THE ROLE OF PAK IN MECHANOTRANSDUCTION IN MESANGIAL CELLS

THE ROLE OF P21-ACTIVATED KINASE IN MECHANICAL STRESS-INDUCED CONNECTIVE TISSUE GROWTH FACTOR UPREGULATION IN MESANGIAL CELLS

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ABSTRACT

Glomerulosclerosis (GS) is the irreversible scarring of glomerular tissue which underlies the development of chronic kidney disease (CKD). Increased intraglomerular capillary pressure (P_{gc}) is a major contributor to the development of GS and can occur in both hypertensive and diabetic patients. With elevated P_{gc}, *in vitro* and *in vivo* evidence suggest that mesangial cells (MC) experience cyclic stretch and secrete pro-fibrotic factors such as connective tissue growth factor (CTGF) which contributes to GS. The signaling pathways that are activated in response to elevated P_{gc} and lead to extracellular matrix (ECM) production in MCs are the main focus of this thesis.

Previous data demonstrated activation of the Rho GTPase, Rac1, with cyclic stretch in MCs. Furthermore, the most characterized effector of Rac1, p21-activated kinase (PAK), has been observed to have a role in endothelial cells (ECs) exposed to mechanical stress. We thus proposed that the Rac1-PAK signaling pathway is involved in mechanical stress signaling in MCs.

Our data demonstrate that Rac1-PAK signaling was activated in response to cyclic stretch and required for stretch-induced CTGF production in MCs. RhoA activation was also regulated by Rac1-PAK signaling, and RhoA/ROCK were observed to mediate CTGF upregulation with stretch. Further investigation on the role of Rac1-PAK signaling and how it regulates CTGF in MCs exposed to stretch, will provide insight into potential therapeutic targets to delay the progression of hypertension-mediated CKD.

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LIST OF ABBREVIATIONS

AID – Autoinhibitory Domain

AP-1 – Activator Protein-1

ASO – Antisense Oligonucleotide

BCE-1 – Basal Control Element-1

CKD – Chronic Kidney Disease

CTGF – Connective Tissue Growth Factor

DOCA – Deoxycorticosterone Acetate

EC – Endothelial Cells

ECM – Extracellular Matrix

EMSA – Electrophoretic Mobility Shift Assay

EMT - Epithelial-to-Mesenchymal Transition

ERK – Extracellular signal-regulated kinase

ESRD – End Stage Renal Disease

GAP – GTPase Activating Protein

GDI – Guanine Dissociation Inhibitor

GEF – Guanine Nucleotide Exchange Factor

GFR – Glomerular Filtration Rate

GIT1 – GRK-interacting targets

GRK – G-protein-coupled receptor kinase

GS – Glomerulosclerosis

JNK – c-Jun N-terminal kinase

LPA – Lysophosphatidic Acid

MAPK – Mitogen Activated Protein Kinase

MC – Mesangial Cells

NEMO – NF-κB essential modifier

NF-kB – Nuclear Factor Kappa-light chain-enhancer of activated B cells

PAK – p21-activated kinase

PBD – p21-binding Domain

PDGF – Platelet Derived Growth Factor

PIX – p21-activated kinase interacting exchange factor

P_{gc} – Intraglomerular Capillary Pressure

Rac1 – Ras-related C3 botulinum toxin substrate 1

RhoA – Ras homolog gene family, member A

ROCK – RhoA-kinase

SHR – Spontaneously Hypertensive

SNGFR – Single Nephron GFR

TGF-β – Transforming Growth Factor

UUO – Unilateral Ureteral Obstruction

VSMC – Vascular Smooth Muscle Cells

VWF – Von Willenbrand Factor

Declaration of Academic Achievement

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award for any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement has been made in the text. I performed all experiments except for the RT-PCR analysis, which was performed by our lab technician, Bo Gao.

1. Chronic Kidney Disease

Chronic kidney disease (CKD) is a major health problem worldwide due to its high prevalence, excessive health care costs, and negative impact on life expectancy and quality of life. CKD has been reported to affect between 1.9 and 2.3 million Canadians (Levin *et al.*, 2008). The progression of CKD is a degenerative process that reduces kidney function, ultimately leading to End Stage Renal Disease (ESRD) and the need for renal replacement strategies such as dialysis and renal transplantation. These therapies are very expensive – in 2000, ESRD patients generated health care costs of around $1.3 billion in Canada, with more costs for these patients than any other disease (Zelmer *et al.*, 2007). Therefore, it is imperative to develop therapeutic strategies to delay the progression of CKD and improve long-term outcomes.

1.1 Hypertension – Induced Chronic Kidney Disease

Two universal risk factors for CKD are diabetes and hypertension (Brosnahan *et al.*, 2010). A 15-year follow up study on 12,000 hypertensive individuals in the U.S. provided evidence that uncontrolled blood pressure is a major risk factor for CKD (Udani *et al.*, 2010). There is considerable evidence that hypertension-mediated renal disease can occur as a result of abnormal glomerular hemodynamics. The afferent arteriole regulates blood flow into the glomerulus (Figure 1), and as a protective mechanism against high blood pressure, it vasoconstricts and increases vascular resistance (Bidani *et al.*, 1987). This initial increase in resistance protects the glomerulus from exposure to elevated hydrostatic pressure in hypertensive individuals and ensures glomerular capillary

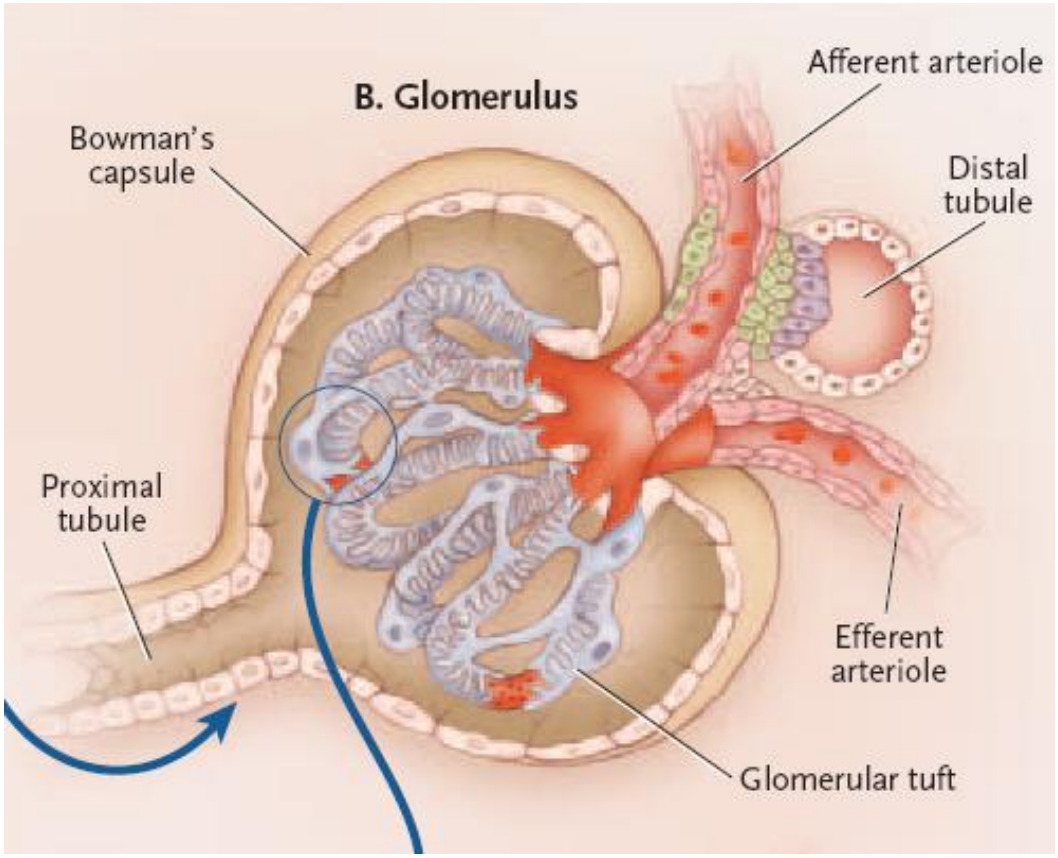

Fig. 1: **Anatomy of the glomerulus.** Figure adapted from Tryggvason *et al.*, 2006

pressure (P_{gc}) is maintained. This autoregulation mechanism is only operational if the blood pressure is within regulatory limits (Anderson *et al.*, 1986). With chronic exposure to elevated blood pressure or during the natural process of aging, vascular remodeling of the afferent arteriole can occur, thereby reducing its ability to regulate glomerular blood flow. This can eventually lead to a rise in P_{gc} and subsequent injury to the "unprotected" glomerulus – a significant event leading to glomerulosclerosis (GS) (Bidani *et al.*, 1987). Similarly, elevated glucose levels in diabetic patients can also disrupt afferent arteriole vasoconstriction which also results in elevated P_{gc}, despite blood pressure within the normal autoregulatory range (Hayashi *et al.*, 1992).

1.2 Role of Elevated P_{gc} in the Progression of Chronic Kidney Disease

Elevated P_{gc} is an important determinant in the progression of CKD by initiating glomerular sclerosis, which has been previously demonstrated in animal models of hypertensive kidney disease (Dworkin *et al.*, 1986). For example, normalizing elevated P_{gc} in uninephrectomized rats using ACE inhibitors was an effective strategy in reducing proteinuria and glomerular lesions. Another model commonly used to mimic hypertensive kidney disease *in vivo* is the rat remnant kidney model (also referred to as the 5/6 nephrectomy model), which is prepared by infarction of two-thirds of the left kidney and removal of the right kidney (Anderson *et al.*, 1986). In this model, there is increased perfusion in the remaining nephrons in order to maintain total kidney glomerular filtration rate (GFR) – a measure of renal function (Anderson *et al.*, 1986; Stevens *et al.*, 2006). Consequently, P_{gc} and single nephron glomerular filtration rate

(SNGFR) are increased and continue to rise with further nephron loss through sclerosis. These rats eventually develop hypertension, proteinuria, and glomerular lesions after 12 weeks, similar to the profile of human CKD (Anderson *et al*, 1986). Normally, the glomerulus, which is comprised of endothelial cells (EC), podocytes, and MCs, is exposed to pulsatile cycles of stretch and relaxation during systole and diastole of the cardiac cycle (Ingram *et al.*, 2000). Therefore, an increase in P_{gc} is transmitted as cyclic mechanical strain to the glomerular cells, most notably the MCs, which results in the deposition and accumulation of ECM within the glomerulus – a key feature of GS (Ishida *et al.*, 1999).

2. Mesangial Cells

Mesangial cells (MC) are microvascular pericytes that constitute the central stalk of the glomerulus and surround the glomerular capillaries, providing architectural support for the capillary tuft (Schlondorff *et al.*, 1987) (Figure 2). Their contractile abilities prevent distension and distortion of the glomerular capillary tuft, and provide another mechanism of GFR regulation (Drenckhan *et al*., 1990; Stockand & Sansom, 1998). Contraction is generated from anchoring filaments bound to the glomerular basement membrane (GBM) at perimesangial angles (area where GBM begins to cover mesangial matrix and MCs instead of ECs) (Figure 3) (Kriz *et al*., 1995). MCs respond to, as well as synthesize, cytokines and growth factors such as TGF-β and PDGF, both of which have been implicated in GS (Choi *et al.*, 1993; Floege *et al.*, 1993).

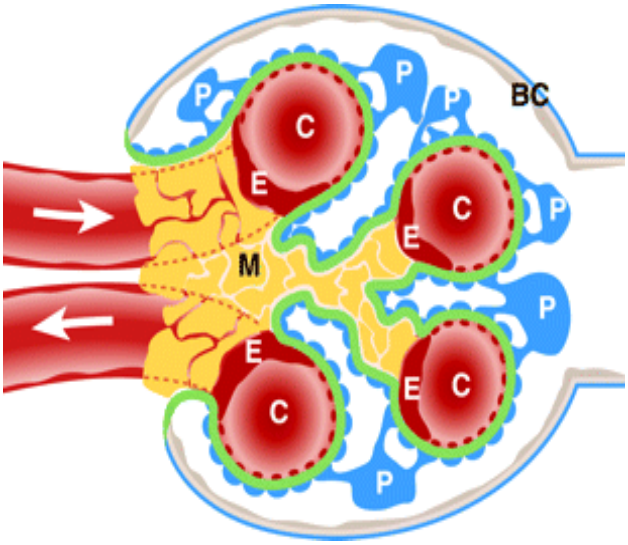

Fig. 2: **Cross-section of the glomerulus.** M = Mesangium; C = Capillaries; E = Endothelial Cells; P = Podocytes; BC = Bowman's Capsule; Figure adapted from Quaggin *et al.*, 2008.

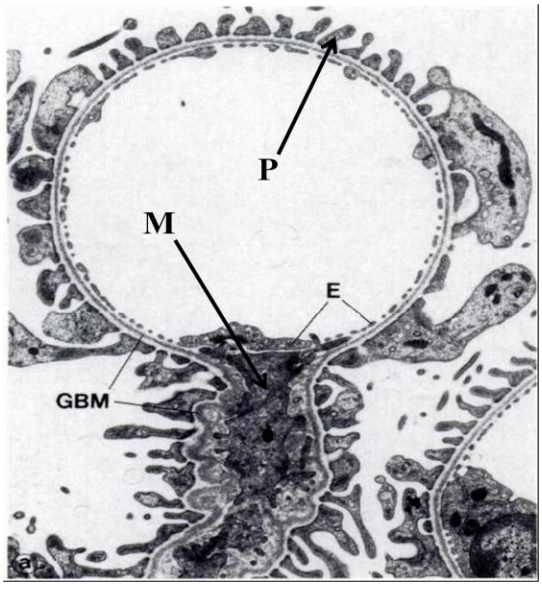

Fig. 3: **Electron microscopic image of the capillary loop.** M = Mesangium; E = Endothelial Cell Processes; P = Podocyte Foot Processes; GBM = Glomerular Basement Membrane; Figure adapted from Kriz *et al*., 1995.

MCs generate and embed in their own ECM, collectively forming the mesangium, which acts as a functionally continuous medium anchoring MCs to the GBM (Kriz *et al*., 1995). The composition and amount of mesangial matrix is tightly controlled in normal glomeruli, but can be markedly altered during disease states such as fibrosis.

2.1 Effects of Mechanical Stress on MCs

The mesangium provides structural stability against the pressure gradient across the glomerular capillary wall (Kriz *et al*, 1995). Since there is no hydrostatic barrier between the mesangium and capillary lumen, intracapillary pressure and its fluctuations can be transmitted to the MCs (Riser *et al*., 2000). By virtue of its location, P_{gc} is transmitted to MCs as mechanical strain in the form of triaxial stretch. It is well established that in response to stretch, MCs proliferate and upregulate ECM components, such as collagens type I and IV, laminin, and fibronectin, perhaps as a protective response to decrease the magnitude of stress on individual cells (Ishida *et al*., 1999; Krepinsky *et al*., 2005; Kriz *et al.*, 1995; Yasuda *et al*., 1996). This accretion of ECM may also be attributed to a decrease in matrix degradation from matrix metalloproteinase inhibition (Yasuda *et al.*, 1996). The excessive deposition of ECM within the glomerulus is a key feature of GS (Ishida *et al*., 1999). These *in vitro* studies parallel what is observed *in vivo* as the aforementioned rat remnant kidney model, which is associated with an increase in P_{gc}, reveals an accumulation of ECM in the mesangial area at 2 weeks from injury (Floege et *al.*, 1992). The signaling pathways that link mechanical stretch with elevated ECM expression have not yet been fully elucidated. Since MCs play an

important role in the progression of CKD, these mechanical stress-activated pathways require further exploration.

2.2 *In Vitro* Mechanical Stress Signaling

A well established method of investigating the effects of mechanical stress *in vitro* is the use of the FlexCell Tension Plus system. Firstly, MCs are grown on flexible-bottom plates coated with an ECM component, such as type I collagen which is used by our lab. Cyclic mechanical strain is then applied by generating a vacuum below the wells, resulting in a non-uniform, biaxial deformation of the base of the plate (Riser *et al*., 1992). This experimental design utilizes cyclic stretch/relaxation phases to reproduce the changes in hemodynamics that MCs experience *in vivo*. In response to mechanical stretch, MCs elongate and align in the direction of the mechanical strain, and actin filaments polymerize and orient along this axis (Cortes *et al.*, 2000). Several signaling pathways have been identified to mediate the stretch-induced production of matrix proteins, especially RhoA and the mitogen-activated protein (MAP) kinases – extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 (Ishida *et al.*, 1999; Krepinsky *et al*., 2003; Zhang *et al*., 2007). Studies have demonstrated that mechanical stress-mediated activation of RhoA and ERK leads to the activation of the transcription factor activator protein-1 (AP-1), which in turn is a key mediator of ECM production (Ishida *et al*., 1999, Krepinsky *et al*., 2003). Furthermore, in response to stretch, MCs exhibit increased transforming growth factor-β (TGF-β) mRNA and protein expression (Hirakata *et al*., 1997). TGF-β is a pro-fibrotic stimulus that leads to the production of ECM proteins and the matricellular proteins thrombospondin-1

(TSP-1) and connective tissue growth factor (CTGF), both of which are implicated in renal diseases (Giehl *et al.*, 2008; Schnaper *et al.*, 2003, Yasuda *et al.*, 1996). These pathways require further investigation to determine how these components are regulated and their contribution to GS.

3. Rho family of GTPases

Several intracellular signaling pathways are activated in response to mechanical stimuli with modifications in RhoGTPase signaling being one example (Chiquet *et al.*, 2009). RhoGTPases are 20 – 30 kDa proteins and classical molecular switches that are able to transduce extracellular signals into downstream signaling pathways (Gao *et al*, 2004). These signaling mediators have been shown to regulate a plethora of processes including cytoskeleton remodeling, cell motility, microvascular permeability, cell cycle progression and have also been implicated in several pathological states including cancer, hypertension, and fibrosis (Tzima *et al.*, 2006; Spindler *et al.*, 2010; Fu *et al.*, 2006; Gao *et al.*, 2004). RhoGTPases cycle between an active, GTP-bound form and an inactive, GDP-bound form (Etienne-Manneville & Hall, 2002). Rho GTPases are regulated by 3 different proteins (Figure 4): Guanine nucleotide exchange factors (GEFs) facilitate GDP for GTP exchange, GTPase activating proteins (GAPs) increase the GTP hydrolytic rate, thereby reducing its activity, and guanine dissociation inhibitors (GDIs) help to maintain Rho proteins in the inactive state (Zhao & Manser, 2005). When bound to GTP,

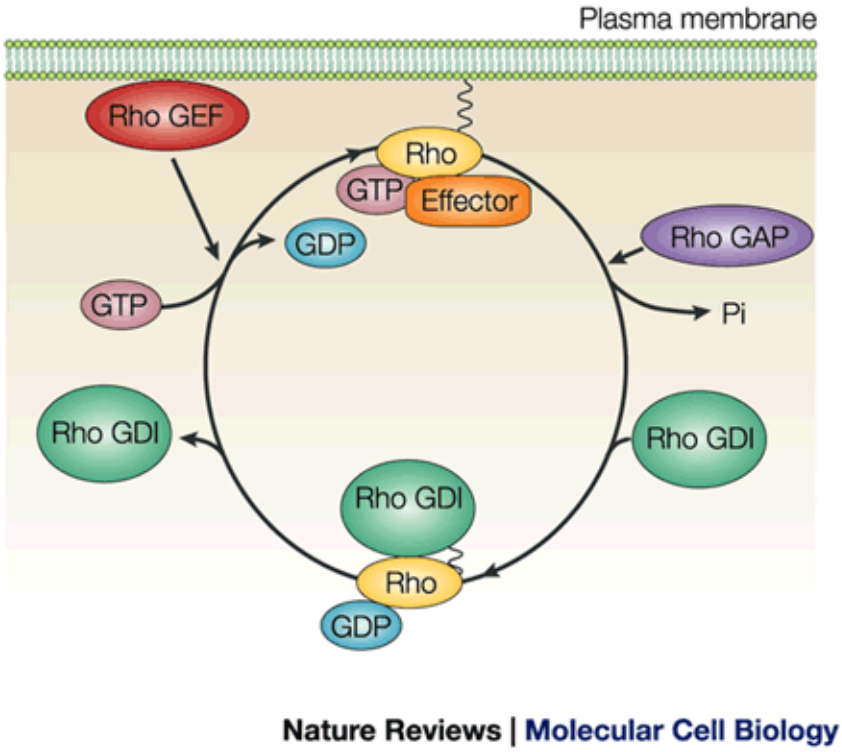

Fig. 4: **Regulation of Rho GTPase family.** GEFs (Guanine Nucleotide Exchange Factor) promote GDP for GTP exchange, GAPs (GTPase Activating Proteins) enhances GTP hydrolysis, and GDIs (Guanine Dissociation Inhibitors) bind and maintain Rho proteins in an inactive state. Figure adapted from Kukata and Kaibuchi *et al.*, 2001.

RhoGTPases are able to act on target proteins until GTP hydrolysis occurs, at which point they then revert back to their inactive GDP-bound form.

3.1 RhoGTPase signaling in MCs

The most characterized members of the Rho family of small GTPases are Rac1, Cdc42, and RhoA. In the context of mechanical stress, both Rac1 and RhoA have been shown to be activated in response to mechanical stress in various cell types including MCs (Krepinsky *et al*., 2003; Qi *et al.*, 2010; Zhang *et al*., 2010). Stretched MCs exhibited RhoA activation which was required for downstream ERK activation and actin stress fiber formation (Krepinsky *et al*., 2003). Inhibition of the well characterized RhoA effector, ROCK (Rho-kinase) with the inhibitor Y27632, also prevented these RhoA-mediated responses. Furthermore, Rac1 was observed to be activated and translocated to the membrane in MCs during stretch, and was also determined to be an important regulator of thrombospondin-1 (TSP-1) and cell spreading (Giehl *et al.*, 2007; Zhang *et al*., 2010). Studies have identified the ability of Rho GTPases to cross-talk between members. Since both RhoA and Rac1 are activated in response to stretch, how these GTPases interact in MCs and what downstream effects they have require further examination.

3.2 Ras-related C3 botulinum toxin substrate 1 (Rac1)

Rac1 plays an important role in many cellular functions including migration, adhesion, proliferation, and reactive oxygen species production as a component of NADPH oxidase (Giehl *et al*., 2007; Qi *et al.*, 2010; Vecchione *et al.*, 2009; Silbert *et al*.,

2008). A majority of these functions can be attributed to its influence on the actin and microtubule cytoskeleton. Studies have shown that Rac1 is activated at the leading edge of migrating cells, where it can promote membrane ruffling and lamellipodia formation through actin rearrangement (Nobes and Hall, 1995; Ridley *et al*., 1992). Similar to RhoA, Rac1 is an important component of mechanotransduction in multiple cell types. Vascular smooth muscle cells (VSMC) exposed to cyclic stretch exhibited Rac1 activation, resulting in enhanced migration and proliferation that were later abrogated with the introduction of a specific Rac1 siRNA (Qi *et al.*, 2010). Vecchione *et al.* (2009) demonstrated augmented Rac1 and NADPH oxidase activity in isolated carotid arteries perfused at high pressures (180 mm Hg). Interestingly, these responses were prevented in arteries overexpressing a dominant negative Rac1 mutant. Our lab has previously demonstrated Rac1 activation in MCs exposed to mechanical stretch which was observed to be dependent on caveolae and EGFR transactivation (Zhang *et al*., 2010). Furthermore, the most characterized effector of Rac1 is p21-activated kinase (PAK), a serine/threonine kinase known to regulate gene expression and mediate cytoskeletal rearrangement through various downstream substrates such as Filamin A and stathmin (Szczepanowska *et al.*, 2009; Vadlamudi *et al.*, 2002; Wittman *et al*., 2004). The different mechanisms by which Rac1 is activated in response to mechanical stress and functions through PAK have not been fully defined in MCs and require further investigation.

3.3 Ras homolog gene family, member A (RhoA)

RhoA is an important regulator of diverse functions including migration, proliferation, and contraction (Etienne-Manneville & Hall, 2002). RhoA has a major

influence on cytoskeletal rearrangement through actin stress fiber and focal adhesion formation (Machesky & Hall, 1997). Downstream effectors of RhoA, including the serine/threonine kinase ROCK, contain a Rho-binding domain that allows GTP-bound RhoA to bind, induce a conformational shift and activation of the effector (Riento & Ridley, 2003; Zhao & Manser, 2005). RhoA/ROCK is implicated in a variety of diseases and inhibition of this signaling cascade has already shown positive effects in renal fibrosis in both cell studies and animal models. MCs pretreated with Y27632 *in vitro* exhibited a decrease in TGF-β-induced fibronectin expression (Peng *et al*., 2008). ROCK inhibition using the drug fasudil was shown to ameliorate GS in Dahl salt-sensitive rats (Nishikimi *et al*., 2004), subtotally nephrectomized, spontaneously hypertensive rats (SHR) (Kanda *et al*., 2003), and DOCA-salt SHR rats (Ishikawa *et al*., 2003) – all models of hypertension-mediated renal disease. In addition to mechanical stress, RhoA is known to be activated by high glucose, which then leads to ECM production *in vitro* (Peng *et al.*, 2008). These data highlight the importance of RhoA in mediating hypertensive-induced CKD and further studies will help to delineate its role in mechanical stress signaling in MCs.

4. p21-activated kinases (PAKs)

PAKs are serine/threonine kinases that function as primary effectors for Rac/Cdc42 GTPases and are involved in many processes involving cytoskeletal rearrangement, such as cell migration, contraction, adhesion, and proliferation (Eppinga *et al.*, 2006; Hinoki *et al.*, 2010). They have also been implicated in regulating gene

expression through the activation of JNK/SAPK and p38 MAP kinase pathways (Zhao *et al.*, 1998). Previous studies have identified several important functions that require PAK, such as the formation of lamellipodia, filopodia, and membrane ruffles, as well as the turnover of actin stress fibers and focal adhesions (Delorme *et al.*, 2007; Manser *et al.*, 1997). In addition, PAK has also been implicated in a variety of pathological processes including tumorigenesis of epithelial cells (Arias-Romero *et al.*, 2010), inflammatory gene expression and activation of the proinflammatory transcription factor NF-kB in ECs (Funk *et al.*, 2010), and vascular remodeling through altered VSMC migration and proliferation (Hinoki *et al.*, 2010). Recent data suggest a role of PAK in mechanical stress signaling, but its expression and function in MCs has yet to be studied.

4.1 PAK Isoforms

The PAK family has been conserved throughout evolution, with six isoforms expressed in humans (Kumar *et al.*, 2009). Different combinations of PAK isoforms homologous to human isoforms are expressed in lower organisms such as yeast, drosophila, and C. elegans. Mammals such as mice have been shown to express all six isoforms of PAK (Molli *et al.*, 2009). Mammalian PAKs are classified into two groups: Group 1 (PAK1, 2, 3 or αPAK, γPAK, βPAK) and Group 2 (PAK4, 5, 6) (Szczepanowska *et al.*, 2009). Each isoform is differentially expressed in mammalian tissues. PAK1 is found to be highly expressed in the brain, muscle, and spleen; PAK2 is ubiquitously expressed; PAK4 is present predominantly in the prostate, colon, and testis; PAK6 is expressed predominantly in the testis, prostate and brain; PAK3 and 5 are

specifically expressed in the brain. A study by Zhu *et al.* (2010) confirmed that PAK1/2 protein and mRNA, but not PAK3, are significantly expressed in the rat glomerulus. PAK has not been identified specifically in MCs and its expression will need to be confirmed in this cell type.

4.2 PAK Structure

Group 1 PAKs have been more characterized in terms of regulation and downstream targets compared to Group 2 PAKs. Group 1 PAKs contain an N-terminus that consists of an autoinhibitory domain (AID) connected with the p21 (Rac/Cdc42) binding domain (PBD) (Nikolic *et al.*, 2008) (Figure 5). The PBD domain is a crucial domain for Rac and Cdc42 binding as it contributes to their overall binding affinity between PAK and these GTPases (Bokoch *et al.*, 2003). The AID associates with the C-terminal catalytic domain and prevents kinase activation. PAK1, 2, and 3 share at least 88% sequence homology within their N-terminal regions, and at least 93% within their C-terminal catalytic domains which suggests they act on similar substrates (Arias-Romero *et al*., 2008). In addition to one common non-canonical SH3-binding site for PIX (PAK-interacting exchangers) family proteins, PAK isoforms differ in the number of proline-rich domains that bind to adaptor proteins via their SH3 domains. PAK1 contains five

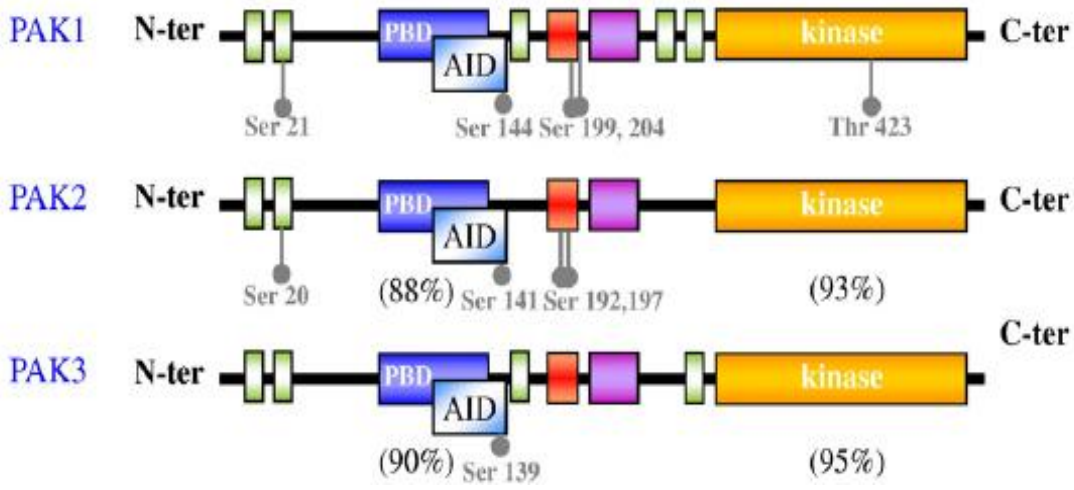

Fig. 5: **Structural features of Group 1 PAKs.** The p21-binding domain (PBD) is associated with the autoregulatory domain (AID). White bars represent proline-rich residues that bind to adaptor proteins containing SH3 domains. Phosphorylation sites are indicated, as well as the percentage of homology in their respective domains compared to PAK1. Figure adapted from Galan-Moya *et al.*, 2009.

proline-rich, SH3-binding motifs, whereas PAK2 and PAK3 contain two and four respectively (Bokoch *et al*., 2003). These motifs enable binding to the adaptor proteins, such as Nck and Grb2 which contribute to PAK membrane translocation (Galisteo *et al*., 1996). PIX proteins are Rac- and Cdc42-specific GEFs, which bind and localize PAK to focal adhesions at the plasma membrane and influence activation (Bokoch *et al.*, 2003; Mott *et al.*, 2005).

4.3 PAK Regulation

PAK is regulated by multiple inputs including the GTPases, Rac1, Rac2, Rac3, Cdc42, calcineurin homologous protein (CHP), TC10, and Wnt-1 responsive Cdc42 homolog-1 (Wrch-1) (Li *et al*., 2010). It is well established that Rac1 and Cdc42 are primary regulators of PAK. Upon activation by these GTPases, PAK undergoes a conformational shift exposing the catalytic domain and subsequent autophosphorylation at several sites (mainly serine and threonine residues) that sustain PAK activation (Chong *et al*., 2001) (Figure 6). The Thr423 residue located in the catalytic domain and exposed upon activation is required for full kinase activation and substrate recognition. Other residues including Ser144 (PAK1) and Ser199 (PAK1) are known autophosphorylation sites that are located in the N-terminus of PAK and proposed to prevent PAK from reverting to its inactive configuration (Chong *et al.*, 2001; Lei *et al.*, 2000). This family of kinases can also be activated through several GTPase-independent mechanisms. PAKs can be regulated by interaction with adaptor proteins, such as Nck and Grb2, through proline-rich motifs found on PAK (Galisteo *et al*., 1996). Binding to Nck has been shown to localize PAK to the plasma membrane, where PAK activation can occur by other

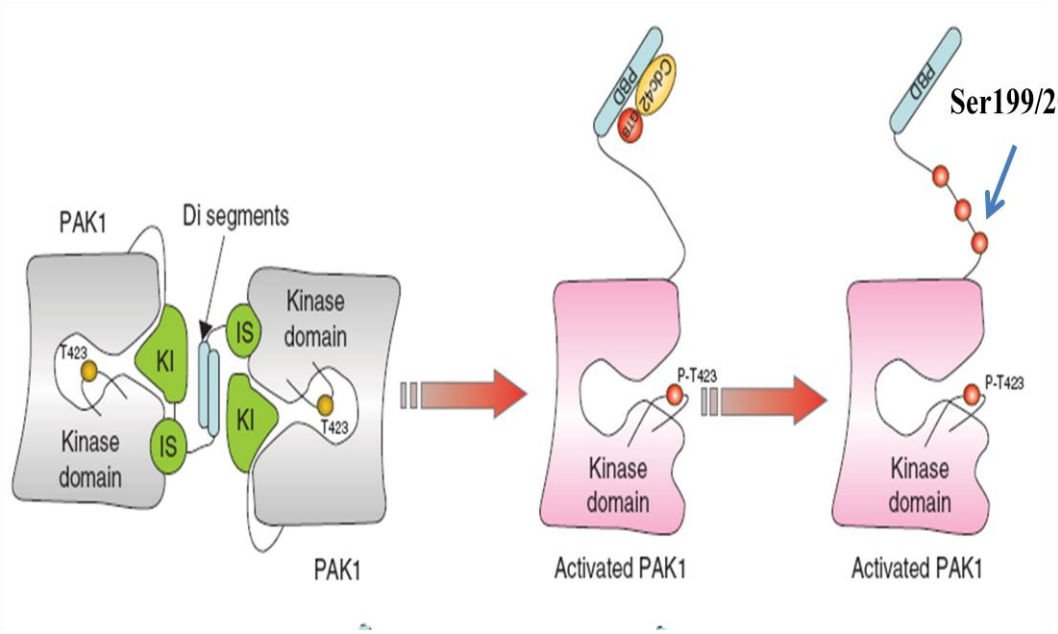

Fig. 6: **Group 1 PAK mechanism of activation.** Inactive PAK exists as a dimer in a trans-inhibited configuration. Once bound to Rac/Ccdc42 GTPase, PAK undergoes a conformational change as a result of autophosphorylation of specific residues. The AID (KI/IS) is released from the C-terminal kinase domain, exposing the kinase loop and promoteing the phosphorylation of the critical residue Thr423. Figure adapted from Li *et al.*, 2010.

PAK monomers, sphingosine and sphingosine-derived lipids or other kinases, such as PDK1 and Akt (Parrini *et al*., 2009; Chong *et al*., 2001; Bokoch *et al*., 1997). PIX, a Rac and Cdc42-specific GEF, can interact with and activate PAK1 independent from, but synergistic with, Rac or Cdc42 binding. PIX mediates PAK localization to focal adhesions via G-protein-coupled receptor kinase (GRK)-interacting targets (GIT1) and promotes PAK activation and focal complex disassembly (Mott *et al.*, 2005; Zhao *et al*., 2000). Inhibition of the association between PAK and PIX using a TAT-fusion protein was sufficient in inhibiting PAK-dependent ERK activation in ECs (Stockton *et al*., 2007). How PAK is regulated in MCs or other cell types exposed to stretch is not clearly known.

4.4 Role of PAK in Mechanical Stress & Kidney Processes

PAK can be activated by a variety of different stimuli including growth factors, cytokines and extracellular stressors (Chan *et al.*, 1999; Dharmawardhane *et al.*, 1997; Woolfolk *et al.*, 2005). In particular, mechanical stress has been demonstrated to influence PAK activity. ECs exposed to laminary and oscillatory flow exhibited an increase in PAK activation which was required for downstream NF-κB activation (Orr *et al.*, 2008). This was similarly seen *in vivo* where regions of arteries exposed to disturbed flow displayed enhanced PAK and NF-κB activation. Injection of a PAK inhibitory peptide decreased NF-κB nuclear translocation at these sites of disturbed flow. Another study demonstrated PAK1/2 expression in the rat glomerulus and their ability to regulate F-actin assembly in rat podocytes (Zhu *et al*., 2010). After transfection of a constitutively active PAK (PAKT423E), decreased actin stress fibers and increased cortical F-actin in

proximity to elongated cellular processes were observed. Conversely, transfection with a kinase-dead PAK mutant (K299R) or a PAK inhibitory construct (PID) led to an increase in stress fibers and decrease in cortical F-actin. Furthermore, PAK activation was also observed in human proximal tubular epithelial cells undergoing myofibroblastic transdifferentiation induced by activated PBMC medium (Patel *et al*., 2005). These studies suggest a potential role for PAK in mechanical stress-induced signaling in MCs, as well as renal fibrosis due to its involvement in pathological processes such as vascular remodeling, inflammation, and myofibroblastic transdifferentiation (Funk *et al.*, 2010; Hinoki *et al.*, 2010).

5. Connective Tissue Growth Factor (CTGF)

CTGF (also referred to as CCN2) is a member of the CCN family of matricellular proteins that interact with the ECM and modulate cellular function (Zhang *et al.*, 2007). This family, named after the first 3 members (cyr61, CTGF, and nov), consists of six members that share a multimodular domain structure. All CCN family members possess a secretory signal peptide at the N-terminus and are abundant in cysteine residues (Gupta *et al*., 2000). CTGF is a heparin-binding protein containing 348 amino acids with an apparent molecular weight of 36 – 38 kDa. The structure of CTGF is comprised of four modules: an insulin-like growth factor-binding domain, a von Willenbrand factor (VWF) type C domain, a thrombospondin type 1 repeat domain, and a C-terminal cysteine knot, heparin-binding domain (Phanish *et al*., 2010). CTGF promotes some of its downstream

functions through binding with integrins and proteoglycans, and has been shown to play a role in adhesion, migration, angiogenesis, apoptosis, and matrix production (Crean *et al*., 2002; Gupta *et al*., 2002; Leask *et al*., 2006). Physiologically, CTGF is important for wound repair, but dysregulation of CTGF can lead to the development of fibrosis (Blom *et al.*, 2001; Igarashi *et al*., 1993).

5.1 CTGF Regulation

CTGF is primarily regulated at the transcription level where it can be induced by multiple stimuli including TGF-β and high glucose in MCs (Chen *et al.*, 2002; Murphy *et al.*, 1998). Similarly, mechanical stress has also been shown to induce CTGF expression in MCs (Riser *et al*., 2000). The promoter sequence for CTGF contains various motifs that allow regulation by diverse proteins and transcription factors. These domains include the Smad-binding site, basal control element-1 (BCE-1), Ets-1-binding element, Sp1 site, and an activator protein-1 (AP-1) binding site (Leask *et al.*, 2006; Phanish, Winn & Dockrell, 2010; Xia *et al.*, 2007). The pro-fibrotic cytokine, TGF-β, a major contributor to GS, is the primary upstream regulator of CTGF and has been shown to require the Smad binding site in MCs and fibroblasts for CTGF induction (Chen *et al.*, 2002; Riser *et al*., 2000; Leask *et al*., 2003). Although the Smad binding site is required for basal expression of CTGF, Smad proteins do not directly bind to their respective binding domain, but instead recruit additional transcription factors to this site (Chen *et al*., 2002). Moreover, BCE-1 and Sp1 sites are proposed to be involved in basal expression of CTGF in fibroblasts (Leask & Abraham, 2006). The BCE-1 site mediates CTGF expression

upon interaction with the Ras/MEK/Erk pathway in response to endothelin-1. The last domain, the AP-1 binding site, is required for CTGF gene expression in response to serum stimulated keloid fibroblasts (Xia *et al.*, 2007). Several studies recognize the ability of mechanical stress to induce CTGF expression, but the underlying mechanisms involved in regulating CTGF in MCs are not well defined.

5.2 RhoGTPases in CTGF regulation

The mechanism in which extracellular stimuli, such as mechanical stress, can regulate CTGF expression can occur through RhoA and Rac1. RhoA has been reported to be important for the basal and stimulated levels of CTGF expression in MCs *in vitro* (Goppelt-Struebe *et al.*, 2001). Using Rho inhibitors such as statins and toxin B, Rho proteins were found to be required for CTGF mRNA expression in MCs treated with lysophosphatidic acid (LPA) (Rho activator) and TGF-β, yet no studies have linked RhoA and CTGF in response to mechanical stress (Hahn *et al.*, 2000, Goppelt-Struebe *et al.*, 2001). Rac1 is also proposed to be a contributor to CTGF regulation. Xu *et al.* (2009) observed that Rac1 inhibition in fibroblasts extracted from fibrotic lesions of scleroderma patients, markedly reduced CTGF mRNA and protein levels compared to control lesions. Furthermore, in human left atrial tissue, angiotensin II-stimulated CTGF production in neonatal cardiomyocytes and fibroblasts was dependent on Rac1 activity (Adam *et al.*, 2010). Despite the prominent role of Rac1 in regulating CTGF, the role of its downstream effector, PAK has not been studied. There is evidence that PAK can mediate cross-talk between the Rho GTPases, Rac1 and RhoA, through the phosphorylation of GEFs that

can modulate RhoA activation and therefore CTGF expression (Zenke *et al*., 2004). PAK mediated JNK/AP-1 activation downstream of Rho GTPase signaling in HeLa cells undergoing an inflammatory response (Naumann *et al*., 1998). Since CTGF contains an AP-1 binding site as previously identified in fibroblasts by Xia *et al.* (2007), the role of PAK and in regulating mechanical stress-induced CTGF expression is probable but whether this occurs in MCs needs to be evaluated.

5.3 Role of CTGF in CKD

There are considerable data that support a role for CTGF specifically in the development of kidney fibrosis. High expression levels of CTGF have been discovered in the glomeruli of patients with IgA nephropathy, diabetic nephropathy, and diffuse lupus nephropathy, suggesting a potential role of CTGF in renal fibrosis *in vivo* (Ito *et al.*, 1998). CTGF was upregulated in MC proliferative lesions, mainly in patients with diabetic nephropathy and IgA nephropathy. In the rat remnant kidney model, there was an increase in CTGF mRNA in interstitial fibroblasts at 4 weeks (Frazier *et al.*, 2000). Human MCs exhibited increased CTGF protein expression when treated with high glucose and TGF-β, which subsequently enhanced the expression of the ECM proteins, fibronectin and collagens I/IV (Murphy *et al.*, 1998). Interestingly, previous studies have demonstrated that long-term exposure of MCs to cyclic mechanical strain (4 hours) and high static pressure (48 hours) induced CTGF mRNA expression, and subsequent accumulation of ECM components (Riser *et al.*, 2000; Hishikawa *et al.*, 2001). Thus far, a few strategies have been employed to investigate the effects of inhibiting CTGF in

animal models of renal disease. Injection of a CTGF antisense oligonucleotide (ASO) into rats that underwent unilateral ureteral obstruction (UUO) showed significantly reduced expression of CTGF, fibronectin, and α1 (I) collagen within the interstitium (Yokoi *et al*., 2004). Furthermore, CTGF ASOs reduced CTGF levels, as well as proteinuria and albuminuria in streptozotocin-induced diabetic mice (Guha *et al*., 2007). These data suggest that CTGF plays an important role in mediating fibrosis *in vitro* and *in vivo*. In particular, there seems to be a strong correlation between mechanical stress and CTGF expression. Therefore, identifying key mediators of CTGF expression in response to mechanical stress can lead to the identification of novel therapeutic targets to delay the progression of GS and the resultant decline in renal function.

RATIONALE, HYPOTHESIS AND OBJECTIVES

Elevated P_{gc} has been shown to be an important contributor to GS in several animal models of CKD (Anderson *et al.*, 1986; Dworkin *et al.*, 1986). CTGF has been identified in a number of renal diseases as well, such as diabetic nephropathy and IgA nephropathy (Ito *et al.*, 1998). MCs exposed to cyclic stretch *in vitro* results in enhanced production of ECM proteins and other components associated with fibrosis, including CTGF (Choi *et al.*, 1993; Ishida *et al*., 1999; Krepinsky *et al*., 2005; Riser *et al.*, 2000; Hishikawa *et al.*, 2001). Therefore, further investigation of the mechanisms in which mechanical stress leads to CTGF upregulation in MCs will allow us to identify potential therapeutic targets to delay the progression of renal disease. Our lab has previously demonstrated Rac1 activation in response to stretch. Furthermore, its main downstream effector, PAK, has been implicated in mechanical stress signaling in ECs and is associated with pathological processes in VSMCs, such as enhanced migration and proliferation (Funk *et al.*, 2010; Hinoki *et al.*, 2010). Therefore, we hypothesized that Rac1-PAK regulates stretch-induced CTGF upregulation through RhoA/ROCK signaling.

<u>The following questions were addressed in this thesis:</u>

1) Is Rac1-PAK activated in response to mechanical stress?
2) Is stretch-induced CTGF upregulation dependent on Rac1-PAK signaling?
3) How does Rac1-PAK regulate stretch-induced CTGF upregulation?

MATERIALS AND METHODS

Mesangial Cell Culture

Glomeruli were obtained from Sprague-Dawley rats by differential sieving to remove tubules. Primary rat MCs were obtained by selection from glomerular outgrowths and were characterized by their stellate appearance, rapid outgrowth in restricted medium and through immunoflourescence staining, which demonstrated the presence of vimentin and absence of Von-Willenbrand factor and cytokeratins. MCs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) supplemented with 20% fetal bovine serum (FBS) (Invitrogen), streptomycin (100 μg/ml) (Invitrogen) and penicillin (100 units/ml) (Invitrogen) at 37°C in 95% air, 5% CO_2. Cells between passages 10 and 19 were used. For all experiments, cells were serum deprived at confluence with DMEM containing 0% FBS and 1% penicillin/streptomycin for 24 hours and pre-treated with various pharmacological inhibitors.

Pharmacological Inhibitors

Drug	Company/Catalogue #	Function	Dose
Curcumin	Sigma/C1386	AP-1 inhibitor	10 μM
IPA3	Tocris/3622	Group 1 PAK inhibitor	30 μM
NSC23766	Calbiochem/553502	Rac1 inhibitor	100 μM
PAK18	EMD/506101	PIX inhibitor	10 μM
Y27632	Calbiochem/688000	ROCK inhibitor	20 μM

Application of stretch/relaxation

MCs were plated onto 6 well plates with flexible, silicon bottoms that were coated with rat tail type I collagen (Sigma, BF-3001C). Cells were grown to confluence and then rendered quiescent by incubation for 24 h in serum-free medium (Hyclone). The flexible membrane in each well was exposed to continuous cycles of stretch/relaxation that were generated by a cyclic vacuum controlled by the computer driven system, Flexcell Tension Plus system, FX-4000T (Flexcell International Corporation), with each cycle being 0.5 s of stretch and 0.5 s of relaxation, for a total of 60 cycles/min (Figure 7). Experiments were performed at a vacuum pressure of -20 kPa which generates an average of 10% elongation along the surface diameter as previously performed (Peng *et al.*, 2010; Awolesi *et al.*, 1995). Stretch of 9.1% was previously demonstrated to induce approximately 13.3% average stretch over each well's surface area (Gilbert *et al.*, 1994). It has been estimated in glomeruli from the rat remnant kidney model that elevated P_{gc} can expand glomerular volume by 12 – 14% (Cortes *et al.*, 1994). Therefore, the stretch parameters in these *in vitro* studies approximate *in vivo* conditions. MCs were stretched for different time intervals between 30 s and 6 hours. Control wells were isolated with reusable rubber stoppers (Flexcell Intl. Corp.) to prevent stretching.

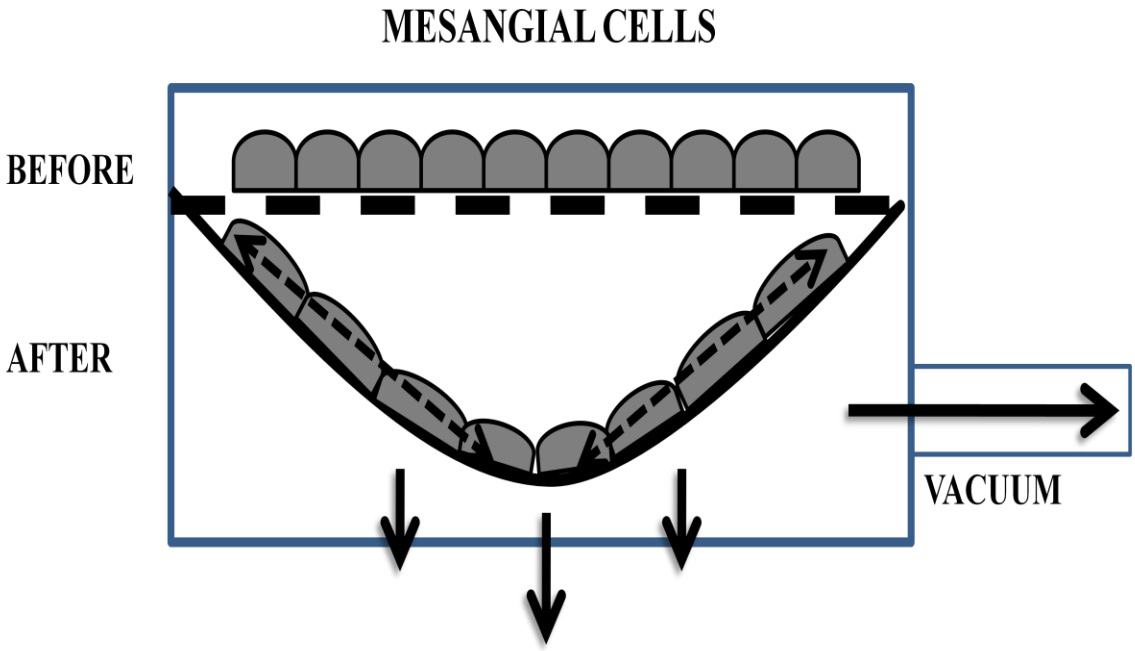

Fig. 7: **Schematic of MCs undergoing mechanical strain using *in vitro* model.** MCs seeded on 6 well plates with silicon bases are placed onto a sealed base plate. A vacuum is generated by a computer-driven system underneath the well, causing a cyclic deformation of the membrane and inducing strain on MCs

Protein Extraction/Western Blots

MCs were lysed using cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 2 mM DTT, 1 mM sodium vanadate, and protease inhibitors added prior to use: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 2 μg/ml aprotinin. Cell lysates were centrifuged at 4°C, 14,000 rpm for 10 min to pellet cell debris. The supernatant containing soluble proteins was equalized for protein concentration using Bio-Rad assay reagent (BioRad), diluted in 5X loading sample buffer, boiled for 5 min, and separated on SDS-PAGE using the Laemlli method. After transfer, nitrocellulose membranes (Amersham, RPN303D) were blocked for 1 h at room temperature with 5% nonfat dry milk in TBST (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween-20). After three washes with TBST, membranes were incubated overnight at 4°C with the primary antibody in 5% bovine serum albumin and TBST. Antibodies used were monoclonal anti-Rac1 (1:500; Cytoskeleton), monoclonal anti-RhoA (1:500; Santa Cruz), monoclonal anti-β-actin (1:5000; Sigma), monoclonal anti-α-Tubulin (1:2000; Sigma), polyclonal anti-phospho-PAK1(Ser199/204)/PAK2(Ser192/197) (1:1000; Cell Signaling), polyclonal anti-PAK1 (N-20) (1:5000; Santa Cruz), polyclonal anti-PAK2 (1:5000; Cell Signaling), polyclonal anti-PAK3 (1:5000; Cell Signaling), and polyclonal anti-CTGF (1:5000; Santa Cruz). Membranes were washed three times with TBST and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase with 5% nonfat dry milk in

TBST for 1.5 h. Membranes were washed again four times in TBST and target protein was detected by enhanced chemiluminescence (Millipore, WBKL50500).

Rho Activity Assay

MCs were lysed within five minutes of adding Rho lysis buffer containing 50 mM Tris-HCl (pH 7.2), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 60 mM n-octyl-glucopyranoside, and protease inhibitors added prior to use: 100 mM PMSF, 10 μg/ml aprotinin, and 2 μg/ml leupeptin. Lysates were cleared of cellular debris by centrifugation at 14,000 rpm for 2 min at 4°C. GTP-bound RhoA (active) was immunoprecipitated by adding supernatant to 20 μg of glutathione-agarose bound, GST-tagged rhotekin Rho binding domain (GST-RBD) (Cytoskeleton, RT01-A), and placed in 4°C for 45 min with gentle rocking. Beads were collected by centrifugation at 14,000 rpm for 30 s at 4°C and an aliquot of supernatant was collected for total RhoA levels to be run later on SDS-PAGE. Beads were washed three times in 500 μl of RhoA wash buffer containing 50 mM Tris (pH 7.2), 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Beads were resuspended in 25 μl of 2X loading sample buffer, boiled for 5 min, immediately vortexed for 5 s, and supernatant was separated on 15% SDS-PAGE, along with total RhoA samples. Monoclonal anti-RhoA antibody (1:500; Santa Cruz) was used to detect pulled-down and total RhoA.

Rac1 Activity Assay

Cells were lysed within five minutes of adding Rac lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 300 mM NaCl, 2% NP-40 and protease inhibitors added prior to use: 100 mM PMSF, 10 μg/ml aprotinin, and 2 μg/ml leupeptin. Lysates were cleared of cellular debris by centrifugation at 14,000 rpm for 2 min at 4°C. GTP-bound Rac1 was immunoprecipitated by adding supernatant to 15 μg of glutathione-agarose bound, GST-tagged p21-activated kinase binding domain (GST-PAK) (Cytoskeleton, PAK02-A) at 4°C for 1 h with gentle rocking. GST-PAK binds active forms of Rac and Cdc42, bound to GTP. Beads were collected by centrifugation at 14,000 rpm for 30 s at 4°C, an aliquot of supernatant was collected for total Rac1 levels, and beads were washed three times in 500 μl buffer containing 25 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$, 40 mM NaCl and protease inhibitors added prior to use: 100 mM PMSF, 10 μg/ml aprotinin, and 2 μg/ml leupeptin. Beads were resuspended in 25 μl of 2X loading sample buffer, boiled for 5 min, and supernatant separated on 15% SDS-PAGE. Polyclonal anti-Rac1 antibody (1:500; Cytoskeleton) was used to detect pulled-down and total Rac1.

Nuclear Extraction

MCs were washed in cold PBS three times. Nuclear extracts were prepared by lysis in cold hypotonic lysis buffer for ten minutes (**Buffer A**: 20 mM Hepes (pH 7.9), 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 0.6% Nonidet P-

40). Cell lysates were homogenized using Dounce homogenizer (Wheaton USA) and sedimented at 14,000 rpm for 30 min at 4°C. Pelleted nuclei were resuspended in 12 µl of resuspension buffer (**Buffer B**: hypotonic Buffer A with 0.42 M $NaCl_2$ and 20% glycerol). The pellet was further resuspended using a pipette tip and rotated for 1 h at 4°C. Samples were centrifuged at 14,000 rpm for 20 min at 4°C and supernatant containing nuclear proteins was collected for calculation of protein concentration using Bio-Rad assay reagent. Equal amounts of nuclear extracts were stored in -80°C without the addition of 5X loading sample buffer for the following EMSA experiments.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were incubated (3 – 5 µg) with 10 fmol of biotin-labeled AP-1 consensus oligonucleotide (Sigma, A9590) for 30 min at room temperature as per LightShift Chemiluminescent EMSA Kit (Pierce, 20148). The reaction mixtures were applied onto a 6% non-denaturing polyacrylamide gel (prepared overnight) for 1.5 h. Samples were then transferred for 1 h to a Nylon membrane (Amersham) and cross-linked using UV-light cross-linker (Spectronics Corp.). Biotin-labeled DNA was detected using a streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate (Pierce).

PAK Activity Assay

MCs were washed with PBS three times and were lysed in Co-IP buffer (50 mM Tris-HCl (pH 7.5), 15 mM EGTA, 100 mM NaCl, 0.1% Triton X-100, 10% Glycerol, 1

mM DTT, 1 mM sodium pyrophosphate, 10 mM NaF, 10 mM sodium pyrophosphate, 10 μg/ml aprotinin, and 2 μg/ml leupeptin) containing 60 mM n-octyl-glucopyranoside for 10 minutes. Cell lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and protein concentration was calculated using Bio-Rad assay reagent (BioRad). Equal amounts of lysate were incubated with 1 μg of polyclonal anti-PAK1 (N-20) antibody (Santa Cruz) and rotated overnight at 4°C. The following day, 20 μl of protein G-agarose slurry (Invitrogen, 15920-010) was added for 1.5 hours at 4°C. Immunobeads bound to PAK1 were pelleted by centrifugation at 4,000 rpm for 1 min at 4°C, and were washed three times with cell lysis buffer and two times with 1X kinase buffer (50 mM HEPES/NaOH (pH 7.5), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM DTT). PAK activity was assayed by incubating the immunobeads with 1X kinase buffer containing 0.5 μg/reaction His-tagged stathmin (Novus Biologicals, NBC1-21276), 200 μM ATP (Cell Signaling, 9804), and 0.2 mM DTT. Reaction mixtures were incubated in a thermomixer for 30 min at 30°C. Samples were then placed on ice and the reaction was stopped by adding 5X loading sample buffer. After boiling for 5 min, samples were then immediately vortexed and briefly centrifuged. The supernatant was run on a 15% SDS-PAGE and probed for anti-phospho-stathmin (Ser16) (Cell Signaling, 3353).

RNA Extraction/Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

MC RNA was extracted using Trizol (Life Technologies BRL) and 2 μg was reverse-transcribed with Superscript II (Life Technologies). The resulting cDNA was used for semi-quantitative PCR amplification of PAK1, 2, and 3. The primer set was

previously used by Zhu *et al.* (2010). PAK1: forward, ggctccttgacagatgtggt; reverse, tgtccctgtgaatgacttgg; PAK2: forward, aacctttgccttctgttcca; reverse, aacatggatggtgtgctcaa; PAK3: forward, tcattgcaccaagaccagag; reverse, gtggtggaattggcattctc. RT products were mixed with PCR buffer containing PAK primers, Taq DNA polymerase, dNTPs, and ddH_2O. Reactions were carried out for 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, with a 10-min final extension at 72°C. PCR products were run on 2% Agarose gel containing ethidium bromide and TAE buffer.

Statistical Analysis

All experiments were repeated at least three times and representative results are shown. Data was quantitated using imaging densitometry with the Scion imaging software. Data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using ANOVA for experiments containing 2 or more groups or time points, and Tukey's post hoc analysis was used to assess which groups were significantly different from one another. Results were considered statistically significant if $P < 0.05$.

RESULTS

Stretch-induced Rac1 activation requires Rac GEFs Tiam-1 and TrioN

To further investigate the role of Rac1 in mechanical stress signaling, we used the only Rac1 specific inhibitor available, NSC23766, which binds and inhibits Rac1 activation by preventing its interaction with two specific GEFs, Tiam-1 and TrioN (Gao *et al.*, 2004). The GEFs that regulate Rac1 activation in our cell system are unknown, so we first tested the effectiveness of NSC23766 to inhibit stretch-induced Rac1 activation. To detect Rac1 activation, a Rac activity assay was performed which uses a fusion protein containing the PBD domain of PAK (site where Rac1 and Cdc42 binds) and enables the pull down of activated, GTP-bound Rac. MCs pre-treated with NSC23766 demonstrated reduced Rac1 activity after 5 min of stretch (Figure 8). This result validated the effectiveness of NSC23766 as a Rac1 inhibitor and therefore allowed us to use this pharmacological inhibitor in subsequent experiments.

Group 1 PAK mRNA and protein are expressed in MCs

To determine PAK1 – 3 mRNA expression in MCs, we used the same DNA primers as Zhu *et al.* (2010) and performed RT-PCR, as well as Western Blot to evaluate the expression of PAK1-3 protein levels. Our results demonstrate that Group 1 PAK mRNA and proteins are expressed in MCs (Figure 9A and B).

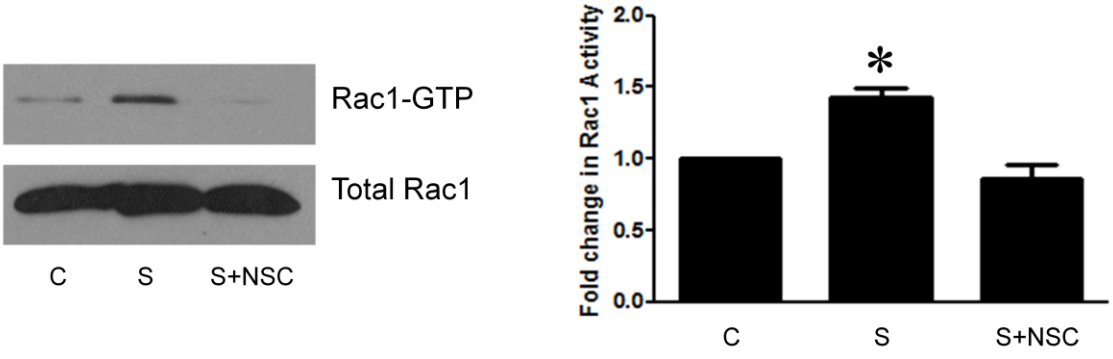

Fig. 8: **Mechanical stretch-induced Rac1 activation is regulated by RhoGEFs, Tiam-1 and TrioN.** MCs were stretched for 5 min with and without pre-treatment with NSC23766 (Rac1-specific inhibitor; 100 μM, 30 min). Activated, GTP-bound Rac1 was pulled-down using GST-PAK beads and Western Blot was performed. Rac1 levels were quantified by densitometry. Data are representative of four independent replicates. *P < 0.05.

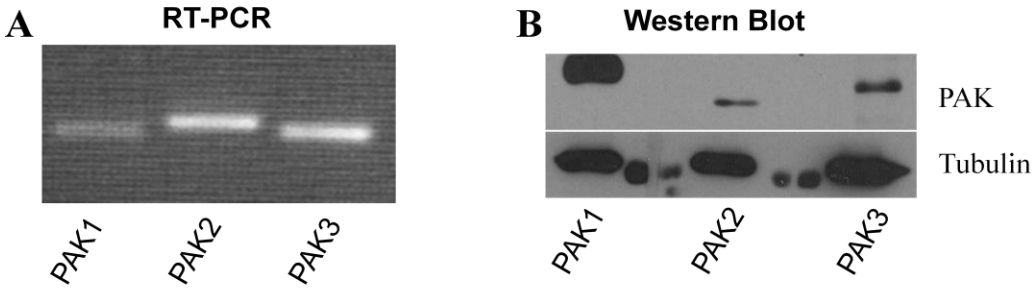

Fig. 9: **Expression of PAK isoforms in rat MCs**. A: mRNA transcript expression of various PAK isoforms. RT-PCR was performed and PAK1, 2, 3 mRNA levels were detected at 35 cycles with an annealing temperature of 41°C. B: Protein expression of PAK isoforms. 50 μg of whole cell lysates of cultured rat MCs were analyzed by immunoblotting.

PAK is phosphorylated in response to mechanical stretch

To determine if PAK is activated with stretch, MCs were stretched and PAK activity was initially assessed by its phosphorylation status. We initially attempted to measure PAK1 phosphorylation through IP/IB, where PAK1 was pulled-down with an anti-PAK1 antibody and IB with an anti-phospho-Serine/Threonine antibody. An anti-phospho-Ser/Thr antibody was used because the activation process of PAK involves the autophosphorylation of serine and threonine residues (Chong *et al.*, 2001). We were unable, however, to detect clear phosphorylation. We next used a more specific antibody that targets the phosphorylation sites, Ser199/204 (PAK1)/Ser192/197 (PAK2). This antibody has been used by several studies as a marker for PAK activation (Woolfolk *et al.*, 2005; Harfouche *et al*., 2005; Zhu *et al*., 2010; Hinoki *et al*., 2010). This PAK antibody is able to detect PAK1 and 2, and possibly PAK3 phosphorylation (same molecular weight as PAK1). Since Rac1 was previously shown to be activated between 30 s to 5 min of stretch, MCs were similarly stretched and whole cell lysates were probed using this phospho-specific antibody. Our results indicate initial PAK phosphorylation at 1 min which further increased at 5 min (Figure 10). In addition, to determine if stretch-induced PAK phosphorylation is dependent on Rac1, MCs were pretreated with NSC23766 and stretched for 5 min. PAK phosphorylation in response to stretch was significantly inhibited with NSC23766 (Figure 11).

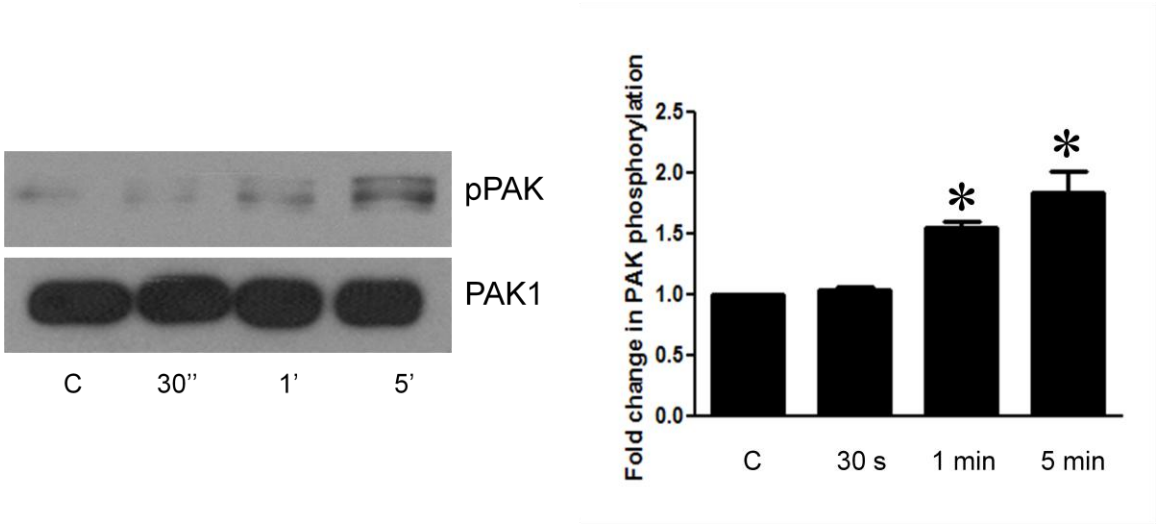

Fig. 10: **PAK phosphorylation occurs with stretch induction.** MCs were stretched from 30 s to 5 min and Western Blot was performed to detect phosphorylated PAK. PAK phosphorylation was quantified using densitometry. Data are representative of four independent replicates. *P < 0.05.

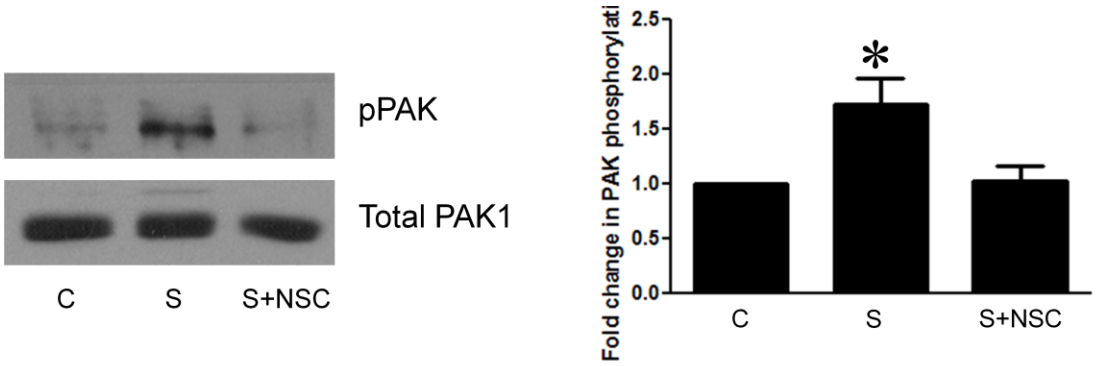

Fig. 11: **Stretched-induced PAK phosphorylation is dependent on Rac1.** MCs were stretched for 5 min with and without pre-treatment with NSC23766 (100 μM, 30 min) and Western Blot was performed to detect phosphorylated PAK. PAK phosphorylation was quantified using densitometry. Data are representative of three independent replicates. *P < 0.05.

PAK activity is enhanced in response to stretch

PAK activity was also evaluated using an *in vitro* kinase activity assay based on a protocol published by Allen *et al.* (2009) and involved using a His-tagged stathmin fusion protein as a substrate. Stathmin was confirmed as a downstream target of PAK in MCs as IPA3 (Group 1 PAK inhibitor) reduced levels of stathmin phosphorylation (Ser16) in response to stretch (Figure 12). IPA3 is a non-ATP-competitive, allosteric inhibitor that covalently binds to the autoregulatory domain of Group 1 PAKs and prevents binding of Cdc42 and Rac1 (Bokoch *et al.*, 2008). Since 5 min of stretch induced maximum PAK phosphorylation, the same duration of stretch was used to examine kinase activity. Furthermore, NSC23766 pre-treatment was used to determine if Rac1 mediates PAK activity. MCs were stretched for 5 min, with and without NSC23766 pre-treatment, and the kinase activity assay was performed for immunoprecipitated PAK1. We observed a significant increase in stathmin phosphorylation in stretched MCs versus control group (Figure 13). This result also suggests a trend toward a decrease in stretch-induced PAK activation with Rac1 inhibition, but this observation needs to be replicated to achieve significance.

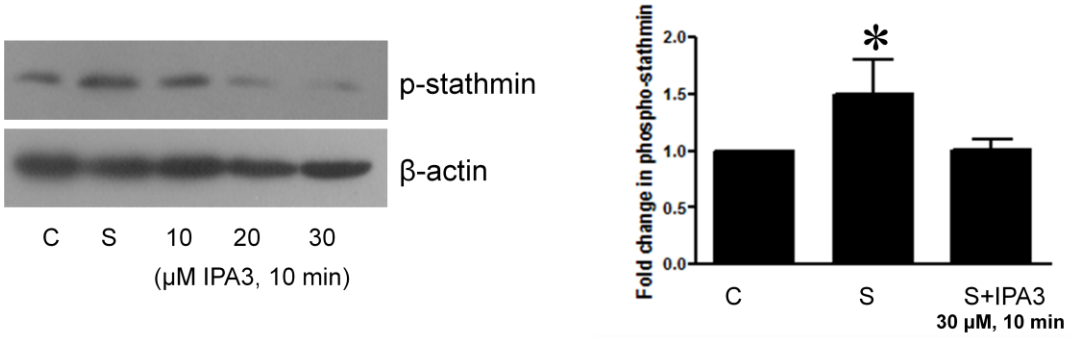

Fig. 12: **PAK mediates stathmin phosphorylation at Ser16 in response to stretch.** MCs were stretched for 1 min with and without pre-treatment with IPA3 at various concentrations (10, 20, and 30 μM) for 10 min, and Western Blot was performed to detect phospho-stathmin (Ser16). Stathmin phosphorylation was quantified using densitometry. Data are representative of three independent replicates. *P < 0.05.

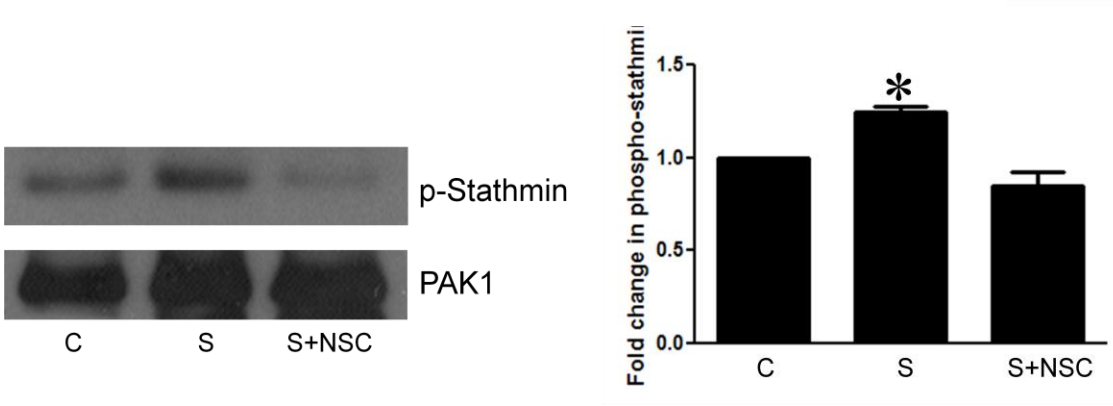

Fig. 13: **Stretch induces PAK1 activation.** MCs were stretched for 5 min with and without pre-treatment with NSC23766 (100 μM, 30 min). PAK1 was immunoprecipitated with anti-PAK1 antibody and incubated with His-tagged stathmin and ATP. Samples were run on SDS-PAGE and IB for phospho-stathmin (Ser16). Stathmin phosphorylation was quantified using densitometry. Data is representative of three independent replicates. *P < 0.05 vs. control.

CTGF is upregulated in response to stretch.

To determine if CTGF protein levels were increased in response to stretch, MCs were stretched at various time points (1, 3, and 6 hours) and CTGF was detected using Western Blot. Our results revealed a time-dependent increase in CTGF production initially at 1 hour and progressed up to 6 hours (Figure 14). Stretching of 1 hour was used in subsequent experiments to induce CTGF upregulation.

Mechanical stress-induced CTGF upregulation requires Rac1-PAK signaling

To ascertain if the Rac1-PAK signaling mediates CTGF upregulation with stretch, NSC23766 and the Group 1 PAK inhibitor, IPA3, were used to inhibit this pathway. MCs pre-treated with NSC23766 and IPA3 exhibited a significant decrease in stretch-mediated CTGF upregulation compared to untreated, stretched MCs at 1 hour (Figures 15 and 16). We next examined if 5 min of stretch, the time point when PAK is activated, is sufficient for inducing CTGF upregulation at the 1 hour time point. MCs stretched for 5 min and left quiescent for 55 min resulted in a significant increase in CTGF levels compared to control MCs (Figure 17).

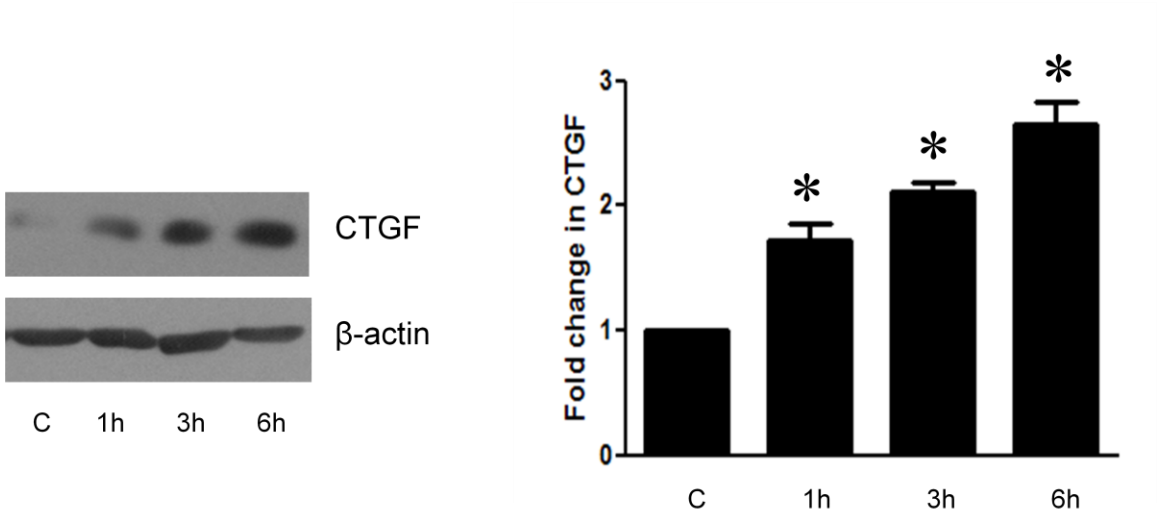

Fig. 14: **CTGF is upregulated by stretch.** MCs were stretched for 1, 3 and 6 h and Western Blot was performed to detect cellular CTGF. CTGF levels were quantified using densitometry. Data are representative of three independent replicates. *P < 0.05.

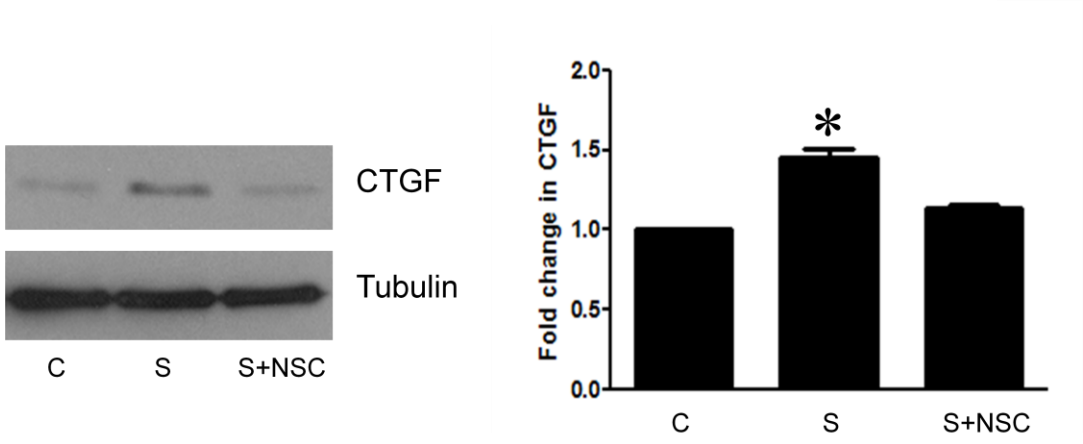

Fig. 15: **Stretched-induced CTGF upregulation is dependent on Rac1 activation.** MCs were stretched for 1 h with and without pre-treatment with NSC23766 (100 μM, 30 min) and Western Blot was performed to detect CTGF. CTGF levels were quantified using densitometry. Data are representative of three independent replicates. *P < 0.05.

RhoA activation is downstream of Rac1-PAK signaling

We next investigated the potential mechanism linking Rac1-PAK signaling to CTGF regulation in response to stretch. To determine if RhoA was involved, MCs were pre-treated with the inhibitors, NSC23766 and IPA3, stretched for 5 min, and RhoA activity assay was performed. Our results reveal that inhibition of Rac1 effectively blocked RhoA activation compared to untreated, stretched MCs (Figure 18). Furthermore, inhibition of PAK reduced stretch-induced RhoA activation below baseline levels (Figure 19).

RhoA/ROCK mediates stretch-induced CTGF upregulation

To confirm if RhoA, and its downstream effector, ROCK, mediates CTGF upregulation with stretch, MCs were pre-treated with the ROCK-specific inhibitor, Y27632, stretched for 1 hour and lysed for CTGF detection. MCs pre-treated with Y27632 demonstrated a significant decrease in CTGF protein levels (Figure 20). To investigate whether AP-1 plays a role in stretch-induced CTGF upregulation, we used curcumin, a component of the spice turmeric that can inhibit AP-1 DNA binding and transcriptional activity, as well as *c-jun* transcription (Park, Lee, & Yang, 2005). MCs pre-treated with curcumin and stretched for 1 hour displayed a significant decrease in CTGF upregulation versus untreated, stretched MCs (Figure 21). To further test whether PAK regulates AP-1 during stretch, we performed an EMSA to examine AP-1 binding to its consensus oligonucleotide after stretch. MCs were thus stretched for 10 min, previously demonstrated to induce AP-1 binding by Krepinsky *et al.* (2002), with and

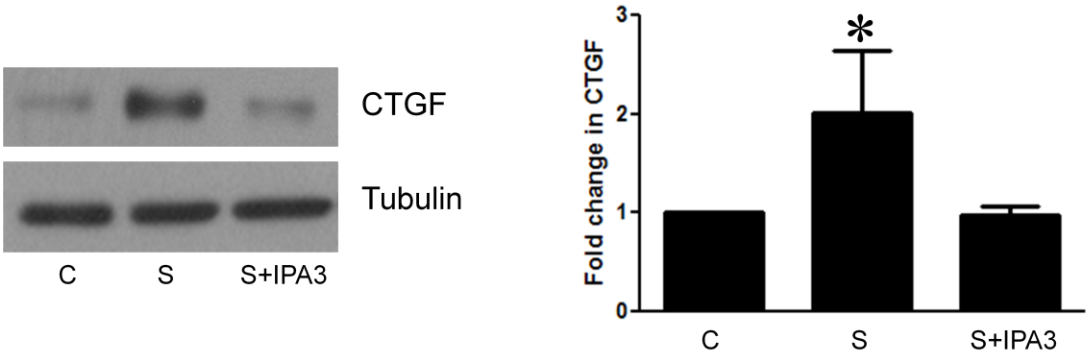

Fig. 16: **Stretched-induced CTGF upregulation is dependent on PAK activation.** MCs were stretched for 1 h with and without pre-treatment with IPA3 (30 μM, 10 min) and Western Blot was performed to detect CTGF. CTGF levels were quantified using densitometry. Data are representative of three independent replicates. *P < 0.05.

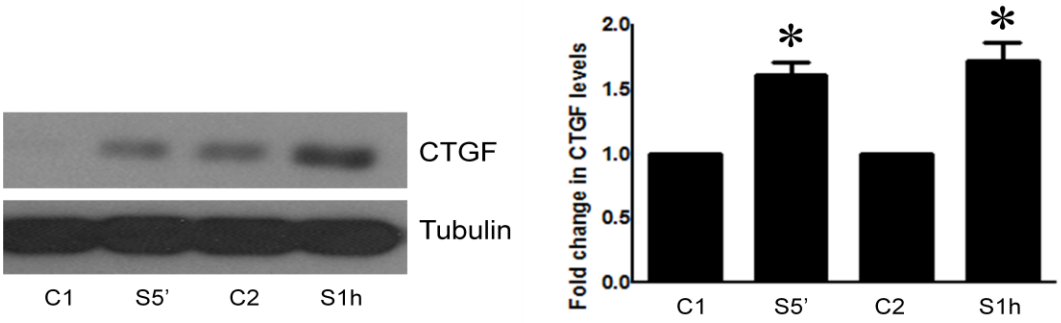

Fig. 17: **Acute stretch with 55 min chase period is sufficient for CTGF upregulation.** MCs were stretched for 1) 5 min and left quiescent for 55 min and 2) 1 hour. Western Blot was performed to detect CTGF. CTGF levels were quantified using densitometry. Fold change in CTGF for each stretch group was determined relative to its own control group (C1 or C2). Data are representative of three independent replicates. *P < 0.05.

without IPA3 pre-treatment. Although a high baseline in control MCs prevented a stretch-induced AP-1 shift previously demonstrated to be significant in our lab, PAK inhibition using IPA3 did not inhibit stretch-induced AP-1 activation (Figure 22). This suggests Rac1/PAK signaling regulates CTGF upregulation independent of AP-1.

PIX is not required for stretch-induced PAK activation and downstream CTGF production

To assess if PIX mediates stretch-induced PAK activation, we used PAK18 (TAT-PIX) which is a peptide containing the cell permeant TAT peptide sequence and an 18 amino acid sequence that corresponds to the PIX-binding motif on PAK (Hashimoto *et al.*, 2010). To determine if PAK18 is effective in inhibiting PAK activation in our system, MCs were pre-treated with this inhibitor and stretched for 1 min, followed by immunoblotting for phospho-stathmin (Ser16). Stathmin was used as a substrate since we previously demonstrated that PAK inhibition using IPA3 reduced phospho-stathmin levels in response to stretch (Figure 11). Our recent data indicates that stathmin phosphorylation via PAK in response to stretch is independent of PIX binding (Figure 23).

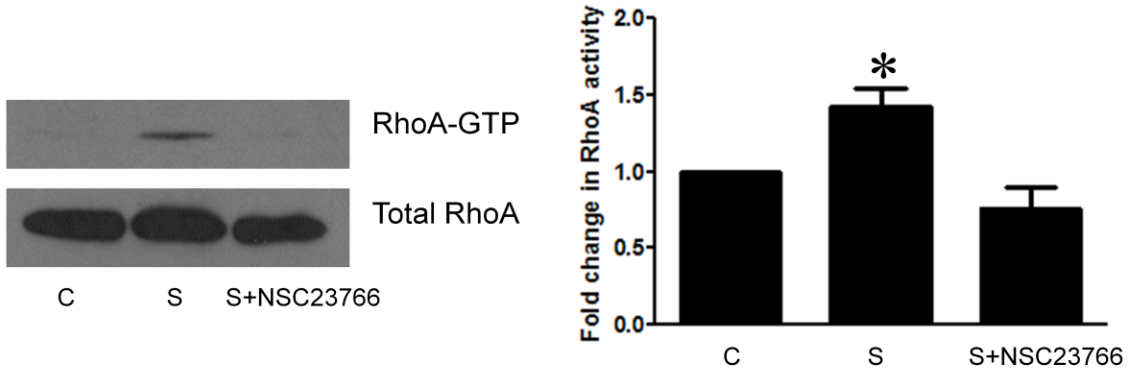

Fig. 18: **Stretch-induced RhoA activation is dependent on Rac1 signaling.** MCs were stretched for 5 min with and without pre-treatment with NSC23766 (100 μM, 30 min). Activated, GTP-bound RhoA was pulled-down with GST-RBD beads and Western Blot was performed to detect RhoA. RhoA levels were quantified using densitometry. Data are representative of five independent replicates. *P < 0.05.

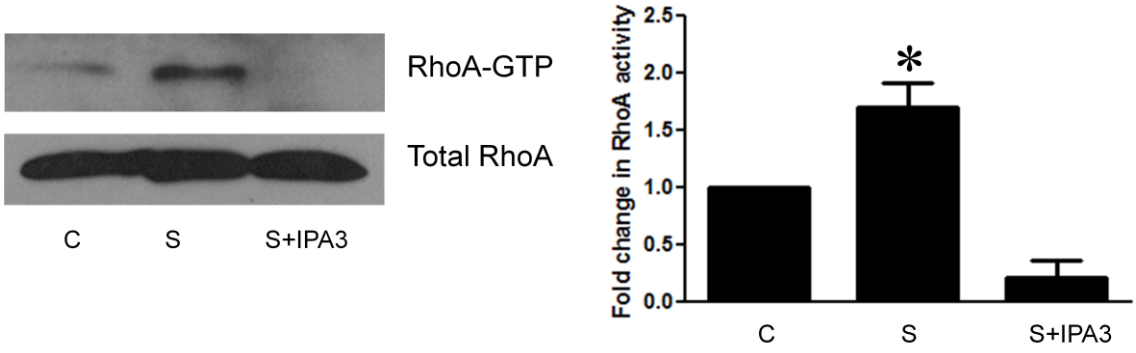

Fig. 19: **Stretch-induced RhoA activation is dependent on PAK signaling.** MCs were stretched for 5 min with and without pre-treatment with IPA3 (30 μM, 10 min). Activated, GTP-bound RhoA was pulled-down with GST-RBD beads and Western Blot was performed to detect RhoA. RhoA levels were quantified using densitometry. Data are representative of three independent replicates. *P < 0.05.

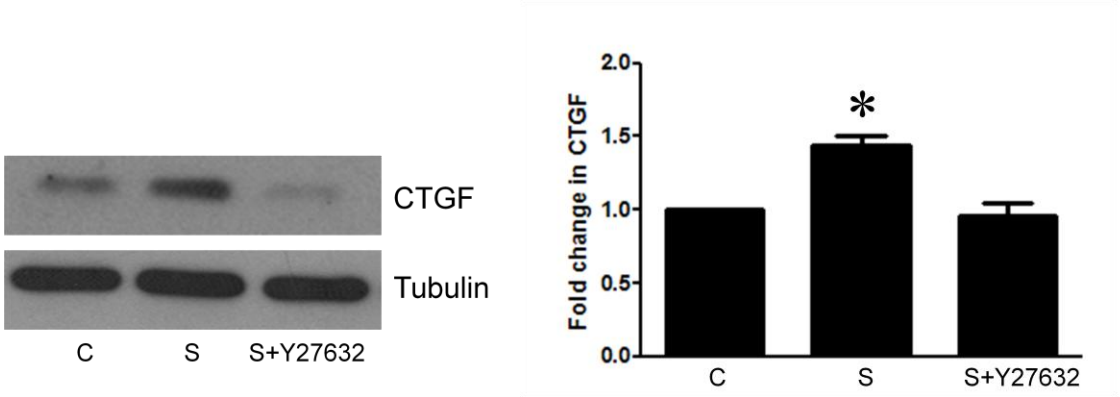

Fig. 20: **Stretch-induced CTGF upregulation is dependent on ROCK activation.** MCs were stretched for 1 h with and without pre-treatment with Y27632 (ROCK inhibitor) (20 μM, 30 min) and Western Blot was performed to detect CTGF. CTGF levels were quantified using densitometry. Data are representative of three independent replicates. *P < 0.05.

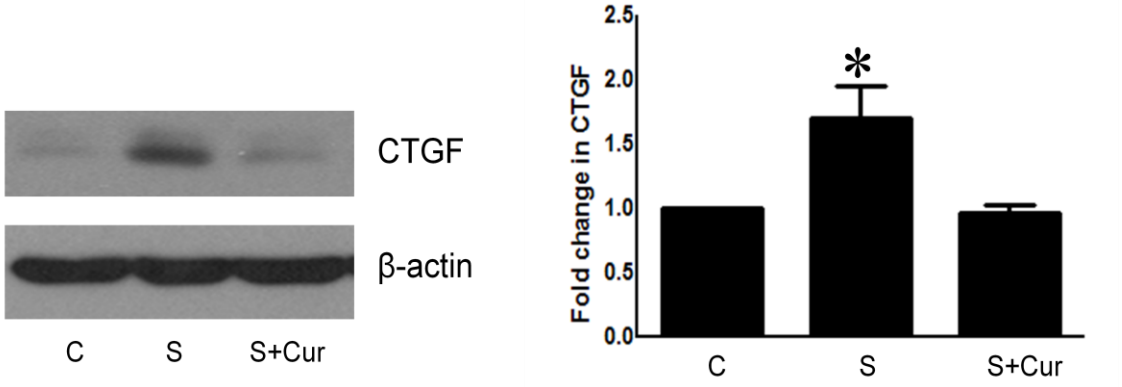

Fig. 21: **Stretch-induced CTGF upregulation is dependent on AP-1 activation.** MCs were stretched for 1 h with and without pre-treatment with curcumin (10 μM, 30 min). Western Blot was performed to detect CTGF. CTGF levels were quantified using densitometry. Data are representative of three independent replicates. *P < 0.05.

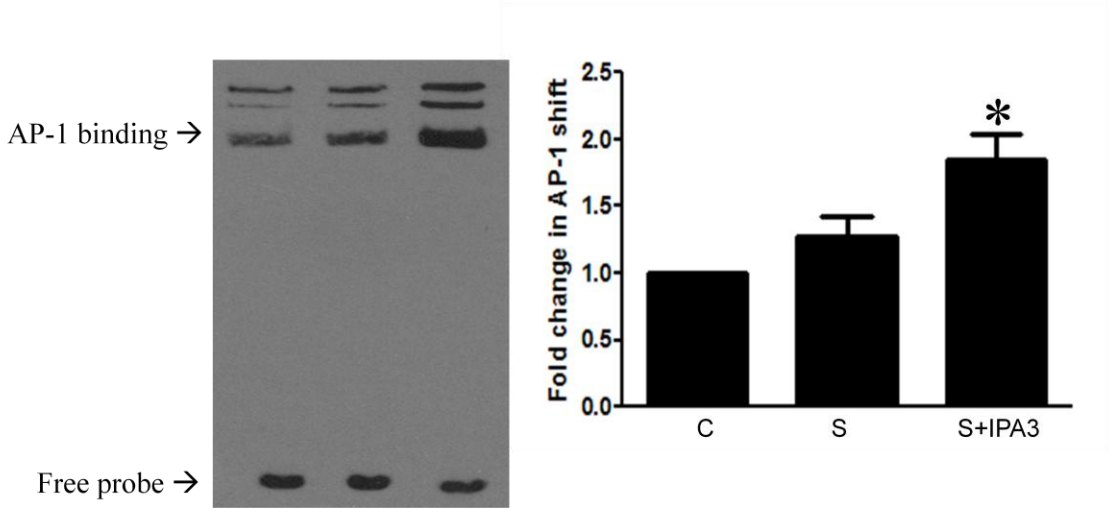

Fig. 22: **Stretch-induced AP-1 activation is independent of PAK**. MCs were stretched for 10 min with and without pre-treatment with IPA3 (30 μM, 10 min) and nuclear proteins were extracted. EMSA was performed using 3 – 5 μg of nuclear extracts and an AP-1 consensus oligonucleotide. AP-1 shift was quantified using densitometry. Data are representative of four independent replicates. *P < 0.05.

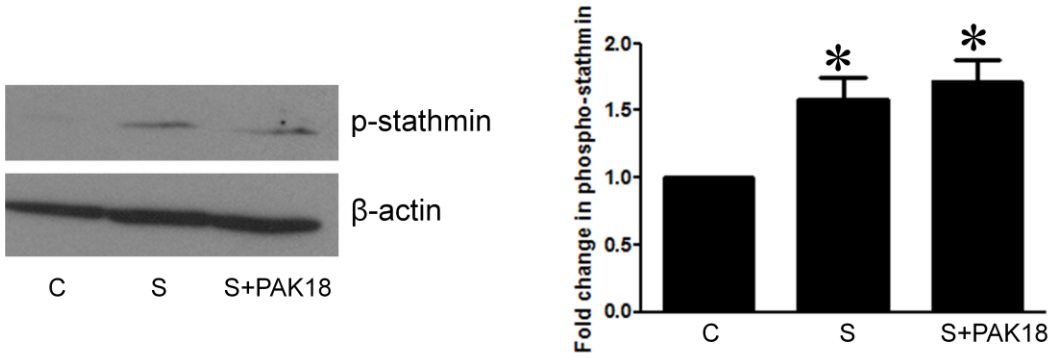

Fig. 23: **PIX is not required for stathmin phosphorylation in response to stretch**. MCs were stretched for 1 min with and without pre-treatment with PAK18 (PIX inhibitor; 10 μM, 1 h) and Western Blot was performed to detect phospho-stathmin. Stathmin phosphorylation was quantified using densitometry. Data are representative of three independent replicates. *P < 0.05 versus control.

Elevated P_{gc} has been shown to be an important contributor to GS in animal models of CKD (Frazier *et al.*, 2000; Ito *et al.*, 1998). In the context of CKD, MCs have been shown to contribute to GS via excessive ECM production, ultimately leading to fibrosis (Ishida *et al*., 1999; Yasuda *et al.*, 1996). ECM secretion occurs in response to pro-fibrotic stimuli, such as TGF-β and mechanical stress. However, it is believed that MCs also secrete other macromolecules, such as CTGF, which also contribute to the development of fibrosis (Krepinsky *et al*., 2005; Kriz *et al.*, 1995; Chen *et al.*, 2002; Riser *et al*., 2000). The mechanisms by which mechanical stress is transduced into fibrotic responses in MCs has not been well elucidated, thus our current study has aimed to further investigate this link between mechanical stress and fibrosis. Our results have identified the Rac1-PAK axis as an important regulator in mechanical stress through the regulation of CTGF production in MCs.

Rac1-PAK is activated in response to stretch in MCs

Although there is considerable evidence implicating Rac1 activation in response to mechanical stress, downstream effectors of Rac1 have not been well studied in the context of mechanotransduction (Qi *et al.*, 2010; Vecchione *et al.*, 2009; Zhang *et al*., 2010). To elucidate the downstream effectors of Rac1, a Rac1-specific inhibitor, NSC23766, which inhibits Rac1 association with the GEFs Tiam-1 and TrioN was utilized (Gao *et al*., 2004). However, whether these GEFs were involved in stretch-induced Rac1 activation was unknown and therefore we tested its effectiveness with a Rac activity assay. MCs pre-treated with NSC23766 showed suppressed Rac1 activation

in response to stretch suggesting involvement of these RhoGEFs in Rac1 activation by stretch.

Of the known downstream Rac1 effectors, PAK was of particular interest since it has been shown to possess a Rac/Cdc42 binding domain (Nikolic *et al.*, 2008) and is expressed in the glomeruli of rats (Zhu *et al*, 2010). Our results have extended the work of Zhu *et al.* (2010) by demonstrating, through RT-PCR and Western Blot, the expression of PAK1, 2, and 3 mRNA and protein in rat MCs. Analysis of the kinetics of stretch-induced PAK phosphorylation in MCs revealed initial PAK phosphorylation after 1 minute of stretch, which was sustained and enhanced at 5 minutes. These observations are similar to the Rac kinetics observed by Zhang *et al*. (2010) and studies completed with oscillatory flow on ECs (Orr *et al*, 2008; Funk *et al*, 2010). PAK activity was assessed by probing for the phosphorylation status of stathmin, a substrate that is phosphorylated when PAK is active. Exposure of MCs to 5 minutes of continual stretch resulted in a significant increase in PAK1-mediated stathmin phosphorylation. Furthermore, inhibition of Rac1 with NSC23766 resulted in reduced PAK phosphorylation, and possibly kinase activity, in response to stretch in MCs. An additional replicate demonstrating NSC23766 inhibition of PAK activity is required for statistical significance. Taken together, we have shown that PAK is involved in mechanotransduction in MCs (Orr *et al*., 2008; Funk *et al,* 2010) and that Rac1 is a primary upstream regulator of PAK in response to stretch.

PAK activation was detected using a phospho-specific antibody that detects phosphorylation on PAK1 (Ser199/204), PAK2 (Ser194/197), and possibly PAK3

(Ser200/205). These residues have been used by several studies as a marker of PAK activity as phosphorylation of these sites is believed to prevent PAK from reverting to an inactive configuration (Woolfolk *et al.*, 2005; Harfouche *et al.*, 2005; Zhu *et al.*, 2010; Hinoki *et al.*, 2010). The limitation of using this antibody is that it is difficult to delineate which specific Group 1 PAK isoform is involved on our proposed pathway. Furthermore, IPA3, the PAK inhibitor we used in our studies, binds to the PAK-AID and prevents Rac/Cdc42-dependent activation of only Group 1 PAKs which possess the AID (Bokoch *et al.*, 2008). As a result, the role of Group 2 PAKs is not evaluated in this study. These factors introduce a question of which PAK isoform is predominantly activated with stretch and regulates CTGF upregulation in MCs, especially since there are relatively few structural differences between PAKs 1-3 in their N-terminal regulatory and C-terminal kinase domains (Galan Moya *et al.*, 2009). However, amongst Group 1 PAKs, it is likely that PAK3 does not play an important role in our proposed model as PAK3 expression and downstream functions are mostly specific to the brain. Additionally, PAK3 knockout in humans is associated with X-linked, nonsyndromic mental retardation, and PAK3 depleted mice develop abnormalities in synaptic plasticity, and deficiencies in learning and memory (Allen *et al.*, 1998; Meng *et al.*, 2005). Conversely, PAK1 and 2 are more characterized in other tissues and observed to mediate a broader range of functions, such as migration and proliferation, and most importantly, mechanotransduction (Funk *et al.*, 2010; Hinoki *et al.*, 2010; Orr *et al.*, 2008).

The second limitation of the antibody used to detect PAK phosphorylation is that there is insufficient evidence correlating the phosphorylation of these targeted sites with

kinase activity. Therefore, to determine whether PAK activity was increased with stretch, an *in vitro* kinase assay was performed using a protocol similar followed by Allen *et al*. (2009). This kinase assay required the use of a substrate downstream of PAK, so stathmin was used as others have demonstrated its phosphorylation at Ser16 by PAK (Wittman *et al*, 2004). Stathmin can be phosphorylated at Ser16 by other kinases as well, including cAMP-dependent protein kinase (PKA) and calmodulin-dependent kinase (le Gouvello *et al.*, 1998), but we verified PAK was an upstream mediator of stathmin phosphorylation after we demonstrated that IPA3 pre-treatment reduced phospho-stathmin levels in response to stretch using Western Blot.

To determine whether PAK1 is specifically involved and if PAK phosphorylation was correlative of activity, PAK1 was immunoprecipitated from MCs stretched for 5 min and the kinase activity assay was performed. Consistent with PAK phosphorylation at 5 min of stretch, there was a significant increase in stathmin phosphorylation at this time point, which represents an increase in PAK1 activity. Whether PAK2 or PAK3 are activated will be determined in future studies.

CTGF is upregulated in response to stretch

In response to 1 hour of cyclic stretch we have observed an increase in CTGF upregulation. This observation is in agreement with other studies that have demonstrated enhanced CTGF mRNA expression and ECM production in MCs exposed to cyclic stretch and high static pressure (Riser *et al*., 2000, Hishikawa *et al*., 2001). In contrast, however, Riser *et al.* (2000) did not observe an increase in CTGF protein levels with

cyclic stretch for a period of 4 to 48 hours. This conflicting data may be attributed to the longer stretch duration or lower frequency of stretch used by this group. *CCN2* is an early response gene that is transiently activated at an early time point with stretch (Goppelt-Struebe *et al*., 2001), which may explain why no CTGF protein was present with longer stretch times. Alternatively, Riser *et al*. (2000) may not have detected the CTGF in the media because of its ability to bind to the cell surface, proteoglycans, or other ECM components via the cysteine knot, heparin-binding domain (Riser *et al*., 2000). They demonstrated that addition of sodium heparin significantly increased the amount of CTGF in the medium of serum-deprived MCs. This supports the notion that secreted CTGF is predominately cell bound. Therefore, the lack of detectable CTGF protein in the medium of stretched cells does not necessarily reflect a reduction in CTGF synthesis. Overall, our data are also consistent with studies performed on smooth muscle cells and fibroblasts, which collectively demonstrate a strong link between mechanical stress and CTGF upregulation (Chaqour *et al.*, 2006; Schild *et al.*, 2002).

Rac1-PAK regulate stretch-induced CTGF

Rac1 and PAK inhibition had led to a significant reduction in CTGF in response to stretch. Stretch for 5 minutes followed by quiescence for 55 minutes in MCs, in the absence of inhibitors, resulted in a significant increase in CTGF in stretched versus control MCs. These results support the importance of Rac1-PAK in the regulation of stretch-induced CTGF regulation and demonstrate that stretch upregulates CTGF production in MCs. In support of this observation, other studies have observed Rac1 and

RhoA as important regulators of CTGF production in MCs in response to TGF-β and LPA (Goppelt-Struebe *et al.*, 2001). For instance, Xu *et al.* (2009) have shown that inhibition of Rac1 with NSC23766 suppressed CTGF expression in fibroblasts extracted from lesions of scleroderma patients, which provides evidence for our proposed link between Rac1-PAK signaling and CTGF production.

Rac1-PAK signaling regulates CTGF via RhoA/ROCK

Analyzing Rac1-PAK regulation of CTGF production led us to determine that inhibition of Rac1 and PAK prevented stretch-induced RhoA activation. Interestingly, PAK inhibition was sufficient for reducing basal RhoA activity. To further investigate the role of RhoA activation in CTGF regulation, we utilized a ROCK inhibitor, Y27632. Pre-treatment with Y27632 led to a significant suppression of stretch-induced CTGF in MCs. These results suggest that Rac1-PAK signaling regulates CTGF production through RhoA/ROCK signaling.

This is consistent with other studies that support RhoA as an important regulator of CTGF production in MCs exposed to stretch (Hahn *et al.*, 2000, Goppelt-Struebe *et al.*, 2001). RhoA/ROCK has been previously implicated in the progression of hypertensive-renal disease in animal models (Nishikimi *et al.*, 2004; Kanda *et al.*, 2003). Based on our data, the influence of RhoA/ROCK on CTGF regulation may play a role in renal disease. Furthermore, RhoA activation by stretch in MCs is believed to be mediated by the Rho-specific GEF, Vav2 (Peng *et al.*, 2010). There is evidence suggesting that PAK can interact with other Rho-specific GEFs, such as GEF-H1 and NET1, and even

GDIs which may subsequently modulate RhoA activity (Zenke *et al.*, 2004; Alberts *et al*., 2005; DerMardirossian *et al*., 2004). Thus, it is possible that stretch induces cross-talk between Rac1 and RhoA, likely through PAK-mediated interactions with Rho GEFs, GDIs, or GAPs, which in turn regulate downstream CTGF production.

Our observations have indicated that both Rac1 and RhoA function cooperatively in response to stretch which is consistent with observations from neonatal rat cardiomyocytes exposed to biaxial stretch (Kawamura *et al.*, 2004). However these data lay in conflict with the dogma that Rac1 and RhoA are mutually antagonistic. Multiple examples of Rac1 and RhoA antagonism exist such as: 1) RhoA/ROCK promotions of actin stress fiber formation in MCs and fibroblasts (Krepinsky *et al.*, 2003; Nobes and Hall, 1995) opposing Rac1/PAK-induced actin stress fiber disassembly in epithelial cells and fibroblasts (Manser *et al.*, 1997; Zhao *et al.*, 1998); 2) Rac1 activation in cell migration on the leading edge of the forming lamellipodia in opposition to RhoA activity at the rear and side of the cell promoting retraction (Pestonjamasp *et al.*, 2006); 3) RhoA repression of Rac1 activation by activation of FilGAP (Ohta *et al.*, 2006) versus over-expression of a constitutively active Rac1 mutant (V12Rac) which inhibits RhoA activation in NIH 3T3 cells (Sander *et al.*, 1999). However, there has been some data which suggest synergistic cooperation between Rac1 and RhoA such as: 1) A requirement for Rac1 activation for Rho-dependent actin stress fiber regulation in Swiss 3T3 cells (Ridley *et al.*, 1992); 2) Dominant negative mutants of RhoA and Rac1 inhibit stress fiber formation and cell alignment in fetal type II epithelial cells exposed to cyclic

stretch (Silbert *et al.*, 2008). It may be that the antagonism between Rac1 and RhoA only operates in localized subcellular areas, such as lamellipodia extension and cell retraction.

RhoA/ROCK has been shown to regulate AP-1 in MCs (Peng *et al.*, 2008). Furthermore, an AP-1 binding site was identified in the *ctgf* promoter sequence (Xia *et al.*, 2007). Based on these data, we investigated whether AP-1 activation was involved in stretch-induced CTGF upregulation. Firstly, we used curcumin which was shown to inhibit AP-1 DNA binding and transcriptional activity, as well as *c-jun* transcription (Park, Lee, & Yang, 2005). Pre-treatment with curcumin significantly decreased CTGF upregulation after 1 hour of stretch which suggests that AP-1 may be involved in this pathway. To determine if PAK can regulate AP-1 activity, an EMSA was performed to examine AP-1 binding to its consensus oligonucleotide as previously demonstrated (Peng *et al.*, 2008). In MCs pre-treated with IPA3, however, there was no significant decrease in AP-1 DNA binding in response to stretch. This is inconsistent with our proposed hypothesis that PAK mediates CTGF production through AP-1. This surprising observation may be due to a lack of specificity of curcumin as noted by other authours, in which curcumin had an inhibitory effect on other signaling components such as NF-κB and ERK in hepatic stellate cells and human tenocytes, as well as PAK1 in a gastric cell line (Buhrmann *et al.*, 2011; Chen and Zheng, 2008; Cai *et al.*, 2009). I was unable to demonstrate significance in stretch-induced AP-1 shift due to a few replicates with high baseline levels. This was not expected based on previous findings in our lab and may indicate variability in cell passage number or experimental technique (Peng *et al.*, 2008). In summary, it is unclear what signaling pathway curcumin acts on to inhibit stretch-

induced CTGF expression, and finally, what transcription factor regulates CTGF downstream of RhoA/ROCK.

PIX is not required for stretch-induced PAK activation and downstream CTGF production

For precise regulation of the numerous processes that involve PAK, multiple GTPase-independent methods of PAK activation are required, such as binding to the PIX/GIT1 complex, which transports PAK to focal adhesion complexes and facilitates PAK activation (Zhao *et al*., 2000; Stockton *et al*., 2007). To assess whether PIX contributes to PAK activation in MCs in response to stretch, we used PAK18 (TAT-PIX), a peptide that prevents interaction between PAK and PIX. Other studies have demonstrated that PIX is important in PAK activity and downstream ERK signaling in ECs treated with VEGF (Stockton *et al*., 2007). Pre-treatment with PAK18 did not demonstrate any significant effect on stretch-induced stathmin phosphorylation, which suggests that MCs do not require PIX for stretch-induced PAK activation. This implies that regulation of PAK may be cell or stimulus-specific, as mechanical stress can activate different signaling pathways compared to growth factors. Overall, our results suggest that PAK activation in response to stretch is primarily GTPase-dependent and PIX-independent.

The role of PAK in renal disease has been only been investigated by a few studies. Sebe *et al.* (2008) examined the role of PAK in human proximal tubular epithelial cells (LLC-PK/AT1) that were stimulated to commence epithelial-to-mesenchymal transition (EMT). Introduction of a dominant negative mutant of PAK into these cells suppressed markers of EMT such as alpha-smooth muscle actin (Sebe *et al.*, 2008). Similarly, PAK phosphorylation was enhanced in human tubular epithelial cells undergoing EMT in response to activated PBMC medium (Patel *et al*., 2005). These studies suggest that PAK has a possible role in the phenotypic transformation of MCs into myofibroblasts, which are a major contributor to tubulointerstitial fibrosis through their enhanced capabilities of ECM synthesis (Grgic *et al.*, 2011). There is evidence that when MCs are exposed to pro-fibrotic stimuli, they take on a myofibroblast-like phenotype (Diah *et al.*, 2008; Simonson, 2007). Similarly to myofibroblasts, these "activated" MCs secrete abnormal ECM components, such as collagen I/IV and fibronectin, and possibly CTGF (Yasuda *et al.*, 1996). The role of PAK in the transformation of MCs to this fibrotic phenotype is unknown and necessitates future studies.

The data presented here establishes a novel signaling pathway in MCs that is activated in response to mechanical stress (Figure 24). We demonstrated that the Rac1-PAK signaling pathway responded to cyclic stretch in MCs. Furthermore, this pathway mediated the upregulation of CTGF through RhoA/ROCK signaling, which is capable of cross-talking with Rac1-PAK to further modulate CTGF expression. Identifying key signaling mediators that regulate CTGF is extremely important as there is considerable

evidence of its pathological role in renal disease. The expression of CTGF is higher in both animal and human models of CKD (Ito *et al.*, 1998; Frazier *et al.*, 2000). Furthermore, inhibition of CTGF *in vivo* has shown positive results in terms of CKD outcomes. Yokoi *et al.* (2004) observed a significant reduction in expression of CTGF, fibronectin, and collagen I in the tubulointerstitium of hypertensive-mediated CKD rats after a CTGF antisense oligonucleotide was injected. CTGF is also considered a much safer therapeutic target than TGF-β itself, since CTGF mediates many TGF-β-induced fibrotic effects. This may prevent adverse effects associated with inhibiting TGF-β and its other physiological functions (Chen *et al.*, 2002; Riser *et al*., 2000; Leask *et al*., 2003). Our data, combined with others, have demonstrated a prominent role for mechanical strain in CTGF regulation in MCs (Riser *et al*., 2000, Hishikawa *et al*., 2001). Therefore, investigating the mechanisms in which PAK regulates CTGF in response to mechanical stress will validate PAK inhibition as a potential therapeutic target for CKD.

PROPOSED PATHWAY

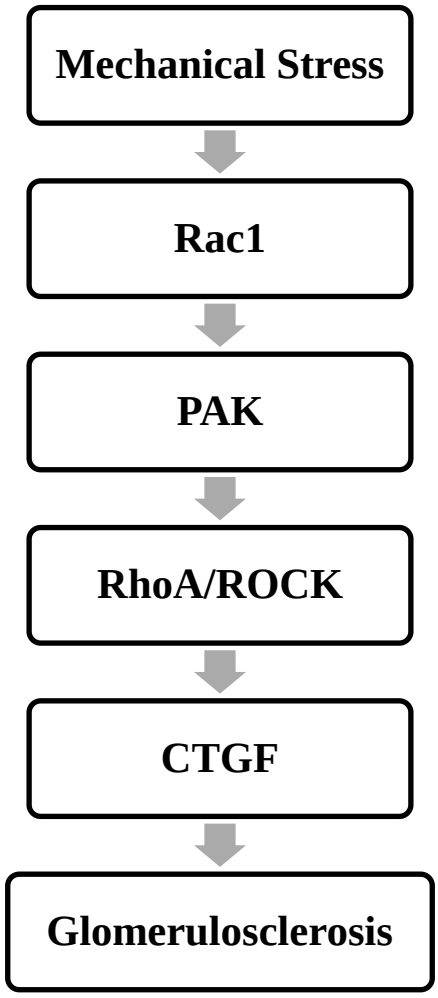

Fig. 24: **Outline of proposed pathway.**

FUTURE DIRECTIONS

Our work here presents the first evidence supporting a role for PAK in both mechanical stress signaling and CTGF regulation in MCs. These results provide a foundation for future *in vitro* and *in vivo* studies on the molecular basis of CKD progression and the effects of mechanical stress in other cell types. These future objectives below will lead to a better understanding of the Rac1-PAK signaling cascade in MCs:

1) Determining the role of each specific PAK isoform
2) Investigate how PAK regulates stretch-induced CTGF upregulation
3) Assess the role of PAK inhibition in an *in vivo* model of CKD

1) Determine the role of each specific PAK isoform

In response to mechanical stress, we observed an increase in PAK phosphorylation, which was detected using an antibody that immunoreacts with residues on PAK1 (Ser199/204), PAK2 (Ser194/197), and possibly PAK3 (Ser200/205). Several studies have used this antibody as a marker for PAK activity, but since this antibody detects phosphorylation of PAK1, 2, and 3, it is difficult to delineate the effects of each isoform. Our method of PAK inhibition also doesn't allow us to evaluate the role of each isoform as it inhibits activation of all Group 1 PAKs. In addition, our data suggests that PAK1 activity is increased in response to stretch, but whether PAK1, or the other PAK

isoforms, are involved in CTGF regulation requires further study. PAK3 is unlikely to be involved due to its involvement in brain-specific processes (Allen *et al.*, 1998; Meng *et al.*, 2005). Conversely, Orr et al. (2008) and Funk *et al.* (2010) observed PAK2 phosphorylation at Ser141 in ECs exposed to mechanical stress. In addition, PAK1 phosphorylation at Ser199/204 and Thr423 occurred in VSMCs in response to Angiotensin II and PDGF, and was also demonstrated in being an important contributor to vascular remodeling (Hinoki *et al.*, 2010). These data suggest that either PAK1 or PAK2 is involved in mediating mechanical stress pathways in MCs. Therefore, to determine which isoform is being activated upon stretch, MCs could be transfected with either PAK1 or 2 siRNA, similarly done by previous studies, and downstream CTGF regulation can be assessed in response to stretch (Beeser *et al.*, 2005; Coniglio *et al.*, 2008). Another approach may involve using previously developed PAK mutants, such as constitutively active or dominant negative forms of PAK1 and PAK2, to further evaluate the role of each isoform (Zhu *et al.*, 2010; Wilkes *et al.*, 2003).

2) Investigate how PAK regulates stretch-induced CTGF upregulation

Previous studies have identified mechanical stress as a regulator of CTGF expression in MCs (Riser *et al.*, 2000; Hishikawa *et al.*, 2001), but the underlying mechanisms involved have not been looked at in detail. Our data provides evidence that Rac1-PAK signaling is an important mediator of stretch-induced CTGF upregulation through RhoA/ROCK as depicted by our proposed model. We hypothesized that RhoA/ROCK can regulate stretch-induced CTGF upregulation through the transcription factor AP-1.

Our results indicated that curcumin, an AP-1 inhibitor, sufficiently blocked stretch-induced CTGF upregulation. After performing an EMSA, it was observed that PAK-mediated CTGF regulation was independent of AP-1. These contrasting results may be attributable to other inhibitory effects of curcumin on signaling mediators such as NF-κB and ERK (Buhrmann *et al.*, 2011; Chein and Zheng, 2008; Cai *et al.*, 2009). Furthermore, previous data show that curcumin can inhibit NF-κB signaling in hepatic stellate cells and human tenocytes (Buhrmann *et al.*, 2011; Chen and Zheng, 2008). Therefore, it is possible that inhibition of stretch-induced CTGF production with curcumin pre-treatment may have occurred through NF-κB signaling. This hypothesis is further supported by data demonstrating that mechanical strain of cultured bladder smooth muscle cells results in the activation and binding of NF-κB to the proximal promoter region of the *CCN2* gene (Chaqour, Yang, and Sha, 2006). In addition, PAK was shown to modulate NF-κB activation in ECs exposed to shear stress (Orr *et al.*, 2008). NF-κB is a heterodimeric protein complex that comprises of two subunits, one of them being p65 which is phosphorylated at Ser526 and translocated to the nucleus upon activation (Orr *et al.*, 2008). To determine whether NF-κB signaling is downstream of Rac1-PAK, MCs can be pre-treated with either NSC27633 or IPA3 prior to stretch, to inhibit Rac1 and PAK respectively, and NF-κB activation can be assessed by examining Ser536 phosphorylation and nuclear translocation of p65 using Western Blot. A significant increase in nuclear and phosphorylated p65 levels would indicate NF-κB activation. Another method to determine if PAK is upstream of NF-κB is to perform an EMSA using an oligonucleotide containing the consensus sequence for the NF-κB binding site

(Chaqour, Yang, and Sha, 2006). Using nuclear extracts from MCs pre-treated with IPA3, we would be able to assess whether PAK regulates NF-κB nuclear translocation and binding to its consensus sequence in stretched MCs. Furthermore, to determine whether NF-κB mediates stretch-induced CTGF upregulation, a cell-permeable peptide containing the NEMO (NF- κB essential modifier) -binding domain can be used (May *et al.*, 2000). NEMO is a regulatory protein required for NF-κB activation. If NF-κB is the main transcription factor involved in PAK-mediated CTGF upregulation, MCs pre-treated with this peptide would exhibit reduced CTGF production in response to stretch. In summary, these experiments will help elucidate whether NF-κB is regulated by PAK and required for CTGF expression in MCs.

3) Assess the role of PAK inhibition in an *in vivo* model of CKD.

We have demonstrated that PAK inhibition leads to the suppression of stretch-induced CTGF upregulation. Given the considerable evidence that indicate CTGF is an important mediator of renal fibrosis, inhibiting PAK may have therapeutic effects in patients with CKD. In order to verify the therapeutic potential of PAK inhibition, we need to assess whether PAK contributes to hypertensive-mediated GS *in vivo*. This can be accomplished by acquiring PAK1-knockout mice model previously used by Allen *et al.* (2009), and performing 5/6 nephrectomy on these mice to induce hypertensive-mediated CKD (Anderson *et al.*, 1986). Whether PAK2 is involved in mediating GS *in vivo* cannot be determined through PAK2-knockout mice as they are embryonically lethal (Asrar *et al.*, 2009). PAK1-knockout mice have normal life span, growth, fertility, and locomotor

behavior, with impairments observed only in mast cell degranulation against allergic responses and in hippocampal synaptic plasticity (Allen *et al.*, 2009; Meng *et al.*, 2005). We will be able to evaluate the role of PAK1 in the progression of CKD associated with elevated P_{gc}, by analyzing parameters of kidney function and the extent of fibrosis. Blood pressure measurements and urine can be collected at different intervals similarly described in studies on remnant kidney mice models (Kren and Hostetter, 1999). In addition, extracted kidneys can be analyzed using Northern and Western Blots to assess whether CTGF mRNA and protein levels are significantly different compared to sham-operated mice. Furthermore, immunohistochemical analysis using an anti-CTGF antibody will allow us to visualize differences in CTGF expression in isolated glomeruli from each mice group (Yokoi *et al.*, 2004).

Further investigation of this mechanical stress signaling pathway will lead to a better understanding of the role of PAK in transducing mechanical stress into fibrotic responses in MCs. Undertaking *in vivo* studies, after elucidating the mechanisms underlying PAK activation and CTGF regulation, will help determine if PAK is a potential therapeutic target for the pathogenesis of GS.

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