affects over 200 million lives worldwide. Type 1 diabetes is considered an autoimmune disease in which the immune system attacks the pancreatic insulin-producing β-cells [1]. More common than type 1 diabetes, insulin resistance causes type 2 diabetes, in which the body is unable to respond to endogenously produced insulin. Ultimately, diabetes results in hyperglycemia, leading to many subsequent complications such as damage to the heart, eyes, kidneys, and nerves [2]. As of 2020, Diabetes Canada has estimated approximately 11.2 million people currently live with either type 1 diabetes, type 2 diagnosed or type 2 undiagnosed diabetes; this number is predicted to increase, reaching 13.5 million by 2030 [3]. In addition, diabetes places a significant economic burden on the Canadian health system, estimated to cost around $3.8 billion in 2020 and predicted to reach $4.9 billion by 2030 [3].

Current common treatment for diabetic patients with DKD is the use of renin-angiotensin-aldosterone system (RAAS) inhibitors. Insulin administration is also used as a treatment for both type-1 and 2 diabetic patients, whereas sodium glucose linked transporter-2 (SGLT-2) inhibitors have recently proven to have beneficial cardiovascular and kidney-related effects in type-2 diabetic patients [4]. Diabetes continues to be the leading cause of renal failure, retinopathy, and limb amputations in adults, particularly in

the elderly and those with other chronic complications such as obesity [5], contributing to 30% of all strokes, 40% of all heart attacks, as well as one of the leading causes for blindness in Canada [6].

Diabetic Kidney Disease

Also known as diabetic nephropathy, DKD is described as the reduction in renal function characterized by an increase in plasma creatinine, proteinuria, and decreased glomerular filtration rate (GFR) [7]. As the leading cause of kidney failure in Canada, DKD patients suffer the highest morbidity and mortality rates of any kidney failure patient group [8]. The consequences of DKD include reduced glomerular filtration rate (below 15 ml/min/1.73 m^2) and advancement to end stage renal disease (ESRD) as individuals in this stage ultimately require dialysis or kidney transplantation [9].

One of the major risk factors for DKD is hyperglycemia and it has been shown that strict glycemic regulation can help slow the progression of this chronic disease, but this is very difficult to achieve [5]. In addition, regulating blood pressure levels via and use of SGLT-2 inhibitors, angiotensin converting enzyme-inhibitors, or angiotensin II receptor blockers in patients with proteinuria is the standard of care for DKD [8]. However, current treatments fail to inhibit disease progression as many patients still reach ESRD despite glucose and blood pressure control. Thus, there is growing interest in novel treatments which target the molecular mechanisms of ESRD development such as mineralocorticoid and endothelin receptor antagonists [8]. With the rising prevalence of diabetes in the Western world, it is estimated that about 40% of diabetics will develop DKD, which

significantly increases the risk of advancing into ESRD [5]. Consequently, finding new therapeutic targets for the treatment of DKD is critical.

Kidney Anatomy

To fully understand the effects of diabetes on the kidney and its involvement in the development of kidney disease, it is helpful to understand the physiology and function of the kidney, in addition to its impact as the body's primary excretory organ.

Through maintaining homeostasis in the body, the kidneys perform a variety of functions vital for survival [10]. These include, but are not limited to, excreting waste in the form of urine, reabsorbing key nutrients such as glucose, water, and sodium, regulating blood pressure, producing and secreting hormonal and vasoactive chemicals including renin, calcitriol, and erythropoietin, and fine-tuning pH through acid-base homeostasis [10]. These functions can occur in the kidney due to the presence of approximately one million functional units known as nephrons, consisting of a renal corpuscle that is attached to the collecting duct and ureter via a tubular system [11]. The renal corpuscle is the subunit of the kidney responsible for filtration, consisting of a tuft of specialized capillaries composed of fenestrated endothelial cells known as the glomerulus which is enclosed by the Bowman’s capsule.

The glomerulus consists of a cluster of capillaries that are held together by the mesangium and the filtration component of the glomerulus consists of visceral epithelial cells known as podocytes and fenestrated endothelial cells which are responsible for charge

and size selective filtration of components from the blood, surrounded by a specialized extracellular matrix (ECM) structure known as the glomerular basement membrane (GBM) [11]. Furthermore, the mesangium is composed of MC, specialized perivascular pericytes involved in the production and secretion of the ECM, primarily composed of proteins such as collagens, laminin, and fibronectin [12]. The ECM anchors MCs to the GBM, providing structural support and integrity to the surrounding glomerular capillaries [12]. Lastly, the nephron's tubular section consists of proximal and distal tubules connected by a loop of Henle, responsible for further processing and excretion of urine through the collecting duct and ureter [11].

DKD Pathophysiology

DKD in humans usually develops over five stages which can take up to thirty years, presenting itself as morphological changes in the glomeruli during the initial phases of the disease [13]. The earliest changes in diabetic glomeruli include hyperfiltration, thickening of the GBM, expansion of the mesangial matrix, decrease in podocytes and effacement of foot processes, and glomerular hypertrophy [14] (Fig. 1). As DKD advances, damage spreads to the tubulointerstium, characterized by interstitial fibrosis and renal tubular atrophy [13]. In the late stages of the disease, these changes in kidney morphology manifest as decreased GFR, proteinuria, and hypertension [14]. The pathophysiology of DKD stems from complicated interactions between hemodynamic and metabolic factors that are activated by chronic hyperglycemia [14]. Many of these mechanisms overlap with the

activation of growth factors including transforming growth factor (TGF)-β1, eventually leading to a decline in kidney function [15].

Early hemodynamic changes such as hyperfiltration and increased perfusion lead to an increase in intraglomerular capillary pressure and manifest as damage to the glomerulus (GBM thickening and expansion of the mesangium) [15]. Hyperglycemia has been shown to induce the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the kidney, driving vascular dysfunction by activation of RAAS, further damaging the kidney [16]. Advanced glycation end-products (AGEs) produced as a result of glucose reacting with amino groups in proteins, nucleic acids, and lipids have been shown to induce pro-inflammatory and pro-fibrotic signaling in the kidney through binding to their corresponding receptors [15]. The RAAS system known for its regulation of hemodynamic factors also contribute to DKD pathogenesis, as angiotensin II (Ang II) has been shown to have direct pro-fibrotic and pro-inflammatory effects [15].

Lastly, MC secrete various growth factors and cytokines in response to stress (inflammation) or stretch factors (glomerular hypertension), including insulin-like growth factors, TGF-β, activins, connective tissue growth factor (CTGF), and vascular endothelial growth factor (VEGF) [17]. This enables crosstalk between MCs, endothelial cells, and podocytes, resulting in a coordinated response to stress in the glomerulus [15]. Due to their role in early glomerular alterations, MCs have been the primary focus of many labs including our own, as they show much potential as targets for novel DKD drug development.

The Role of TGF-β1 in DKD

The TGF-β superfamily are highly pleiotropic molecules including TGF-β1, activins, bone morphogenic proteins (BMPs), and growth differentiation factors [18]. From the three TGF-β isoforms expressed in mammals, TGF-β1 is considered the dominant isoform in the kidney [19]. They are responsible for controlling the expression of genes involved in key developmental and physiological functions including inflammation, fibrosis, cell apoptosis, and proliferation [20]. TGF-β1 is the most abundant cytokine produced by resident renal cells, shown to function as a pathologic regulator of renal fibrosis [22]. Synthesized in an inactive form, TGF-β1 contains a prodomain which is cleaved but remains associated with the mature domain to form the small latent complex (SLC) [21].

Furthermore, the SLC forms a large latent complex in the ECM through interaction with other latent TGF-β binding proteins (LTBP) [22]. Release of TGF-β1 from the LTBP by proteases and environmental cues leads to the activation of TGF-β1 signaling [22]. Canonically, active TGF-β1 binds to a constitutively active kinase (type II TGF-β1 receptor), leading to the recruitment of the type I TGF-β1 receptor and subsequent phosphorylation of downstream receptor-associated small mothers against decapentaplegic (Smad) proteins, Smad2 and Smad3 [22]. Furthermore, phosphorylated Smad2 and Smad3 bind to and form an oligomeric complex with Smad4, translocating into the nucleus where the complex interacts with numerous co-activators and/or repressors to modulate the transcription of target Smad-responsive genes [22] (Fig. 2).

TGF-β1 is also able to signal through Smad-independent pathways (non-canonical), leading to the activation of p38, extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK), Jun Kinase, Rho-GTPases and integrin linked kinases, among others [23]. TGF-β1's function in the development of renal fibrosis is well established and hypothesized to occur through three processes including the upregulation of ECM proteins, upregulation of matrix metalloprotease inhibitors leading to decreased matrix degradation and increased epithelial-mesenchymal transition (EMT) leading to a pro-fibrotic phenotype [24]. Inhibition of TGF-β1 via neutralizing antibodies, antisense TGF-β1 oligodeoxynucleotides, soluble human TβRII (sTβRII.Fc) and specific inhibitors to TβR kinases (GW788388 and IN-1130) effectively inhibits the progression of renal fibrosis in a variety of experimental kidney disease models [22].

Nonetheless, the use of monoclonal TGF-β1 neutralization antibodies in clinical studies investigating DKD and steroid-resistant focal segmental glomerulosclerosis has not been as effective as predicted [25 & 26], most likely due to its role as an anti-inflammatory and anti-tumorigenesis cytokine as well as the pleiotropic effects of TGF-β1 signalling in various tissues [18 & 26]. As a result, research focus has been shifted towards indirectly targeting TGF-β1 via alternative members of the TGF-β family such as activins, as their inhibition may prove to be a more feasible and efficacious treatment option for DKD progression.

The Role of Activins in DKD

Activins are dimeric proteins and members of the TGF-β superfamily, consisting of two inhibin β subunits connected by disulfide bonds [27]. Different activin isoforms include homodimeric ActA ($\beta_A – \beta_A$), ActB ($\beta_B – \beta_B$), heteromeric activin AB (ActAB) ($\beta_A – \beta_B$), as well as activins C, D, and E [27]. These glycoproteins have been shown to regulate growth and differentiation in many biological systems, including secretion of several anterior pituitary hormones, erythropoiesis, neural cell survival, and early embryonic development [28]. Heteromeric receptor complexes mediate activin's biological activity, consisting of type I (ActR-I) and type II receptors (ActR-II) characterized by an intracellular serine/threonine kinase domain [20].

Activin first binds to the ActR-II, which exists in the cell membrane as an oligomeric form with activated kinase, recruiting and forming a complex with ActR-I, including activin receptor-like kinases (ALK) 4, ALK5, and ALK7 (ActA binds to ALK4, whereas activin B acts via ALK4 and ALK7) [29]. Furthermore, ActR-II activates ActR-I by phosphorylating the GS domain, leading to downstream activation of Smads resulting in the transcriptional activation of Smad responsive genes, similar to TGF-β1 [28] (Fig. 2). More recently, activins have been studied as autocrine and paracrine factors that are prominent contributors to the profibrotic and inflammatory responses within the kidneys, liver, and lungs in response to injury [20].

ActA has been the most studied isoform due to its abundance and potential role in various renal diseases and associated complications. Kidney development is partly

regulated via ActA as its expression can mostly be detected during fetal kidney development [30]. However, increased ActA in circulation has been observed in human DKD where it was correlated with markers of kidney injury and reduced kidney function [31]. ActA has been shown to activate renal interstitial fibroblasts by promoting proliferation, differentiation, and matrix protein production, acting as a paracrine factor [32]. Most importantly, expression and secretion of ActA is induced via TGF-β1 [32], a widely accepted key mediator of fibrosis in DKD, where TGF-β1-induced profibrotic effects were reduced by follistatin treatment or overexpression of truncated type II activin receptor in renal fibroblasts [32]. In addition, ActA was shown to have a critical role in the HG profibrotic response in MCs and tubular cells [33]. Lastly, data from animal chronic kidney disease (CKD) models, as well as human kidney tissues have shown upregulation of ActA expression which were associated with an increase in either circulating or renal ActA [34 & 31]. These data suggest a prominent role for ActA in promoting HG-induced profibrotic responses in the kidney.

Studies have shown that circulating levels of ActA and ActB are often co-elevated in disease [36 & 37]. While much focus has been on ActA, a recent study by Sun et al. [35] detected the upregulation of INHBB in three various models of kidney fibrosis including DKD, in addition to human kidneys with fibrosis, localized to the proximal tubular epithelial cell region of tubules. It was hypothesized that proximal tubular cell derived INHBB acts as a paracrine factor to induce local fibroblast activation, leading to promoting fibrosis in the tubulointerstitial region. ActB signalling has also been shown to potentially

play an important role in the conversion of normal bile duct fibroblasts to scar fibroblasts when investigating benign bile duct scar formation [38]. In a murine model of ischemia-reperfusion injury (IRI), a difference between the kinetics of ActA and ActB in response to renal injury was demonstrated and it was hypothesized that ActB initiates and ActA potentiates renal injury after IRI respectively [39].

De Kretser et al. demonstrated in a large cohort of acute respiratory failure patients that ActA and ActB levels are often elevated and predictive of the risk of death [36]. In another study investigating idiopathic pulmonary fibrosis, ActB was highly expressed in the human and mouse fibrotic lung [40]. ActB has also been shown to promote hepatic fibrogenesis and a potential circulating biomarker and potent promotor of liver fibrosis [24]. LPS-induced inflammation was shown to increase ActB in mouse liver tissues, leading to the induction of CTGF, a principal inducer of liver fibrogenesis [41]. These data suggest that similar to ActA, ActB also contributes to the profibrotic phenotype observed in DKD, since the two structurally related proteins share 63% identity and 87% similarity [43], as well as multiple common activator protein-1 (AP-1) sites within the promoters of inhibin βA and inhibin βB, a known transcription factor upregulated in DKD [24].

Activin Targeted Therapies

Follistatin (FST) is a ubiquitously expressed glycoprotein which binds to TGF-β superfamily members, inhibiting their profibrotic and proinflammatory actions via internalization and degradation [42]. Although most potent towards ActA, FST also neutralizes several other TGF-β family members with lower affinity, including myostatin

(GDF8), GDF9, ActB, ActC, TGF-β3 and BMPs 2, 4, 6, and 7 [43 & 27]. Although FST does not neutralize TGF-β1, it has been shown to inhibit the TGF-β1-induced profibrotic response via activin inhibition, in addition to protection against both basal and glucose-induced matrix production. Alternate splicing of the *fst* gene results in the synthesis of two major isoforms FST-288 and FST-315, which function to neutralize local cell-surface bound and circulating TGF-β family members, respectively [43 & 27].

The binding of two FST molecules to one activin dimer sterically hinders the receptor binding sites, leading to internalization and degradation of activins via the lysosomal pathway [43 & 27]. In the type 1 diabetic Akita mouse model, administration of exogenous FST has shown therapeutic potential in protecting against the progression of early DKD, characterized by glomerular hyper-filtration, albuminuria, and glomerulosclerosis [33]. Although promising, recent data illustrate that a higher dose of FST (10μg vs 5μg) does not lead to improved outcomes in kidney function and renal fibrosis in a CKD murine model [44], which suggests a narrow therapeutic window for FST.

Alternatively, ActRII ligand traps are fusion proteins of extracellular domain of ActRIIA or ActRIIB and the Fc portion of IgG1. They have been shown to inhibit renal fibrosis in CKD mice [34], in addition to increasing bone mass and preventing cancer-induced bone destruction in models of myeloma and breast cancer [45]. Unlike FST, ligand traps are more potent towards other TGF-β family members, such as GDF11 and several BMPs [46], which have been associated with adverse effects such as an increase in

hematocrit and the development of hypertension in early phase trials, as well as mucocutaneous bleeding [46 & 47].

Neutralizing antibodies targeting either the type II receptors or activins themselves have also been used to inhibit activin signaling. Receptor targeting antibodies have been developed to promote skeletal muscle growth through their inhibition of myostatin [48], whereas the ActA neutralizing antibody REGN2477 has been tested in patients with specific bone and muscle disorders [49 & 50]. However, neither method of inhibition has been assessed in renal models.

Interventions that target one or a small subset of ActRIIA/IIB ligands are becoming more commonly recognised as the most effective way of achieving a desired outcome with minimum risk of incurring significant off-target effects [51]. TGF-β family member propeptides are important mediators of growth factor synthesis and function, aiding in the folding, disulfide bond formation, and export of their mature dimers. Activins are synthesized as large precursor molecules with the N-terminal prodomain mediating the folding of the C-terminal mature domain [51 & 52]. After cleavage and secretion, the prodomain remains noncovalently associated, stabilizing the activin dimer and increasing its half-life extracellularly, although not affecting its biological activity.

In contrast, TGF-β1, myostatin and GDF-11 bind to their prodomains with high affinity and are secreted from the cell in a latent form [51 & 52]. Several studies have demonstrated that myostatin and TGF-β1 are naturally inhibited by their own propeptides *in vivo* [53 & 54]. Furthermore, Chen et al. have managed to specifically inhibit activins

using their prodomains through mimicking the adaptations that confer latency to TGF-β1 and myostatin [51]. This was achieved by covalently linking the prodomain chains via fusion proteins from the Fc regions of mouse IgG2A, in addition to substituting "fastener" residues from the myostatin prodomain, leading to the stable dimerization of the activin prodomains. The modified activin prodomains, also referred to as propeptides, have been shown to potently and specifically inhibit ActA signaling, demonstrated by the reversal of activin-induced wasting of skeletal muscle, heart, liver, and kidneys in a mouse model of activin-induced cachexia [55].

Study Objectives

As outlined above, evidence supports an important role for ActA in the induction of fibrosis in DKD, but whether other activins contribute is unknown. With recent data suggesting a role for ActB, we suggest that other activins may potentially play a role in DKD progression. Since ActD has not been detected in mammals [56] and ActC & ActE are characterized as hepatokines [57], **I hypothesize that under hyperglycemic conditions, similar to ActA, secretion of ActB from resident kidney cells induces profibrotic signaling and ECM upregulation, contributing to DKD progression.**

Aim 1 – *Determine the contribution of activins to HG-induced profibrotic effects in MC and RF.*

1A. *Determine the relative potency of ActA & ActB in Smad3 activation and ECM upregulation.*

1B. *Determine if HG treatment leads to ActA & ActB upregulation in MC and RF.*

1C. *Determine if ActB inhibition attenuates HG-induced activation of Smad3 and upregulation of ECM proteins in MC.*

1D. *Determine if ActA and/or ActB regulate HG-induced activation of non-canonical MRTF-A and SRF signalling pathway in MC.*

1E. *Determine if activins regulate HG-induced activation of the epidermal growth factor receptor (EGFR), a mediator of profibrotic signalling.*

Aim 2 – *Determine the effects of ActB stimulation in RFs.*

2A. *Determine if ActA or ActB inhibition attenuates HG-induced activation of Smad3 and upregulation of ECM proteins in RF.*

Aim 3 – *Confirm activin propeptide functionality and specificity in MCs for future use in vivo.*

3A. *Confirm specificity towards ActA vs. ActB.*

3B. *Confirm if activin inhibition leads to attenuation of HG-induced activation of Smad3 and upregulation of ECM proteins.*

3C. *Purify activin propeptide for further use in vivo.*

Materials and Methods

Cell Culture

Primary MC extracted from the glomeruli of C57BL/6J mice were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), streptomycin (100 µg/ml) and penicillin (100 µg/ml) at 37˚C in 95% O2, 5% CO2. Rat primary RFs (Catalog No. R2071, Cell Biologics) were cultured in 1:1 DMEM/F12 media supplemented with 10% FBS, streptomycin (100 µg/ml) and penicillin (100 µg/ml) at 37˚C in 95% O2, 5% CO2. Cells from passages 10 to 13 were used for transfection experiments, whereas passages 10 to 17 were used for all other experiments. MCs and RFs were serum deprived in 1% bovine serum albumin, streptomycin (100µg/mL), and penicillin (100µg/mL) for 24 hrs following transfections and prior to any experimental drug treatments. Reagents used for treatments included glucose (30 mM), TGF-β1 (varying concentrations, R&D Systems), recombinant mouse ActA, ActB, and ActAB (varying concentrations, R&D Systems), monoclonal mouse ActA, ActB neutralizing antibody (MAB3381, MAB659, R&D Systems), and recombinant mouse FST-288 (769-FS, R&D Systems).

Animal Models

Animal studies were carried out in accordance with the principles of laboratory animal care and McMaster University (Hamilton, ON, Canada) and Canadian Council on Animal Care guidelines. Male type 1 diabetic C57BL/6-Ins2Akita/J mice or corresponding

WT mice (Jackson Laboratory, Bar Harbor, ME, USA) at 7-8 weeks of age were housed under standard conditions with free access to regular chow and water and sacrificed after 40 weeks, as previously described in the original studies (ethics approval number 18-07-30). Male CD1 mice (Charles River), 9 weeks of age, underwent a left nephrectomy. At 10 weeks of age, diabetes was induced by a single intraperitoneal (IP) injection of streptozotocin (STZ) at 200 mg/kg whereas control mice were injected with an equal volume of citrate buffer. Mice were sacrificed after 12 weeks of diabetes as previously described in the original studies (ethics approval number 14-11-48) [76]. Kidney tissues were harvested and kept in liquid nitrogen. For human studies, kidney biopsy samples with a diagnosis of DKD were obtained. Normal kidney tissues surrounding resected renal cancers were used as controls. Tissue was obtained after approval by the local research ethics board (ethics approval number 2010-159).

Protein Extraction and Western Blotting

Cells were lysed with cell lysis buffer containing protease and phosphatase inhibitors (10 µg/ml phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml leupeptin, 2 µg/ml aprotnin in lysis buffer). Cellular debris was separated from cell lysate by centrifugation at 14,000 rpm for 10 minutes at 4°C. Normalized concentrations of proteins were obtained from the supernatant of the sample and supplementary lysis buffer and protein solubility buffer (PSB) were added to each sample. MC cytoplasmic/nucleus protein extraction were carried out using hypotonic lysis buffer containing 20mM HEPES (pH 7.6), 20% glycerol, 10mM NaCl, 1.5mM MgCl2, 0.2 mM EDTA, 0.1% NP40, 2mM DTT, 1mM sodium

vanadate, 1mM PMSF, 1 μg/ml leupeptin, and 2 μg/ml aprotinin. Cell lysates were centrifuged (500rpm, 10min, 4oC), the pellet containing nucleus was suspended in buffer and sonicated, and supernatant (cytoplasmic extract) was collected, protein concentration normalized, and boiled in SDS-PAGE sample loading buffer. Proteins were separated using SDS-PAGE and transferred onto nitrocellulose membranes. Nonspecific binding was blocked with 5% skim milk in TBST at room temperature for one hour. The blots were exposed to Hyperfilm ECL reagent and densitometric analysis was carried out using ImageJ.

ELISA (Enzyme Linked Immunosorbent Assay)

Conditioned media from MCs and RFs were collected after treatment with HG for 48 hours. The amount of ActA and ActB in the media was determined through the activin A (R&D Systems, DAC00B) and activin B (MyBioSource, MBS088878) ELISA kits.

Immunohistochemistry

Immunohistochemistry was conducted on 4 μm paraffin-embedded kidney sections which were deparaffinized and probed for ActB using the activin B beta B subunit antibody (R&D Systems, 1:50, antigen retrieval using proteinase K, 40 μg/mL, 10 min, 37˚C). Images were taken at 20x and 40x magnification. Quantification was completed using Image J software.

RNA Extraction and qPCR

Total RNA was extracted using TRIzol (Life Technologies, Carlsbad, CA), reverse transcription was performed, and cDNA was analyzed by real-time polymerase chain reaction using a SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA). Amplification using primers for ActA and ActB, with 18S as internal control was measured using a ViiA 7 Sequence Detector (Life Technologies). The relative change in gene expression was determined using Ct values from 18S. ΔCt values were calculated based on the results obtained and normalized to the average of the control samples (ΔΔCt), in which then a linear transformation using the formula, $2^{-\Delta\Delta Ct}$ was followed. mRNA levels were measured using specific primer sets listed in Table 1.

Transient Transfections

For luciferase experiments, MCs were plated in 12-well plates in triplicates at 60%-70% confluence and transfected with 0.5 µg of SMAD3-responsive luciferase construct (pGL3-CAGA12-luc) along with 0.05 µg pCMV β-galactosidase (Clonetech) using Effectene (Qiagen) (pGal3-luc). After treatment, MCs were lysed with 1X Reporter Lysis Buffer (Promega, Madison, WI) and kept in -80°C overnight. Cells were thawed on ice the following day and luciferase activity was measured on clarified cell lysate using the Luciferase Assay System (Promega) with a luminometer (Junior LB 9509, Berthold). β-galactosidase activity, used to normalize for transfection efficiency, was measured in clarified cell lysates using the β-Galactosidase Enzyme Assay System (Promega) with a

plate reader absorbance set at 420nm (SpectraMax Plus 384 Microplate Reader, Molecular Devices).

siRNA experiments were completed according to protocol. MCs were plated on a 6-well plate at 50-60% confluence and transfected with ALK7 or MRTF-A siRNA. Media was changed after 18 hours to allow the cells to recover, and cells were serum deprived in 1% bovine serum albumin overnight prior to treatments. To confirm siRNA knockdown, q-PCR was performed to measure ALK7 or MRTF-A mRNA.

Production of Activin A (ProA) and B (ProB) Propeptides

The propeptides were produced by transient transfection of plasmid DNA obtained from Chen et al. (MIMR-PHI Institute of Medical Research, Clayton, Australia) [51] into HEK-293T cells using calcium phosphate precipitate method. 12 hours post-transfection, media was changed to allow cells to recover. After 48 hours, conditioned medium (CM) was collected from transient transfected HEK293T cells expressing either ProA, ProB, or empty vector (EV). For purification, HEK293T cells transfected with either ProA or ProB were scaled up in Excell293 media with 2 mg/l puromycin, 6 g/l Glucose, 2 mmol/l Glutamax-I (Life Technologies), and 0.2 mmol/l Butyric acid (Sigma Aldrich) for 72 hours. To purify the recombinant protein, conditioned media was first centrifuged at 3000 rpm at 4 °C for 10 minutes and the supernatant clarified using a 0.45 μm filter (PALL Life Sciences). The supernatant was loaded onto a chromatography column (Bio-Rad) with protein G Sepharose 4 Fast Flow resin (Cytiva) and eluted using 50mM citrate (pH 2.5) and neutralized with Tris buffer (pH 8.5). The absorbance of recoveries and yields of the

activin propeptides were measured by Nanodrop spectrophotometer 1,000 (Thermo Fisher Scientific) at a wavelength of 280 nm.

Statistical Analysis

Student's t-test or one-way ANOVA were used to compare the means between two or more groups of data. Significant differences between multiple groups (post hoc) were analyzed using Tukey's HSD with $p \leq 0.05$. Data is presented as mean ± SEM.

Results

ActA and ActB are Upregulated in Rodent and Human DKD

In a previous study [33], the increase of ActA expression was confirmed in different models of DKD, in addition to kidney tissues from patients with tissue diagnosis of DKD. Similarly, immunohistochemical analysis confirmed the significant increase of ActB in DKD mice models (Akita and STZ-CD1) and patients (Fig. 3A). In addition, INHBA and INHBB mRNA was significantly increased in both DKD models (Fig. 3B & 3C), further supporting the increase of ActA and ActB in the disease. Lastly, data analysis from the Nephroseq database (www.nephroseq.org) revealed the significant increase of INHBA and INHBB expression in two models of DKD, in addition to the significant increase of INHBA expression in the tubulointerstitium of DKD patients (Fig. 3D & 3E). These results suggest that renal ActA and ActB are induced in DKD.

HG-Induced Activin Signaling Occurs via Smad2/3 Pathway Rather than Smad1/5 in MC

Canonically, activins are known to signal via activin receptors ALK4 or ALK7 to activate the Smad2/3 pathway (Fig. 4A & S1A). However, a recent study has suggested that activins can also signal via the BMP receptor ALK2, causing activation of the Smad1/5 pathway [59]. In this study, to determine if HG-induced activins can also activate the Smad1/5 pathway, we first confirmed the increase in Smad1/5 activation after 24h of HG treatment (Fig. 4B). Furthermore, unlike treatment with the BMP inhibitor gremlin1 (500 ng/ml), treatment with SB431542 (5uM), the inhibitor of ALK5, ALK4 and ALK7, or follistatin did not attenuate HG-induced Smad1/5 activation, measured by pSmad1/5 expression and BRE-luc promoter activity, suggesting that activins do not contribute to HG-induced Smad1/5 activation in MC (Fig. 4C & 4D).

HG Increases ActA & ActB and ActB Regulates ECM Production in MC

Earlier studies suggest that one of the reasons for the induction of ActA and ActB in DKD is due to hyperglycemia and the downstream release of growth factors such as TGF-β1. To investigate this, we treated MC with HG or TGF-β1 which revealed increased INHBA and INHBB mRNA in treated cells measured via qRT-PCR (Fig 5A & 5B). In addition, cell culture supernatant from MC treated with HG showed increased ActA and ActB secretion measured via ELISA (Fig. 5C). These results confirm the increased production and secretion of ActA and ActB by MC in response to HG.

Our lab's previous data has shown that the inhibition of ActA via neutralizing antibody and ALK4 siRNA inhibition resulted in the attenuation of HG-induced upregulation of pSmad3 and ECM protein expression [33]. Here, we show that inhibition of ActB signaling via neutralizing antibody or ALK7 siRNA has similar effects, in addition to significant attenuation of HG-induced CAGA12 promoter activity, suggesting that similar to ActA, ActB also contributes to the HG response in MC (Fig. 5D-5F).

ActA and ActB Regulate HG induced MRTF-A/SRF Activation in MC

Myofibroblast differentiation caused by TGF-β1 is thought to be a prominent component of fibrosis [62]. Mechanical stress and TGF-β1 are shown to activate Myocardin related transcription factor-A (MRTF-A), resulting in myofibroblast-like cells which produce excessive ECM, characterized by increased α-smooth muscle actin (α-SMA) expression. Although it's generally assumed that myofibroblasts are derived from resident fibroblasts [63], in various types of glomerular injury, the MC may acquire characteristics of a myofibroblast. These "activated" MCs are shown to be proliferating and have acquired fibroblast-like properties, characterized by the production and secretion of interstitial collagens in addition to normal mesangial matrix constituents [64]. As one of the key mediators of α-SMA expression, MRTF-A binds and activates serum response factor (SRF), leading to the transcriptional regulation of genes involved in contractile functions through binding to CArG boxes present in the promoters [65]. Recent data has shown long-term (> 1 week) glucose-induced activation of the Rho-kinase signalling pathway results in an increased F/G-actin ratio and subsequent activation of SRF and MRTF-A [66]. In

addition, MRTF-A deficiency was shown to attenuate renal fibrosis in a murine model of DKD [67].

Upregulation of MRTF-A/SRF signaling due to external stimuli such as HG and TGF-β1 have been shown in previous studies. In our study, inhibition of activins via FST or specific neutralizing antibodies led to an attenuation of HG-induced upregulation of nuclear/active MRTF-A/SRF proteins (Fig. 6A-6C). Treating MC with ActA or ActB led to an increase in MRTF-A/SRF nuclear proteins (Fig. 6D) which regulate α-SMA expression in MC, as inhibition of MRTF-A/SRF signaling via siRNA leads to the attenuation of ActA or ActB-induced α-SMA promoter activity (Fig. 6E & 6F). To support this, specific inhibition of ActA or ActB via neutralizing antibodies leads to a significant attenuation of HG-induced α-SMA protein expression in MC (Fig. 6G & 6H). These results suggest that ActA and ActB regulate HG-induced MRTF-A/SRF signaling non-canonically, in addition to downstream α-SMA expression.

Furthermore, previous studies [69] have identified EGFR as a possible key target gene of ActA signaling in MC. However, the inhibition of activins via FST did not attenuate HG-induced activation of EGFR, suggesting that EGFR phosphorylation is not regulated by HG-induced activin signaling in MC (Fig. 6I).

HG Induces ActA but not ActB Production to Activate Smad2/3 Signaling and ECM upregulation in RF

Fibroblast activation via secreted factors such as TGF-β1, ActA, and ActB lead to downstream Smad3 activation (Fig. S1B) and contribute to interstitial fibrosis in DKD. In RF, HG treatment resulted in increased secretion of ActA but not ActB (Fig. 7A). Currently it is postulated that proximal tubular cell derived INHBB acts as a paracrine factor to induce local fibroblast activation, characterized by increased ECM production. To support this, in our study, RF treated with ActA or ActB show an "activated" phenotype, revealed by the increase in ECM production such as FN and CTGF (Fig. 7B). To further confirm that only ActA secretion was increased in RF after HG treatment, unlike ActB inhibition, specific ActA inhibition attenuated HG-induced activation of Smad3 and upregulation of Col-1 and FN proteins (Fig. 7C & 7D).

Modified Activin Propeptides Effectively Inhibit Activin/HG-induced Signaling in Vitro

A previous study [51] investigated ProA and ProB for their ability to suppress activin-mediated release of follicle stimulating hormone. This revealed that ProA potently inhibited ActA signaling, whereas ProB inhibited both ActA and ActB signaling. To confirm this, HEK293T cells were transfected with expression vectors for ProA or ProB and inhibition of activin signaling was measured via CAGA12 promoter activity. Transfection of the ProA expression vector only inhibited ActA-induced CAGA12 promoter activity, whereas transfection of the ProB expression vector inhibited both ActA

and ActB induced CAGA12 promoter activity (Fig. 8A & 8B). Furthermore, to verify the propeptide's functionality in MC and RF, we aimed to test their ability to inhibit HG induced upregulation of FN and pSmad3 in MC and RF. Therefore, MC and RF were treated with CM from ProA or ProB-expressing HEK293T cells (CM-ProA & CM-ProB), or CM from cells transfected with the empty vector (CM-EV), revealing that both propeptides were able to significantly inhibit HG-induced activation of Smad3 and upregulation of FN (Fig. 8C - 8E), similar to experiments performed with neutralizing antibodies. These results confirm the functionality of the propeptides and suggest the feasibility for their application *in vivo.*

Discussion

Globally, the largest cause of ERSD has been identified as DKD, which leads to changes in the deposition of ECM proteins in the mesangium, in addition to other pathological changes to various compartments of the kidneys. Since current treatment options only slow disease progression and DKD prevalence will continue to increase, there is a major need for new therapeutic targets. Targeting TGF-β1 as a therapeutic means of alleviating DKD is no longer considered a viable treatment due to the associated harmful side effects. Targeting ActA and/or ActB would be an indirect and thus more feasible way of attenuating the effects of both TGF- β1 and activins in DKD. However, current therapeutic options also inhibit other members of the TGF-β family, which could cause

unwanted adverse side effects. Therefore, specific inhibition would provide desired patient outcomes without the risk of adverse events.

Current literature suggests that ActA is the main activin contributing to profibrotic events. TGF-β1 induces the secretion of ActA more than other activin members, where FST treatment attenuates TGF-β1 induced profibrotic responses [32]. Increased ActA expression has also been observed in several *in vivo* DKD models, in addition to increased levels in the circulation and kidney [34 & 31]. In humans, systemic ActA was shown to be increased starting at stage 2 CKD, before elevations in any other measured biomarkers, showing its clinical relevance [60]. In our study, ActA mRNA and protein secretion were increased by HG treatment in MC, RF, and murine DKD models. In addition, ActA was more potent when inducing CAGA12 promoter activity in MCs and ECM proteins including Col-1, FN, and CTGF in RFs compared to ActB. Treatment of RF and MC with ProA resulted in attenuation of HG-induced profibrotic effects, similar to ActA inhibition via ALK4 siRNA or neutralizing antibody [33]. These results suggest that specific ActA inhibition can potentially be an effective therapeutic option for DKD patients. Future experiments will aim to investigate the effects of ProA treatment *in vivo*.

ActB appears to also contribute to profibrotic events, although not as potently as TGFβ-1 or ActA. Similar to ActA, ActB mRNA and protein were increased after HG treatment in MC, but not RF, as well as in human and murine DKD tissues. Treatment with ActB was shown to induce CAGA12 promoter activity and led to a significant upregulation of pSmad3 and ECM protein expression. Although ActB seems to be a less relevant

contributor to profibrotic events when comparing its abundance and potency relative to ActA., ActB inhibition attenuated HG-induced profibrotic effects in MC and RF, suggesting that ActB may have a small however critical role to play. In support of this, it has been revealed that INHBB acts as a paracrine factor to induce local fibroblast activation, leading to promoting fibrosis in the tubulointerstitial region [35].

Distinct functionality of ActB has also been shown in inflammatory-induced anaemia via regulation of hepcidin expression, a function distinct from ActA [61]. This may be due to differential signalling, as both ActA and ActB exert their actions through Smad 2/3 signaling, whereas ActB is also able to cross-activate noncanonical Smad1/5/8 signaling to induce hepcidin expression in hepatocytes [61]. However, activation of the Smad1/5/8 pathway by ActB is not consistent across all cell types, as in our study, activin inhibition via FST did not attenuate HG-induced Smad1/5 activation in MC. Interestingly, HG treatment in RF did not result in increased ActB secretion, although treatment with ActB had profibrotic effects. Therefore, we hypothesize that in the kidney, release of ActB from resident cells such as MC and tubular epithelial cells in response to hyperglycemia leads to the activation of fibroblasts, characterized by increased activation of Smad2/3 and ECM secretion, resulting in tubulointerstitial fibrosis in DKD (Fig. 9). Altogether, our data along with previous studies suggest the promotion of glomerular injury and fibrosis by ActB. Therefore, we postulate the enhanced effectiveness of combined inhibition of ActA and ActB as a therapeutic option to attenuate kidney fibrosis in DKD.

A common feature linked to renal dysfunction in various kidney disease is tubular changes such as interstitial fibrosis, characterized by the accumulation of interstitial fibroblasts that differentiate into myofibroblasts and actively synthesize ECM [32]. TGF-β1 is considered a potent activator of RFs, where disruption of TGF-β1 signalling was shown to improve renal interstitial fibrosis [32]. The factors that contribute to renal fibrosis such as ActA and ActB, on the other hand, have yet to be fully studied in RFs. Currently, fibroblast activation is thought to be due to secreted factors from tubular cells. In our study, in addition to showing that RFs can synthesize and secrete ActA after HG treatment, ActA inhibition leads to attenuation of HG-induced effects, further supporting the effectiveness of ActA inhibition as a therapeutic option for kidney fibrosis. To support this, ActA has been shown to promote cell proliferation, expression of Col-1 mRNA, and the production of α-SMA in rat kidney fibroblasts in addition to primary cultured renal interstitial fibroblasts where these effects were attenuated after blockade of activin signaling by overexpression of truncated type II activin receptor [32]. The effects of ActB on RFs however have not been studied previously, here we observe a significant induction of pSmad3 and upregulation of Col-1, FN, and CTGF protein expression.

Results from our study show the upregulation of nuclear MRTF-A and SRF expression after treatment with HG, ActA, or ActB, which was attenuated after inhibition of ActA and/or ActB., suggesting that both activins contribute to HG-induced activation of MRTF-A/SRF signaling. Furthermore, we show that ActA and ActB contribute to HG-induced upregulation of α-SMA levels in MC via regulation of MRTF-A/SRF signaling.

These results suggest activin inhibition can potentially attenuate the activated phenotype in MC during DKD, leading to reduced glomerular injury and fibrosis.

Akt activation has been shown to play a critical role in cell proliferation, survival, and metabolism. Phosphoinositide 3-kinase (PI3K), an upstream mediator of Akt, is activated when its regulatory subunit interacts with phosphotyrosine residues of activated growth factor receptors such as EGFR [68]. Various stimuli including glucose can indirectly transactivate EGFR, leading to downstream activation of the transcription factor AP-1 and upregulation of TGF-β1, in addition to collagen I production in MCs [68]. ActA signalling has been suggested to regulate EGFR expression in hepatocytes [69] and oral cavity squamous cell carcinoma tumors [70]. Novel results from our study suggest that in MCs, HG-induced phosphorylation of EGFR is independent of activin signalling. Whether this is also true for tubular epithelial cells and RFs requires further investigation.

The TGF-β family ligands are known to mainly signal through two canonical pathways of Smad transcription factors, depending on the type 1 receptor activated in the signaling complex [71]. Generally, signal transduction via ALK4, 5, and 7 lead to the activation of the Smad2/3 pathway, whereas the Smad1/5 pathway is activated by the type 1 receptors ALK1, ALK2, ALK3 and ALK6. Recently, it was discovered that ActA and ActB can activate Smad1/5 through mutated and wild type ALK2 [72 & 61]. Another study has shown that similar to BMPs, ActA and ActB can induce apoptosis in myeloma cells through ALK2 binding and Smad1/5 activation [73]. Collectively, these studies suggest that activins are dual specificity TGF-family ligands that can activate both Smad branches.

In our study, we show that HG treatment increases Smad1/5 activation after 24h. However, this increase is not due to activins as FST treatment had no effect on the activation of Smad1/5. It is quite possible that HG-induced Smad1/5 activation is due to increased BMP4 secretion and ALK3 activation, as it has been reported in rat MC [74].

As mentioned previously, specific inhibition of activins as a therapeutic option for kidney fibrosis would be most efficient due to the minimum risk of incurring significant off-target effects. Modified activin propeptides are becoming more recognized for their ability to inhibit ActA and/or ActB specifically and effectively *in vivo*. In our study, we confirm that ProB inhibits both ActA and ActB induced signaling, whereas ProA is more targated towards ActA, for which the specific reason is still unknown and requires further investigation. In addition, we confirm the functionality of the propeptides *in vitro*, characterized by their ability to attenuate HG-induced profibrotic effects. Altogether, our data suggest that the propeptides will effectively attenuate kidney fibrosis *in vivo.*

Conclusion

In conclusion, we suggest that under hyperglycemic conditions, in addition to ActA, ActB contributes to the profibrotic response in DKD. In this study, ActB expression was increased in rodent and human DKD tissue, as well as in MC treated with HG or TGF-β1, leading to increased Smad activation and ECM upregulation. In RF, only ActA secretion was increased after HG treatment. However, we show that treatment of RF with ActB has similar effects. Our investigation also reveals that ActA and ActB can trigger an "activated" phenotype in MC similar to myofibroblast differentiation, leading to increased α-SMA

expression. Lastly, we confirm the efficacy of ProA and Prob in inhibition of Act A and ActA/B signaling respectively, as well as the functionality of ProA and ProB in inhibiting MC and RF HG-induced Smad3 activation, providing rationale for their further testing *in vivo.* Based on our findings, we suggest that the combined inhibition of ActA and ActB will be more effective in attenuating kidney fibrosis, providing an alternative therapeutic option for DKD patients.

Appendix

Table 1. Primers used for qPCR experiments.

Gene	Forward Primer Sequence	Reverse Primer Sequence
18S	GCCGCTAGAGGTGAAATTCTTG	CATTCTTGGCAAATGCTTTCG
Mouse INHBA	TCATCACGTTTGCCGAGTC	CACGCTCCACCACTGACA
Mouse INHBB	CGTCTCCGAGATCATCAGC	CAGGACATAGGGGAGCAGTT

Table 2. Antibodies used for western blot experiments.

Antibody	Dilution/Amount	Source
Flag-M2	1:1000	Sigma
pSmad3 [Ser423/425]	1:2000	Novus
total Smad3	1:2000	Abcam
collagen-1α1 (Col-1)	1:10000	Abcam
Tubulin	1:2000	Sigma
connective tissue growth factor (CTGF)	1:2000	Santa Cruz Biotechnology
fibronectin (FN)	1:2000	BD Transduction Laboratories
phospho-epidermal growth factor receptor (pEGFR Y1068)	1:1000	Sigma

total EGFR	1:2000	Cell Signalling
MRTF-A	1:1000	Abcam
SRF	1:1000	Cell Signalling
Lamin B	1:1000	Santa Cruz Biotechnology
pSmad1/5 [Ser463/465]	1:1000	Cell Signalling
total Smad1/5	1:1000	Cell Signalling
α-SMA	1:5000	Thermo Fisher Scientific

Figure 1

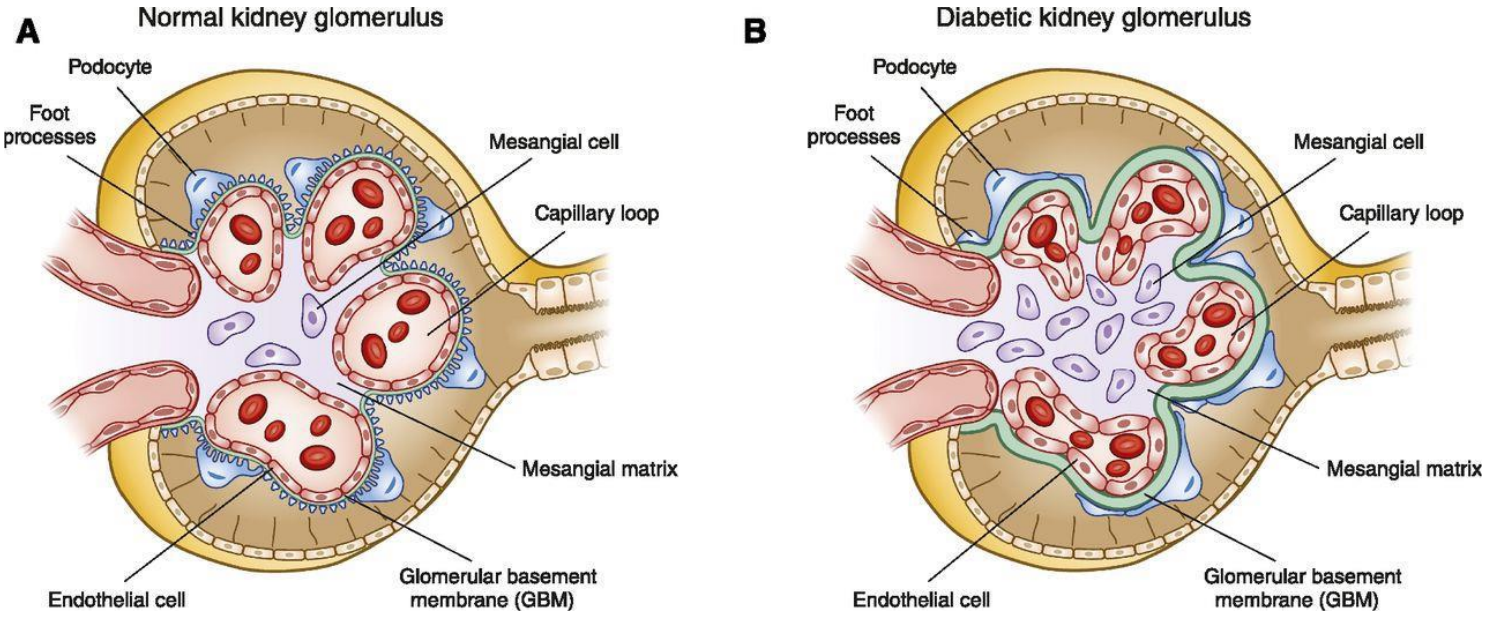
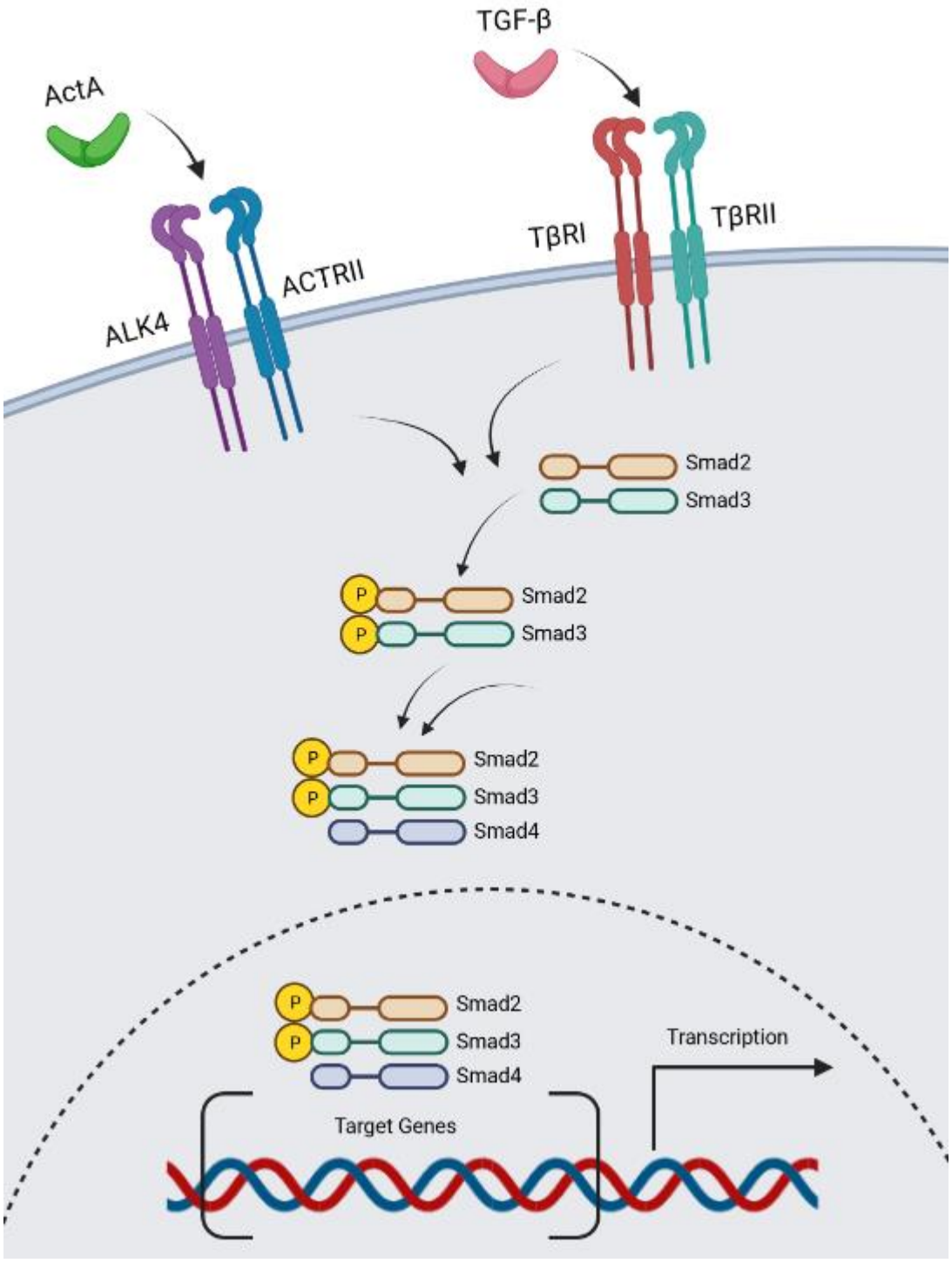


Figure 1. Glomerular changes during DKD progression. DKD gives rise to a variety of early structural changes in the glomerulus such as glomerular hypertrophy, expansion of the mesangium, thickening of the GBM, and loss of podocytes and foot processes. Published with permission from Alicic et al. [14].

Figure 2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TβRI
TβRII
ACTRII
ALK4
Smad2
Smad3
P
Smad2
P
Smad3
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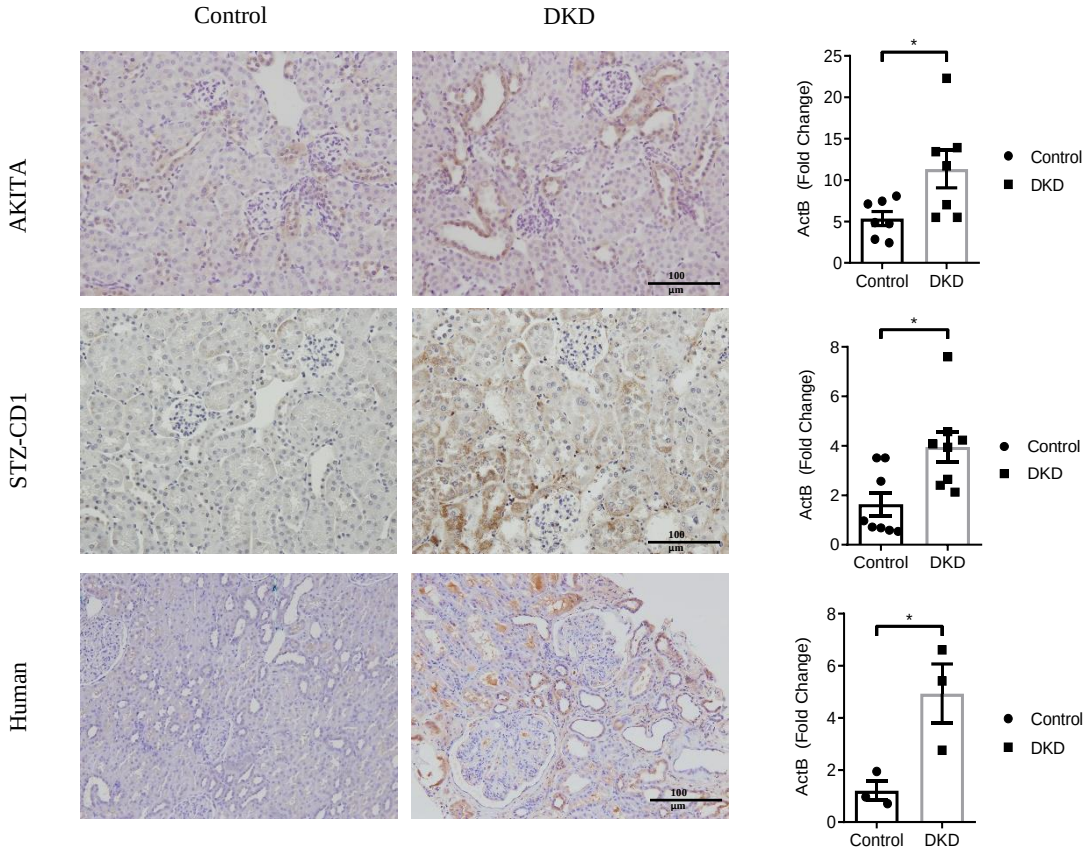
Smad2
P
Smad3
Smad4
P
Smad2
P
Smad3
Smad4
Transcription
Target Genes

Figure 2. Canonical ActA and TGF-β1 signaling pathway. ActA (green) and TGF-β1 (red) bind to their own respective type 2 receptors, recruiting and auto phosphorylating their corresponding type 1 receptor. This leads to the intracellular phosphorylation of Smads2/3 and nuclear translocation via Smad4. Binding of the nuclear Smad2/3/4 complex to Smad-responsive genes leads to transcriptional regulation of genes involved in ECM production. Image created using Biorender.

Figure 3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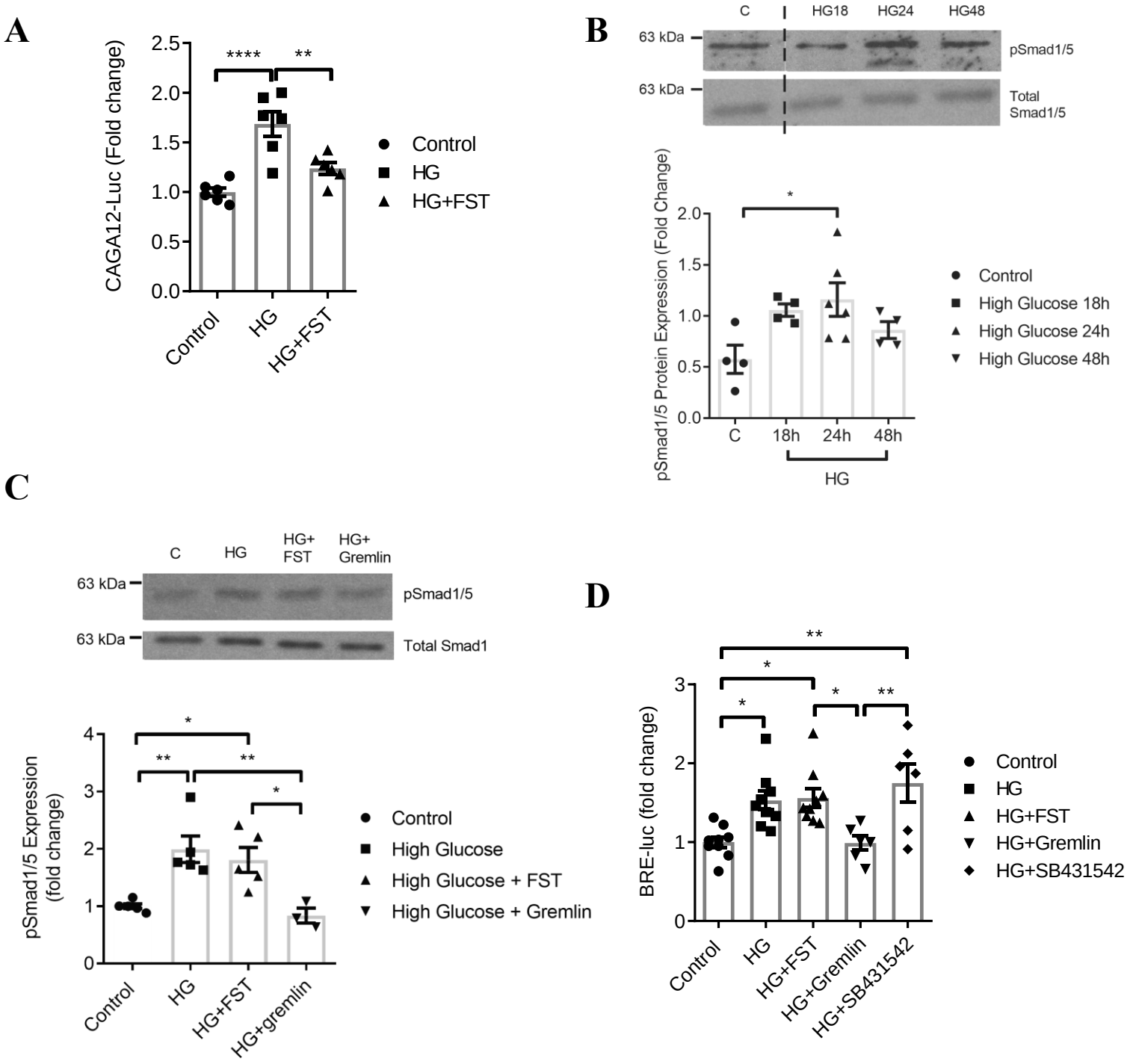
Control
DKD
AKITA
STZ-CD1
Human
100 µm
ActB (Fold Change)
Control
DKD
B
mRNA Expression (Fold Change)
INHBA
INHBB
Control
AKITA
C
STZ-CD1
D
Median-centred log2 INHBA
db/db C57BLKS
eNOS-deficient C57BLKS db/db
Human Glomerulus
Human Tubulointerstitium
E
Median-centred log2 INHBB

Figure 3. ActA and ActB are upregulated in rodent and human DKD. **(A)** Immunohistochemistry (IHC) for activin B was done in different models of DKD including type 1 diabetic Akita mice assessed at 40 weeks (n=7) and CD-1 mice made diabetic by high-dose STZ for 12 weeks (n=8), in addition to kidney tissue from control patients and patients with a tissue diagnosis of DKD (n=3), showing increased activin B in diabetic kidneys (data represent n±SEM, p*<0.05). Upregulation of INHBA and INHBB mRNA in the Akita (30w, n=8) **(B)** and STZ induced diabetic CD-1 (n=13) mice kidneys **(C)** measured via q-PCR (data represent n±SEM, p*<0.05). Data from the Nephroseq (www.nephroseq.org, University of Michigan, Ann Arbor, MI, USA) databases were extracted to examine gene expression in mouse and human kidney. INHBA **(D)** and INHBB **(E)** expression data were obtained from the datasets: Hodgin Diabetes Mouse Glom (db/db C57BLKS and eNOS-deficient C57BLKS db/db), Woroniecka Diabetes Glom (Human Glomerulus), and Woroniecka Diabetes TubInt (Human Tubulointerstitium). Statistical significance is presented as p-values computed using Welch's t test and q values (exported from Nephroseq) corrected using the Benjamini–Hochberg method (p*<0.05, p** < 0.01, p***<0.001).

Figure 4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CAGA12-Luc (Fold change)
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**
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HG+FST
B
C
HG18
HG24
HG48
63 kDa
pSmad1/5
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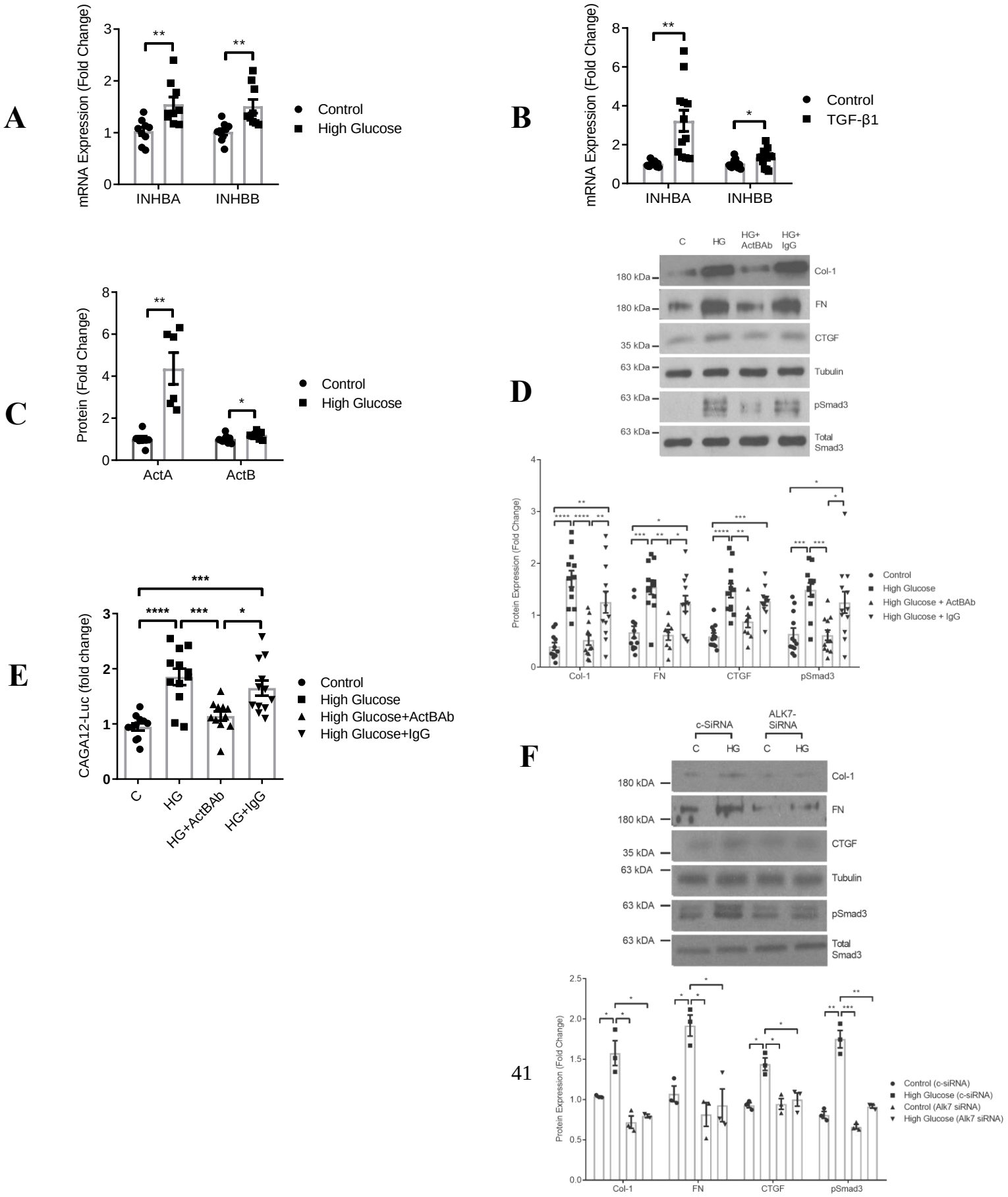
pSmad1/5 Protein Expression (Fold Change)
High Glucose 18h
High Glucose 24h
High Glucose 48h
HG
C
HG+ FST
HG+ Gremlin
Total Smad1
pSmad1/5 Expression (fold change)
High Glucose
High Glucose + FST
High Glucose + Gremlin
HG+gremlin
D
BRE-luc (fold change)
HG+Gremlin
HG+SB431542

Figure 4. HG induced activin signaling occurs via Smad2/3 pathway rather than Smad1/5. **(A)** Increased CAGA12 promoter activity due to HG induced activin signaling. MCs were treated with HG or HG+FST (300 ng/mL) for 48h and induction of downstream CAGA12 promoter activity was measured via luciferase assay (data represent n±SEM, n=6, p**<0.01, p****<0.0001). **(B)** Increased activation of Smad1/5 pathway after 24h HG treatment. MCs were treated with HG for 18h (n=4), 24h (n=6), and 48h (n=4) and phosphorylation of Smad1/5 was determined via western blot (data represent n±SEM, p*<0.05). **(C)** Unlike BMP inhibition via gremlin, activin inhibition via FST fails to attenuate HG-induced activation of Smad1/5. MCs were treated with HG (n=5), HG+FST (300 ng/mL, n=5), or HG+Gremlin (500 ng/mL, n=3) for 24h and phosphorylation of Smad1/5 was determined via western blot (data represent n±SEM, p*<0.05, p**<0.01). **(D)** Activin and activin receptor inhibition fails to attenuate HG-induced upregulation of BRE promoter activity. MCs were treated with HG (n=9), HG+FST (300 ng/ml, n=9), HG+Gremlin (500 ng/ml, n=6), or HG+SB431542 (5 uM, n=6) for 48h (data represent n±SEM, p*<0.05, p**<0.01).

Figure 5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mRNA Expression (Fold Change)
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INHBB
Control
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INHBB
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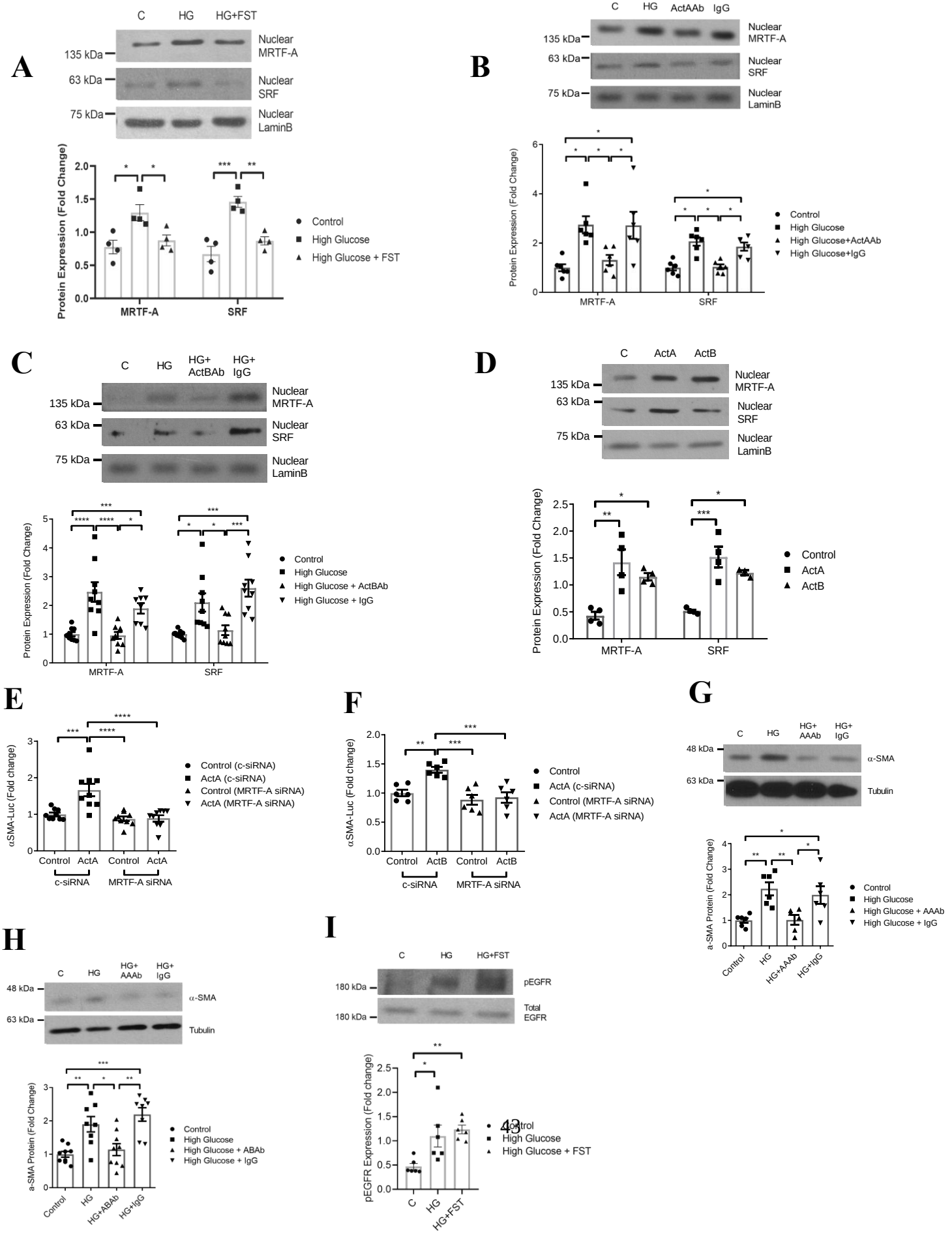
C
Protein (Fold Change)
ActA
ActB
Control
High Glucose
D
C
HG
HG+ ActBAb
HG+ IgG
180 kDa
Col-1
FN
35 kDa
CTGF
63 kDa
Tubulin
pSmad3
Total Smad3
Protein Expression (Fold Change)
Col-1
FN
CTGF
pSmad3
Control
High Glucose
High Glucose + ActBAb
High Glucose + IgG
E
CAGA12-Luc (fold change)
C
HG
HG+ActBAb
HG+IgG
Control
High Glucose
High Glucose+ActBAb
High Glucose+IgG
F
c-SiRNA
ALK7-SiRNA
C
HG
180 kDA
Col-1
FN
35 kDA
CTGF
Tubulin
pSmad3
Total Smad3
Protein Expression (Fold Change)
Control (c-siRNA)
High Glucose (c-siRNA)
Control (Alk7 siRNA)
High Glucose (Alk7 siRNA)

Figure 5. HG increases ActA and ActB and regulates ECM production in MC. MCs were treated with HG for 48h **(A)** or 1 ng/mL of TGF-β1 for 24h **(B)** and INHBA and INHBB mRNA were measured via q-PCR (data represent n±SEM, n=12, p*<0.05, p** < 0.01). **(C)** MCs were treated with HG for 48h and activin secretion was measured using ELISA (data represent n±SEM, n=6, p*<0.05, p** < 0.01). **(D)** ActB inhibition attenuates HG-induced upregulation of Col-1, pSmad3, FN, and CTGF protein expression, in addition to CAGA12 promoter activity **(E)**. MCs were treated with HG, HG and ActBAb (3.5 ug/mL), or HG and monoclonal pre-adsorbed IgG (3.5 ug/mL) for 48h. Downstream protein expression was measured via western blot and induction of downstream CAGA12 promoter activity was measured via luciferase assay (data represent n±SEM, n=12, p*<0.05, p**<0.01, p***<0.001, p****<0.0001). **(F)** siRNA mediated inhibition of ALK7 attenuates HG-induced upregulation of Col-1, pSmad3, FN, and CTGF protein expression. MCs were transfected with either c-siRNA or ALK7 siRNA (50 nM) and treated with HG for 48h. Protein expression was (data represent n±SEM, p*<0.05, p**<0.01, p***<0.001).

Figure 6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C
HG
HG+FST
135 kDa
63 kDa
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Nuclear MRTF-A
Nuclear SRF
Nuclear LaminB
Protein Expression (Fold Change)
MRTF-A
SRF
Control
High Glucose
High Glucose + FST
B
HG+ ActAAb
HG+ IgG
High Glucose+ActAAb
High Glucose+IgG
C
HG+ ActBAb
High Glucose + ActBAb
High Glucose + IgG
D
ActA
ActB
E
αSMA-Luc (Fold change)
Control (c-siRNA)
ActA (c-siRNA)
Control (MRTF-A siRNA)
ActA (MRTF-A siRNA)
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MRTF-A siRNA
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High Glucose + AAAb
High Glucose + IgG
H
HG+ ABAb
High Glucose + ABAb
High Glucose + IgG
I
180 kDa
pEGFR
Total EGFR
pEGFR Expression (Fold change)

Figure 6. ActA and ActB regulate HG induced MRTF-A/SRF activation and downstream α-SMA upregulation. **(A)** HG induced upregulation of nuclear MRTF-A and SRF is attenuated after activin inhibition. MCs were treated HG or HG+FST (300 ng/ml) for 48h. After extraction of nuclear proteins, MRTF-A and SRF protein expression was measured via western blot (data represent n±SEM, n=4, p*<0.05, p**<0.01, p***<0.001). The induction of nuclear MRTF-A and SRF protein expression with HG treatment is attenuated after ActA **(B)** (n=6) and ActB **(C)** (n=9) inhibition. MCs were treated with HG, HG+ActAAb (3 ug/ml), HG+ActBAb (3.5 ug/ml), or HG+monoclonal pre-adsorbed IgG (3-3.5 ug/mL) for 48h. After extraction of nuclear proteins, MRTF-A and SRF protein expression was measured via western blot (data represent n±SEM, p*<0.05, p**<0.01, p***<0.001, p****<0.0001). **(D)** The induction of nuclear MRTF-A and SRF protein expression via ActA and ActB. MCs were treated with ActA or ActB (20 ng/ml) for 48h. After extraction of nuclear proteins, MRTF-A and SRF protein expression was measured via western blot (data represent n±SEM, n=4, p*<0.05, p**<0.01, p***<0.001). ActA and ActB induce upregulation of α-SMA via MRTF-A/SRF pathway, assessed by the activation of the α-SMA luciferase promoter construct. MCs were transfected with either c-siRNA or MRTF-A siRNA (100 nM) and treated with 20 ng/ml of ActA **(E)** (n=9) or ActB **(F)** (n=6) for 24h (data represent n±SEM, p*<0.05, p**<0.01, p***<0.001, p****<0.0001). HG induced upregulation of α-SMA is attenuated by activin inhibition. MCs were treated with HG, HG+ActAAb (3 ug/ml) (**G**), HG+ActBAb (3.5 ug/ml) **(H)**, or HG+monoclonal pre-adsorbed IgG (3-3.5 ug/mL) for 24h (data represent n±SEM, p*<0.05, p**<0.01, p***<0.001, p****<0.0001). **(I)** Activin inhibition fails to attenuate HG induced

phosphorylation of EGFR. MCs were treated with HG or HG+FST (500 ng/ml) for 48h and pEGFR protein expression was measured via western blot (data represent n±SEM, n=6, p*<0.05, p**<0.01).

Figure 7

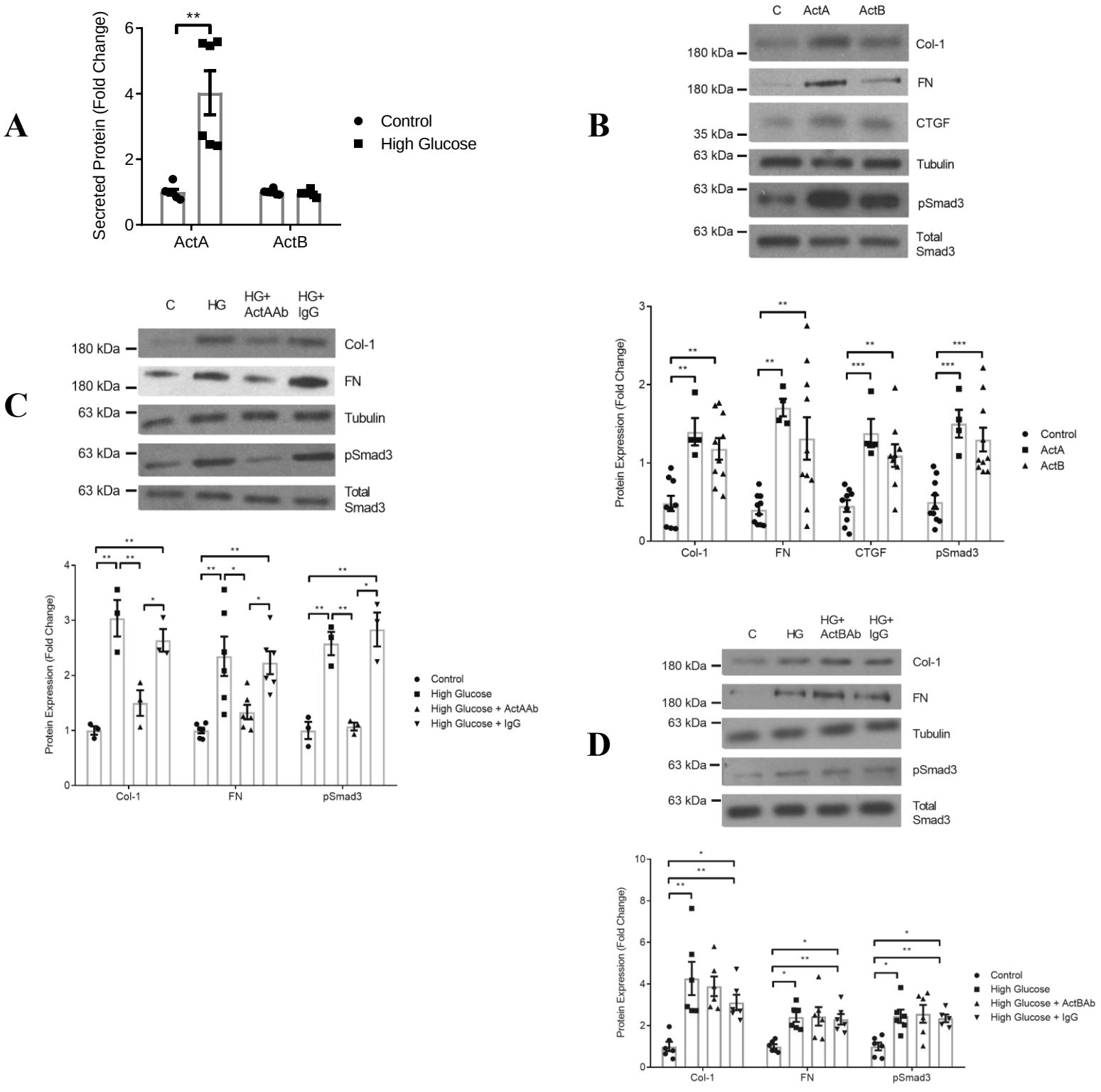

A
Secreted Protein (Fold Change)
ActA
ActB
Control
High Glucose
B
C
ActA
ActB
Col-1
FN
CTGF
Tubulin
pSmad3
Total Smad3
C
HG
HG+ ActAAb
HG+ IgG
Protein Expression (Fold Change)
High Glucose + ActAAb
High Glucose + IgG
D
HG+ ActBAb
High Glucose + ActBAb

Figure 7. HG induces ActA but not ActB production to activate Smad2/3 signaling and ECM upregulation in RF. **(A)** HG treatment leads to an increase in ActA but not ActB protein secretion in RF. RFs were treated with HG for 48h and activin secretion was measured using ELISA (data represent n±SEM, n=6, p*<0.05, p** < 0.01, p****<0.0001). ActA and ActB induce downstream pSmad3, Col-1, CTGF, and FN protein expression in RF. RFs were treated with 10 ng/ml of ActA (n=4) or ActB (n=10) for 48h and downstream protein expression was measured via western blot (data represent n±SEM, p*<0.05, p**<0.01, p***<0.001). ActA inhibition attenuates HG-induced upregulation of Col-1, pSmad3, and FN protein expression protein expression in RF **(C**), whereas ActB inhibition has no effect **(D**). RFs were treated with HG, HG+ActAAb (3 ug/mL), HG+ActBAb (3.5 ug/mL), or HG and monoclonal pre-adsorbed IgG (3-3.5ug/mL) for 48h. Downstream protein expression was measured via western blot (data represent n±SEM, n=6, p*<0.05, p**<0.01).

Figure 8

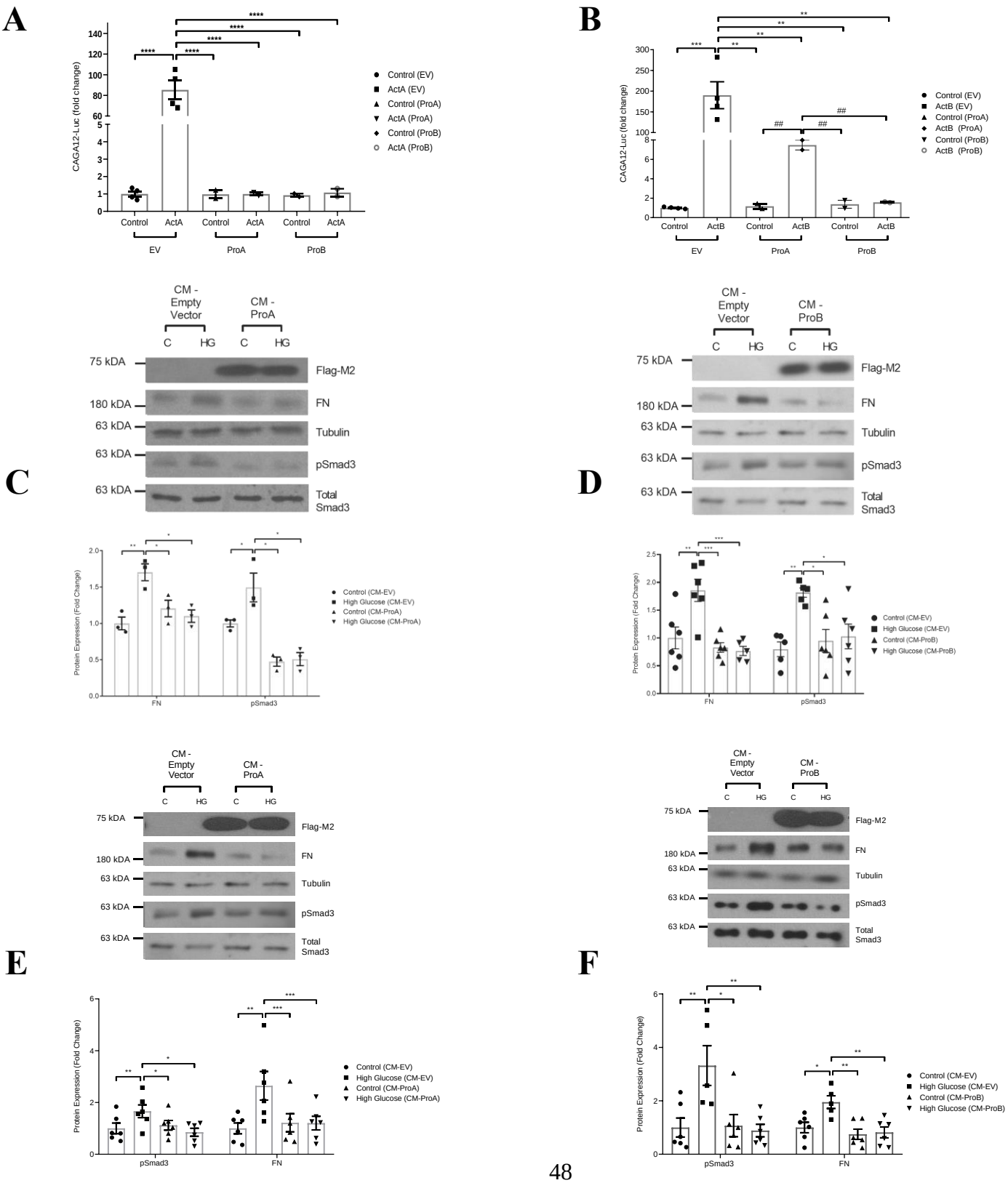

A
CAGA12-Luc (fold change)
Control (EV)
ActA (EV)
Control (ProA)
ActA (ProA)
Control (ProB)
ActA (ProB)
Control
ActA
EV
ProA
ProB
B
CAGA12-Luc (fold change)
Control (EV)
ActB (EV)
Control (ProA)
ActB (ProA)
Control (ProB)
ActB (ProB)
Control
ActB
EV
ProA
ProB
C
CM - Empty Vector
CM - ProA
C
HG
75 kDA
Flag-M2
180 kDA
FN
63 kDA
Tubulin
pSmad3
Total Smad3
Protein Expression (Fold Change)
Control (CM-EV)
High Glucose (CM-EV)
Control (CM-ProA)
High Glucose (CM-ProA)
FN
pSmad3
D
CM - Empty Vector
CM - ProB
Protein Expression (Fold Change)
Control (CM-EV)
High Glucose (CM-EV)
Control (CM-ProB)
High Glucose (CM-ProB)
E
CM - Empty Vector
CM - ProA
Protein Expression (Fold Change)
Control (CM-EV)
High Glucose (CM-EV)
Control (CM-ProA)
High Glucose (CM-ProA)
pSmad3
FN
F
CM - Empty Vector
CM - ProB
Protein Expression (Fold Change)
Control (CM-EV)
High Glucose (CM-EV)
Control (CM-ProB)
High Glucose (CM-ProB)

Figure 8. Modified activin propeptides effectively inhibit activin/HG induced signaling in vitro. **(A & B)** ProA inhibits ActA induced CAGA12 promoter activity more effectively compared to ActB, whereas ProB inhibits both ActA and ActB induced CAGA12 promoter activity. HEK293T cells were transfected with 0.001 ug/ml of EV, Pro.A, or Pro.B propeptide vector and treated with either ActA or ActB (5 ng/ml) for 24h. Induction of downstream CAGA12 promoter activity was measured via luciferase assay (data represent n±SEM, n=2, p*<0.05, p**<0.01, p***<0.001, p****<0.0001, $p^{\#\#}$ <0.01 through unpaired T-test). ProA and ProB inhibit HG induced upregulation of FN and pSmad3 in MC **(C & D)** and RF **(E & F)**. HEK293T cells were transfected with 0.1 ug/ml of EV, ProA, or Pro.B propeptide vector for 48h and Conditioned medium (CM) was collected. Furthermore, MCs and RFs were treated with CM-EV, CM-ProA, or CM-ProB and HG for 48h and downstream protein expression was measured via western blot (data represent n±SEM, n=6, p*<0.05, p**<0.01, p***<0.001).

Figure 9

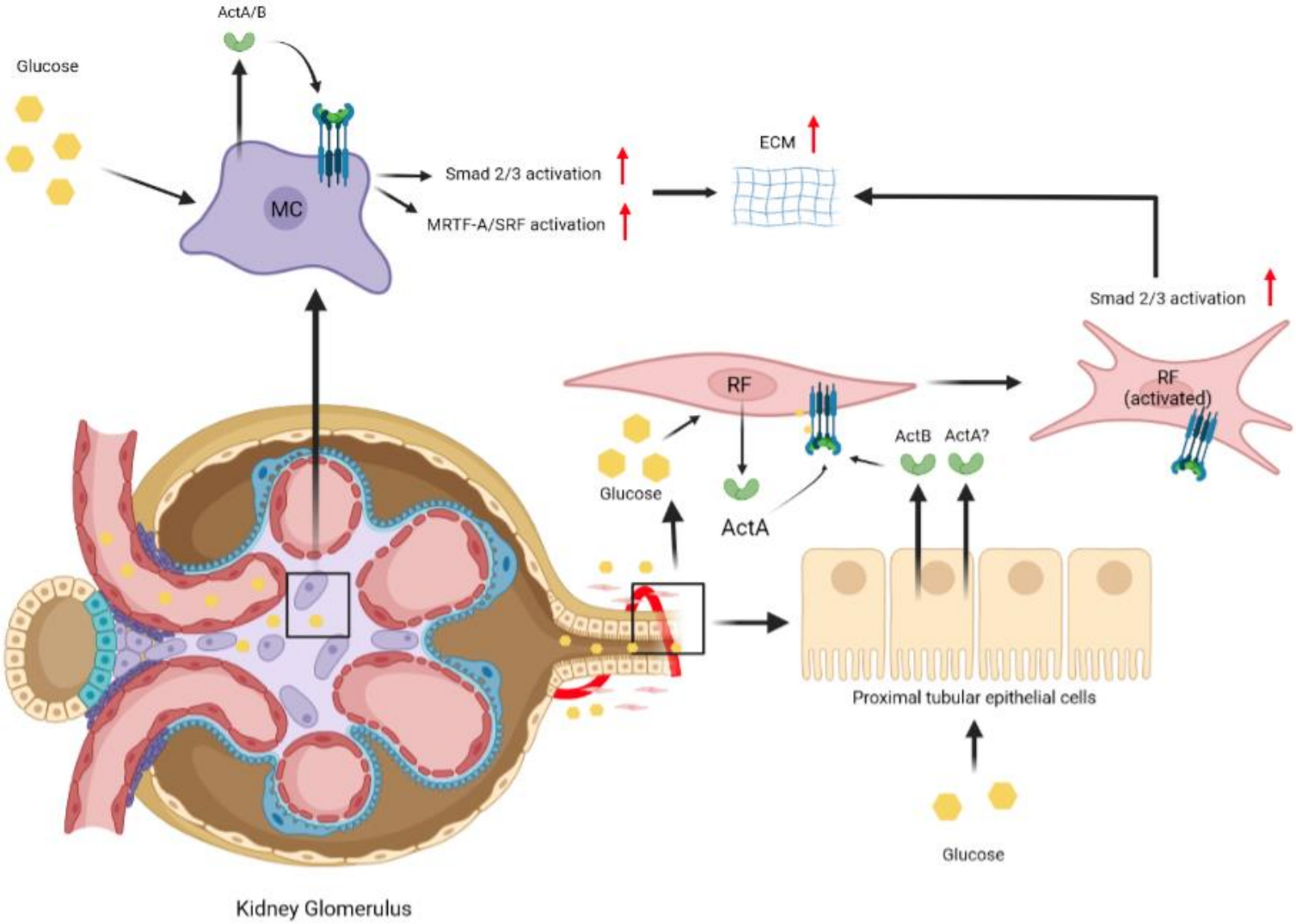
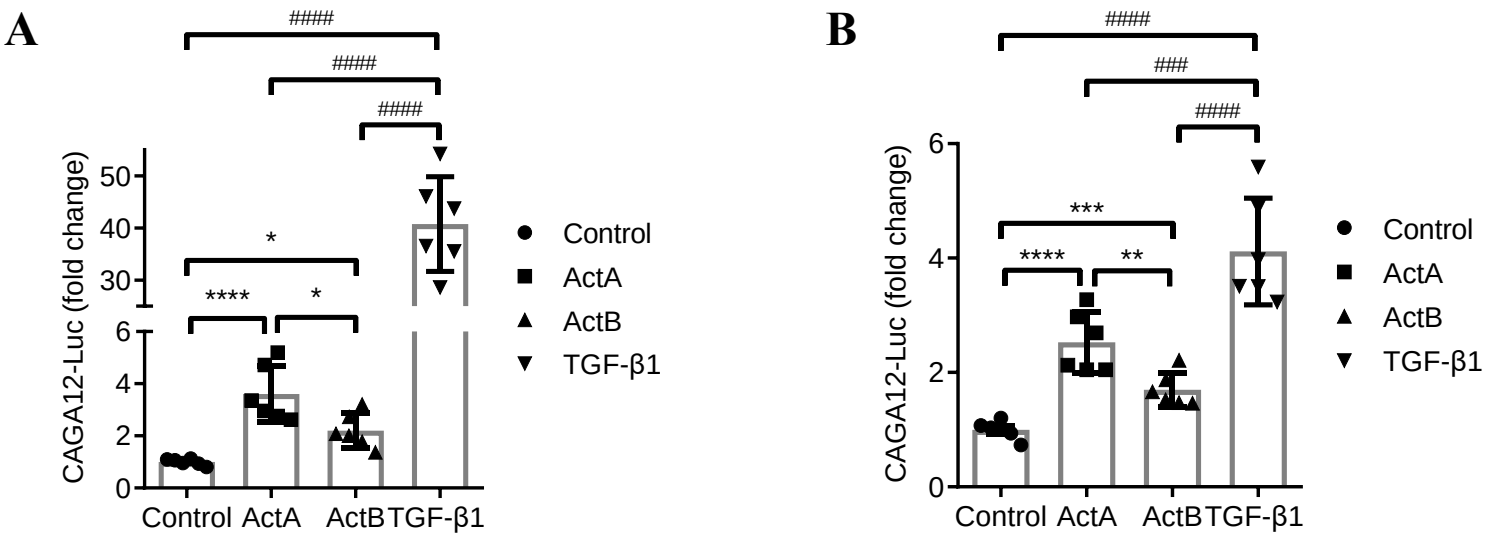

ActA/B
Glucose
MC
Smad 2/3 activation
MRTF-A/SRF activation
ECM
Smad 2/3 activation
RF
RF
(activated)
ActB
ActA?
Glucose
ActA
Proximal tubular epithelial cells
Glucose
Kidney Glomerulus

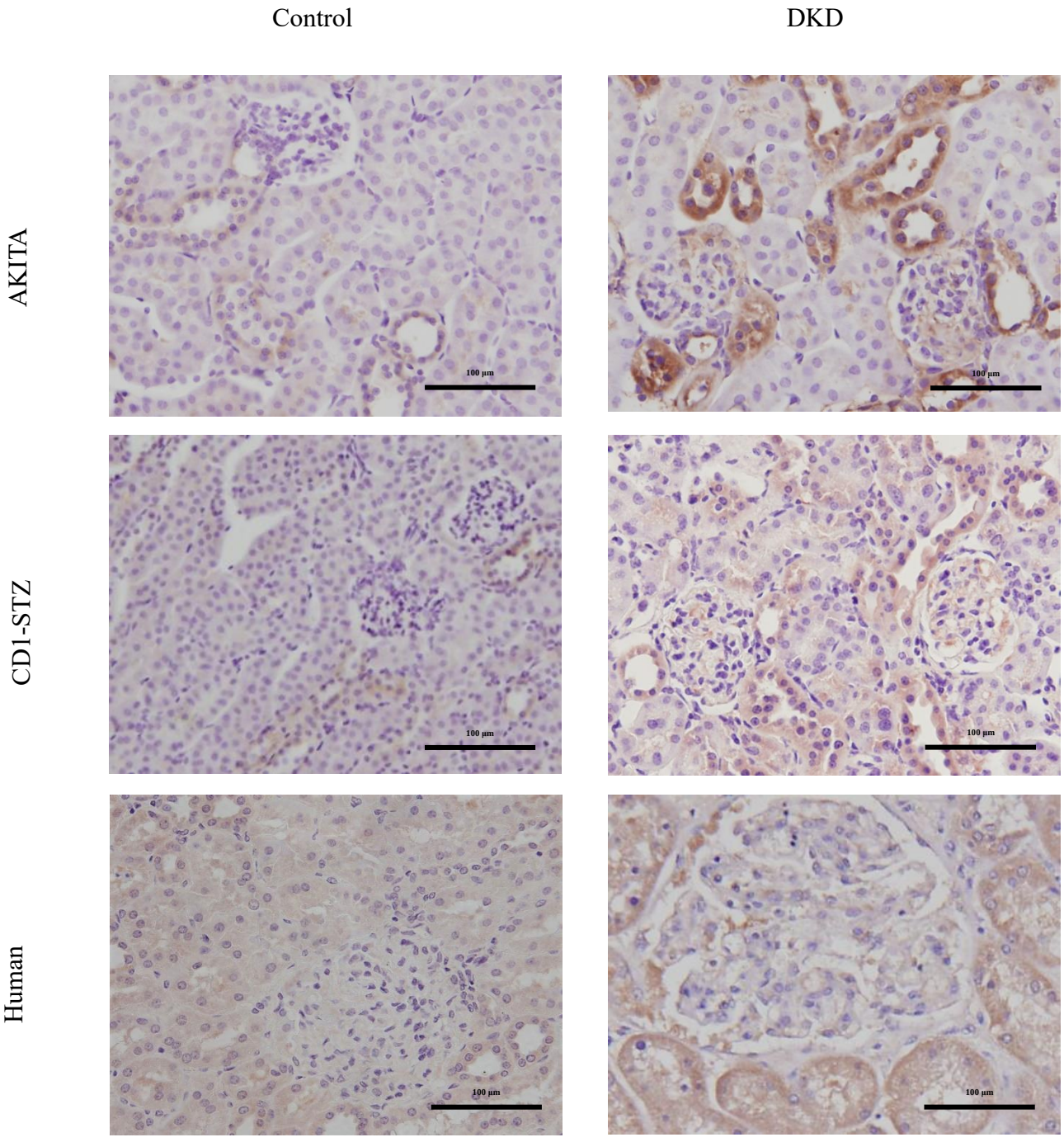
Figure 9. Schematic representation of proposed mechanism for ECM upregulation by kidney cells due to HG-induced activin secretion. Prolonged exposure of MC, RF, and proximal tubular epithelial cells to glucose leads to the secretion of ActA/B, ActA, and ActB, respectively. This results in increased activation of profibrotic pathways such as Smad2/3 and MRTF-A/SRF and ECM upregulation in MC and RF. Whether ActA is also secreted from proximal tubular epithelial cells is currently unknown. Image created using BioRender.

Supplementary Figure 1



Supplementary Figure 1. TGF-β1 is the most potent inducer of Smad3 activation in MC and RF, followed by ActA & ActB, respectively. MCs and RFs were treated with TGF-β1, ActA, or ActB (2 ng/ml) for 24h and induction of downstream Smad3 activation and CAGA12 promoter activity was measured via luciferase assay (data represent n±SEM, n=6, p*<0.05, p**<0.01, p***<0.001).

Supplementary Figure 2



Supplementary Figure 2. Glomerular ActB Staining in the AKITA and STZ-CD1 DKD models, in addition to kidney tissue from control patients and patients with a tissue diagnosis of DKD.

References

1. Wild, S., et al. “Global Prevalence of Diabetes: Estimates for the Year 2000 and Projections for 2030.” Diabetes Care, vol. 27, no. 5, May 2004, pp. 1047–53. DOI.org (Crossref), doi:10.2337/diacare.27.5.1047.
2. World Health Organization. Diabetes, 2018, https://www.who.int/news-room/fact-sheets/detail/diabetes.
3. Diabetes Canada. Diabetes in Canada: Backgrounder, https://www.diabetes.ca › Advocacy-and-Policy.
4. Dhatariya K. Diabetes: the place of new therapies. Ther Adv Endocrinol Metab. 2018;10:2042018818807599. Published 2018 Oct 30. doi:10.1177/2042018818807599
5. Andersen, A. R., et al. “Diabetic Nephropathy in Type 1 (Insulin-Dependent) Diabetes: An Epidemiological Study.” Diabetologia, vol. 25, no. 6, Dec. 1983. DOI.org (Crossref), doi:10.1007/BF00284458
6. Nathan, David M. “Long-Term Complications of Diabetes Mellitus.” New England Journal of Medicine, vol. 328, no. 23, June 1993, pp. 1676–85. DOI.org (Crossref), doi:10.1056/NEJM199306103282306.
7. Gross, Jorge L., et al. “Diabetic Nephropathy: Diagnosis, Prevention, and Treatment.” Diabetes Care, vol. 28, no. 1, Jan. 2005, pp. 164–76. PubMed, doi:10.2337/diacare.28.1.164.
8. Johnson, Stacy A., and Robert F. Spurney. “Twenty Years after ACEIs and ARBs: Emerging Treatment Strategies for Diabetic Nephropathy.” American Journal of

Physiology-Renal Physiology, vol. 309, no. 10, Nov. 2015, pp. F807–20. DOI.org (Crossref), doi:10.1152/ajprenal.00266.2015.

9. Gheith, Osama, et al. “Diabetic Kidney Disease: World Wide Difference of Prevalence and Risk Factors.” Journal of Nephropharmacology, vol. 5, no. 1, 2016, pp. 49–56.
10. Preuss, H. G. “Basics of Renal Anatomy and Physiology.” Clinics in Laboratory Medicine, vol. 13, no. 1, Mar. 1993, pp. 1–11.
11. Kriz, Wilhelm, and Brigitte Kaissling. “Structural Organization of the Mammalian Kidney.” Seldin and Giebisch’s The Kidney, Elsevier, 2013, pp. 595–691. DOI.org (Crossref), doi:10.1016/B978-0-12-381462-3.00020-3.
12. Schlöndorff, Detlef, and Bernhard Banas. “The Mesangial Cell Revisited: No Cell Is an Island.” Journal of the American Society of Nephrology, vol. 20, no. 6, June 2009, pp. 1179–87. DOI.org (Crossref), doi:10.1681/ASN.2008050549.
13. Fioretto, Paola, and Michael Mauer. “Histopathology of Diabetic Nephropathy.” Seminars in Nephrology, vol. 27, no. 2, Mar. 2007, pp. 195–207. DOI.org (Crossref), doi:10.1016/j.semnephrol.2007.01.012.
14. Alicic, Radica Z., et al. “Diabetic Kidney Disease: Challenges, Progress, and Possibilities.” Clinical Journal of the American Society of Nephrology, vol. 12, no. 12, Dec. 2017, pp. 2032–45. DOI.org (Crossref), doi:10.2215/CJN.11491116.
15. Forbes, J., et al. “Diabetic Nephropathy: Where Hemodynamics Meets Metabolism.” Experimental and Clinical Endocrinology & Diabetes, vol. 115, no. 02, Feb. 2007, pp. 69–84. DOI.org (Crossref), doi:10.1055/s-2007-949721.
16. Pacher, Pál, et al. “Nitric Oxide and Peroxynitrite in Health and Disease.” Physiological Reviews, vol. 87, no. 1, Jan. 2007, pp. 315–424. DOI.org (Crossref), doi:10.1152/physrev.00029.2006.
17. Wolf, G. “New Insights into the Pathophysiology of Diabetic Nephropathy: From Haemodynamics to Molecular Pathology.” European Journal of Clinical Investigation,

vol. 34, no. 12, Dec. 2004, pp. 785–96. DOI.org (Crossref), doi:10.1111/j.1365-2362.2004.01429.x.

18. Meng, Xiao-Ming, et al. “TGF-Î²/Smad Signaling in Renal Fibrosis.” Frontiers in Physiology, vol. 6, Mar. 2015. DOI.org (Crossref), doi:10.3389/fphys.2015.00082.
19. Pelton, R. W., et al. “Immunohistochemical Localization of TGF Beta 1, TGF Beta 2, and TGF Beta 3 in the Mouse Embryo: Expression Patterns Suggest Multiple Roles during Embryonic Development.” Journal of Cell Biology, vol. 115, no. 4, Nov. 1991, pp. 1091–105. DOI.org (Crossref), doi:10.1083/jcb.115.4.1091.
20. Antsiferova, Maria, and Sabine Werner. “The Bright and the Dark Sides of Activin in Wound Healing and Cancer.” Journal of Cell Science, vol. 125, no. 17, Sept. 2012, pp. 3929–37. DOI.org (Crossref), doi:10.1242/jcs.094789.
21. Rifkin, Daniel B. “Latent Transforming Growth Factor-β (TGF-β) Binding Proteins: Orchestrators of TGF-β Availability.” Journal of Biological Chemistry, vol. 280, no. 9, Mar. 2005, pp. 7409–12. DOI.org (Crossref), doi:10.1074/jbc.R400029200.
22. Feng, Xin-Hua, and Rik Derynck. “SPECIFICITY AND VERSATILITY IN TGF-β SIGNALING THROUGH SMADS.” Annual Review of Cell and Developmental Biology, vol. 21, no. 1, Nov. 2005, pp. 659–93. DOI.org (Crossref), doi:10.1146/annurev.cellbio.21.022404.142018.
23. Zhang, Ying E. “Non-Smad Pathways in TGF-β Signaling.” Cell Research, vol. 19, no. 1, Jan. 2009, pp. 128–39. DOI.org (Crossref), doi:10.1038/cr.2008.328.
24. Wang, Wansheng, et al. “Transforming Growth Factor-Beta and Smad Signalling in Kidney Diseases. Review Article.” Nephrology, vol. 10, no. 1, Feb. 2005, pp. 48–56. DOI.org (Crossref), doi:10.1111/j.1440-1797.2005.00334.x.
25. Vincenti, Flavio, et al. “A Phase 2, Double-Blind, Placebo-Controlled, Randomized Study of Fresolimumab in Patients With Steroid-Resistant Primary Focal Segmental

Glomerulosclerosis.” Kidney International Reports, vol. 2, no. 5, Sept. 2017, pp. 800–10. DOI.org (Crossref), doi:10.1016/j.ekir.2017.03.011.

26. Voelker, James, et al. “Anti–TGF- β 1 Antibody Therapy in Patients with Diabetic Nephropathy.” Journal of the American Society of Nephrology, vol. 28, no. 3, Mar. 2017, pp. 953–62. DOI.org (Crossref), doi:10.1681/ASN.2015111230.
27. de Kretser, David M., et al. “The Roles of Activin A and Its Binding Protein, Follistatin, in Inflammation and Tissue Repair.” Molecular and Cellular Endocrinology, vol. 359, no. 1–2, Aug. 2012, pp. 101–06. DOI.org (Crossref), doi:10.1016/j.mce.2011.10.009.
28. Namwanje, Maria, and Chester W. Brown. “Activins and Inhibins: Roles in Development, Physiology, and Disease.” Cold Spring Harbor Perspectives in Biology, vol. 8, no. 7, July 2016, p. a021881. DOI.org (Crossref), doi:10.1101/cshperspect.a021881.
29. Tsuchida, Kunihiro, et al. “Activin Isoforms Signal through Type I Receptor Serine/Threonine Kinase ALK7.” Molecular and Cellular Endocrinology, vol. 220, no. 1–2, May 2004, pp. 59–65. DOI.org (Crossref), doi:10.1016/j.mce.2004.03.009.
30. Ritvos, Olli, et al. “Activin Disrupts Epithelial Branching Morphogenesis in Developing Glandular Organs of the Mouse.” Mechanisms of Development, vol. 50, no. 2–3, Apr. 1995, pp. 229–45. DOI.org (Crossref), doi:10.1016/0925-4773(94)00342-K.
31. Bian, Xiaohui, et al. “Senescence Marker Activin A Is Increased in Human Diabetic Kidney Disease: Association with Kidney Function and Potential Implications for

Therapy." BMJ Open Diabetes Research & Care, vol. 7, no. 1, Dec. 2019. PubMed Central, doi:10.1136/bmjdrc-2019-000720.

32. Yamashita, S. "Activin A Is a Potent Activator of Renal Interstitial Fibroblasts." Journal of the American Society of Nephrology, vol. 15, no. 1, Jan. 2004, pp. 91–101. DOI.org (Crossref), doi:10.1097/01.ASN.0000103225.68136.E6.
33. Zhang, Dan, et al. "The Caveolin-1 Regulated Protein Follistatin Protects against Diabetic Kidney Disease." Kidney International, vol. 96, no. 5, Nov. 2019, pp. 1134–49. DOI.org (Crossref), doi:10.1016/j.kint.2019.05.032.
34. Agapova, Olga A., et al. "Ligand Trap for the Activin Type IIA Receptor Protects against Vascular Disease and Renal Fibrosis in Mice with Chronic Kidney Disease." Kidney International, vol. 89, no. 6, June 2016, pp. 1231–43. DOI.org (Crossref), doi:10.1016/j.kint.2016.02.002.
35. Sun, Yanyan et al. "Tubule-derived INHBB promotes interstitial fibroblast activation and renal fibrosis." The Journal of pathology vol. 256,1 (2022): 25-37. doi:10.1002/path.5798
36. de Kretser, David, et al. "Serum Activin A and B Levels Predict Outcome in Patients with Acute Respiratory Failure: A Prospective Cohort Study." Critical Care, vol. 17, no. 5, 2013, p. R263. DOI.org (Crossref), doi:10.1186/cc13093.
37. Li, Q., et al. "Prevention of Cachexia-like Syndrome Development and Reduction of Tumor Progression in Inhibin-Deficient Mice Following Administration of a Chimeric

Activin Receptor Type II-Murine Fc Protein." Molecular Human Reproduction, vol. 13, no. 9, June 2007, pp. 675–83. DOI.org (Crossref), doi:10.1093/molehr/gam055.

38. Deng, Shi-Kang, et al. "Activin B Signaling May Promote the Conversion of Normal Fibroblasts to Scar Fibroblasts." Medicine, vol. 99, no. 24, June 2020, p. e20253. DOI.org (Crossref), doi:10.1097/MD.0000000000020253.
39. Fang, Doreen Y. P., et al. "The Role of Activin A and B and the Benefit of Follistatin Treatment in Renal Ischemia-Reperfusion Injury in Mice." Transplantation Direct, vol. 2, no. 7, July 2016, p. e87. DOI.org (Crossref), doi:10.1097/TXD.0000000000000601.
40. Myllärniemi, Marjukka, et al. "Upregulation of Activin-B and Follistatin in Pulmonary Fibrosis – a Translational Study Using Human Biopsies and a Specific Inhibitor in Mouse Fibrosis Models." BMC Pulmonary Medicine, vol. 14, no. 1, Dec. 2014, p. 170. DOI.org (Crossref), doi:10.1186/1471-2466-14-170.
41. Kanamori, Yohei, et al. "Regulation of Hepcidin Expression by Inflammation-Induced Activin B." Scientific Reports, vol. 6, no. 1, Dec. 2016, p. 38702. DOI.org (Crossref), doi:10.1038/srep38702.
42. Hashimoto, Osamu, et al. "A Novel Role of Follistatin, an Activin-Binding Protein, in the Inhibition of Activin Action in Rat Pituitary Cells." Journal of Biological Chemistry, vol. 272, no. 21, May 1997, pp. 13835–42. DOI.org (Crossref), doi:10.1074/jbc.272.21.13835.
43. Hedger, M. P., and D. M. de Kretser. "The Activins and Their Binding Protein, Follistatin—Diagnostic and Therapeutic Targets in Inflammatory Disease and Fibrosis." Cytokine & Growth Factor Reviews, vol. 24, no. 3, June 2013, pp. 285–95. DOI.org (Crossref), doi:10.1016/j.cytogfr.2013.03.003.
44. Mehta, Neel, et al. "Follistatin Protects Against Glomerular Mesangial Cell Apoptosis and Oxidative Stress to Ameliorate Chronic Kidney Disease." Antioxidants & Redox Signaling, vol. 31, no. 8, Sept. 2019, pp. 551–71. DOI.org (Crossref), doi:10.1089/ars.2018.7684.
45. Chantry, Andrew D., et al. "Inhibiting Activin-A Signaling Stimulates Bone Formation and Prevents Cancer-Induced Bone Destruction in Vivo." Journal of Bone and Mineral

Research, vol. 25, no. 12, Dec. 2010, pp. 2633–46. DOI.org (Crossref), doi:10.1002/jbmr.142.

46. Aykul, Senem, and Erik Martinez-Hackert. “Transforming Growth Factor-β Family Ligands Can Function as Antagonists by Competing for Type II Receptor Binding*.” Journal of Biological Chemistry, vol. 291, no. 20, May 2016, pp. 10792–804. DOI.org (Crossref), doi:10.1074/jbc.M115.713487.
47. Tao, Jessica J., et al. “First-in-Human Phase I Study of the Activin A Inhibitor, STM 434, in Patients with Granulosa Cell Ovarian Cancer and Other Advanced Solid Tumors.” Clinical Cancer Research, vol. 25, no. 18, Sept. 2019, pp. 5458–65. DOI.org (Crossref), doi:10.1158/1078-0432.CCR-19-1065.
48. Morvan, Frederic, et al. “Blockade of Activin Type II Receptors with a Dual Anti-ActRIIA/IIB Antibody Is Critical to Promote Maximal Skeletal Muscle Hypertrophy.” Proceedings of the National Academy of Sciences, vol. 114, no. 47, Nov. 2017, pp. 12448–53. DOI.org (Crossref), doi:10.1073/pnas.1707925114.
49. Wentworth, Kelly L., et al. “Therapeutic Advances for Blocking Heterotopic Ossification in Fibrodysplasia Ossificans Progressiva.” British Journal of Clinical Pharmacology, vol. 85, no. 6, June 2019, pp. 1180–87. DOI.org (Crossref), doi:10.1111/bcp.13823.
50. Saitoh, Masakazu, et al. “Myostatin Inhibitors as Pharmacological Treatment for Muscle Wasting and Muscular Dystrophy.” JCSM Clinical Reports, vol. 2, no. 1, Oct. 2017. DOI.org (Crossref), doi:10.17987/jcsm-cr.v2i1.37.
51. Chen, Justin L., et al. “Development of Novel Activin-Targeted Therapeutics.” Molecular Therapy, vol. 23, no. 3, Mar. 2015, pp. 434–44. DOI.org (Crossref), doi:10.1038/mt.2014.221.
52. Makanji, Yogeshwar, et al. “Generation of a Specific Activin Antagonist by Modification of the Activin A Propeptide.” Endocrinology, vol. 152, no. 10, Oct. 2011, pp. 3758–68. DOI.org (Crossref), doi:10.1210/en.2011-1052.
53. Matsakas, Antonios, et al. “Molecular, Cellular and Physiological Investigation of Myostatin Propeptide-Mediated Muscle Growth in Adult Mice.” Neuromuscular

Disorders, vol. 19, no. 7, July 2009, pp. 489–99. DOI.org (Crossref), doi:10.1016/j.nmd.2009.06.367.

54. Qiao, Chunping, et al. "Myostatin Propeptide Gene Delivery by Adeno-Associated Virus Serotype 8 Vectors Enhances Muscle Growth and Ameliorates Dystrophic Phenotypes in Mdx Mice." Human Gene Therapy, vol. 19, no. 3, Mar. 2008, pp. 241–54. DOI.org (Crossref), doi:10.1089/hum.2007.159.
55. Walton, Kelly L., et al. "Activin A–Induced Cachectic Wasting Is Attenuated by Systemic Delivery of Its Cognate Propeptide in Male Mice." Endocrinology, vol. 160, no. 10, Oct. 2019, pp. 2417–26. DOI.org (Crossref), doi:10.1210/en.2019-00257.
56. Oda, S., et al. "Molecular Cloning and Functional Analysis of a New Activin β Subunit: A Dorsal Mesoderm-Inducing Activity in Xenopus." Biochemical and Biophysical Research Communications, vol. 210, no. 2, May 1995, pp. 581–88. DOI.org (Crossref), doi:10.1006/bbrc.1995.1699
57. Sekiyama, Kazunari, et al. "Activin E Enhances Insulin Sensitivity and Thermogenesis by Activating Brown/Beige Adipocytes." Journal of Veterinary Medical Science, vol. 81, no. 5, 2019, pp. 646–52. DOI.org (Crossref), doi:10.1292/jvms.19-0036.
58. Gold, Elspeth, et al. "Activin C Antagonizes Activin A in Vitro and Overexpression Leads to Pathologies in Vivo." The American Journal of Pathology, vol. 174, no. 1, Jan. 2009, pp. 184–95. DOI.org (Crossref), doi:10.2353/ajpath.2009.080296.
59. Olsen, Oddrun Elise et al. "Activins as Dual Specificity TGF-β Family Molecules: SMAD-Activation via Activin- and BMP-Type 1 Receptors." Biomolecules 10.4 (2020): 519. Crossref. Web.
60. Lima, Florence, et al. "Serum Bone Markers in ROD Patients across the Spectrum of Decreases in GFR: Activin A Increases before All Other Markers." Clinical Nephrology, vol. 91, no. 4, Apr. 2019, pp. 222–30. PubMed Central, doi:10.5414/CN109650.
61. Besson-Fournier, Céline, et al. "Induction of Activin B by Inflammatory Stimuli Up-Regulates Expression of the Iron-Regulatory Peptide Hepcidin through Smad1/5/8

Signaling.” Blood, vol. 120, no. 2, July 2012, pp. 431–39. Silverchair, doi:10.1182/blood-2012-02-411470.

62. Shiwen, Xu, et al. “A Role of Myocardin Related Transcription Factor-A (MRTF-A) in Scleroderma Related Fibrosis.” PLOS ONE, edited by Qiang Ding, vol. 10, no. 5, May 2015, p. e0126015. DOI.org (Crossref), doi:10.1371/journal.pone.0126015.
63. Mack, Matthias, and Motoko Yanagita. “Origin of myofibroblasts and cellular events triggering fibrosis.” Kidney international vol. 87,2 (2015): 297-307. doi:10.1038/ki.2014.287
64. Johnson, R J et al. “The activated mesangial cell: a glomerular "myofibroblast"?.” Journal of the American Society of Nephrology : JASN vol. 2,10 Suppl (1992): S190-7. doi:10.1681/ASN.V210s190
65. Fintha, Attila, et al. “Characterization and Role of SCAI during Renal Fibrosis and Epithelial-to-Mesenchymal Transition.” The American Journal of Pathology, vol. 182, no. 2, Feb. 2013, pp. 388–400. DOI.org (Crossref), doi:10.1016/j.ajpath.2012.10.009.
66. Swärd, Karl, et al. “Emerging Roles of the Myocardin Family of Proteins in Lipid and Glucose Metabolism.” The Journal of Physiology, vol. 594, no. 17, Sept. 2016, pp. 4741–52. PubMed Central, doi:10.1113/JP271913.
67. Xu, Huihui, et al. “Myocardin-Related Transcription Factor A Epigenetically Regulates Renal Fibrosis in Diabetic Nephropathy.” Journal of the American Society of Nephrology, vol. 26, no. 7, July 2015, pp. 1648–60. DOI.org (Crossref), doi:10.1681/ASN.2014070678.
68. Uttarwar, L., et al. “HB-EGF Release Mediates Glucose-Induced Activation of the Epidermal Growth Factor Receptor in Mesangial Cells.” American Journal of

Physiology-Renal Physiology, vol. 300, no. 4, Apr. 2011, pp. F921–31. DOI.org (Crossref), doi:10.1152/ajprenal.00436.2010.

69. Haridoss, Srividyameena, et al. "Activin A Is a Prominent Autocrine Regulator of Hepatocyte Growth Arrest." Hepatology Communications, vol. 1, no. 9, 2017, pp. 852–70. Wiley Online Library, doi:https://doi.org/10.1002/hep4.1106.
70. Tsai, Chi-Neu, et al. "Activin A Regulates the Epidermal Growth Factor Receptor Promoter by Activating the PI3K/SP1 Pathway in Oral Squamous Cell Carcinoma Cells." Scientific Reports, vol. 9, no. 1, Mar. 2019, p. 5197. www.nature.com, doi:10.1038/s41598-019-41396-7.
71. Yadin, David et al. "Structural insights into BMP receptors: Specificity, activation and inhibition." Cytokine & growth factor reviews vol. 27 (2016): 13-34. doi:10.1016/j.cytogfr.2015.11.005
72. Hatsell, Sarah J et al. "ACVR1R206H receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A." Science translational medicine vol. 7,303 (2015): 303ra137. doi:10.1126/scitranslmed.aac4358
73. Olsen, Oddrun Elise et al. "BMPR2 inhibits activin and BMP signaling via wild-type ALK2." Journal of cell science vol. 131,11 jcs213512. 11 Jun. 2018, doi:10.1242/jcs.213512
74. Chen, Cheng et al. "The role of the BMP4/Smad1 signaling pathway in mesangial cell proliferation: A possible mechanism of diabetic nephropathy." Life sciences vol. 220 (2019): 106-116. doi:10.1016/j.lfs.2019.01.049
75. Borahay, Mostafa A., et al. "Signaling Pathways in Leiomyoma: Understanding Pathobiology and Implications for Therapy." Molecular Medicine, vol. 21, no. 1, Jan. 2015, pp. 242–56. DOI.org (Crossref), doi:10.2119/molmed.2014.00053.
76. Van Krieken, Richard et al. "Inhibition of SREBP With Fatostatin Does Not Attenuate Early Diabetic Nephropathy in Male Mice." Endocrinology vol. 159,3 (2018): 1479-1495. doi:10.1210/en.2018-00093

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