TGFβ Receptor I Transactivation Mediates Stretch-Induced Pak1 activation and CTGF Upregulation in Mesangial Cells

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Increased intraglomerular pressure is an important pathogenic determinant of kidney fibrosis in the progression of chronic kidney disease, and can be modeled by exposing glomerular mesangial cells (MC) to mechanical stretch. MC produce extracellular matrix and profibrotic cytokines, including connective tissue growth factor (CTGF) when exposed to stretch. We show that p21-activated kinase 1 (Pak1) is activated by stretch in MC and in vivo in a model marked by elevated intraglomerular pressures. Its activation is essential for CTGF upregulation. Rac1 is an upstream regulator of Pak1 activation. Stretch induces transactivation of the type I transforming growth factor β1 receptor (TβRI) independently of ligand binding. TβRI transactivation is required not only for Rac1/Pak1 activation, but also leads to activation of the canonical TGFβ signaling intermediate Smad3. We show that Smad3 activation is an essential requirement for CTGF upregulation in MC under mechanical stress. Pak1 regulates Smad3 C-terminal phosphorylation and transcriptional activation. However, a second signaling pathway, that of RhoA/Rho-kinase and downstream Erk activation, is also required for stretch-induced CTGF upregulation in MC. Importantly, this is also regulated by Pak1. Thus, Pak1 serves as a novel central mediator in the stretch-induced upregulation of CTGF in MC.
**Introduction**

Elevated glomerular capillary pressure (Pgc) is a central pathogenic determinant of progression of chronic kidney disease of varying etiology. Increased Pgc transmits to mesangial cells (MC), which provide architectural support for the glomerular capillary tuft, as mechanical stretch (Riser et al., 2000a). In vitro, cyclic application of vacuum to plates with deformable wells is used to model the effects of stretch. MC grown on matrix and subjected to cyclic stretch/relaxation increase matrix protein synthesis (Riser et al., 1992), providing a model system to study mechanical stretch-induced signaling in MC. Two key mediators of matrix protein synthesis and glomerular fibrosis are the profibrotic cytokines transforming growth factor \( \beta 1 \) (TGF\( \beta 1 \)) and connective tissue growth factor (CTGF). The latter mediates at least some of the profibrotic effects of TGF\( \beta 1 \), but it also has independent and additive effects on matrix upregulation (Phanish et al., 2010). CTGF upregulation is associated with the severity of kidney fibrosis, and inhibition of its expression is protective (Inoue et al., 2003; Ito et al., 2011). CTGF upregulation by stretch has been demonstrated in cells including MC (Chaqour and Goppelt-Struebe, 2006; Hishikawa et al., 2001), but the molecular mechanism by which this occurs is not well defined.

The p21-activated kinases (Paks) are a family of 6 serine-threonine kinases categorized into 2 subfamilies. Group 1 (Pak 1-3) and group 2 (Pak 4-6) differ in structure and regulation, with only group 1 being activated by binding to Rho GTPases. Group 1 Paks have been implicated in regulation of fundamental cellular processes including actin reorganization, motility, apoptosis, and oncogenesis (Eswaran et al., 2008). Pak1 expression has been shown in whole kidney (Kichina et al., 2010) and Pak1 and 2 in glomerular podocytes (Zhu et al., 2010).
The expression of Paks in MC has not been defined. Although Pak1 activation may be GTPase-independent, the most well described activators are the Rho family GTPases Cdc42 and Rac1, 20-24kDa proteins which cycle between an active GTP-bound form and an inactive GDP-bound form (Bishop and Hall, 2000; Burridge and Wennerberg, 2004). A conformational change induced by GTP binding allows Rho proteins to interact with and activate downstream targets by relieving intramolecular autoinhibitory interactions (Bishop and Hall, 2000). Basally, Pak1 exists in an autoinhibited inactive dimeric conformation which is released after binding of the active GTPase, allowing autophosphorylation of the active segment of Pak 1. Phosphorylation of T423 and S141 of Pak1 are important for maximal activation (Kichina et al., 2010).

Pak1 mediates signaling in a context specific manner. No studies have examined the role of Pak1 in MC, but Pak isoforms were shown to be activated in response to mechanical signals (Iliev et al., 2009; Shifrin et al., 2012). In vascular endothelial cells Pak2 was suggested to be a proinflammatory factor in atherogenesis through its matrix-specific activation of JNK and NF-κB and resulting increase in vascular permeability in response to shear (Funk et al., 2010; Iliev et al., 2009; Orr et al., 2007; Orr et al., 2008). Pak1 mediated actin cytoskeleton reorganization in sheared pulmonary endothelial cells (Birukov et al., 2002) and apoptosis in response to tensile forces in NIH3T3 and HEK cells (Shifrin et al., 2012). The role of Pak1 in the MC response to stretch and in matrix upregulation remain undefined and are addressed by our studies.

Increased TGFβ upregulation and release into medium has been shown to regulate cell responses to more prolonged exposure to mechanical stimuli (Gruden et al., 2000; Wipff et al., 2007; Yasuda et al., 1996). TGFβ signals through heteromeric complexes of type I and II transmembrane serine/threonine kinase receptors (TβRI, TβRII) (Wrighton and Feng, 2008). The cytoplasmic domain of TβRII is constitutively active. Upon ligand binding, it complexes with,
phosphorylates and thereby activates TβRI. The Smad protein family are the paradigmatic downstream mediators of TβRI activation. Active TβRI recruits receptor-regulated Smads (2 and 3) and phosphorylates them at a highly conserved C-terminal SSXS motif. These then dissociate from the receptor, associate with the common mediator Smad4, and move as a complex to the nucleus to bind Smad Binding Elements (SBE) in target gene promoter regions, usually in cooperation with other transcription factors (Geisinger et al., 2012; Wrighton and Feng, 2008). TGFβ may also signal through non-Smad proteins. Direct Pak1 activation by TGFβ has thus far only been seen in cancer cells (Luettich and Schmidt, 2003; Wang et al., 2006). However, in HEK293 cells a role for TGFβ receptors in regulating Pak1 signaling by nucleating assembly of a Cdc42/PIX (Pak-interacting Rac1/Cdc42 exchange factors) and Pak complex was suggested, although the functional relevance of this association was not demonstrated (Barrios-Rodiles et al., 2005).

Interestingly, ligand-independent activation of a receptor, known as transactivation, which has long been described for protein tyrosine kinase receptors (Correa-Meyer et al., 2002; Hua et al., 2003; Krepinsky et al., 2005a), has recently also been described for the TβR (Burch et al., 2010; Little et al., 2010). Indeed, CTGF induction in myoblasts by the G-protein coupled receptor (GPCR) agonist LPA required TβRI transactivation (Cabello-Verrugio et al., 2011). Stretch-induced ligand-independent TβRI activation has not yet been described. In our current studies, we identify a novel role for Pak1 as a central mediator of CTGF upregulation in MC in response to stretch. Not only was Pak1 activation effected by ligand-independent transactivation of the TβRI, but Pak1 regulated activation of two parallel but necessary signaling cascades. Thus, both Smad3 and RhoA/Rho-kinase activation downstream of Pak1 effected upregulation of CTGF. Pak1 thus represents an interesting and novel target for therapeutic evaluation in chronic
renal fibrotic disease.
Results

Stretch leads to Pak activation in MC and remnant kidneys

Glomerular podocytes have been shown to express Pak1 and Pak2, with only weak expression of the Pak3 transcript (Zhu et al., 2010). Since Pak expression has not been assessed in MC, we first sought to identify which of the group I Pak isoforms are expressed in these cells. Figure 1A shows that Pak1 is the predominant isoform expressed, with significantly less Pak2 and Pak3 identified after immunoblotting 20\mu g of total cellular protein. To identify whether Pak is activated by stretch, MC were stretched for the times indicated in Figure 1B/C. Pak activation was assessed by immunoblotting with an antibody which detects Pak only when autophosphorylated at S141 (Kichina et al., 2010). Stretch induces early Pak activation, within 30s, which is sustained to 30min and followed by a decrease with longer periods of stretch. This antibody detects phosphorylation of all of the group I Pak isoforms. To more specifically identify which is being activated, isoform-specific antibodies were used to immunoprecipitate Pak from total cell lysate, followed by immunoblotting for phospho-S141. Figure 1D shows that both Pak1 and Pak2 are activated by stretch, with no activation of Pak3. Pak2 activation, however, is somewhat delayed compared to that of Pak1. In Supplementary Figure 1A we confirmed that Pak1 activation remains elevated to 1h with stretch.

To determine whether Pak activation can also be seen in vivo, we used a model of chronic kidney disease achieved by reduction in renal mass, the remnant kidney. This model is characterized by both systemic hypertension and increased intraglomerular pressures. This intraglomerular hypertension transmits to MC as mechanical stretch, and is a critical determinant of glomerular sclerosis in this model (Anderson et al., 1986; Cortes et al., 1994). Figure 1E
shows that Pak1 and Pak2 protein expression are increased in remnant kidneys. While Pak3 expression was detected, requiring prolonged exposure of immunoblots, no change was observed in the remnant kidney. Importantly, Pak activation, as assessed by phosphorylation, was also significantly increased in remnant kidneys. It should be noted that, with a longer period of stretch (16h), we also observed increased Pak1 and Pak2 expression in MC, with Pak1 upregulation being significantly greater than that of Pak2 (Supplementary Figure 1B). This pattern is in keeping with the upregulation of Pak2 in remnant kidneys seen to be primarily in the tubular epithelial cells rather than glomeruli (see below). We used immunohistochemistry to localize Pak upregulation. Figure 1F shows significant Pak1 upregulation in glomeruli as well as in injured (dilated) tubules. By immunofluorescence, Pak1 significantly colocalizes with mesangial cells as identified by their marker Thy1.1 (Supplementary Figure 1C). Pak2 immunohistochemistry (Figure 1G) also shows increased expression in remnant kidneys. However, the increase appeared to be primarily in tubules, with expression in glomeruli appearing similar to control kidneys. Activation of Pak was assessed by immunohistochemistry for phospho-Pak S141. Figure 1H shows increased Pak phosphorylation, particularly in glomeruli, but also seen to a lesser degree in tubules. Given both the predominant expression and activation of Pak1 by mechanical stretch in MC, its upregulation and more importantly its activation in glomeruli of remnant kidneys, we chose to focus on the role of this isoform in further studies.

CTGF upregulation by stretch is dependent on Pak1

The profibrotic cytokine CTGF, not normally expressed in rodent or human glomeruli (Wahab et al., 2001), is upregulated in the hypertensive remnant kidney (Inoue et al., 2003; Phanish et al., 2010). In human hypertensive nephrosclerosis, its upregulation was associated
with the severity of renal fibrosis (Ito et al., 2011; Leask et al., 2009). We confirmed CTGF upregulation by immunohistochemistry in our remnant kidneys, where it was seen predominantly in areas of glomerular sclerosis. Upregulation was also observed in injured, dilated tubules and in some cells located between tubules, likely representing myo/fibroblasts (Figure 2A). Although not investigated here, it is possible that Pak1 and/or 2 may also regulate the development of renal interstitial fibrosis through upregulation of CTGF in both tubular and myo/fibroblast cells. Indeed, CTGF upregulation in tubular epithelium was shown to be important to the development of interstitial fibrosis in the remnant kidney model (Okada et al., 2005) and to mediate TGFβ-induced matrix upregulation in renal fibroblasts (Duncan et al., 1999).

CTGF upregulation by mechanical stress has also been shown in several cell types including MC (Chaqour and Goppelt-Struebe, 2006; Hishikawa et al., 2001; Riser et al., 2000b), but mechanistic studies are limited. Furthermore, the role of Pak1 in CTGF or matrix upregulation has not as yet been examined. We were thus interested in assessing whether Pak1 might contribute to fibrotic signaling, and to CTGF upregulation in particular, in this model. We first confirmed that stretch increased CTGF expression. As shown in Figure 2B, CTGF protein was upregulated within 1h of stretch, in keeping with its known characteristics as an early response gene (Phanish et al., 2010). Upregulation was sustained to 6h. Subsequent studies used 1h of stretch. To determine whether Pak might play a role in CTGF upregulation, we used the nonselective group I Pak inhibitor IPA3. We first confirmed that this blocked Pak1 activation by stretch in MC (Supplementary Figure 2A). IPA3 efficiently blocked CTGF upregulation by stretch (Figure 2C). To implicate the Pak1 isoform, we used several approaches. We first overexpressed the kinase dead Pak1 H83,86L/K299R which functions as a dominant negative protein, and assessed its impact on CTGF upregulation. Figure 2D shows that overexpressed
dominant negative Pak1, identified by immunoblotting for its tag Myc, inhibited stretch-induced CTGF induction. Dominant negative Pak1 did not have any significant basal effect on CTGF expression (Supplementary Figure 2B). Similarly, downregulation of the expression of Pak1 using siRNA also prevented stretch-induced CTGF upregulation (Figure 2E). Successful Pak1 downregulation is shown by immunoblotting. Furthermore, overexpression of constitutively active (ca)Pak1 rescued the inhibition of CTGF upregulation seen with IPA3 (Supplementary Figure 2C). The degree of caPak1 overexpression likely mediated the lack of full restoration of CTGF expression. Last, since CTGF has been shown to induce the synthesis of the matrix protein fibronectin (Phanish et al., 2010; Riser et al., 2000b), the effects of Pak1 downregulation on stretch-induced production of the matrix protein fibronectin was assessed. As shown in Supplementary Figure 2D, stretch increased fibronectin protein both in the cell lysate and in the medium. This was inhibited by Pak1 siRNA. Taken together, these data show that CTGF and subsequent matrix protein upregulation by stretch is dependent on Pak1 in MC.

Pak1 activation is mediated by Rac1

Pak1 is well known to be activated by the GTPases Cdc42 and Rac1 (Eswaran et al., 2008). We have previously shown Rac1 to be activated by stretch with kinetics similar to those of early Pak activation (Zhang et al., 2010). Furthermore, Rac1 has been implicated in CTGF upregulation by angiotensin II in cardiac cells and in scleroderma fibroblasts (Adam et al., 2010; Xu et al., 2009). We thus sought to determine whether Rac1 lies upstream of Pak1 activation in mediating stretch-induced CTGF upregulation. We first assessed the efficacy of the commercially available Rac1 inhibitor NSC23766 in our system. Figure 2F confirmed that this
inhibitor blocked Rac1 activation by stretch, enabling its use in further studies. Pak phosphorylation was also blocked by this inhibitor (Figure 2G). To specifically confirm inhibition of Pak1 phosphorylation and activity, we conducted a Pak1 activity assay. A kinase reaction was carried out using immunoprecipitated Pak1 in which its ability to phosphorylate the well-known Pak substrate stathmin, provided as a recombinant protein, was assessed. Figure 2H confirms that Rac1 inhibition prevented Pak1 activation by stretch. We then assessed the effects of this inhibitor on CTGF upregulation. As expected, NSC23766 prevented the stretch-induced increase in this cytokine (Figure 2I). Thus, Rac1 activation lies upstream of Pak1 activation and CTGF upregulation.

CTGF upregulation is mediated by RhoA and Erk signaling

CTGF expression is tightly controlled, with the mechanisms of regulation being highly context and cell specific (Cicha and Goppelt-Struebe, 2009; Phanish et al., 2010; Samarin et al., 2009). However, a common mediator which appears to not be specific to any particular cell type is activation of the small GTPase RhoA. Our previous work showed RhoA to be activated by stretch in MC at early time points (Krepinsky et al., 2003). To determine whether RhoA signaling is also required for stretch-induced CTGF upregulation, we used the cell-permeable RhoA inhibitor exoenzyme C3 transferase. Figure 3A shows that this prevented stretch-induced CTGF upregulation. Inhibition of the downstream RhoA effector Rho-kinase with Y-27632 also blocked stretch-induced CTGF upregulation (Figure 3B).

RhoA/Rho-kinase effects on F-actin polymerization regulate the transcription factors serum response factor (SRF) and myocardin related transcription factor (MRTF). SRF was
required for shear stress-induced CTGF upregulation in endothelial cells through binding to its consensus element at -3791 (Muehlich et al., 2007). We obtained both the -4.5kb and -4.5kb mSRF (mutated SRF site) CTGF promoter luciferase constructs from Dr. Goppelt-Struebe (Muehlich et al., 2007). Contrary to that seen in endothelial cells, however, both constructs were activated equally by stretch in MC (Figure 3C). Furthermore, although stretch increased the transcriptional activation of SRF assessed using an SRF-responsive luciferase plasmid, this was not inhibited by Pak1 siRNA (Figure 3D). These findings suggest that SRF is not involved in CTGF regulation by stretch in MC. MRTF-A, although not assessed for CTGF (also known as CCN2), was found to regulate stretch-induced increases in the related gene CCN1 (Hanna et al., 2009). Our data, however, show that MRTF-A siRNA did not inhibit CTGF upregulation by stretch (Figure 3E).

Other mediators shown to regulate CTGF by stretch include NF-κB in bladder smooth muscle cells and PI3K-JNK, but not Erk, signaling in osteoblast-like cells (Chaqour et al., 2006; Xiao et al., 2011). Our studies using the NF-κB inhibitor SC514 excluded a role for NF-κB in CTGF upregulation (Figure 3F). However, inhibition of Erk activation with the MEK inhibitor U0126 completely prevented CTGF promoter luciferase upregulation by stretch (Figure 3G). Collectively, these data indicate that RhoA and Erk activation, but not SRF, MRTF-A or NF-κB, are required for stretch-induced CTGF upregulation in MC. Last, we assessed whether RhoA and Erk were activated in the remnant kidney. Using cortical lysates, we performed a RhoA activity assay as described in Methods and assessed Erk activation by immunoblotting for its phosphorylation. Supplementary Figure 3 shows that both are activated in the remnant kidney.
Pak1 mediates Erk and RhoA activation by stretch

Since we found both Erk and RhoA, as well as Pak1, to be required for CTGF upregulation by stretch, we were interested in assessing whether Pak1 could function as a central mediator of both of these signaling proteins. We first assessed the role of Pak1 in Erk activation. Figure 4A shows that Pak1 downregulation with siRNA inhibited Erk activation by stretch, as assessed by Erk phosphorylation on T202/Y204. Our data showing Rac1 to be upstream of Pak1 activation implied that its inhibition should also prevent Erk activation. Figure 4B shows that the Rac1 inhibitor NSC23766 did indeed block stretch-induced Erk activation. We next assessed the effects of Pak inhibition on RhoA activation. Figure 4C shows that the Pak inhibitor IPA3 prevented stretch-induced RhoA activation. Similarly, inhibition of upstream Rac1 activation with NSC23766 also blocked stretch-induced RhoA activation (Figure 4D). Interestingly, inhibition of RhoA unexpectedly attenuated stretch-induced Pak1 activation (Supplementary Figure 4). RhoA activation was also shown to contribute to the activation of Pak1 in vascular smooth muscle cells (Wang et al., 2009). These data support a bidirectional relationship between RhoA and Pak1 activation in MC.

TGFβ receptor I transactivation mediates ligand-independent Rac1/Pak1 activation by stretch

Having established that Rac1/Pak1 activation are central to the upregulation of CTGF by stretch, we next sought to identify the upstream mediator of activation of these GTPases. TGFβ has been shown to activate Pak1 in cancer cells (Luettich and Schmidt, 2003; Wang et al., 2006), and it activates both Rac1 and RhoA in MC (Hubchak et al., 2009). We were thus interested in determining whether TGFβ may be a proximal mediator in this signaling cascade. We thus first
assessed the effects of two structurally distinct TβRI inhibitors, SB431542 and GW788388. Figures 5A,B show that both inhibitors prevented stretch-induced Rac1 activation by stretch. Downstream activation of Pak1 and RhoA, as well as upregulation of CTGF, were also blocked by inhibition of TβRI (Figures 5C-F).

The Smad protein family are the paradigmatic downstream mediators of TβRI activation. Since our inhibition studies implicated activation of TβRI by stretch, we next assessed activation of the receptor-regulated Smad3 by immunoblotting for its C-terminal SSXS phosphorylation. Figure 6A shows that Smad3 was activated by stretch. As expected after activation, Smad3 was also seen to translocate to the nucleus in response to stretch (Supplementary Figure 5). Smad3 activation was dependent on TβRI signaling as shown by complete inhibition by the TβRI inhibitor SB431542 (Figure 6B). The distinct TβRI inhibitor GW788388 also prevented stretch-induced Smad3 activation (not shown).

To determine whether stretch induces TGFβ secretion to mediate activation of its receptors within the timeframe of our studies, we incubated cells with a neutralizing TGFβ1 antibody or a control antibody prior to stretch. As seen in Figure 6C, TGFβ1 neutralization had no effect on stretch-induced Smad3 activation. Since TβRI activation in response to stretch may also occur through proteolytic action of a metalloprotease to induce release of latent TGFβ1 (Wipff et al., 2007; Wipff and Hinz, 2008), we preincubated MC with the general metalloprotease inhibitor GM6001. Similar to that seen with TGFβ1 neutralization, metalloprotease inhibition had no effect on stretch-induced Smad3 activation (Figure 6D). To further confirm the absence of TGFβ secretion into the medium by stretch, we performed an
ELISA on conditioned medium after 30min of stretch, a time point at which Pak1 and Smad3 are robustly activated. We assessed both active TGFβ1 as well as total TGFβ1, the latter by acid activation of the samples. No increase in TGFβ1 in the media was observed (not shown). These data imply that stretch induces the transactivation of TβRI in a ligand-independent manner.

CTGF upregulation by stretch requires Smad3

Both basal and TGFβ-induced CTGF upregulation in MC require a Smad binding element at -173 (Chen et al., 2002). Whether Smad3 is required for stretch-induced CTGF upregulation, however, is unknown. We isolated primary MC from Smad3 knockout (KO) mice and their wild-type (WT) counterparts. Absence of Smad3 in KO cells was confirmed by immunoblotting (Figure 6E). We then determined the effect of Smad3 deletion on CTGF upregulation by stretch. While somewhat higher basal expression of CTGF protein was observed in Smad3 KO cells, CTGF protein upregulation by stretch was almost completely inhibited in the absence of Smad3 (Figure 6F). CTGF transcript upregulation and promoter activation, the latter assessed by luciferase activity, were only seen in WT cells (Figures 6G,H). These data show that Smad3 is essential for stretch-induced upregulation of CTGF. We further assessed the temporal relationship between Pak, Erk and Smad3 activation in relation to CTGF upregulation. Supplementary Figure 6 shows that activation of these upstream signaling mediators of CTGF occur within 30min, with increases in CTGF protein seen shortly after this, within 1h.

Stretch-induced Smad3 activation is regulated by Rac1/Pak1 activation

Our data thus far indicated that Smad3 and Rac1/Pak1 activation are required for CTGF
upregulation by stretch. To our knowledge, no data indicating positive cross-talk between these signaling mediators is available. To determine whether cross-talk exists, we first tested the effects of inhibiting Rac1/Pak1 on Smad3 activation. Interestingly, inhibition of Rac1 with NSC23766 prevented Smad3 C-terminal phosphorylation by stretch (Figure 7A). Similarly, inhibition of Pak activation by IPA3 also prevented Smad3 activation (Figure 7B), as did Pak1 downregulation by siRNA (Figure 7C). To determine whether Smad3 transcriptional activity was also dependent on Pak1 activation, we used two different Smad3-responsive luciferase constructs. p3TP-lux contains 3 consecutive TPA response elements (TREs) and a portion of the plasminogen activator inhibitor-1 (PAI-1) promoter region. CAGA_{12}-luc contains 12 repeats of the CAGA Smad binding element driving luciferase expression. Stretch activated both p3TP-lux and CAGA_{12}-luc (Figure 7D-F). Both kinase dead Pak1 and Pak1 downregulation by siRNA prevented p3TP-lux activation (Figure 7D,E), and CAGA_{12}-luc activation was also prevented by Pak1 siRNA (Figure 7F). Furthermore, when stretch-induced p3TP-lux activation was suppressed by Pak1 siRNA, caPak1 was able to restore Smad3 transcriptional activation of this promoter (Supplementary Figure 7). These data establish an upstream requirement for Pak1 in Smad3 phosphorylation and transcriptional activation.

We have previously shown that TGFβ-induced Smad3 C-terminal phosphorylation is unaffected by Rho-kinase inhibition (Peng et al., 2008). Given that Pak1 mediates activation of both Smad3 and RhoA/Rho-kinase signaling, both of which contribute importantly to CTGF upregulation, we assessed whether Rho-kinase regulates Smad3 activation. As seen in Figure 7G, stretch-induced Smad3 phosphorylation was unaffected by the Rho-kinase inhibitor Y-27632. However, Smad3 transcriptional activation, as assessed by CAGA_{12}-luc activation, was partially
prevented by Rho-kinase inhibition (Figure 7H). We previously showed that Rho-kinase mediated stretch-induced Erk activation, and Erk may modulate Smad3 transcriptional activity through phosphorylation on the Smad3 linker region (Hayashida et al., 2007; Krepinsky et al., 2005b). Figure 7I shows that inhibition of Erk activation with the MEK inhibitor U0126 similarly partially prevented Smad3 transcriptional activation. These data suggest that Pak1 is a central mediator of two signaling mediators, Smad3 and RhoA/Erk which integrate to effect CTGF upregulation in response to mechanical stress in MC. Whether Erk also functions through activation of additional co-transcription factors is yet to be determined. The overall schematic of this proposed signaling pathway is shown in Figure 8.
Discussion

A greater understanding of the molecular mechanisms regulating matrix production and thereby glomerular sclerosis is essential to enable identification of new potential therapeutic targets for treatment of chronic renal disease. Our study presents several novel findings. Most importantly, we have identified a key role for Pak1 in the regulation of the profibrotic cytokine CTGF, a mediator of matrix upregulation in its own right, as well as an important mediator of the effects of the well-known profibrotic cytokine TGFβ1 (Phanish et al., 2010). We have also identified a novel role for ligand-independent TβRI transactivation in the profibrotic response to stretch. Importantly, Pak1 regulates both Smad (Smad3) and non-Smad (RhoA/Rho-kinase/Erk) signaling downstream of the TβRI. Pak1 thus stands as the central mediator of two important signaling cascades required for CTGF upregulation. These studies serve as the foundation for assessing Pak1 as a potential modifiable target in the treatment of chronic renal disease.

Of the group 1 Paks, only Pak2 has thus far been linked to fibrosis. Activated by TGFβ in mesenchymal cells, one group has suggested that Pak2 may be important to the development of renal interstitial fibrosis. However, in vivo studies assessing this in the unilateral ureteral obstruction model were performed with an inhibitor of c-Abl, a kinase downstream of Pak2 in these cells (Wang et al., 2005; Wang et al., 2010). While our studies showed that Pak2 was also activated by stretch in MC, its upregulation in remnant kidneys was seen primarily in tubular cells, leading us to focus on a potential role for Pak1 in profibrotic signaling. It is possible, however, that Pak isoform specificity exists for the different cell types within a kidney, such that
Pak1 may contribute to glomerular sclerosis and Pak2 to interstitial fibrosis. Indeed, studies performed primarily by one group showed cell specific activation of Pak2 in mesenchymal, but not epithelial or mesangial cells by TGF\(\beta\) (Hough et al., 2012; Wang et al., 2005; Wilkes et al., 2003; Wilkes et al., 2005). Interestingly, in epithelial cells Pak2 was actually inhibitory to TGF\(\beta\) signaling, possibly through direct interaction with and inhibition of Smad2/3 (Yan et al., 2012).

Although our study is the first to link Pak1 to matrix regulation, a role for its upstream activator Rac1 has been suggested. In MC derived from integrin \(\alpha_1\) knockout mice, increased Rac1 activation was associated with increased collagen IV production (Chen et al., 2007), and TGF\(\beta\)-induced collagen I expression was mediated by Rac1 in MC (Hubchak et al., 2009). CTGF upregulation by angiotensin II in cardiac cells and in scleroderma fibroblasts (which are characterized by elevated Rac1 activity) was also decreased by Rac1 inhibition (Adam et al., 2010; Xu et al., 2009). One study has shown a role in vivo for Rac1 in matrix upregulation. Here, fibroblast-specific Rac1 deletion prevented bleomycin-induced skin fibrosis (Liu et al., 2008). Rac1 may also contribute to injury and fibrosis through its role in regulating NADPH oxidase activity and hence ROS generation. Indeed, we previously showed that stretch-induced ROS production, mediated by the NADPH oxidase system including Rac1, regulates RhoA activation (Zhang et al., 2010). This suggests that ROS also contribute to CTGF upregulation. We confirmed this in Supplementary Figure 8A which shows that the antioxidant N-acetylcysteine (NAC) prevented stretch-induced CTGF upregulation. ROS also contribute to the upregulation of CTGF by stretch in kidney tubular epithelial cells (Sonomura et al., 2012). Rac1 thus offers an additional potential treatment target worthy of investigation in a chronic renal fibrosis model.

Our data showed that T\(\beta\)RI transactivation, independent of ligand binding, mediates the
activation of Rac1/Pak1. Indeed, while increased TGFβ transcript levels and secretion into the medium have been demonstrated in stretched MC and other cells, this occurs with much longer periods of stretch (Gruden et al., 2000; Riser et al., 1998; Sakata et al., 2004; Zheng et al., 2001). In MC, increased latent and active TGFβ1 were only seen after 48-72h of stretch (Riser et al., 1996), and none was observed at 3, 6 or 12h (Yasuda et al., 1996). The earliest TGFβ secretion noted in non-MC was at 4h in stretched airway smooth muscle cells (Mohamed and Boriek, 2010). Thus, TGFβ secretion in MC occurs much later than activation of the signaling pathways (minutes) and upregulation of CTGF (1h) which we have observed.

The molecular mechanism underlying TβRI activation of Rac1/Pak1 is unknown. We have previously shown that the epidermal growth factor receptor (EGFR) is an important upstream regulator of Rac1 activation by mechanical stress in MC (Zhang et al., 2010). Since TGFβ activation of the EGFR has been described (Murillo et al., 2005), we assessed whether the TβRI might signal through the EGFR. Supplementary Figure 8B-D shows that in stretched MC, TβRI inhibition prevents EGFR transactivation, and that EGFR inhibition prevents CTGF upregulation. The nature of the cross-talk between TβRI and EGFR in stretched MC, however, remains to be elucidated.

Direct Pak1 activation by TGFβ has thus far only been seen in cancer cells (Luettich and Schmidt, 2003; Wang et al., 2006), although a screening study in HEK293 cells using protein overexpression suggested that TGFβ receptors may scaffold the assembly of a Rho GTPase (Rac1/Cdc42), PIX (Rac1 GEF) and Pak complex (Barrios-Rodiles et al., 2005). However, the functional significance of this interaction was not clear, and whether TGFβ receptors serve as a
scaffold for Pak1 activation in stretched MC is unknown. Pak2 association with either TβRI or TβRII was not seen despite its activation by TGFβ1 in mesenchymal cells (Wilkes et al., 2003), suggesting both cell and isoform specificity for the mechanism of Pak regulation through TβR activation.

CTGF is a tightly regulated early response gene, regulated primarily at the level of transcription, with the mechanisms of regulation being highly context and cell specific (Cicha and Goppelt-Struebe, 2009; Phanish et al., 2010; Samarin et al., 2009). Although CTGF upregulation by mechanical stress has been shown in MC (Hishikawa et al., 2001; Riser et al., 2000b), the mechanism of its upregulation was unknown. We now show a central role for Pak1 in mediating its expression in response to stretch, through its coordination of Smad3 and RhoA signaling. While RhoA activation appears to be a common mediator of CTGF upregulation not specific to any particular cell type, its effects were thought to be mediated by regulation of the transcription factor SRF (Muehlich et al., 2007). However, our studies did not support a role for SRF in regulation of CTGF induction in MC, contrary to that seen in endothelial cells (Muehlich et al., 2007). Similarly, the transcription factor MRTF-A which regulates stretch-induced increases in the related gene CCN1 (Hanna et al., 2009) and is also regulated by changes in F-actin polymerization (Olson and Nordheim, 2010), was not found to mediate CTGF upregulation in our system.

Our previous data showing that stretch-induced activation of Erk and its upstream kinase Raf-1 were dependent on RhoA/Rho-kinase cytoskeletal effects (Krepinsky et al., 2003; Krepinsky et al., 2005b) led us to assess whether Erk might be involved in CTGF upregulation. Although Erk has been found to not mediate stretch-induced CTGF expression in osteoblasts
(Xiao et al., 2011), CTGF induction in response to other stimuli including endothelin-1 and TGFβ has required Erk (Gosmain et al., 2005; Recchia et al., 2009). Indeed, our data showed that Erk is required for CTGF upregulation by stretch in MC, providing a mechanistic link between Pak1/RhoA signaling and CTGF gene regulation. It should be noted, however, that Pak1 may also regulate Erk activation through direct phosphorylation and thereby activation of Raf-1 (Eswaran et al., 2008). The transcription factor regulated by Erk to mediate CTGF upregulation in stretched MC remains to be determined. AP-1, regulated by both Erk and JNK signaling, is one possibility, since it is both activated by mechanical stress in MC (Ingram et al., 2000) and required for CTGF upregulation in some settings (Recchia et al., 2009; Xia et al., 2007). In support of this, Supplementary Figure 9 shows that the JNK inhibitor SP600125 attenuates stretch-induced CTGF protein upregulation and activation of the CTGF promoter. Inhibition of stretch-induced JNK activation is also confirmed. Our data using Smad3-deficient MC have identified an absolute requirement for Smad3 in stretch-induced CTGF upregulation, and show for the first time that Pak1 may facilitate Smad3 C-terminal phosphorylation and transcriptional activation. Interestingly, Pak1/Smad3 activation were mediated by ligand-independent TβRI activation. Recently, Smad3 has also been implicated in stretch-induced CTGF expression in inner meniscus cells, but this study did not define whether TGFβ1 secretion was involved. How Pak1 functions to regulate Smad3 C-terminal phosphorylation, a well recognized target of the activated TβRI, is not known. Interestingly, Pak2 has been found to directly associate with Smad 3 and to phosphorylate S375 in epithelial cells. This attenuated Smad3 interaction with the TβRI, thereby inhibiting its activation (Yan et al., 2012). Given the differential effects of Pak1 and
Pak2 on TGFβ signaling in different cell types, it is possible that Pak1 may also interact with Smad3 and serve to assist its recruitment to the TβRI for phosphorylation. Indeed, Pak1 interaction with TβRI has been reported (Barrios-Rodiles et al., 2005). Although far less likely, direct C-terminal phosphorylation of Smad3 by Pak1 would also need to be excluded. Pak1 may also modulate Smad3 transcriptional activation through its regulation of Erk, which is known to phosphorylate Smads 2/3 on their linker region. This phosphorylation may either inhibit or enhance Smad-mediated transcriptional activity in a context-dependent manner (Hayashida et al., 2003; Hough et al., 2012; Leask and Abraham, 2004). In our system, Erk activation downstream of Pak1 contributed to Smad3 transcriptional activation. Pak1 thus regulates Smad3 activation at multiple levels.

In conclusion, we show that Pak1 is a central mediator of stretch-induced CTGF upregulation in MC. Both Smad3 and RhoA/Rho-kinase/Erk signaling pathways are necessary downstream of Pak1. Our study provides a strong foundation for the assessment of Pak1 inhibition as a novel antifibrotic strategy in chronic renal disease.
Materials and Methods

Cell Culture

Sprague-Dawley primary rat and mouse MC were obtained from glomeruli of rats or mice (Smad3 knockout or their corresponding wild-type, 129SvEv/C57BL/6, obtained from Dr. Gauldie, McMaster University, Hamilton, Canada) by differential sieving and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 units/ml, all Life Technologies Inc, Burlington, ON, Canada) at 37°C in 95% air, 5% CO2. Experiments were carried out using cells between passages 6-15. To apply mechanical stretch, MC were plated onto 6-well plates with flexible bottoms coated with bovine type I collagen (Flexcell International Corp., Hillsborough, NC, USA). At confluence, cells were serum deprived for 24h. They were then exposed to continuous cycles of stretch/relaxation by vacuum controlled by software (Flexercell 4000, Flexcell International Corp.), with each cycle being 0.5s of stretch (10%) and 0.5s of relaxation, for a total of 60 cycles/min. Pharmacologic inhibitors were added as follows prior to stretch: IPA3 (30 μM, 10min), SB431542 (5 μM, 30min), both Tocris, Burlington, ON, Canada); NSC23766 (100 μM, 30min), Y-27632 (10 μM, 30min), GM6001 (20 μM, 1h), PD168393 (0.5 μM, 30min), SP600125 (20 μM, 1h), all EMD Millipore, Mississauga, ON, Canada; SC 514 (25 μM, 1h, Cayman Chemical, Burlington, ON, Canada); C3 transferase (2 μg/ml, 6h, Cytoskeleton, Denver, CO, USA); GW788388 (5 μM, 30min), U0126 (10 μM, 30min), N-acetylcysteine (NAC, 4mM, 30min), AG1478 (1 μM, 30min), all Sigma, Oakville, ON, Canada.
Protein Analysis

Cells were lysed and protein extracted as published (Krepinsky et al., 2003). Lysates were centrifuged at 4°C, 14,000rpm for 10min. Supernatant (50μg) was separated by SDS-PAGE, and Western blotting performed. Antibodies used were monoclonal collagen I (1:1000, Sigma), goat polyclonal CTGF (1:2000, Sant Cruz Biotechnology Inc, Santa Cruz, CA, USA), phoso-Erk T202/Y204 (1:1000, Cell Signaling, Boston, MA, USA), monoclonal myc (1:1000, Santa Cruz), polyclonal Pak1 (1:1000, Santa Cruz), polyclonal Pak2 (1:1000, Cell Signaling), polyclonal Pak3 (1:1000, Cell Signaling), polyclonal phoso-Pak S141 (1:1000, Abcam, Toronto, ON, Canada), polyclonal phoso-Smad3-S423/425 (1:3000, EMD Millipore), polyclonal Smad3 (1:500, Abcam), neutralizing anti-TGFβ (R&D Systems, Burlington, ON, Canada), anti-rabbit isotype control (Jackson ImmunoResearch, West Grove, PA, USA), monoclonal tubulin (0.5μg/ml, Sigma), polyclonal phoso-JNK-Thr183/Tyr185 (1:1000, Cell Signaling), monoclonal fibronectin (1:5000, BD Biosciences, Mississauga, ON, Canada). For assessment of fibronectin in the medium, conditioned media was harvested after stretch for 24h, centrifuged at 4000rpm to remove cell debris and 15μg run on SDS-PAGE after boiling in PSB.

For immunoprecipitation experiments, cells were lysed, clarified and equal amounts of lysate incubated overnight with 2μg primary antibody rotating at 4°C, followed by 25μl of protein G-agarose slurry for 1.5h at 4°C. Immunoprecipitates were extensively washed, resuspended in 2x sample buffer, boiled, and analyzed by immunoblotting. For Pak activity assays, the immunoprecipitated Pak was incubated in kinase buffer containing 200μM ATP, 0.2mM DTT and 0.5μg His-Stathmin (Novus Biologicals, Littleton, CO, USA) for 30min at 30°C. Activity was detected by phosphorylation of Stathmin at S16 (1:500, Cell Signaling).
RhoA and Rac1 pull-down assays were performed according to manufacturer’s specifications (Cytoskeleton kits). Briefly, cells were lysed in hypertonic buffer and GTP-bound RhoA or Rac1 was immunoprecipitated from cleared lysate with 30μg of glutathione-agarose bound GST-tagged rhotekin RhoA binding domain for RhoA or Pak-binding domain (PBD) for Rac1. Beads were washed and the immunoprecipitate resolved on 15% SDS-PAGE. Membranes were probed with monoclonal anti-RhoA (1:500, Santa Cruz) or monoclonal anti-Rac1 antibody (1:500, Cytoskeleton). Lysate (40μg) was also probed for RhoA or Rac1 to ensure equality across conditions.

Transfection and siRNA

Myc-Pak1 H83,86L/K299R (dominant negative (dn)Pak1) or Myc-Pak1 H83,86L/T422E Pak1 (constitutively active (ca)Pak1), kindly provided by Dr. A. Mak, Queen’s University, Canada) was transfected in subconfluent cells using Fugene according to manufacturer’s instructions (Promega, Madison, WI, USA). On-target plus SMART pool siRNA for Pak1, Pak2 and MRTF-A and control non-targeting siRNA were obtained from Dharmacon (Pittsburgh, PA, USA). MC were transfected with 100nM siRNA using Lipofectamine RNAiMAX (Life Technologies) at 60% confluence. Protein expression was used to assess efficacy of downregulation by RNAi.

Real-Time PCR

Snap frozen kidney cortex was homogenized in Trizol and total RNA extracted according to manufacturer’s instructions (Life Technologies). RT was performed using standard methods
and cDNA analysed using real-time PCR for fibronectin, collagen Iα1 or CTGF, with values normalized to 18S.

**TGFβ ELISA**

After stretch for 30min, medium was harvested and debris removed by centrifugation at 4°C, 4000rpm. Media was stored at -80°C until processing. TGFβ was measured according to manufacturer’s instructions using a kit from Promega. Both untreated and acid-activated samples were assayed, representing active and total TGFβ respectively.

**Luciferase Assay**

Constructs used were: CTGF promoter luciferase (pGL3-CTGF 4.5kb), both wild-type and mutated at the -3791 SRF site (pGL3-CTGF 4.5kb SRFmut), both kindly provided by Dr. M. Goppelt-Struebe (University of Erlangen-Nuremberg, Germany), the Smad3-responsive luciferase constructs p3TP lux (in pGL2) and pGL3-CAGA12-luc, kindly provided by Dr. J. Massague (Sloan-Kettering Institute, NY, USA) and Dr. M. Bilandzic (Prince Henry’s Institute, Australia) respectively. pSRF-luc was obtained from Stratagene.

MC plated to 80% subconfluence were transfected with 1μg of the promoter-luciferase construct and 0.1μg pCMV-β-galactosidase (β-gal) (Clontech, Mountain View, CA, USA) using LipofectAMINE (Qiagen, Toronto, ON, Canada) in 6-well stretch plates. MC were serum-deprived overnight 24h after transfection prior to stretch. Cells were lysed with Reporter Lysis Buffer (Promega) using one freeze-thaw cycle, and luciferase and β-gal activities measured on
clarified lysate using specific kits (Promega) with a Berthold luminometer and a plate reader (420nm) respectively. β-gal activity was used to adjust for transfection efficiency.

**Animals**

Experiments were conducted in accordance with McMaster University and Canadian Council on Animal Care guidelines. Male Sprague-Dawley rats (Charles River, Montreal, QC, Canada) weighing 200g underwent a two-stage 5/6 nephrectomy. In the first stage, 2 of the 3 branches of the left renal artery were ligated to induce infarction of 2/3 of the kidney. This was followed one week later by a right nephrectomy. Control rats underwent sham operation, consisting of a laparotomy and manipulation of the renal pedicles. Housing was in a temperature controlled facility with free access to standard chow and water. There were 4 rats per group, with sacrifice 4 weeks after surgery. Remnant kidneys developed significant hypertension as assessed by volume pressure recording (Coda 2, Kent Scientific, Torrington, CT, USA).

For immunoblotting, renal cortex was homogenized in lysis buffer and processed as described above. For immunohistochemistry (IHC), 4μm paraffin sections were deparaffinized and heat-induced epitope retrieval performed. Primary antisera used were rabbit Pak1 (1:50, Santa Cruz), rabbit Pak2 (1:75, Abcam), rabbit phospho-Pak S141 (1:50, Abcam), and goat CTGF (1:500, Santa Cruz). For immunofluorescence, cortical sections stored in OCT were processed as previously described (Wu et al., 2007). Primary antisera used were polyclonal Pak1 (1:50, Santa Cruz) and monoclonal Thy1.1 (1:50, BD Biosciences).

**Statistical Analysis**
Statistical analyses were performed with SPSS20 for Windows using one-way ANOVA, with Tukey’s HSD for post-hoc analysis. For IHC analysis, data was analysed using a linear mixed model, random effects. A P-value < 0.05 (two-tailed) was considered significant. Data are presented as the mean ± standard error of the mean, and number of repetitions denoted as “n=”.
Acknowledgements

J. Krepinsky is the guarantor for this manuscript, and gratefully acknowledges the support of the Kidney Foundation of Canada (KFOC) and St. Joseph’s Healthcare for their support of nephrology research. G. Chen was supported by a Father Sean O’Sullivan Postdoctoral Fellowship. H.W. Schnaper was supported by NIDDK grant RO1 DK49362. We would like to thank the following for providing constructs: Dr. A Mak, Queen’s University, Canada for Pak1 constructs, Dr. M. Goppelt-Struebe, University of Erlangen-Nuremberg, Germany for CTGF promoter luciferase (pGL3-CTGF 4.5kb), both wild-type and mutated at the -3791 SRF site (pGL3-CTGF 4.5kb SRFmut), Dr. J. Massague, Sloan-Kettering Institute, NY, USA for p3TP lux, and Dr. M. Bilandzic, Prince Henry’s Institute, Australia for pGL3-CAGA12-luc.
Reference List


Okada, H., Kikuta, T., Kobayashi, T., Inoue, T., Kanno, Y., Takigawa, M., Sugaya, T.,


Figure Legends

Figure 1. Pak1 is activated by stretch in MC and upregulated in remnant kidneys. (A) Immunoblotting for Pak1, 2 and 3 was performed with isoform-specific antibodies. Pak1, 2 and 3 are expressed in MC, with Pak1 expression being the greatest (20 μg protein was run). (B,C) MC were stretched for the indicated times (30 sec to 5 min) in (B) and to 60 min in (C) and Pak activation assessed by phosphorylation on S141 (*p<0.05 control vs others, n=7 for A and B). The pPak S141 antibody recognizes all 3 isoforms. MC were stretched for 5 min to assess Pak activation in subsequent studies. (D) To assess activation of each isoform, an isoform-specific antibody was used for immunoprecipitation after stretch for 5 min for Pak1 and 3, or to 30 min for Pak2. This was probed for pPak S141 using the same antibody as in B/C. Both Pak1 and Pak2 were activated by stretch, although Pak2 activation was only seen after 30 min. No Pak3 activation was seen even after 30 min of stretch (not shown). (E-H) Analysis of a model of chronic kidney disease characterized by both systemic and intraglomerular hypertension, the 5/6 nephrectomized or remnant rat. This was created by ligation of 2 of 3 renal artery branches to one kidney, and removal of the contralateral kidney, to produce a 5/6 reduction in renal mass or the “remnant kidney”. (E) Pak1 and 2 expression are upregulated in remnant kidneys, with no significant change in expression of Pak3. Increased Pak activation, as assessed by its S141 phosphorylation, is seen in remnant kidneys. (F-H) Immunohistochemistry of control or remnant kidney tissue. (F) Pak1 was significantly increased in remnant kidneys in glomeruli as well as in injured (dilated) tubules. Tubular staining is identified by red arrows. The black arrows indicate possible staining in interstitial fibroblasts. (G) Pak2 was also increased, although upregulation
was localized predominantly to tubules, with some tubules having a greater increase than others. The black arrow identifies a tubule with greater Pak2 upregulation, and the black arrowhead one with a lower degree of upregulation. **(H)** Phospho-Pak S141, an indicator of Pak activation, was significantly increased in glomeruli in remnant kidneys as well as in some tubules, the latter identified by black arrows.

**Figure 2.** Rac1-induced Pak1 activation mediates CTGF upregulation by stretch. **(A)** CTGF expression in control and 4-week remnant kidneys was assessed by immunohistochemistry. CTGF was not expressed in control rats, but was upregulated in sclerosed areas of glomeruli in remnants and in damaged tubules. **(B)** CTGF was upregulated by stretch in MC (*p<0.05 control vs others, n=6). For subsequent experiments assessing CTGF protein, 1h of stretch was used. **(C)** CTGF upregulation was blocked by the Pak1-3 inhibitor IPA3 (‡p<0.001 vs others, n=6). **(D)** Kinase dead Pak1, “dnPak1”, prevented CTGF upregulation by stretch (*p<0.05 vs others, n=3) as compared to MC transfected with the empty vector pcDNA. Immunoblotting for Myc identified successful overexpression of the construct. **(E)** Pak1 siRNA blocked CTGF upregulation by stretch as compared to non-targeting control siRNA (†p<0.01 stretch vs others, n=3). Immunoblotting for Pak1 showed successful downregulation by siRNA. **(F)** Stretch-induced Rac1 activation at 5min (which we previously showed was an early maximal time of activation) was blocked by the Rac1 inhibitor NSC23766, ensuring functionality of the inhibitor (*p<0.05 vs others, n=4). **(G)** NSC23766 blocked stretch-induced Pak activation, assessed by Pak phosphorylation on S141 (†p<0.01 vs others, n=6). **(H)** Inhibition of the Pak1 isoform by NSC23766 was confirmed using a kinase activity assay. After stretch, Pak1 was
immunoprecipitated and incubated with the Pak substrate His-stathmin. Its phosphorylation on Ser 16 was assessed by immunoblotting using a pStathmin S16 antibody (*p<0.05 vs others, n=4). (I) CTGF upregulation by stretch was blocked by Rac1 inhibition with NSC23766 (‡p<0.001 vs others, n=6).

Figure 3. **CTGF upregulation is mediated by RhoA/Rho-kinase and Erk signaling, but not by SRF, MRTF-A or NF-κB.** (A,B) CTGF upregulation by stretch was blocked by the RhoA inhibitor C3 and downstream Rho-kinase inhibitor Y27632 (†p<0.01 vs others, n=6 for B). (C) Comparison of stretch-induced activation of the CTGF promoter luciferase construct with or without mutation at the SRF binding site at -3791 (“mSRF”). This site was shown to be important for shear stress-induced CTGF upregulation. CTGF promoter luciferase activation is assessed after 6h of stretch (found to be a reliable time of induction in preliminary studies). Both promoters responded equally to stretch, indicating that this site is not important for CTGF upregulation in MC (*p<0.05 S vs controls, n=3). (D) Although stretch increased activation of an SRF-luciferase reporter, this was not inhibited by downregulation of Pak1 with siRNA (*p<0.05 vs controls, n=3). (E) MRTF-A siRNA did not inhibit CTGF promoter activation (*p<0.05 vs controls, n=3). (F) NF-κB inhibition with SC-514 also did not prevent CTGF promoter activation (*p<0.05 vs control, n=4). (G) CTGF promoter luciferase activation was blocked by inhibition of MEK, an upstream kinase required for Erk activation (†p<0.01 vs others, n=4).

Figure 4. **Pak1 mediates Erk and RhoA activation by stretch.** (A) Stretch-induced Erk activation (after 10 min of stretch), as assessed by its phosphorylation, was inhibited by Pak1 downregulation with siRNA (*p<0.05 S vs others, n=3). (B) Inhibition of upstream Rac1 by
NSC23766 also blocked stretch-induced Erk activation (*p<0.05 vs others, n=2). (C) RhoA activation was assessed by immunoprecipitation of the active, GTP-bound RhoA by GST-Rhotekin (Cytoskeleton). Stretch-induced RhoA activation (after 5 min of stretch) was blocked by the Pak inhibitor IPA3 (†p<0.01 vs others, n=6). (D) Inhibition of Rac1 by NSC23766 also blocked stretch-induced RhoA activation (*p<0.05 vs others, n=5).

**Figure 5. TGFβRI is required for stretch-induced Rac1/Pak1/RhoA activation and CTGF upregulation.** (A) Stretch (5 min)-induced Rac1 activation was blocked by the TGFβRI inhibitor SB431542 (*p<0.05 vs others, n=6), (B) as well as by a second chemically distinct inhibitor GW788388. (C) Both inhibitors blocked Pak1 S141 phosphorylation, which was assessed on immunoprecipitated Pak1 (*p<0.05 vs others, n=3). (D) Stretch-induced RhoA activation was also blocked by TGFβRI inhibition with SB431542 (*p<0.05 vs others, n=4). (E,F) Stretch-induced CTGF upregulation was blocked by both TGFβRI inhibitors (‡p<0.01 vs others, n=6 in (E) and †p<0.01 vs others, n=4 in (F)).

**Figure 6. TGFβ-independent Smad3 activation is required for CTGF upregulation by stretch.** (A) Stretch induced Smad3 C-terminal SSXS (S423/425) phosphorylation within 30 min, and this was sustained to 3h (*p<0.05 stretch vs control, n=5). (B) Stretch-induced Smad3 phosphorylation was blocked by the TGFβRI inhibitor SB431542 (†p<0.01 vs others, n=4). (C) Smad3 phosphorylation was not blocked by a neutralizing TGFβ antibody (10µg/ml, added 1h prior to stretch). Nonspecific control rabbit antibody did not have any effect. (D) Stretch-induced Smad3 phosphorylation was not blocked by the metalloprotease inhibitor GM6001. (E) Smad3 knockout (KO) MC isolated from Smad3 KO mouse kidneys were confirmed to lack Smad3.
expression. (F) CTGF induction by stretch was significantly reduced in Smad3 KO compared with their wild-type (WT) counterpart MC (†p<0.01 vs others, n=6). Somewhat higher basal expression of CTGF was observed in KO cells. Statistics for all graphs in this figure compare stretch to control for KO and WT separately. (G) Stretch (6h)-induced CTGF transcript upregulation, as assessed by real-time PCR, was abrogated by Smad3 deficiency (†p<0.01 vs others, n=6). (H) Stretch-induced CTGF promoter activation was also prevented by Smad3 deletion (†p<0.01 vs others, n=3).

Figure 7. Smad3 C-terminal phosphorylation and transcriptional activity require Rac1/Pak1 activation, but not RhoA signaling. (A) Smad3 phosphorylation in response to stretch (30 min) was blocked by the Rac1 inhibitor NSC23766 (*p<0.05 vs others, n=5). (B) Stretch-induced Smad3 phosphorylation was prevented by the Pak inhibitor IPA3 (*p<0.05 vs others, n=4), as well as (C) by downregulation of Pak1 using siRNA (*p<0.05 vs others, n=3). (D) Smad3 luciferase reporter (p3TP-lux) activation by stretch was prevented with overexpression of kinase dead Pak1 (“dnPak1”). pcDNA is the empty vector (†p<0.01 vs others, n=6). (E) p3TP lux activation by stretch was also inhibited by Pak1 siRNA (*p<0.05 vs others, n=3). (F) Activation of another Smad3-sensitive luciferase reporter, CAGA12-luc, which contains 12 repeats of the CAGA Smad binding element driving luciferase expression, was also inhibited by Pak1 siRNA (†p<0.01 S vs others, n=5). (G) Smad3 C-terminal phosphorylation in response to stretch (30 min) was not blocked by the Rho-kinase inhibitor Y-27632. (H) Smad3 transcriptional activation, assessed by activation of the Smad3-sensitive CAGA12-luc reporter, was partially inhibited by Y-27632 (‡p<0.01 S vs con, *p<0.05 vs S, n=6). (I) Stretch-induced
Smad3 transcriptional activation was also partially inhibited by the MEK inhibitor U0126 (†p<0.01 S vs con, *p<0.05 vs S, n=4).

**Figure 8. Summary of the proposed role of Pak1 in mediating stretch-induced CTGF upregulation.** Pak1 activation is dependent on TβRI transactivation and activation of Rac1. Pak1 regulates activation of the complementary signaling mediators Smad3 and RhoA/Erk. We previously showed RhoA/Rho-kinase mediated Raf/MEK/Erk pathway activation through its cytoskeletal effects (Krepinsky et al., 2005b). Erk contributes to Smad3 transcriptional activation. Whether it also enables CTGF upregulation through activation of transcriptional cofactors is yet to be determined.
Figure 1

A

B

C

D

IP Pak1

IP Pak2

IP Pak3
Figure 1 continued

G  Pak2       Con        5/6

40x

60x

H  pPak S141  Con        5/6

40x

60x
Figure 2 continued

D

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Figure 2 continued

**H** Pak1 activity assay

![Immunoblot showing Pak1 activity assay](image)

**I** CTGF and Tubulin

![Immunoblot showing CTGF and Tubulin](image)

**Graphs**

- **H** Pak1 activity assay:
  - Fold change in Pak1 activity
  - Con, S, S+NSC

- **I** CTGF:
  - Fold change in CTGF
  - Con, S, S+NSC

*p* and ‡ indicate statistical significance.
Figure 3

A

B

C

D

E

F

CTGF

Tubulin

Con  S  S+Y27632

Con  S  S+C3

†

Con  S  S+Y

Con  S  S+Y

* *

Con  S  S+Y

Con  S  S+Y

* *

Con  S  S+Y

Con  S  S+SC514
Figure 3 continued

![Graph showing fold change in CTGF 4.5kb Luciferase Activity](image)

- Con
- S
- S+U0126

Fold Change in CTGF 4.5kb Luciferase Activity

Significance indicator: +
Figure 4

A

Con S Con S Con S
No siRNA Con siRNA Pak1 siRNA

B

Con S S+IPA3
RhoA-GTP Total RhoA

C

Con S S+NSC
RhoA-GTP Total RhoA

D

Con S S+NSC
RhoA-GTP Total RhoA

E

Con S S+IPA3
Fold change in RhoA activity

F

Con S S+NSC
Fold change in RhoA activity

* p < 0.05
† p < 0.001

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Figure 5

A  Rac1-GTP
      Total Rac1
    Con  S  S+SB

B  Rac1-GTP
      Total Rac1
    Con  S  S+GW

C  IP Pak1
          pPak
    Con  S  S+SB  S+GW

D  RhoA-GTP
        Total RhoA
    Con  S  S+SB

* Indicates significant difference.
Figure 5 continued

**E**

- CTGF
- Tubulin

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**F**

- CTGF
- Tubulin

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**Graphs**

- **Fold Change in CTGF**
  - **E**: [Graph Image]
  - **F**: [Graph Image]
Figure 6

A

Con | 30' | 1h | 3h
---|---|---|---
pSmad3 | Total Smad3

B

Con | S | S+SB
---|---|---
pSmad3 | Total Smad3

C

Con | S | S+αT | Con | S | S+αRab
---|---|---|---|---|---
pSmad3 | Total Smad3

D

Con | S | S+GM6001
---|---|---
pSmad3 | Total Smad3

E

KO | WT
---|---
Smad3 | Tubulin
Figure 6 continued

**F**

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**CTGF**

**Tubulin**

**G**

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**Fold Change in CTGF mRNA**

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<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smad3 KO</td>
<td></td>
<td></td>
<td>Smad3 WT</td>
<td></td>
</tr>
</tbody>
</table>

†

**Fold Change in CTGF 4.5kb Luciferase Activity**
Figure 7

A. Western blot analysis showing the expression levels of pSmad3 and Total Smad3 with Con, S, and S+NSC conditions.

B. Western blot analysis showing the expression levels of pSmad3 and Total Smad3 with Con, S, and S+IPA3 conditions.

C. Western blot analysis showing the expression levels of pSmad3, Pak1, and Tubulin under No siRNA, Con siRNA, and Pak1 siRNA conditions.
Figure 7 continued

D

![Graph showing fold change in p3TP Lux activity for different conditions](image)

E

![Graph showing fold change in p3TP Lux activation for different treatments](image)

F

![Graph showing fold change in CAGA4-Luciferase activation for different conditions](image)

G

![Image showing Western blot for pSmad3 and Total Smad3](image)

H

![Graph showing fold change in CAGA4-Luciferase activation for different conditions](image)

I

![Graph showing fold change in CAGA4-Luciferase activation for different conditions](image)
Figure 8

Stretch

TGFβRI

EGFR

Rac1

Pak1

RhoA

Rho-kinase

via effects on actin stress fibers

pRaf

MEK

pSmad3

pErk

CTGF promoter ↑

increased matrix synthesis